Evaluation of the mechanism of activity of extracts of *Pterocarya fraxinifolia* (Juglandaceae) trees used to replace antibiotic growth promoters in pig production in Europe

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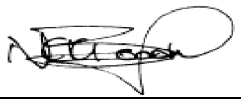
Declaration

I declare that the experimental work described in this thesis is my original work (except where the input of other is acknowledged), conducted in the Phytomedicine Programme, Department of Paraclinical Sciences, Faculty of Veterinary Sciences, University of Pretoria, and has not been submitted in any other form to any University or academic institution.

Signature: 

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List of abbreviations

EU	European Union
WHO	World Health Organization
GIT	Gastrointestinal tract
SD	Sabouraud dextrose
SDP	Spray-dried plasma
CPA	*Clostridium perfringens* Alpha toxin
CPB	*Clostridium perfringens* Beta toxin
ETX	Epsilon
ITX	Iota
O	Somatic antigens
K	Capsular or micro capsular antigens
H	Flagella antigens
F	Fimbriae antigens
EEC	European Economic Community
UK	United Kingdom
USA	United States of America
AGP	Antibiotic growth promoters
MLS	Macrolide-lincosamide-streptogramin
RNA	Ribonucleic acid
tRNA	Transcriptional ribonucleic acid
50S	50 Subunit
30S	30 Subunit
C55	Carbon 55
NGP	Natural growth promoters
NSP	Non-starch polysaccharide
ETEEC	Enterotoxaemia Escherichia coli
EFSA	European Food Safety Authority
CEF	Chloroform, ethyl acetate, formic acid
EMW	Ethyl acetate, methanol, water
BEA	Benzene, ethyl acetate, ammonia
DPPH	2, 2-diphenyl-1-picryl hydrazyl
ABTS	2, 2ô azinobis-(3-ethylbenzothiazoline-6-sulphonic acid)
TEAC	Trolox equivalent antioxidant capacity
OH	Hydroxyl
ROO	Peroxyl radical

INT	*p*-iodonitrotetrazolium
NADH	Nicotinamide adenine dinucleotide
MIC	Minimum inhibitory concentration
ATCC	American type culture collection
MH	Muller-Hinton
ATP	Adenosine triphosphate
NF	Nuclear factor
IL-1	Interleukin-1
TNF-Ŭ	Tumour necrosis factor
NFkB	Nuclear factor kappa B
15-LO	15-lipoxygenase
MEM	Minimal essential medium
DMSO	Dimethylsulphoxide
PBS	Phosphate buffer solution
MTT	3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium
NMR	Nuclear magnetic resonance
^{1}H	Hydrogen
MS	Mass spectroscopy
^{13}C	Carbon 13
MHz	Megahertz
TSA	Tryptic soy agar
Rf	Retention/retardation factor
EC_{50}	Effective concentration
IC_{50}	Inhibitory concentration
LC_{50}	Lethal concentration
PF	*Pterocarya fraxinifolia*

Abstract

The emergence of microorganisms resistant to antibiotics has led to the ban on many of the antibiotic feed additives used in animal production. The Swann Report and World Health Organisation (WHO) recommended that precautionary measures be established by governments to adopt a proactive approach to reduce the levels of antibiotic use in animals and put in place surveillance mechanisms to detect resistance. This led to the limitation and in some cases the total ban on the use of antibiotic growth promoters (AGP) in animal production. The first countries to enforce the ban included Denmark, Sweden and the United Kingdom. The main reason that led to the banning of antibiotics in Europe was the risk of antibiotic resistance transfer to human pathogens. Several research initiatives have been established to look into other alternative sources that can be used as antibiotics. Plants have received a lot of attention in this regard and phytogenic feed additives and/or antibiotics are now a focal point as new alternatives.

In the European Commission funded research projects Rumen (QLK5-CT-2001-00992) and REPLACE (FOOD-CT-2004-506487), a catalogue/database of plant samples were established through the collection of plant material from six geographical locations in three European countries. *Pterocarya fraxinifolia* fed to pigs and poultry had good activity in replacing antibiotic growth promoters. Plants growing in Denmark, Germany and Poland had widely diverging activities in animal studies. *Pterocarya fraxinifolia* trees growing in locations in Denmark, Germany and Poland had activity levels ranging from high to medium and inactive according to *in vivo* results provided by Dr Ole Hojberg. It would make sense to isolate and characterize the antimicrobial compounds so that this can be used to select populations with a high activity.

The original aim of this project, following the success achieved in the *in vivo* trials using un-extracted plant material, was to isolate and characterize the antimicrobial compounds from *P. fraxinifolia* trees growing in different areas in Europe, active against relevant pathogens in order to facilitate the selection of tree populations with good activity without having to carry out further animal experiments. A secondary aim was to investigate alternative mechanism of activity to replace antibiotic feed additives in the REPLACE programme. A supplementary aim was to find out what *in vitro* method can be used to predict the *in vivo* activity of different tree populations.

Antimicrobial activity was generally moderate for *Pterocarya fraxinifolia* extracts; the excellent activity was in the extracts from trees growing in Denmark and Germany (MIC 0.02 to 0.16 mg/ml). Activity against microaerophillic organisms (*Clostridium perfringens* and *Campylobacter jejuni*) was also good (MIC ranged = 0.02 to 0.63 mg/ml). The important aspect is that there were poor correlations between the antimicrobial activity of extracts from different populations and the *in vivo*

activity of plants from different populations against the different pathogens. This indicates that antimicrobial activity may not be the mechanism of activity of plants included in the feed of the animals. In general the antimicrobial activities of the solvent-solvent derived fractions were lower than that of the crude extracts pointing to synergistic activities between different compounds in the crude extract. In considering the antimicrobial activity of the crude extracts, there was a low correlation between *in vivo* activity of the plant and *in vitro* activity of the pathogens that are usually considered to play an important role had very low R^2 values, (*E. coli* K88 = 0.0047, *C. jejuni* = 0.0809*, C. perfringens* = 0.1092*).* Therefore other mechanisms of activity were also considered.

Antioxidant compounds are known to disrupt bacterial cell membrane function and structure and may also influence the response of the host to the pathogen. There was excellent correlations between the antioxidant activity of the crude acetone extract and the *in vivo* activity of plants from different populations (R^2= 0.8167). The correlation was even better in the case of the polar water/methanol fraction (R^2 = 0.8746). Because the content of the gastrointestinal track consists of an aqueous solution, it is understandable that polar compounds may be readily solubilized from the plant material included in the diet.

The anti-inflammatory activity was determined using the 15-lipoxygenase enzyme assay. The crude extracts and the solvent-solvent derived fractions of *P. fraxinifolia* had moderate activity for both leaf and fruit samples, with EC_{50} ranging from 4.47 ± 0.15 to 7.19 ± 0.05 mg/ml. There was not such a good correlation between anti-inflammatory activity and *in vivo* activity of the crude acetone extract as was the case for antioxidant activity (R^2 =0.3685). It is interesting that there was a better correlation between *in vivo* activity and anti-inflammatory activity for the hexane fraction (R^2 = 0.5536). This may indicate that non-polar compounds are involved with anti-inflammatory activity.

The crude acetone extracts of *P. fraxinifolia* trees growing in Denmark, Germany and Poland were relatively toxic with cellular toxicity LD_{50} varying between 0.77 and 1.75 µg/ml. The lack of toxicity in the animal trials may possible be due to the lower solubility and/or lower bioavailability of toxic metabolites in the aqueous gastro intestinal fluid. Hence *in vitro* toxicity may not always equate to whole organism toxicity.

The TLC bioautograms of plant extracts revealed the presence of several antimicrobial compounds in the plant extracts. The isolation and characterization of the compound(s) from *P. fraxinifolia* was performed using bioassay-guided procedures for antibacterial activity using open column chromatography with silica gel and Sephadex LH 20 as stationary phases. Five compounds were isolated and sufficient quantities of two were available for structure elucidation as *β*-sitosterol and

pentacosanol. All five isolates had weak antimicrobial activity when compared to the crude extract. The isolation did not yield any novel compound of importance. It was also very disappointing that the compounds isolated had such low antimicrobial activity. Although bioassay guided fractionation was used up to a certain point the assumption that compound present in high concentration in active fractions were the active compound, may have been wrong.

As has already been stated there were good correlations between *in vivo* activity and antioxidant activity, intermediate correlations with anti-inflammatory activity and poor correlations with antimicrobial activity. An alternative mechanism for *in vivo* activity could be anti-inflammatory activity or stimulation of the immune system (via antioxidant activity) based on good correlations obtained. Another positive aspect is that it appears that direct killing of pathogens by the plant extract is not a major factor responsible for the *in vivo* activity.

If the factors above are indeed the main factors responsible for *in vivo* activity, there may be decreased development of resistance by pathogens against antimicrobial compounds present in the extracts. Additional animal and *in vitro* studies are required to confirm the results that antioxidant activities of extracts are the best predictor of activity in animal studies. This may facilitate the selection of plant material from different tree populations for replacing antibiotic feed additives in animal production.

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Chapter 1

1.1 Introduction

Antibiotics are commonly fed to animals in livestock production systems to prevent disease and metabolic disorders. Many of these mainstream antibiotic feed additives are products developed for human pharmaceutics (Rochfort *et al*, 2008). An enormous number of antibiotics have been described in literature but many are highly toxic for clinical use. Antibiotics are generally low to medium (100-1500) molecular weight compounds that exhibit a variety of chemical structures, physical and biological properties (Crosby, 1991). There are over 7000 compounds that have been described in terms of their source, mode of action and chemical structure (Crosby, 1991). The feed additives used currently on the animal markets are mainly those with antimicrobial, endectocides and anticoccidial activity, drugs which are all synthetic derivatives of natural products (Rochfort *et al*, 2008).

Additives are now available in varying forms, from direct-fed to slow release boluses which significantly improves the efficiency of the diet even in low quality feeds. The range of additives used in the animal production industry is quite broad (vitamins, trace minerals and growth promoters). They also include a range of auxiliary substance that may not be essential in nutrition but play a role in improving palatability (Wallace & Chesson, 1995). The majority of the industrial animal feed formulations are composed of plant derived materials. The major components of feeds are cereals and their by-products, plant material which are considered proteinaceous. There are lesser components like animal by-products in feeds but the vitamins, trace metals, growth promoters and synthetic amino acids are all added to generally complete the feed. Probiotics and enzymes are also now known to feature as additives (Wallace & Chesson, 1995).

There is a growing concern worldwide on the prevalence of antibiotic resistance and this has led to the development and emergence of resistant bacteria and genes (van den Bogaard & Stobberingh, 2000). Approximately 50% of all antibacterial agents used in Europe annually in human health are also used in veterinary medication for therapy and prevention of bacterial infections. They are also used as animal feed additives to promote growth and enhance feed efficacy (van den Bogaard & Stobberingh, 2000). Nearly all of the antibiotics used for veterinary purposes are given orally as feed additives to livestock by way of mixing it into the food, water or at times fed directly to the animals.

The introduction of antibiotics in the veterinary practise has led to an increase in the resistance of pathogenic bacteria and an increase in faecal flora (Wallace, 2004). In recent years public concern

over the routine use of antibiotics in livestock nutrition has increased due the emergence of resistant bacteria agents that represent a risk to human health (Benchaar, *et al*. 2008). The banning of antibiotic feed additives by the European Union (EU) in 2006 has directed the focus to finding new natural antibiotic feed additive alternatives. Plant extracts offer a unique opportunity in this regard since they contain secondary metabolites which have antimicrobial properties and thus making them potential alternatives to conventional antibiotics (Wallace, 2004; Benchaar *et al*., 2008). This prompted the EU to invest in the Framework 6 REPLACE program which aims to screen plants for a range of activities including antibacterial, nematocidal and immune stimulating effects (www.rowett.ac.uk/rumen_up/RumenUp).

The plant kingdom is a reservoir of a variety of chemical compounds and serves as source of new pharmaceutical products. Nearly all plants can be expected to protect themselves against disease and animal herbivores through the use of these chemical compounds stored by the plants. Plants compounds are been considered as possible natural alternatives (Steiner, 2006). There are large numbers of herbs and spices that are now considered as natural growth promoters in animal nutrition. Herbs like oregano and cinnamon have shown broad activity *in vitro* against pathogenic bacteria like *E. coli*, *Salmonella spp* and *Clostridium perfringens* (Kamel, 2000). Some of the enormous number of compounds that plants produce as natural protection against pathogen and insect attack may also be toxic to animals while others may not be. The diversity of plants growing worldwide along with their pharmacological uses offer a possibility of finding novel chemical agents with efficacious antibiotic properties. It has been stated that natural products and their derivatives form about 50% of most drugs in use with about 25% of them been derived from plants (Farnsworth, 1984 & Harborne, 1998).

Through the European Commission funded research projects Rumen (QLK5-CT-2001-00992) and REPLACE (FOOD-CT-2004-506487), a catalogue of plant samples were established through the collection of plant material from six geographical locations in three European countries and around the world. *Pterocarya fraxinifolia,* one of the plant species from the catalogue, fed to pigs and poultry had good activity in replacing antibiotic growth promoters. Leaves and fruit extracts from *P. fraxinifolia* trees growing in Denmark, Germany and Poland had widely diverging activity in animal studies. For this reason it would make sense to isolate and characterize the antimicrobial compounds so that this can be used to select tree populations with a high activity in replacing antibiotic growth promoters and establish the possible mechanism of activity for *P. fraxinifolia* as replacement for antibiotic growth promoters and this was the reason why *P. fraxinifolia* was selected for this study.

In vivo trials on non-extracted plant material of *Pterocarya fraxinifolia*

The influence of *Pterocarya fraxinifolia* plant material was tested on the growth performance of piglets six weeks post weaning. In brief a standard weaner diet was formulated and four experimental treatments were prepared consisting of; i) Control (standard diet), ii) control plus 0.5 % plant material, iii) control plus 1 % plant material and iv) control plus 2 % plant material. A total of 192 piglets from 48 litters were included in the trial. There were twelve replicates per treatment group, each consisting of a pen with four piglets each. The piglets were weaned at 28 ± 2 days and moved to the pens for the initiation of the experiment. The piglets were kept separate in each holding pen for the duration of the experiment and no mixing of piglets was allowed. The piglets were fed *ad libitum* (Hojberg *et al*, 2008).

The trail was conducted over a six week period commencing on the day of weaning. The feed intake of each pen was recorded daily and the individual body weight of the piglets was recorded weekly. In the first two weeks of the study the consistency of the faeces in the each pen was recorded. Faecal samples from randomly selected piglets from each pen were taken on days 5, 13 and 41 of the experiment and this was collected directly from the rectum for analysis (Hojberg *et al*, 2008).

The weekly feed intake improved significantly from day 5 post weaning in the four treatments used in this study and contained to increase through days 15, 20 and 28. No decrease in feed intake was observed for the duration of the experiment. The daily feed intake was also good and no decrease was observed, with the highest daily feed intake occurring from days 7-14, 14-21 and 21-28. The live body weights of the piglets increased significantly from the first day post weaning, almost doubling in body weight after 28 ± 2 days. The daily weight gain of the piglets was notably significant on days 14-24 but the overall weight gain post 28 days was less but still significant. There was a significant drop in faecal score 6 days post weaning in all four treatment parameters, with significant decrease in faecal coliform counts and pH on day 14 through to day 28 of the trail (Engberg, 2008 & Hojberg *et al*, 2012).

The inclusion of *P. fraxinifolia* plant material in the feed of piglets post weaning has growth promoting effects and demonstrates significant levels of antimicrobial activity of the non-extracted plant material from different regions of Europe. This was evident in the in vivo trials and in vitro assays conducted using the non-extracted plant material of *P. fraxinifolia*.

Following the success of the *in vivo* trials, it was suggested that the potential active compounds in the plant material responsible for the antimicrobial and growth promoting effects be identified and characterised and elucidate their potential mode of action.

1.2 Hypothesis

Plants contain antimicrobial agents which are active against animal and human pathogenic bacteria. They also contain antioxidant and anti-inflammatory compounds that could be used to manage and decrease the severity of diseases caused by antimicrobial agents. These active compounds can be isolated and characterized to yield potential drugs with novel structures and activity for use in treating and protecting humans and animals against pathogenic bacteria, and also against certain inflammatory diseases. Moreover, the plant extracts or compounds can also be used to enhance animal productivity, to bring about animal welfare.

1.4 Aims/objectives

1.4.1 Aim

The original aim of this project was to isolate and characterize the antimicrobial compounds from *P. fraxinifolia* trees growing in different areas, active against *aureus* relevant pathogens in order to facilitate the selection tree populations with good activity without having to carry out animal experiments. A secondary aim was to investigate alternative mechanisms of activity to replace antibiotic feed additives in the REPLACE programme.

1.4.2 Objectives

- To evaluate *in vitro* antimicrobial activity of *P. fraxinifolia* trees growing in different areas of Europe against selected Gram-positive (*Staphylococcus aureus* and *Clostridium perfringens*) and Gram-negative bacteria (*Campylobacter jejuni* and *E. coli strain* K88) and fungal yeast (*Candida albicans*) and mould (*Aspergillus fumigatus*).
- To determine the antioxidant and anti-inflammatory activity as possible alternative mechanisms of action and also the cytotoxicity of *P. fraxinifolia* extracts.
- To isolate the bioactive compound from the plant and determine the structure and antimicrobial activity of the isolated compounds.
- To determine the correlation between *in vivo* and *in vitro* activity of extracts from different tree populations against *E.coli* K88, *Clostridium perfringens* and *Campylobacter jejuni*
- To discuss the possible mode of actions and variation of activity between extracts of tree populations from different areas.

Chapter 2

2. Literature review

2.1 Introduction

Medicinal plants have been the subject of study based on human curiosity and need. The World Health Organisation (WHO) define medicinal plants as any plant containing substances that can be used for therapeutic purposes or which are precursors of chemo-pharmaceutical semi-synthesis (WHO, 1979). Plant derived products are present in fourteen of the fifteen therapeutic categories of pharmaceutical preparations that are currently used and recommended by medical practitioners and form an important part of the health care system in the eastern and now also in the western world (Phillipson & Anderson, 1989). According to Perumal *et al* (1999), there are 119 secondary plant metabolites derived from plant which are used globally as drugs/medicine. The selection of a suitable plant for pharmacological study is a very important and decisive step. This involves taking into account the traditional use, chemical content, toxicity or randomised selection and several other criteria (Soejarto, 1996).

Diets are formulated for livestock production with one goal in mind and that is to enhance the performance of the animal. In swine production the dietary ingredients influences not only the growth of the pig but also the microflora which inhabit the gastrointestinal tract (GIT) (Miguel & Pettigrew, 2005). There are several factors that affect microflora in the gut and these may range from alterations in the external environment such as housing, change in the composition of diet to, the introduction of new pen-mates. According to Miguel & Pettigrew, (2005) these changes can result or manifest as subclinical digestive disturbances or decreased production performance resulting in clinical infections, diseases and eventually death of the pig. Miguel & Pettigrew, (2005) further affirms that diet composition is probably the most important factor for microbial activity in the gastrointestinal tract monogastric animals.

2.1.1 Pig production systems

Pigs are known scientifically as *Sus scrofa*, (Fig. 2.1) they are polytocous species (Oliver & Leus, 2008). The ancestral roots goes back to the wild boar and are considered as a subspecies of the wild boar but some say it is a distinct species in its own right described by Carl Linnaeus, in 1758. The litter size varies depending on parity and several other factors. The reproductive cycle is relatively short which allows for several generations to be present in a given farrowing herd (Madec *et al*, 2001). The current production system ranks pigs according to age and physiological stage. There are three stages or categories to follow; weaning, growing or finishing pigs, pregnant or

nursing sows and boars. The holistic approach towards the pig unit tends to prevail over consideration of individual pigs. As a consequence pigs are identified individually and this makes for appropriate herd management (Madec *et al*, 2001).

The economic pressure on pig production has resulted in intensified production methods in order to decrease the production costs and this has caused the use of cheap and at times harmful methods during weaning to try and increase the quality of the pig products (Mahan, 1993). In the past decade the world pig population has increased above the 868 million number that was recorded in the nineties. Pig production in developed countries has become a specialized activity and as a result production has increased and been concentrated in the hands of specialist and large scale producers (Bhat *et al*, 2010). The growth of the pig population depends on a number of factors such as, good animal husbandry practises, control of disease, proper nutrition, good housing, proper selection of breed conducive to stress in the environment, improved marketing facilities and market demands (Bhat *et al*, 2010).

Figure 2.1: Pigs in production line (www.wikipedia.org/swineproduction)

2.1.2 Weaning of pigs

Pigs are capable of extremely rapid growth after weaning but there are many factors that limit the extent to which this potential is expressed. There are several factors that affect weaning of pigs, weaning age, weaning weight, weaning stress, health status, low feed intake, diet composition, digestive immaturity and the environment (Mahan & Lepine, 1991). About 75% of all pig farmers wean the pig at 3 to 4 weeks of age. It has been said that early weaning imposes a lot of stress on all domestic animals. The younger and small pigs have a less developed digestive systems and a

lower body fat content (Mahan, 1993). At weaning the piglet faces three challenges; first, there are major changes in the food supply, secondly the piglet must cope with the change in its physical environment and thirdly the psychological stress that accompanies moving and mixing of pigs (Pluske *et al*, 2003).

Early weaning of piglets is always often accompanied by serve growth defects and diarrhoea. Weaning is a multi-factorial process and post-weaning anorexia and under nutrition are major aetiological factors (Lalles *et al.,* 2007). During weaning there is great change in the magnitude and exposure of piglets to environmental agents derived from food and potential pathogenic organisms (Lalles *et al.,* 2007). Under natural conditions weaning is a gradual process that normally occurs at and is not complete until piglets are 10-12 weeks of age. Now piglets are weaned at 3-4 weeks and as a result several defects occur, which hamper growth development of the piglets and leads to metabolic disorders.

The first few weeks after the weaning period is regarded as a crucial stage in pork production and there are nutritional strategies that can be put into place early in the weaning process to reduce stress and the severity of in growth defects and disorders (Pluske *et al*, 2003). The adequate intake of supplemented nutrients before weaning can assist in preparing the digestive system of the pig for the digestion of complex carbohydrates and proteins and also promote weight gain (Lalles *et al*, 2007).

Gastro-intestinal tract (GIT) disorders, infections and diarrhoea increases at the time of weaning in young pigs. This causes large economic losses in the pig industry. To curb this problem antibiotics growth promoters are used (Lalles, 2008). There are several changes that occur due to weaning but the most striking consequence of post weaning disorders is a rapid mucosal atrophy of the small intestines which losses 20-30% of its weight within 2 days post-weaning. There is also a reduction of the villous height and surface area along the small intestine and the activity of most pancreatic and brush boarder enzymes are decreased (Lalles, 2008). Many changes in structure and function of the intestine throughout weaning have been documented and well-studied. Most of the changes are highly dependent on the level of feed intake (Lalles, 2008).

Several dietary management strategies have been researched over the last decade and it has been shown that protein sources like skim-milk and whey are good but expensive for sustaining high growth performance in weaned pigs. Less expensive options like spray-dried plasma (SDP) incorporated at levels of 4-6% into starter diets are more commercially viable. Other energy sources like glucose, lactose and starch incorporated into starter diets improves small intestine structure.

Amino acids, organic acids, probiotics and probiotics, plant extracts and natural substances are all potential areas of interest for controlling post weaning diarrhoea and GIT disorders (Lalles, 2008). Gut microbiota exerts a huge impact on the nutritional and health status of the host through the modulation of the immune and metabolic functions. The micro-biome provides additional enzymatic activity involved in the transformation of dietary compounds which exert significant effects on the intestinal environment modulating gut microbiota composition and function (Laparra & Sanz, 2009).

2.1.3 Managing gut health

In nature bacteria and fungi (a group of yeasts) are microorganisms with a large diversity of species that can readily colonize a habitat containing a steady supply of nutrients and energy for growth and maintenance (Apajalahti, 2005 & Steiner, 2005). The gastrointestinal tract of mammals is one such environment that receives the first inoculum of microorganism and these microorganisms can enter into various degrees of symbiotic relationships with the host. The microflora that forms as a result of these relationships in a healthy animal is collectively referred to as the normal flora (Sorum & Sunde, 2001).

According to Conway (1994), a stable gut health is based on three factors: Host physiology, Diet, and Microflora (gut ecosystem). It is the interactions between these three factors that bring stability to gut health and any disturbance caused by environmental factors or stress such as a drop in feed or hygiene conditions may significantly affect gut health (Steiner, 2005). Gut microflora play a central role within the gut ecosystem, gut lumen and mucosal tissue are popular sites for microbial colonization. The digestive tract of neonates is devoid of microorganism but this change once they are exposed to the environment and a versatile microflora begins to develop (Steiner 2005).

The microflora may exert a number of effects in the gut of the animal, which will in turn lead to a positive or negative impact on the health of the host animal. A stable gut health helps to assist in protecting and maintaining the host animalôs defence against pathogenic invasion. The gut represents the largest organ within a host animal defence system. The digestive system of ruminants is different from that of non-ruminants (Fig. 2.2) and thus the structure of the digestive system determines the sites of the intestine in which both the nutritional and physicochemical requirements are right for the growth of microorganisms (Apajalahti, 2005).

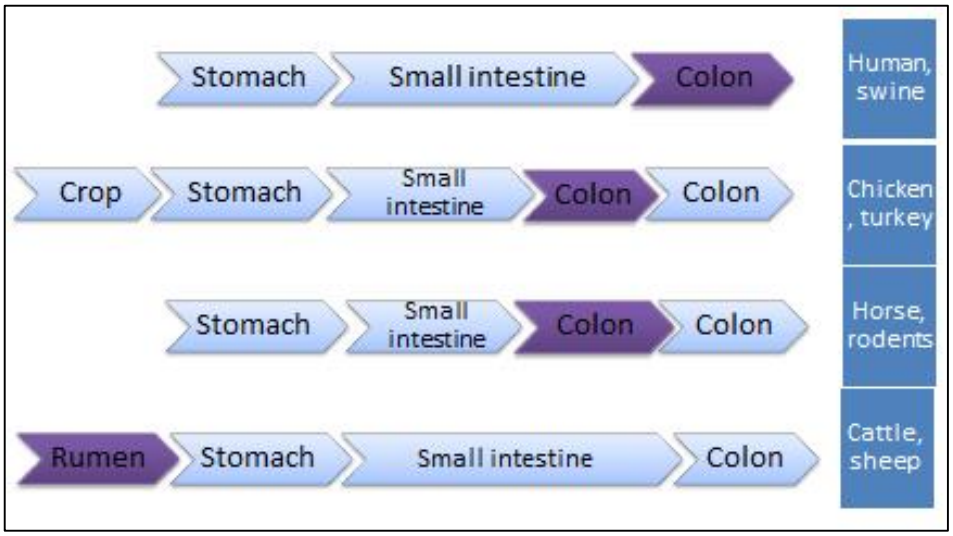


Figure 2.2: Relative intensity of microbial colonization (purple colour) in the gut system of various animals (adopted and modified from Apajalahti, 2005)

The intestinal microflora of the pig is very complex and diverse making it difficult to study qualitatively and quantitatively. The pig gut flora has been extensively studied with regard to the changes that occur in the gut environment due to pH changes and organic acid metabolism due to the breakdown of fermentable carbohydrates (Straw *et al*, 2006). The difference in diet composition has also been linked to add to the diversity of the gut microflora.

2.1.4 Pig diseases

Diseases of the GIT that affect pigs between neonatal and finishing stages continue to be one of the greatest factors that limit the efficiency and profitability of pig production in the world (Straw *et al*, 2006). Despite the advances in the development of new vaccines and antimicrobial products, the awareness of antimicrobial resistance and the ban or limited use of antimicrobial growth promoters has countered these new advances (Straw *et al*, 2006).

There are several diseases that affect pigs and they range from polysystemic, respiratory, circulatory, musculoskeletal central nervous system and gastrointestinal disease to name but a few. A detailed discussion of the many disease that affect pigs can be found in ñAn outline of swine diseasesòby Cowart and Casteel (2001). Clostridia enteritis is one of the major diseases that affect pigs. This condition leads to bloody diarrhoea, progressive emaciation and dehydration.

The bacterial species responsible for this condition is *Clostridium perfringens* type A and C. The major toxins produced are alpha (CPA), beta (CPB), epsilon (ETX) and iota (ITX) (Straw *et al*, 2006). *C. perfringens* type A is part of the normal flora of the pig intestine and is responsible for the

cause of enteric diseases in neonatal and weaned pigs. It occurs mostly in the first 0-4 days after birth and may cause mortality of the herds (Cowart & Casteel, 2001). Piglets develop creamy diarrhoea within 48 hours of birth and this may last for the first five days. Many of the piglets do recover from these infections but they appear stunted in growth (Straw *et al*, 2006).

The pathogen is consumed shortly after birth due to its presence in the soil. The bacteria once ingested attaches to the jejuni villi and invades the entire gastrointestinal tract. The bacterium produces toxins that contribute to intestinal necrosis leading to death via secondary bacteraemia and toxaemia. The treatment of this condition is ineffective once the clinical signs are evident but the administration of antitoxins may help in acute and sub-acute cases (Cowart & Casteel, 2001).

Post weaning scours is also known to occur. Naturally weaning is a gradual process of dietary change from milk to solid food and it is not complete naturally until 10-12 weeks of age. Modern pig production does not allow for this gradual process and weaning occurs 1-3 weeks of age. A three week old pig has an immature digestive and thermoregulatory systems and its immunity is at its lowest at this time, which make it highly susceptible to stress and disease (Cowart & Casteel, 2001).

The most common bacteria seen in post weaning scours are *E. coli* and the strains vary depending on the breed in production. The species of *E. coli* known to cause this condition determined serotypically are O (somatic), K (capsular or micro-capsular), H (flagella) and F (fimbriae) antigens (Straw *et al*, 2006). *E. coli* k88 strain is the major cause of neonatal and young piglet diarrhoea and the causative strain produces one or more enterotoxins which adhere to the small intestinal mucosa in pigs.

During such an infection the newly weaned pig does not feed for a few days post weaning and it eventually starves to death. Prior to starvation the undigested sugars and starches creates an osmotic gradient in the gut and the undigested food then provides a substrate for pathogenic bacteria. These pathogenic bacteria then pass through the stomach to the small intestines. This condition is normally prevented and controlled through proper weaning and nutritional management and immunization (Cowart & Casteel, 2001)

2.1.5 Feed and feed additives

The improvement of the world supply of animal products means the intensification of some of the animal production systems, but not all parts of the world are capable of adapting themselves to the challenge. Animal production in the western world has improved significantly over the last 30 years. These drastic improvements have not extended to the pig meat production industry but it has seen

an increase of almost 50% (Wallace & Chesson, 1995). A well balanced, good quality feed is needed at all times for efficient production.

A feed additive is defined by the European Feed Additive Directive 70/524/EEC as any substance or preparations containing any substance which, when added or incorporated into feeding stuff, is likely to affect its characteristics or livestock production (Steiner, 2009). Feed additives are vital in their role in promoting feed utilization and efficiency. The improvements are a small part of the picture when it comes to additives; the methods that are applied should contribute not only to increase production of superior meat quality but should also conform to the increasingly severe food safety regulations (Steiner, 2009).

Additives are now available in various forms, from direct-feed to slow release molecules which can substantially improve the efficiency of the diet and lead to increased animal production. The range of additives is very broad and includes vitamins, trace metals, growth promoters, disease preventing agents and auxiliary substances (Wallace & Chesson, 1995).

There are several principles behind feed formulation of low-cost diets for monogastric animals. It starts with the determination of growth and production characteristics of the animals in question, followed by their tissue requirements for the specific nutrient quantified (Moughan *et al*, 2000). The nutrient requirements are then translated into dietary specifications that are used to set the minimum limits to inclusion of the nutrients in the feed. These parameters are set to ensure that the feed is palatable, non-toxic and does not induce digestive disorders (Moughan *et al*, 2000). The improvement of efficiency and profitability of animal production by means of animal nutrition remains an important economic priority.

2.1.6 Antibiotics in animal feeds

The potential of therapeutic antibiotic feed additives was first realised when chickens were feed fermented offal from chlortetracycline produced by *Streptomyces aureofacieus.* Since then several other forms of antibiotics have been introduced ever since then as a result of intensive animal farming (Butaye *et al*, 2003).

These products improved feed quality and increased animal growth and decreased incidences of mortality through disease. The mechanism of action for growth promotion through the use of growth promoters is still not well understood but there are four proposed hypothesis that try to explain their action are listed below (Feighner *et al,* 1987) (Fig. 2.8);

- Nutrients may be protected against bacterial destruction,
- Absorption of nutrients may improve because of a thinning of the small intestinal barrier,
- The antibiotics may decrease the production of toxins by intestinal bacteria, and
- There may be reduction in the incidence of subclinical intestinal infections (Feighner *et al,* 1987).

The use of antibiotics as feed additives is a hallmark in modern husbandry. In the past all antibiotics were allowed for use even though many did not enhance growth and some were too expensive (Butaye, *et al.*, 2003). In the 1960s the first talks on the potential dangers of using antibiotic as feed additives gave rise to the Swann report in the United Kingdom (UK). At first the use of antibiotic were restricted and could be used following certain guidelines set out by the Swann Report. The use of penicillin, tetracycline, tylosin and sulphonamides as growth promoters were discontinued after the Swann Report (Butaye, *et al.*, 2003).

The US food and drug administration in 1950 approved the use of sub therapeutic levels of antibiotics in animal feeds (Buchanan *et al.,* 2008). Production animals are fed antibiotics to prevent disease and promote growth but this has resulted in the development of antibiotic resistant bacteria. As a result several countries have banned the use of sub-therapeutic antibiotics in the production of animals (Buchanan *et al.,* 2008). The UK banned the use of penicillin and tetracycline for growth promotion in the 1970s. Sweden and Denmark banned all growth promoting antibiotics in 1986 and 1999. The US banned the use of enrofloxacin in 2005 and the EU banned 6 of the 13 known antibiotics used as feed additives in 2006 (Buchanan *et al.,* 2008). The EU ban also extended to all of the antibiotic growth promoters (AGP) that are also used in human health/medicine (Wegener, 2003).

Bacteria from animals spread to food products during slaughter and processing. The use of antibiotics for growth promotion in food animals has led to the creation of major food-animal reservoirs of bacteria and the development of resistance to last-resort antibiotics (Wegener, 2003). There have not been major side effects to the banning of these antibiotics used in animal production in terms of weight or mortality of livestock. In Denmark there was an increase in antimicrobial treatment for diarrhoea in the post-weaning period after the complete termination of AGPs (Wegener, 2003).

A few of the antibiotics used today as growth promoters in animal feeds have been well investigated and many are known to limit growth of Gram-positive bacteria (Butaye *et al*., 2003). Much research is been put into this due to the fact that many of the antibiotics active on these bacterial species in livestock are also known to cause major problems in humans due to infections caused by these

rumen multi-resistant Gram-positive bacteria (Butaye *et al*., 2003). Differentiation between susceptibility and resistance of micro-organisms to antibiotic growth promoters are commonly based on several clinical, pharmacological and microbiological criteria (Butaye *et al*., 2003).

2.1.7 Types of antibiotics commonly used in animal production

In the animal farming industry antibiotics are used for three main purposes; therapeutically, prophylactically and also as growth promoters (Barton, 2000). Therapeutic treatment involves the use of antibiotics to treat animals for a specific over a specified period. Prophylactic treatment involves moderate to high doses of antibiotics, which are often given (but not limited to) in food and water for a specified period of time (Barton, 2000).

Prophylactic use has its problems in terms of the antibiotic used and the duration of use. Growth promoter use is an area of high concern as many have been regarded as potential compromisers of the efficacy of some key human antibiotics and the duration of use may span the entire life time of the treated animal (Barton, 2000).

The regulatory controls vary from country to country; there are strict regulatory guidelines over which antibiotics can be used in food-producing animals. In the UK and other EU countries antibiotics are authorised as either veterinary medicinal products or zoo-technical feed additives while other EU countries and those outside the EU also have their own regulations (Barton, 2000).

There are several groups of antibiotics ranging from penicillin (ƀ-lactam), macrolides (erythromycin, spiramycin and tylosin), peptides, glycolipid, cephalosporin and quinoaxline to name a few (Table 2.1). The modes of action differ from group to group, some target and inhibit cell wall synthesis, DNA and protein formation, while others completely disintegrate the cell membrane (Barton, 2000 & Chowdhury *et al*, 2009). According to a review by Chowdhury *et al* (2009) the different groups of antibiotics and their antimicrobial agents have resistance ranges of between 2-100% with macrolides as the highest.

Table 2.1: Antibiotic growth-promoters that are still allowed (no date added) and those that have been banned for use by the EC (Butaye *et al*., 2003)

Antibiotic	Banned since	Antibiotic group	Related therapeutics	Mechanism of action
Bambermycin		Glycolipid		Inhibition of cell wall synthesis
Bacitracin	1999	Cyclic peptide	Bacitracin	Inhibition of cell wall synthesis
Monensin		Ionophore		Disintegration of cell membrane
Salinomycin		Ionophore		Disintegration of cell membrane
Virginiamycin	1999	Streptogramin	Quinupristin/dalfopristin	Inhibition of protein synthesis
Tylosin	1999	Macrolide	Erythromycin & others	Inhibition of protein synthesis
Spiramycin	1999	Macrolide	Erythromycin & others	Inhibition of protein synthesis
Avilamycin		Orthosomycin	Everninomycin	Inhibition of protein synthesis
Avoparcin	1997	Glycopeptide	Vancomycin,teicoplanin	Inhibition of cell wall synthesis
Ardacin	1997	Glycopeptide	Vancomycin,teicoplanin	Inhibition of cell wall synthesis
Efrotomycin		Elfamycin		Inhibition of protein synthesis
Olaquindox	1999	Quinoxaline		Inhibition of DNA synthesis
Carbadox	1999	Quinoxaline		Inhibition of DNA synthesis

Bambermycin (an aminoglycoside) (Fig. 2.3) is a complex glycolipid antibiotic produced by *Streptomyces* species with the main component consisting of moenomycin A and a phosphorus-containing glycolipid (Butaye *et al*, 2003). This antibiotic used as growth-promoting antibacterial agent in animal feeds works by inhibiting the synthesis of peptidoglycan.

It inhibits the peptidoglycan polymerase by impairing the transglycolase activities of the penicillin-binding proteins which blocks the formation of murein polysaccharide strands. The spectrum of activity is primarily against Gram-positive microorganisms but to some extent it also inhibits some Gram-negative bacteria (Butaye *et al*, 2003).

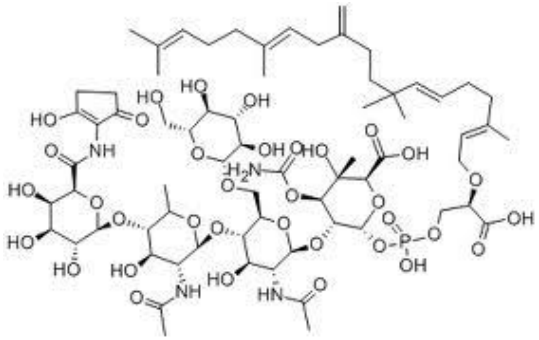


Figure 2.3: Structural representation of bambermycin (www.wikipeida.com/images).

Streptogramins is an antibiotic that belongs to the macrolide-lincosamide-streptogramin (MLS) group (Fig. 2.4) and consists of two components, an A component and a B component which act synergistically. It is produced by *Streptomyces virginiae* as a natural mixture containing two chemically different components (streptogramin A and virginiamycin M ï streptogramin B).

The mechanism of action is through binding to the bacterial 23S rRNA of the 50S ribosome subunit to form a stable dalfopristin-ribosome-quinupristin complex, which irreversibly inhibits protein synthesis resulting in bacterial cell death. The components when used individually cause bacteriostasis (Butaye *et al*, 2003 & Cocito *et al*, 1997).

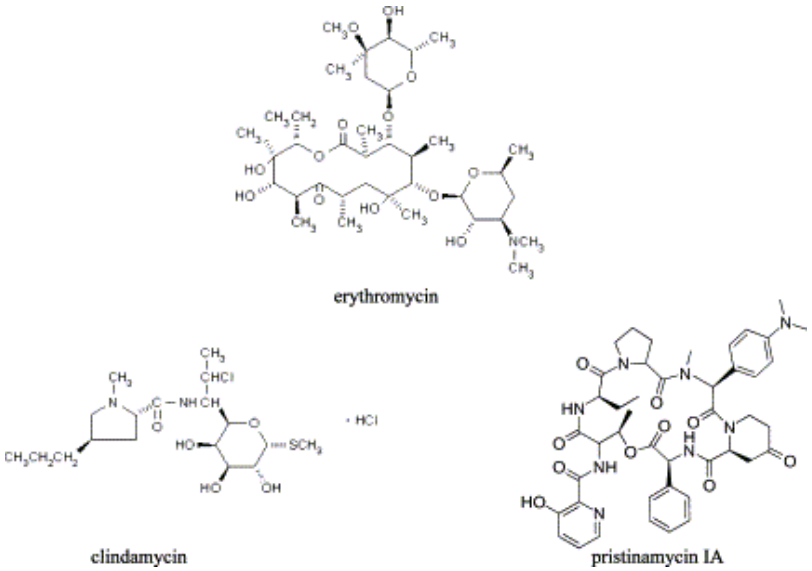


Figure 2.4: Chemical structure of members of the macrolide group of antibiotics (www.wikipeida.com/images).

Avilamycin (Fig. 2.5) is an antibiotic that belongs to the group of oligosaccharides and it is used as a growth promoter. It is produced by *Streptomycin virdocgromogenes* made up of a mixture of several

major and minor components. It acts by binding with the 30S subunit of the ribosome and interferes with the polypeptide-synthesizing function by affecting the attachment of aminoacyl-tRNA to the ribosome but is also known to bind to the 50S subunit (Butaye *et al,* 2003).

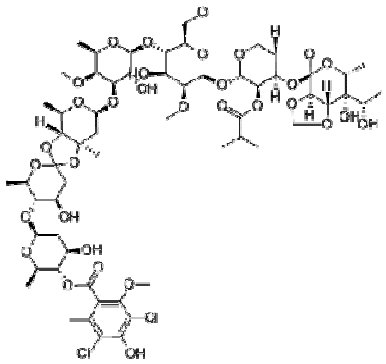


Figure 2.5: Avilamycin structure (www.wikipeida.com/images).

Bacitracin (peptides) (Fig. 2.6) is a polypeptide antibiotic produced by *Bacillus licheniformis*. This antibiotic is made up of a mixture of several major components with the most important been A, B and C, and at least 13 minor components. It is used as a growth promoter and also for topical application in human and veterinary medicine (Butaye *et al,* 2003). It is active against Gram-positive bacteria but also has a similar antibacterial spectrum to that of penicillin. This antibiotic forms a complex with C55-isoprenyl pyrophosphate, the dephosphorylation of this complex by C55-isoprenyl pryophosphatase recycles the carrier *N*-acetylmuramyl peptapeptide and thus inhibit cell wall formation (Butaye *et al*, 2003).

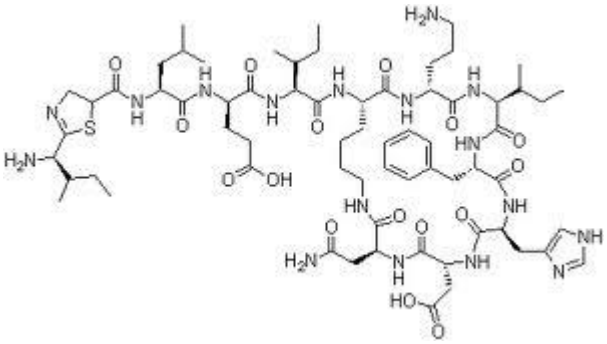


Figure 2.6: Structure of Bacitracin (www.wikipeida.com/images).

Ionophore antibiotics (Fig. 2.7) are produced by *Streptomyces* species but other species of *Nacardiopsis, Nocardia* and *Actinomadura* are also known to produce this antibiotic. This antibiotic works by interfering with the natural ion transport system of both prokaryotic and eukaryotic cells.

They lower the energy barrier for the trans-membrane transport of ions and catalyse an electro-neutral cation-proton exchange across the barrier by getting rid of the Ca^{2+} , Mg^{2+} , K^{+} and Na^{+} gradients leading to cell death (Butaye *et al*, 2003).

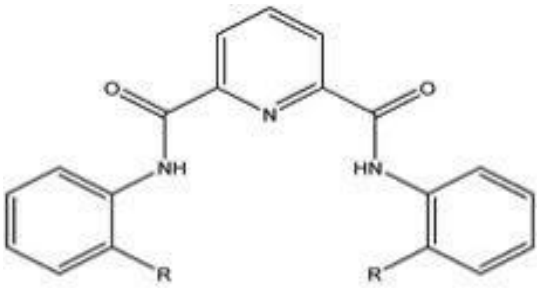


Figure 2.7: Structure of three ionophores bond together (www.wikipeida.com/images).

2.1.8 Antibiotic residues

Controls on the use of antibiotic in animals until recently only focused on the control of residues in the tissues of the treated animals. The concerns about these residues have now shifted to revolve around allergic reactions or the possible side effects of the antibiotic on the flora of the human gut (Barton, 2000).

Since the Swann reporte in 1969 noted penicillin residues in milk triggered allergic reactions in sensitised individuals, confirmed cases were very rare and the adverse reactions to antibiotic have been linked to hypersensitivity.

There is still yet very little information on the effect of antibiotic residues on the bacterial flora of the human intestinal tract (Barton, 2000). Many of the developed countries have set up monitoring systems to survey animal tissues for residues. They have established acceptable calculated daily intake for humans to what they call a ñno-effect levelò

2.1.9 Antibiotic resistance in bacteria

The resistance of bacteria to antibiotics associated with their use in animals has raised major concerns. There are concerns about the transfer of antibiotic resistant pathogens and genes from animal enteric flora to human pathogens (Barton, 2000). There is also the issue of reduced efficacy of antibiotic therapy in animals with resistant bacteria.

There is very little data on antibiotic resistance in bacterial isolates from animals. However due to concerns there has been some surveillance set up to monitor such bacteria since the 1970s in the

UK, Sweden, France, Denmark and USA (Barton, 2000). The difficulty in the reports generated by such surveillance is that there is a lack of standardisation from laboratory to laboratory and country to country and the lack of agreed interpretive criteria.

The biggest concern about antibiotic resistance in animal isolates of bacteria is directed more toward enteric bacteria such as *E.coli*, salmonella, thermophilic campylobacters and enterococci. There is comprehensive information on the antibiotic resistance in *E. coli* and salmonella to tetracycline, oxytetracycline and ampicillin but very little is known about thermophilic campylobacters and enterococci (Barton, 2000, Hinton et al, 1985). Erythromycin-resistant campylobacters have been reported in pigs (Moore et al, 1996).

2.1.10 Antibiotic mode of action

There have been at least four major mechanisms of action (Fig. 2.8) proposed to explain AGP-mediated growth enhancements (Niewold, 2007). According to Dibner and Richards (2004), there have been several studies on the mechanism of action focusing mainly on the interactions between the antibiotic and the gut microbiota.

The direct effects of AGPs on the microflora can be used to explain the decline in competition for nutrients and reduction in microbial metabolites that depress growth (Dibner & Richards, 2004). The reduction in gut wall and villus lamina propria has been used to explain the enhanced nutrient digestibility observed in antibiotic growth promoters (Dibner & Richards, 2004).

The reduction in microflora and its effects may be the underlying mechanism for the beneficial effects of antibiotics. Other mechanisms of action have also been described by Gaskins *et al*, (2002) and Page (2006). The main mode of action of organic acids, probiotics, probiotics and phytogenics is that they modify and stabilise the gut microflora by providing comfortable conditions for beneficial microorganisms, while inhibiting the growth of pathogenic species (Steiner, 2006).

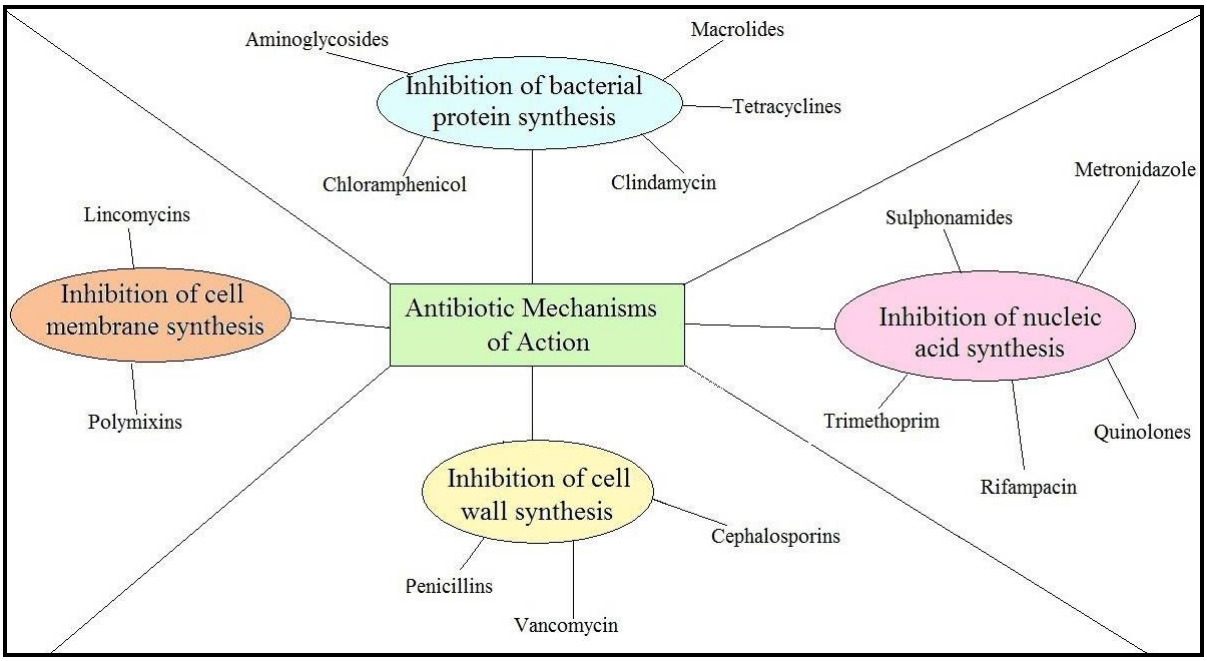


Figure 2.8: Antibiotic mechanisms of action. These are the four proposed mechanisms of action (Niewold, 2007)

2.1.11 Natural growth promoters- alternatives to antibiotics

There are several initiatives giving attention to developing alternatives to counteract the drawbacks associated with the ban of AGPs. There were two large EU programs aimed at developing practical alternatives to antibiotic use in animal feed and replacing the use of synthetic antibiotic in animals namely the Rumen-Up project and Feed for pig health (Wallace, 2005 & Rochfort *et al*, 2008).

Currently there are a large number of natural growth promoters (NGP) on the market; these include organic acids, immune modulators, probiotics, probiotics, feed enzymes and phytogenics. Each of these products has the potential to exert an effect on the gut health and growth performance (Steiner, 2005). According to Steiner (2005), there are comprehensive reviews that prefer the use of NGP as alternatives to AGP in animal nutrition. The main focus should be directed to the application, potential effects and mode of action.

2.1.11.1 Organic acids

Acidifiers also known as organic acids have been considered as a promising alternative to replace AGP due to fact that they are natural and safe metabolites of plant and animal tissues and their use have already been proven and well established (Steiner, 2005). Microbial carbohydrate fermentation in the gut of animals also produce them (Steiner, 2005). Acidifiers cause a reduction in pH values in the gut creating unfavourable conditions for the potential pathogen (Steiner, 2005).

2.1.11.2 Probiotics

The concept of *probiotics* was first established by Elie Metchnikoff, after observing bacteria in fermented milk products that were said to capable of controlling bacterial fermentation in the intestinal tract of men (Metchnikoff, 1907, 1908). The modern concept was derived about 40 years ago by a researcher named Park in1974. The definition was restricted to ña live microbial feed supplement which beneficially affects the host animal by improving intestinal microbial balance (Gatesoupe, 1999).

Since its inception probiotics have been applied in animal nutrition but their use is different from that in humans. There are now up to 21 probiotic preparations authorised by the EU and out of those only about 13 of them have been approved for use in swine production (Simon, 2005).

The beneficial effects of feed additives are generally explained by modification of the intestinal bacteria and their interaction with the host animal (Simon, 2005). The beneficial effects of probiotics are based on its ability to modify gut microflora and thus it is a requirement for the probiotic to make it into the gut in its viable form. Probiotics differ in their susceptibility to temperature and pH.

There are currently many microorganisms used as probiotics in animal nutrition. Their mode of action is based on three factors; competitive exclusion, bacterial antagonism and immune modulation. These factors can be used at different stages of development from gestation right through to weaning of livestock (Simon, 2005).

Probiotics are compounds, other than dietary nutrients that act as substrates for beneficial microbial organisms in the gut. The potential value of probiotics has been the subject of many studies in relation to managing gut health and the prevention of enteric infections (Straw ***et al***, 2006).

Probiotics are known to achieve their beneficial effects in two ways; firstly, sugar based compounds can be broken down and fermented by bacteria giving them a competitive advantage and secondly, mannos-sugar containing compounds added to the diet result in binding with pathogenic bacteria with mannos-specific lectins in fimbriae. This cascade results in reduced attachment of pathogenic bacteria to receptor sites on the gut musical cells (Straw ***et al***, 2006).

2.1.11.3 Enzymes

In various industries there is large scale usage of enzymes in the production system, these include the milk processing, alcohol production and diagnostics. Enzymes are known bio-catalytic protein

that initiate or accelerate specific biological reactions. Their use goes as far back as the 1980s (Steiner, 2005 & Wallace 2008).

Feed enzymes degrade specific substrates through the cleaving of defined bonds in a molecule and are thus classified according to the type of substrate they act on. Due to high specificity of enzymes to their substrate its application as a feed additive should be based on the ingredients that make up the feed (Simon, 1998).

Non-starch polysaccharide- degrading enzyme (NSP) are been used enzyme feed additives due to their abundant nature in cereals and oilseed meals. There are a number of soluble and non-soluble NSP and they may exert different anti-nutritional effects in livestock (Simon, 1998). The mode of action is best described in figure 2.9.

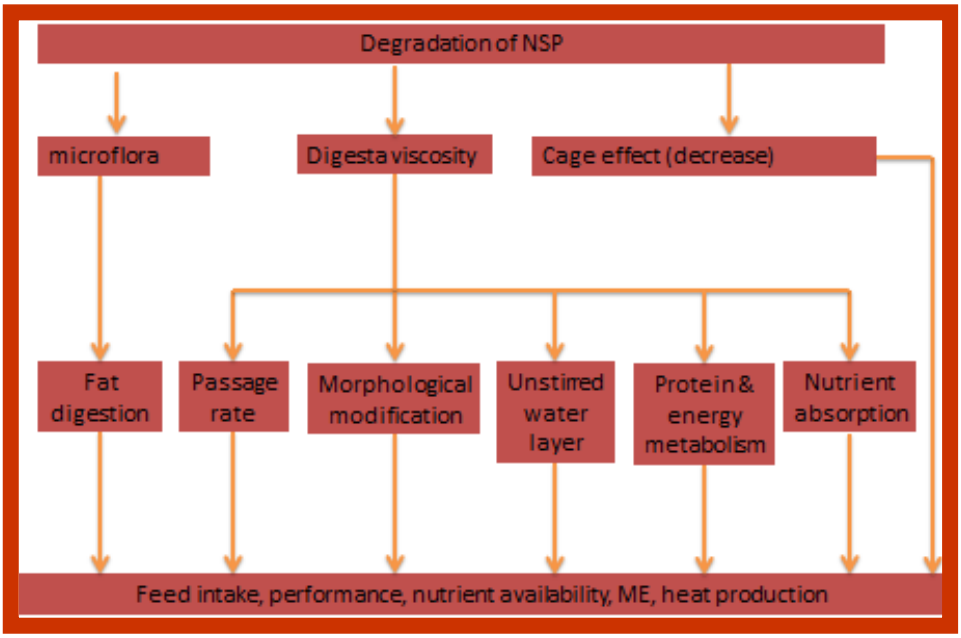


Figure 2.9: Mode of action of NSP enzymes (Simon, 1998)

2.1.11.4 Phytogenics

The ban on feed additives has put a spot-light on alternatives such as probiotics, enzymes and plant extracts as the next or new alternative to replace feed additives (Wallace, 2008). Plant extracts have been used for several decades as a natural remedy to prevent ailments in humans. Herbs, spices and plant extracts have been proposed and reviewed as the new alternatives for in-feed antibiotics in livestock raising (Michiels *et al*., 2009).

Plants produce secondary metabolites (see appendix A figure 10.1) which are a group of chemicals that are not involved in the primary biochemical processes of plant growth and reproduction but are

probably essential in the protection of the plant from pathogens (Bodas *et al*., 2008; Jouany & Morgavi, 2007). Several of these secondary metabolites good antimicrobial activity and thus could be used as alternatives to antibiotics in livestock production (Bodas *et al*., 2008). The range of bioactive compounds on plants is huge and this provides endless potentials for the use of plant extracts or compounds isolated from these extracts as good alternatives to antibiotic feed additives (Wallace, 2008).

In vivo work has been carried out by several researchers on the use of plant extracts as feed additives. The use of potato (*Solanum tuberosum*) extracts has been documented and researched with proven records showing improved performance in the weaning of pigs and reduced the pathogenic bacteria in faeces (Jin *et al*., 2008). Many of the success stories in regard to the use of plant extracts as potential feed additives are based on the use of essential oils (Bodas *et al.,* 2008 & Buchanan *et al*., 2008). To date the most extensive work done on the use of plant extracts to enhance livestock production has been conducted by the ñRumen-upòand ñREPLACEòframework five and six project (http://www.rowett.ac.uk/rumen_up/.).

Phytogenic feed additives are products derived from plants used in animal feeds to promote and improve the productivity of livestock not based on nutritional value in the first place. This class of feed additives have gained increased interest due the ban on most of the antibiotic feed additives. Plant derived feed additives are a relatively new class of feed additives and knowledge on these class is still limited with regard to their mode of action and some aspects of their application. They also vary widely with respect to their botanical origin, processing and composition (Windisch *et al*, 2007).

The content of the active substance when plant derived compounds are used as feed additives also vary widely and this can be attributed to the plant part used, harvesting season, geographic area of growth, and the technique for processing (Windisch *et al*, 2007). All these factors play an important role when considering phytogenic feed additives.

The core mode of nutritional action in livestock is probably improved stimulation of digestive secretions, bile, mucus and enhanced enzyme activity and several *in vitro* studies have proven this to true (Lee *et* al, 2003, Rao *et al*, 2003, Platel & Srinivasan, 2004 and Jang *et al,* 2004). Phytogenic feed additives have also been reported to stimulate secretion of mucus, which an effect thought to impair adhesion of pathogens (Jamroz *et al,* 2006). Many of the studies done on phytogenics support the hypothesis that plant derived feed additives may favourably affect gut functions.

Herbs and spices have been known to exert antimicrobial effects *in vitro* against some very important pathogens including fungi (Adam *et al*, 1998, Hammer *et al*, 1999, Si *et al*, 2006). Some studies *in vivo* in livestock have also demonstrated efficacy (Jamroz *et al*, 2003 and Mitsch *et al*, 2004). Besides efficacy the use of phytogenics in livestock not only has to be safe for the animal but also the user and the consumer of the animal product and the environment (Windisch *et al*, 2007). According to Papatsiros *et al* (2011), the interest in phytogenic feed additives will continue to grow in the years to come, but proper growth can only be achieved through the development of well-designed clinical trials to prove its safety and efficacy. The cost-benefit evaluation is important to support farmers by making the production system more cost effective.

The feed industry is now looking for efficacious, safe and cost-effective additives that have clearly outlined mode of action and proven benefits. Plant-derived compounds have a considerable potential to fulfil this demand (Steiner, 2009). The lack of knowledge in several aspects of phytogenics regarding the consistency of trail results and mechanisms of action needs to be looked at more seriously. More animal trials with plant compounds and their blends are necessary to investigate the effect of these phytogenic compounds in the performance of pigs and other livestock (Steiner, 2009).

The mode of action of phytogenics appear to be highly versatile and far from been based on a simple flavouring or antimicrobial effect (Steiner, 2009). Phytogenics have been proven to be highly effective in poultry production but their addition as feed additives will have to fulfil the strict registration and regulatory guidelines in respect to safety and efficacy for the animal, the environment and especially the consumer.

2.1.11.5 Immune stimulants

Animals that are healthy are protected against pathogenic infections because of their efficient immune system. It is known that a large number of immune cells are associated with the gut and help modulate the immune responses of the animal. In young animals the immune system develops slowly after birth, making them targets to gastrointestinal infections in times of stress and thus leading to the slow function of the immune system (Allen *et al*, 2001).

Many studies have been done to support strategies in the development of immune stimulating agents in animal feeds. In 1995 a report by Dritz *et al* (1995) showed an increase in the daily gain and average daily feed intake from 17-21% to 21-23% after the addition of ß-glucans at a lower dosage of 0.025% of the diet. In poultry Fleischer *et al*, (2000) and Den Hartog *et al*, (2005) proved

that adding yeast ɓ-glucans to broiler feeds as a supplement improved growth performance in infected birds in the first week of the experiment. The history of the use of immune stimulates in animals also cover fields of aquaculture and ruminants (Steiner, 2006).

There is evidence that suggests that dietary compounds of plant origin used as foods, play a key role in disease prevention through multi-factorial actions involving modulation of the immune system. Many classes of secondary plant metabolites have been found to have a positive influence on the immune system of animals *in vivo*. Epidemiological evidence has indicated that a high intake of foods rich in phenolics and polyphenols improves and strengthens the immune system (Cowan, 1999; Katerere & Eloff, 2005).

2.1.11.6 Plants as antibiotic/antimicrobial agents

Plant chemistry or phytochemistry has developed greatly and has become a distinct discipline encompassing natural product organic chemistry and plant biochemistry. There is an insurmountable amount and variety of organic substances that accumulate in plants through their physiological functioning as by products known as secondary metabolites (Hoffman & Evans, 1911).

The useful qualities of these metabolites have been recognized and there are currently many attempts and research dedicated to the isolation and characterization of these compounds (Eloff and McGaw, 2014). Plants produce these secondary compounds (Appendix 1, Figure 10.1) in defence to attack against microbes and insects. Many of these compounds can also be toxic to animals and humans alike (Wallace, 2004).

2.1.12 Major groups of antimicrobial compounds from plants

The unlimited ability of plants to synthesize metabolites with an enormous diversity of structures make plants a valuable source for new compounds for drug discovery (Table 2). Many of these compounds are secondary metabolites which serve as plant defence mechanism against predation by microorganism, insects and herbivores (Cowan, 1999). Many of these compounds give plants their odour, colour and flavour and some are used in the formulation of medicines. It is estimated that there are about 250,000 species of plants and only a small percentage is known and used by humans and other animal species as medicine, but evidence indicate there is more (Cowan, 1999).

Table: 2.2: The major classes of antimicrobial compounds that occur in plants (Cowan, 1999).

Class	Subclass	Example(s)	Mechanism
Phenolics	Simple phenols	Catechol	Substrate deprivation
		Epicatechin	Membrane disruption
	Phenolic acids	Cinnamic acid	
	Quinones	Hypericin	Bind to adhesins, complex with cell wall, inactivate enzymes
	Flavonoids	Chrysin	Bind to adhesins
	Flavones		Complex with cell wall
		Abyssinone	Inactivates enzymes
	Flavonols	Totarol	Unknow mode of action
	Tannins	Ellagitannin	Bind to proteins
			Bind to adhesins
			Enzyme inhibition
			Substrate deprivation
			Complex with cell wall
			Membrane disruption
			Metal ion complexation
	Coumarins	Warfarin	Interaction with eukaryotic DNA (antiviral activity)
Terpenoids, essential oils		Capsaicin	Membrane disruption
Alkaloids		Berberine	Intercalate into cell wall and/or DNA
		Piperine	
Lectins and polypeptides		Mannose-specific agglutinin	Block viral fusion or adsorption
		Fabatin	Form disulphide bridges
Polyacetylenes		8S-Heptadeca-2(*Z*),9(*Z*)-diene-4,6-diyne-1,8-diol	Unknown mode of action

2.1.13 *Pterocarya fraxinifolia* (Lam.) Spach

Pterocarya fraxinifolia (Lam.) Spach. (Caucasian walnut) (Fig. 2.14) is a fast growing tree indigenous to the Caspian shores in the northern parts of Iran, south-western Asia and Anatolia (Sheykholislami & Ahmadi, 2009). The leaves of this plant are used as an anaesthetic, as dyeing and antifungal agent and it has been used to treat diarrhoea. The local people in Iran use the leaves to also catch fish. The tree grows to about 20-30 m in height with a circumference of up to 3 m. It has a thick short bole supporting widely spreading branches.

The plant bears fruit in August, it has pinnate leaves that can exceed 60 cm in length, comprising of 7-27 sessile leaflets. The flowers appear in April, the male catkins are thick and green and the female is longer with less dense flowers bearing red styles forming fruiting catkins of about 30-50 cm long. The green winged nuts are about 1.8 cm wide. According to Akhani and Salimian (2003), *P. fraxinifolia* is a thermophilous tree that grows in mostly flooded forests and valleys with water at an altitude of below 1000 m (Browicz, 1982). This plant grows best on flat ground or shallow slopes and the climate associated with the distribution of this tree includes mild winters and mild humid summers. It grows in mixed stands with other species and rarely grows in pure stands (Sheykholislami & Ahmadi, 2009).

This plant is a characteristic species of the *Pterocaryo-Alnetum glutinose* and *Pterocaryo-Fraxinetum angustifoliae* ecological forest types and is associated with several other plant species in the forest of the central Caspian coast (Rastin, 1983 & Kutbay *et al*, 1999). According to the local people *P. fraxinifolia* grows with living memory and they believe that cutting is inauspicious (unlucky) has persevered them till today. The vernacular name of this plant come from the Arabic root word ñRahmaniòmeaning ñGodôs giftò According to Akhani and Salimian (2003), *P. fraxinifolia* is a beautiful tree with a wide crown, pendulous fruiting spikes and large pinnate leaves has made it an attractive garden and street plant. The species has long been cultivated in gardens across Europe (Wijnands, 1989).

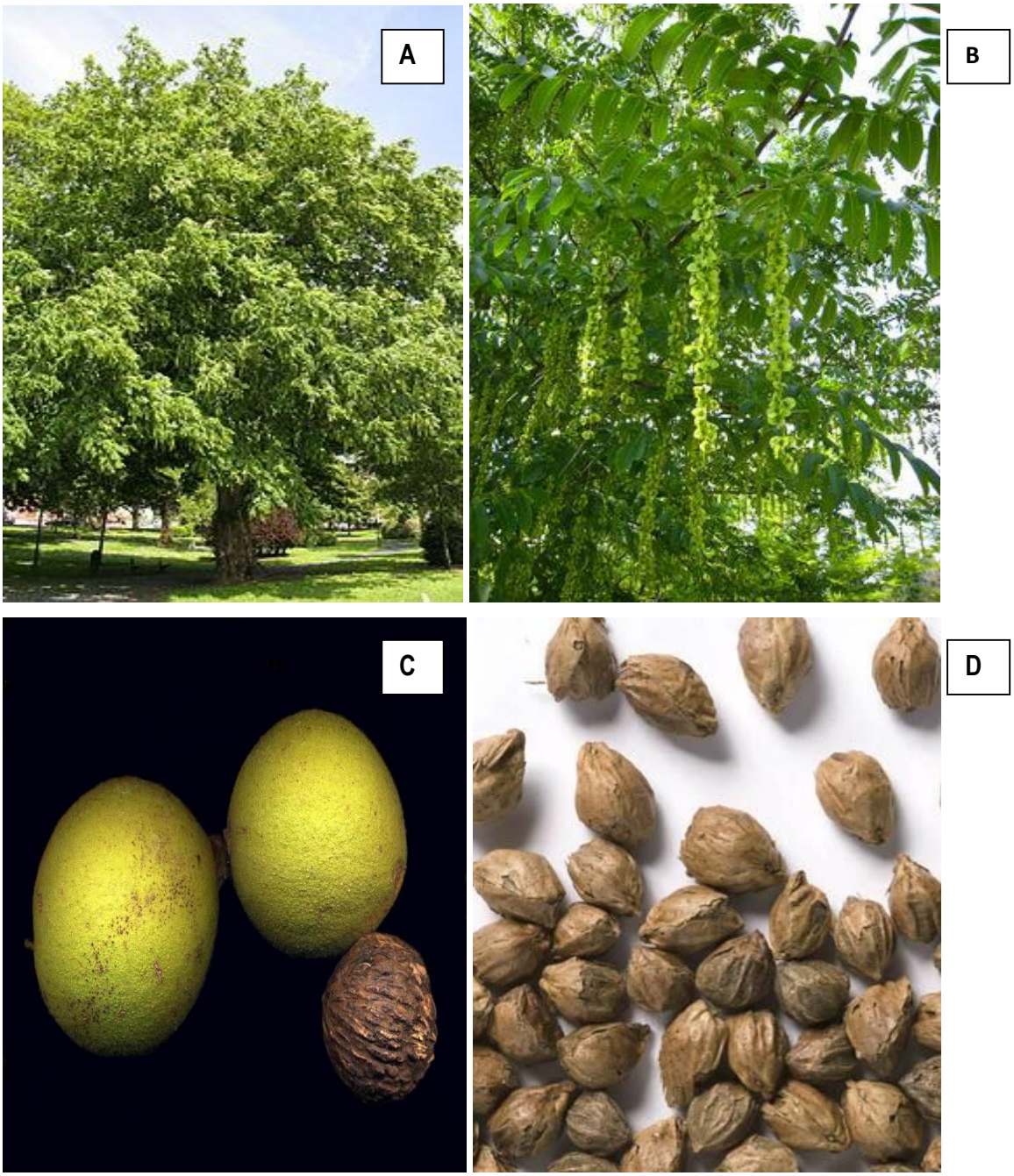


Figure 2.14: *Pterocarya fraxinifolia* A) The adult plant, B) the flowers, C) green walnuts and D) dried walnut (www.wikipedia.com/)

2.1.13.1 Classification of P. Fraxinifolia

Kingdom: Plantae

Order: Fagales

Family: Juglandaceae

Genus: *Pterocarya*

Species: *fraxinifolia*

Juglone (5-hydroxy-1,4-naptoquine) (Fig. 2.15) is a nephthoquinone compound that is present in the leaves and hulls of *P. fraxinifolia* and it is apparently to date to only compound isolated from this plant. Juglone has been reported as a toxic agent in Juglandaceae and has been exploited for catching fish (Aynehchi *et al*, 1973).

It is found in a reduced form as 4-ɓ-D-glucoside in the green parts of the plant and is formed by hydrolysis and oxidation during extraction process (Aynehchi *et al*, 1973). Juglone is well known for its effect on other plants and many plants cannot be grown under walnut trees that exude juglone by its roots.

Figure 2.15: The structure of juglone previously isolated (www.wikipeida.com/images).

2.1.14 Bacterial species used in this study

The genus *Escherichia* is a Gram-negative anaerobic rod. The species includes normal inhabitants of the gastrointestinal tract and strains causing a broad variety of intestinal and extra-intestinal diseases in swine (Straw *et al*, 2006). *Escherichia coli* (ETEC) strains are regularly associated with neonatal diarrhoea in human, live and domestic livestock. There are two virulence factors that work in combination to produce the diarrhoeic condition, their ability to adhere to the intestinal mucosa and the production of heat labile/stable enterotoxins (Vazquez *et al*, 1996). Post weaning diarrhoea in piglets is caused by the *E. coli* strain that expresses K88 fimbriae (Zhang *et al*, 2010).

The economic losses in the swine industry associated with intestinal diseases are high and according to Alexander *et al* (1994), diarrhoea represents 11% of post-weaning mortality in piglets. *E. coli* (ETEC) accounts for 50% of about 10 million and more piglets that die annually worldwide (Gyles, 1994 & Owusu-Asiedu *et al*, 2003). The K88 strain has two plasmids one encodes for genes of the fimbriae on the bacterial surface and the second encoding for genes that produce toxins that trigger diarrhoea (Blomberg, 1992). The present awareness of the problems associated with the

excessive use of antibiotics and the lack of success with k88 specific vaccines for post weaning of piglets to prevent diarrhoea are required (Blomberg, 1992).
Campylobacter jejuni has been recognised as an important cause of diarrhoea and enteritis in humans. This pathogen is carried in the intestinal tract of pigs, chickens and cattle. *C. jejuni* is an enteropathogen transmitted by food.

In 1997, 2648 people were infected during food-borne outbreaks of *C. jejuni* (Ono and Yamamoto 1999), Campylobacteriosis is the most frequent reported zoonotic disease in humans in the European Union (EU) in recent years (Santini *et al*, 2010). Poultry meat is a frequent source if sporadic campylobacteriosis. In 2007, 26% of samples of fresh broiler chickens and 25% of broiler flocks in the EU tested positive for *Campylobacter* (Westrell *et al*, 2009) and over 46 0000 cases of infections were reported in the UK alone out of 190 000 cases in the whole of the EU (EFSA, 2006).

The prevalence of *Campylobacter jejuni* and other *Campylobacter* isolates has been raised in conventionally grown broilers to about 66% and organically frown broilers to about 89% according to studies conducted. *C. jejuni* is the most prominent species in both conventionally and organically grown broilers. Their antimicrobial resistance rates vary according to different broiler production types. Conventionally raised broiler harbour more antimicrobial-resistant *Campylobacter* strains than organically grown broilers (Luangtongkum, *et al*, 2006).

Clostridium perfringens is a food borne, highly opportunistic pathogen. These bacteria are large Gram-positive, spore forming rods and strict anaerobes. They are primary pathogens of pigs and are mainly found in the intestines. There are two major types that cause enteric diseases in pigs, type A and C. There are several types each with their own specific toxin disease causing agent (Straw *et al*, 2006). The Centre for Disease Control in the USA in 1999 estimated that this pathogen was responsible for causing annual food-borne illnesses which has resulted in over 200 000 suspected cases, 41 hospitalization and 7 deaths (Mead *et al*, 1999). The economic costs associated with. *C. perfringens* incurred food illnesses were estimated as $12.5 billion in 1995 (Buzby and Roberts, 1997 and Novak and Juneja, 2002).

Necrotic enteritis is disease in poultry that has a high economic and animal welfare cost. It has become prevalent in the European Union due to the banning of antibiotic growth promoters. Necrotic enteritis estimates vary widely from 1 to 50% of animals per flock. This disease affects a variety of bird species and it is a significant disease in commercially farmed poultry. At low levels the organism is non-pathogenic but any alterations in the gastrointestinal tract (GIT) due to feed type or unfavourable conditions in gut health, leads to proliferation of the organism and a serious onset of

necrotic enteritis. This condition left untreated can produce mortality rates of up to 2% per day in flocks (McDevitt, *et al,* 2006).

The causative organism of necrotic enteritis is *Clostridium perfringens* in the gut. It has been estimated that about 37% of all broilers grown in North America are infected with necrotic enteritis. Contrary to that studies conducted in Norway on the incidence of necrotic enteritis in broiler flocks over a twenty-year period indicated was averaged at 1 to 2% of the broiler flock. At its peak necrotic enteritis in Norway was about 35%. There are very few studies that exist that have measured directly the incidence of necrotic enteritis in any country. In study surveys of necrotic enteritis in broiler flocks conducted in UK over ten year ago revealed that 33% of farmers at one time or another had necrotic enteritis outbreak, about 12.7% of farmers reported that the disease had occurred in their last flocks (McDevitt, *et al,* 2006).

Outbreaks of necrotic enteritis in broilers happen at 17-18 days of age and if this is left untreated always lead to high mortality in flocks. The factors that predispose broilers to necrotic enteritis are not known but any factor that causes stress in broilers could alter their intestinal environment putting broilers at risk of the disease. With recent advances in research about the induction and aetiology of necrotic enteritis, a number of novel treatments are possible but there is still rigorous laboratory and field condition tests required to confirm the clinical efficacy. An example of such treatment is the use of probiotics (chapter 2 section 2.1.11.2), these are agents used to manipulate the gut microflora and reduce *C. perfringens* colonisation (McDevitt, *et al,* 2006).

In the chapters that follow the phytochemical composition, antimicrobial, antioxidant, anti-inflammatory activities and the cytotoxicity of *P. fraxinifolia* crude acetone extracts and fractions from *P. fraxinifolia* trees growing in six different areas in Europe will be determined.

Chapter 3

The antioxidant activity and chemical composition of *Pterocarya fraxinifolia* acetone extracts and solvent-solvent derived fractions

Preface

The plant species selected for this study had been evaluated for activity *in vivo* with results indicating that the plant had good potential as a feed growth promoter. Preliminary screening of the plant species was done to confirm earlier investigations, when plant material was added to animal feed. The chemical composition and antioxidant activity of the selected plant species is also dealt with in this chapter. Antioxidants essentially reverse several conditions associated with immune deficiencies and inflammatory diseases.

3.1 Introduction

The plant kingdom harbours a practically inexhaustible amount of active compounds which could be invaluable in the management of many diseases (Parekh & Chanda, 2007). Plants are used medicinally in different countries and are a rich source of many potent and useful compounds that can be used as antimicrobial agents (Srivastava *et al*, 1996). A few decades ago scientific investigations and information on the therapeutic potential of plant materials were limited (Lin *et al*, 1999). Since then research on the use of plants as new leads for drug development has advanced and continue to advance beyond expectations.

Drugs from plants can be categorised into two broad spectra, firstly they can be included in complex mixtures that contain a variety of compounds or they can be used as pure chemically defined actives compounds (Taylor *et al*, 2001). The screening of plant extracts and products for activity has shown that higher plants represent a potential source for development of novel drugs (Afolayan, 2003). Pharmacological investigations are important when dealing with plants that are intended for therapeutics.

Pharmacognosy is defined as the botanical, biological and chemical study of the therapeutic value of compounds in plants (Taylor *et al*, 2001). Since the 19th century the critical and systematic study of the chemistry and biological activity of natural products have been developed and new technologies have made it easier and faster to determine the novel constituents of plant extracts and also enabled the production of detailed biological and chemical profiles of plant materials from just using a few milligram of the natural product (Taylor *et al*, 2001).

Screening of natural products is highly important but it also has many problems associated with it. The most common problem that is encountered, according to Farnsworth (1993), is that all plant extracts are a mixture of several compounds which can be subject to variation in concentration (Farnsworth, 1993). On the other hand *in vitro* procedures also have their problems in that some compounds may show good activity in an *in vitro* assay and if metabolised *in vivo,* may have no activity at all. There is strong evidence that the antimicrobial activity of plants depend on the mixture of different compounds based on synergism. In some cases the active compounds isolated from plant extracts may have much lower activity than the expected activity based on the activity of crude extracts or fractions (Eloff and McGaw, 2014)

Phenolics are a group of aromatic phytochemical compounds characterised by the presence of one or more hydroxyl groups. These phenolic compounds are generally potent free radical scavengers *in vitro* and have been said to provide *in vivo* antioxidant protection against inter and intra cellular damage. They are also able to modulate various inflammatory mediators in the inflammation process. Several other benefits have been reported with regard to the use of phenolic compounds in various physiological conditions (De Groot, 1994 & Grace, 1994).

In the past decade there has been an increased interest in polyphenols especially in the health and food industry. This is due to the discovery of their biological activities and possible role in the control and prevention of chronic diseases caused by oxidative stress (George *et al*, 2005). Most of the known polyphenols are able to trap free radicals. These free radicals pose a danger in that they may lead to the oxidation of nucleic acids, proteins and lipids that may lead to degenerative conditions. Antioxidant compounds inhibit the oxidative mechanisms (Millier *et al*, 1993).

Antioxidant compounds have been known to exert their effect as scavengers of free radicals. The correlation between antioxidant and antibacterial activity is in the interactions of the bacterial cell membrane and the antioxidant compound. Antioxidant compounds are known to cause changes in the physical properties of bacterial membranes to produce their desired biological effect (Caturla, *et al*, 2003). Any changes on the physical properties of phospholipids within the cell membrane can have dramatic consequences on the bacterial cell metabolism and viability.

It has been studied and well documented that antioxidants such as catechins isolated from the tea plant is known to produce leakage in *E. coli* isolated membranes and this reinforce the antibacterial activity of antioxidant compounds (Caturla, *et al*, 2003). There are several studies that give account of many plant extracts rich in antioxidant compounds and the same compounds have been reported to be responsible for *in vitro* antibacterial activity (Fernandez-Lopez *et al*, 2005). Well known

antioxidant compounds like ascorbic acid and other natural flavonoids have been attracting a lot of attention not only for their antioxidant activity but also as agents for anti-inflammatory and antibacterial activity due to their lipid-peroxidation effects (Fernandez-Lopez, *et al*, 2005). In order to characterise antioxidant activity of a plant extract it is always desirable to evaluate a range of activities using a battery of tests and hence the determination of phenolic content in *P. fraxinifolia* is essential for evaluating its antioxidant capabilities.

There are various methods that have been developed to monitor and compare the antioxidant activity of plant extracts. These methods require specialized equipment and technical skills for analysis and interpretation. Some of these methods involve the use of electron spin resonance and chemiluminescence to measure radical scavenging activity of the antioxidant against free radicals (Millier *et al*, 2000). The aim of this chapter was to determine the antioxidant activity and the chemical composition of *Pterocarya fraxinifolia*.

3.2 Materials and methods

3.2.1. Plant collection

As part of the EC funded research projects Rumen (QLK5-CT-2001-00992) and REPLACE (FOOD-CT-2004-506487), a collection of plant samples have been established by collecting plant material around six geographic locations in Europe; Aberdeen (UK), Hohenheim (DK), Leon, Reading and Dublin and Viborg (DK). The plants were all selected based on literature studies, communication with ethnobotanist and botanical gardens. Only the leaves and fruits of the plants were collected for this study but the general collection included parts/fractions of the whole plant to leaves, roots, flowers and fruits. The plant materials were collected at different stages of maturity, dried and milled into fine powder and stored in the dark at room temperature. Detailed information on the selected plant can be obtained from the Rumen-Up and REPLACE collection list (www.rowett.ac.uk/rumen_up.).

Finely ground *Pterocarya fraxinifolia* leaf (Specimen number, D058R, D052R, and D054R), fruit (Specimen number, D056R and D053R) and leaf and fruit mixture (Specimen number, D021R) plant materials were obtained from Dr Ole Højberg, Aarhus University, Faculty of Agricultural Sciences, Department of Animal Health, Welfare and Nutrition, Denmark (Table 3.1). The activity in the *in vivo* experiments of the different samples were also provided by Dr Hojberg (unpublished data)

Table 3.1: Plant samples used for this study, the country of origin, the plant parts received (marked with X) and activity levels of *in vivo* animal experiments

Code	Country	Year	Leaves	Fruits	Mass	Activity level*
GFL	Germany	2005/2006	X	X	500 g	Medium-high
PL	Poland	2008	X		15 g	Inactive
PF	Poland	2008		X	25 g	Very low
DL1	Denmark	2008	X		500 g	Medium-high
DF	Denmark	2008		X	500 g	Medium
DL2	Denmark	2008	X		500 g	Medium

GFL- Germany Fruit & Leaf, PL- Poland Leaf, PF- Poland Fruit, DL1- Denmark Leaf1, DF- Denmark Fruit and DL2- Denmark Leaf2. *Activity levels: High = 4; Medium = 3; Low = 2; Very-low =1.

3.2.2 Extraction of plant material

In several publications the Phytomedicine Programme has found that acetone is the best overall extractant because it extracts compounds with a wide range of polarities (Eloff 1998a & Eloff et al., 2005). A standard procedure of extraction used in these publications was applied in the preparation of the plant extracts. Finely ground plant material (3 g) of the different samples was weighed into several 40 ml polyethylene centrifuge tubes. 30 ml of acetone were added into the tubes. The tubes were vigorously shaken by hand and then on an orbital shaker for at least 30 minutes. The tubes were centrifuged for 10 minutes at approximately 3000 rpm. The supernatant was filtered using premoistened Whatman No.1 filter paper. The extraction was repeated three times on the same sample. The filtrate was placed into pre-weighed glass containers. The solvent was allowed to evaporate under a stream of air in a fume hood at room temperature, leaving dry plant extract residues in the glass containers. The extract residues were weighed and re-dissolved in acetone to yield 10 mg/ml solutions for further analysis.

3.2.3 Solvent-solvent fractionation of extracts

The solvent-solvent fractionation procedure is known to simplify extracts by separating the components of the extracts based on their polarity. In this study the method developed by the US National Cancer Institute was used with slight modification to suite this study (Suffness & Douros, 1979). Finely ground plant material (200 g) from each region was extracted with 2000 ml of acetone; the mixture was shaken for a few hours and left over night. The resulting extract was filtered and dried in a rotary evaporator under reduced pressure. The procedure was repeated three times. The dried acetone extract was dissolved in a 1:1 mixture of chloroform and water in a separating funnel and collected. The resulting water fraction was mixed with an equal volume of butanol in a

separating funnel to yield the water and butanol fractions. The chloroform fraction was dried to remove all chloroform and equal volumes of hexane and 10% water/methanol mixture was placed into a separating funnel and collected yielding the hexane fraction. The 10% water/methanol mixture was then further diluted to 35% by adding water. An equal volume of chloroform was added and placed in a separatory funnel to yield the water/methanol fraction and the chloroform fraction. To investigate the chemical differences 10 mg of each extract was weighed into a vial and made up to a contraction of 10 mg/ml for each tree sample.

3.2.4 Analysis of plant extract for preliminary screening

3.2.4.1 Thin layer chromatography

Chemical constituents of the extracts were separated on aluminium-backed thin layer chromatography (TLC) plates (Merck, silica gel 60 F_{254}). A volume of 10 µg representing 100 µg of dry mass was chromatographed for each sample. The TLC plates were developed under saturated conditions with one of the three eluent systems developed in our laboratory, i.e., ethyl acetate/methanol/water (40:5.4:4): [EMW] (polar/neutral); chloroform/ethyl acetate/formic acid (5:4:1): [CEF] (intermediate polarity/acidic); benzene/ethanol/ammonia hydroxide (18:2:0.2): [BEA] (non-polar/basic) (Kotze and Eloff, 2002). Separated chemical compounds on the chromatograms were detected using acidified vanillin (0.1 g vanillin: 28 ml methanol: 1ml sulphuric acid) as a spray reagent. After spraying, the chromatograms were carefully heated at 105°C in an oven to allow for optimal colour development.

3.2.4.2 Determining the total phenol content

Antioxidant activity of a plant extract is often associated with the phenolic compounds present in the plant. The total phenol content of the plant was determined using a colorimetric assay based method that was first described by Singleton and Rossi in1965 with modifications to suit this particular study. In brief 500 µl of the crude acetone extracts (5 mg/ml) was mixed with 500 µl of Folin-Ciocalteu's reagent and allowed to stand for 5 minutes. 500 µl of sodium carbonate (20%) solution was added to each sample test tube followed by the addition of distilled water to make up a total volume of 5 ml in each test tube. The test tubes were incubated in the dark at room temperature for two hours. The absorbance was measured at 725 nm using plastic cuvettes. Gallic acid (5 mg/ml) was used as a standard and a calibration curve of gallic acid was constructed and the quantity of phenols present in each extract was expressed as a percentage of gallic acid equivalents of the extracts calculated from the regression equation of the calibration line ($y = 0.4265x$; $R^2 = 0.98$) which was linear from 0 to 10 mg/ml.

3.2.4.3 Antioxidant activity (Polyphenol quantification & Trolox assay)

Qualitative antioxidant screening to determine the number of antioxidant compounds present was employed using 2, 2-diphenyl-1-picryl hydrazyl (DPPH) (Takao *et al*., 1994). TLC plates loaded with 100 µg of each extract were developed in the solvent systems mentioned above and sprayed with 0.2% DPPH in methanol. Compounds separated by TLC with antioxidant activity were visualized as yellow bands against a purple background (Bors *et al*., 1992). Quantification of antioxidant activity was by spectrophotometric means using two radicals, ABTS and DPPH.

In the ABTS method, the Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchromane-2-carboxylic acid) equivalent antioxidant capacity (TEAC) assay (Re *et al*., 1999) was determined. This was based on the scavenging of the ABTS radical into a colourless product by antioxidant substances. The blue/green chromophore $ABTS^{+}$ was produced through the reaction between ABTS and potassium persulfate. The absorbance was read at 734 nm using an Epoch Biotek microplate reader. Trolox is a Vitamin-E analogue and was used as a standard in this assay. Percentage change in absorbency was calculated by using the formula:

[(Initial absorbency of $ABTS^{+}$ - New Initial absorbency of $ABTS^{+}$)/Initial absorbency of $ABTS^{+}$)]× 100

The curves were plotted with the dependent variable being the percentage change in absorbency and the independent variable being the different concentrations at which test substances were analysed. Mathematical comparison of antioxidant activity of different plant extracts was done by dividing the slope obtained for extract to that of Trolox to get the Trolox equivalent antioxidant capacity (TEAC). An extract with a TEAC value of 1 indicates an antioxidant equivalent to that of Trolox. Decrease or increase of antioxidant activity is depicted by a lower or higher value of TEAC, respectively.

3.2.4.4 Scavenging effect assay

The scavenging effect of DPPH free radical was determined using a modified method of Hatano et al (1988). In this assay 50 µl of various concentrations (0.02 mg/ml to 2.5 mg/ml) of the sample extracts were mixed with 200 µl of methanolic solution containing DPPH radicals (5 mg/ml). The mixture was shaken and left to stand in the dark for stable absorption. The reduction of DPPH radical was measured using a microplate reader (Epoch, Biotek) and reading the absorption at 517 nm after 5 minutes.

The scavenging effect was calculated as percentage of the DPPH discolouration using the equation:

% scavenging effect = [(ADPPH – AS)/ (ADPPH)] x 100

AS is the absorbance of the sample extract when incubated with DPPH and ADPPH is the absorbance of the DPPH solution. The standard reference compounds used were BHA and Ŭ-tocopherol.

3.3 Results

3.3.1 Extraction

3.3.1.1 Acetone extraction and solvent-solvent fractionation

The highest yield of extracts following the initial acetone extraction procedure after the solvent-solvent fractionation was obtained in the hexane fraction (300 mg/g) from samples of trees growing in Poland and Germany followed by chloroform and hexane fractions of samples from trees growing in Denmark (220 mg/g and 200 mg/g). The quantity of material extracted (Table 3.2) and the percentage extracted from *Pterocarya fraxinifolia* trees growing in six different regions differed substantially (Fig. 3.1). The hexane fraction yielded the highest (1310 mg/g) overall mass extracted in the solvent-solvent derived fractions (Fig. 3.1).

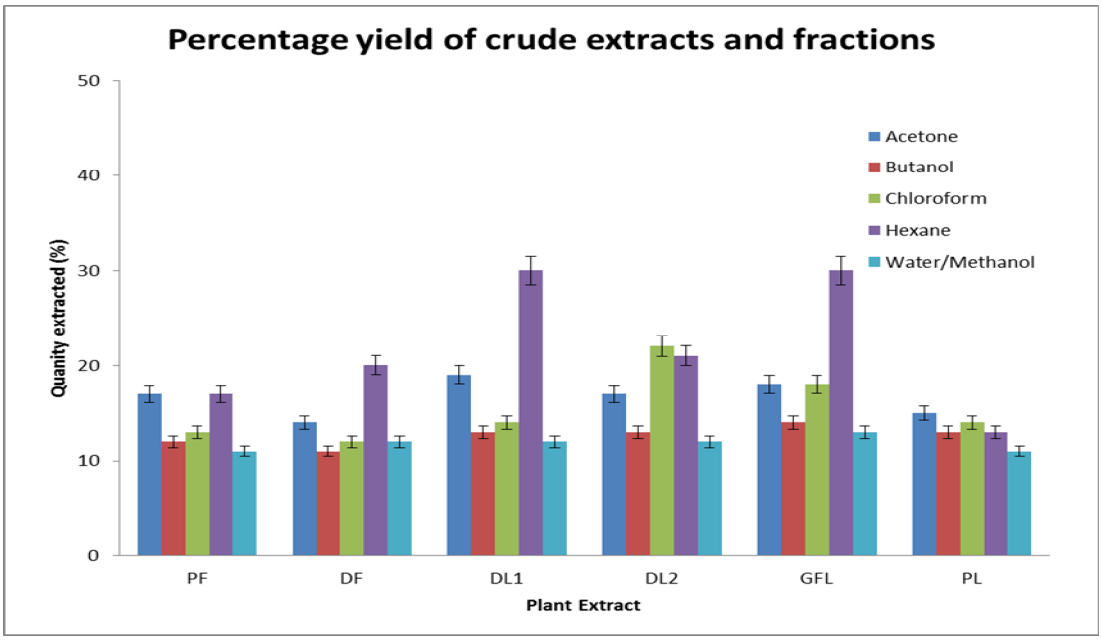


Figure 3.1: Quantity extracted of each plant material for the acetone extracts and the solvent-solvent derived fractions (PF-Fruit extract-Poland**;** DF-Fruit extract-Denmark**;** DL1-Leaf extract-Denmark; DL2- Leaf extract -Denmark**;** GFL-Leaf+fruit extracts-Germany**;** and PL-Leaf extract-Poland).

Table 3.2: Quantity of plant material in mg/g present in acetone extract and the different solvent-solvent derived fractions obtained for each plants sample from trees growing in Denmark, Germany and Poland. (PF-Fruit extract-Poland**;** DF-Fruit extract-Denmark**;** DL1-Leaf extract-Denmark; DL2- Leaf extract -Denmark**;** GFL-Leaf+fruit extracts-Germany**;** and PL-Leaf extract-Poland).

Plant ID	Crude (mg/ml)	Fractions (mg/ml)			
	Acetone	Butanol	Chloroform	Hexane	Water/Methanol
PF	170	120	130	170	110
DF	140	110	120	200	120
DL1	190	130	140	300	120
DL2	170	130	220	210	120
GFL	180	140	180	300	130
PL	150	130	140	130	110

3.3.2 TLC analysis of the plant extracts

Crude extracts and solvent-solvent derived fractions of *Pterocarya fraxinifolia* from trees growing in the six different areas were analysed by TLC and sprayed with vanillin sulphuric acid spray reagent. The chemical profile of the crude extract and solvent-solvent derived fractions is indicated in figure 3.2. The chromatogram indicate that most of the compounds present in the acetone crude extracts and the solvent-solvent fractions varied, from very polar, to non-polar, to intermediate polarity. The fraction depicting the most compounds was the chloroform fractions from trees growing in Denmark and Germany. They indicated an array of compounds ranging from very polar to non-polar compounds (Fig. 3.2). The crude acetone extracts of trees growing in Denmark, Germany and Poland contained more polar to intermediate-polar compounds. The hexane fraction of trees growing in Denmark, Germany and Poland contained a lot of acidic compounds, very few compounds were observed in the butanol and water-methanol fractions from trees growing in the three countries (Fig. 3.2).

3.3.3 Total phenol content

The total phenolic content of *P. fraxinifolia* acetone extracts from the different areas of Europe was different from one area to the other. The highest phenol content was observed in the leaf extracts of trees growing in Denmark. Both leaf samples from trees growing in Denmark indicated high levels of phenols. The sample from trees growing in Germany (mixture of leaves and fruit) had high levels of phenols. The rest of the samples from trees growing in the other areas had relatively low levels of phenols (Table 3.3).

3.3.4 Number of antioxidant compounds (DPPH qualitative assay)

The qualitative antioxidant activity is depicted in the chromatograms in figure 3.3 below. The acetone crude extracts from trees growing in Denmark and Germany had the highest number of separated antioxidant compounds. The chloroform and water-methanol solvent-solvent derived fractions had very few antioxidant compounds. It was not unexpected that the hexane fractions contained no antioxidant compounds separated by the TLC systems used because antioxidant activity is usually associated with polar compounds. The purple coloured compounds on the TLC chromatograms visualized with vanillin sulphuric acid had moderate antioxidant activity when R_f value were compared with the DPPH TLC plates. The blue compound on the TLC chromatograms had poor antioxidant activity as seen by the low intensity of the yellow colour on the DPPH TLC plates (figure 3.1(iii) and 3.3(v)). The TLC and DPPH chromatograms show the presence of antioxidant compounds with varying degrees of activity as seen by the intensity of the yellow colour on the DPPH chromatograms.

3.3.5 The Trolox Equivalent Antioxidant Concentration (TEAC quantitative assay) and phenolic content

In the TEAC antioxidant assay (Table 3.3), extracts of *Pterocarya fraxinifolia* from trees growing in Denmark and Germany had generally moderate free radical scavenging activity when compared with extracts from trees growing in Poland. The quantitative TEAC assay indicated moderate EC_{50} values for the plant samples collected from trees growing in Poland (EC_{50} of 3.39 ± 0.16 mg/ml) compared to the standard, L-ascorbic acid (EC_{50} = 1.59 ± 0.80 mg/ml) which denotes the highest antioxidant activity. The lower the EC_{50} of a substance the more effective its free radical scavenging effect. *Pterocarya fraxinifolia* extracts from trees growing in different areas from Denmark, Germany and Poland had moderate antioxidant free radical scavenging activity.

Table 3.3: The Trolox Equivalent Antioxidant Concentration (TEAC) and phenolic content of *Pterocarya fraxinifolia* plant materials from Denmark, Germany and Poland (PF-Fruit extract-Poland; DF-Fruit extract-Denmark; DL1-Leaf extract-Denmark; DL2- Leaf extract -Denmark; GFL-Leaf+fruit extracts-Germany; and PL-Leaf extract-Poland).

Plant	**Voucher specimen**	**Extract yield**	**phenol**	**Antioxidant values**	
	number	**(%)**	**Quantity (%)**	**TEAC**	**EC_{50} ± SEM, mg/ml**
PF	DO53R	17	10	0.018	3.39 ± 0.16
GFL	DO21R	18	59	0.032	0.26 ± 0.09
DF	DO56R	14	30	0.018	0.32 ± 0.12
DL2	DO58R	17	60	0.03	0.22 ± 0.08
PL	DO52R	14	24	0.021	1.02 ± 0.13
DL1	DO54R	19	55	0.027	0.14 ± 0.06
L-ascorbic acid		N/A*	N/A*	N/A*	1.59 ± 0.08

*N/A = Not available

3.3.6 DPPH Free Radical Scavenging Effect

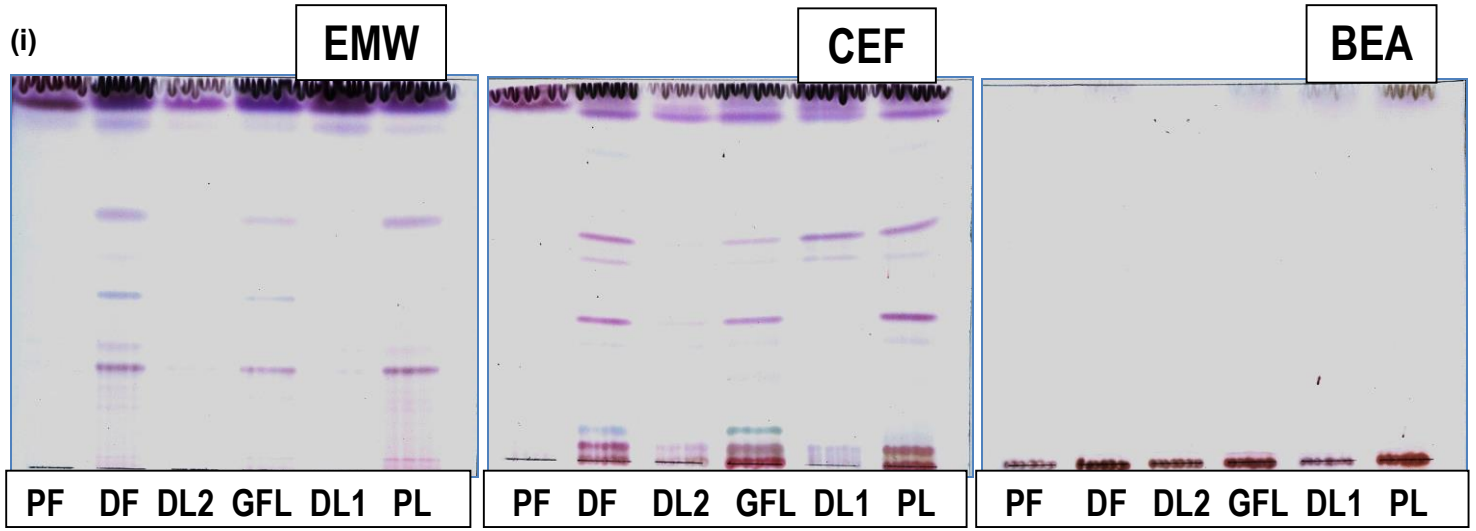
In this assay the results are expressed as the percentage ratio of the absorbance in the decrease of the DPPH radical in solution in the presence of the extract. The scavenging effect on DPPH is indicated by a concentration-dependent activity (Fig. 3.4, 3.5 & 3.6). The crude acetone extracts and solvent-solvent derived fractions of the plant samples from trees growing in Germany and Denmark all had very high scavenging activity even at a low concentration of 0.02 mg/ml (Figs. 3.4, 3.5 & 3.6).

At the highest concentration assayed, the scavenging effect was also high for samples from trees growing in the other areas. The plant samples from trees growing in Denmark and Germany had scavenging effects ranging from 20% at 0.02 mg/ml to 80% at the highest concentration assayed (2.5 mg/ml). When 3 ml of each plant extract from trees growing in the different areas were combined the scavenging effect increased from 0.08% at 0.02 mg/ml to 73.4% at 2.5 mg/ml and the DPPH was reduced to a yellow colour in all the wells. Comparing the results to that of the standard reference compounds used (BHA: 96% at 3 mg/ml and Ŭ-tocopherol: 93% at 7.5 mg/ml) the scavenging activity of samples of *P. fraxinifolia* trees growing in the different areas had a higher DPPH free radical scavenging activity.

The correlation between antioxidant activity and *in vivo* test results show that extracts from Poland have similar activity *in vitro* and both extracts had good antioxidant activity and low activity *in vivo*. The correlation between the extracts from Denmark show that the leaf extracts have better activity compared to the fruit extracts of *P. fraxinifolia* trees from the same country (Figure 3.7). The leaf

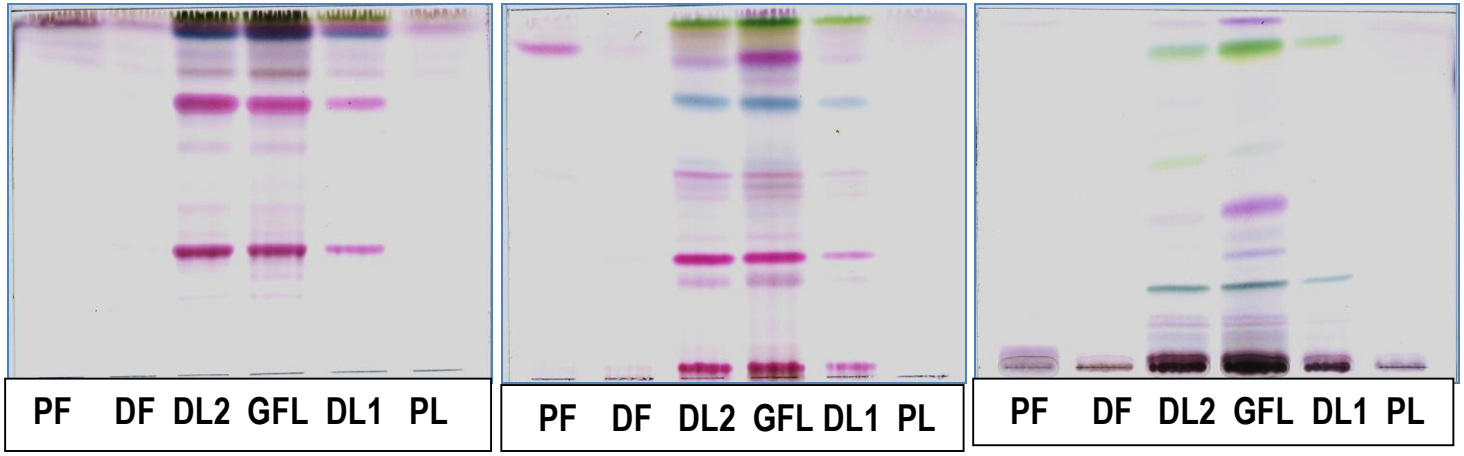
extracts (DL1 and DL2) had good antioxidant activity but, the second extract had better activity when compared with the other two extracts from Denmark, even though the fruit extract had better phenol content by percentage, than the both leaf extracts, it had poor relative activity *in vivo*.

(i)



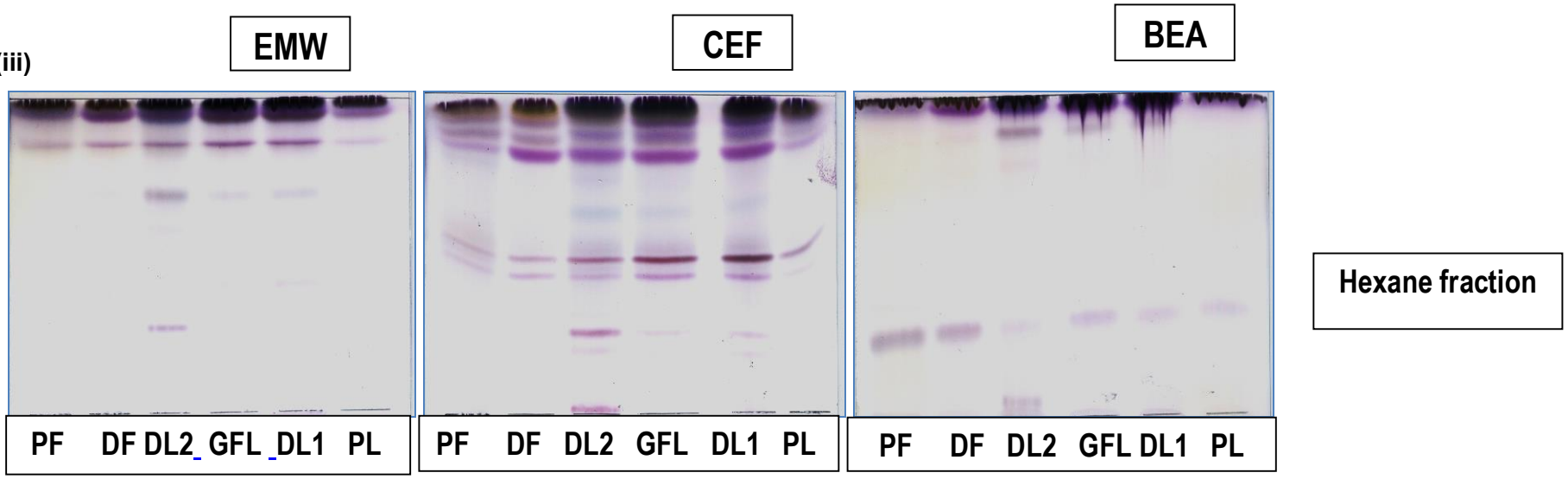
Crude Acetone extract

(ii)



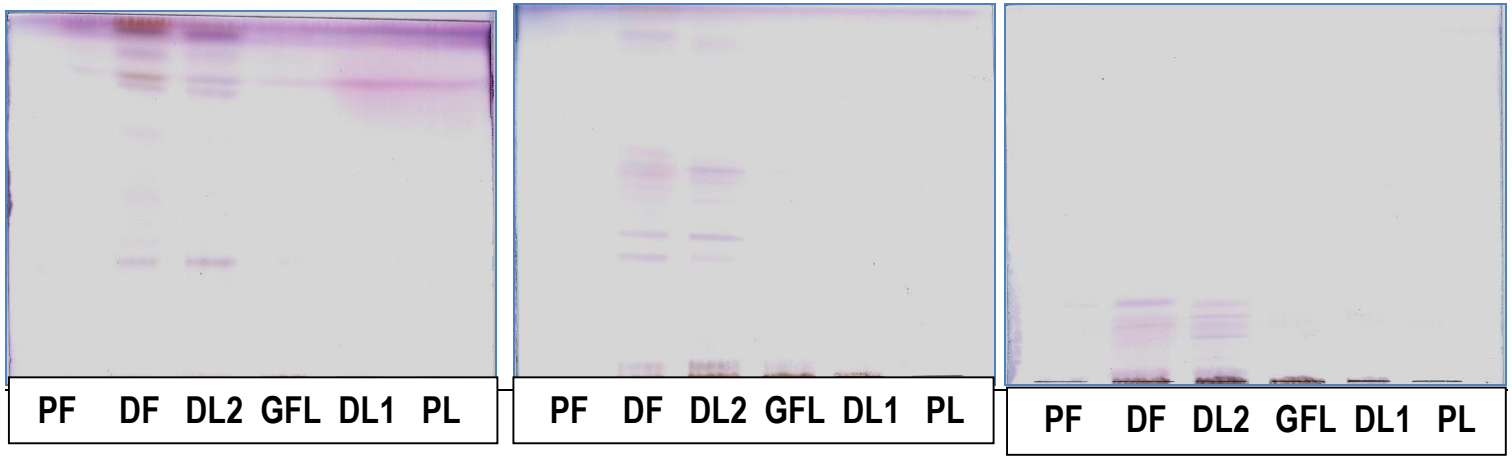
Chloroform fraction

(iii)



Hexane fraction

(iv)



Water-Methanol fraction

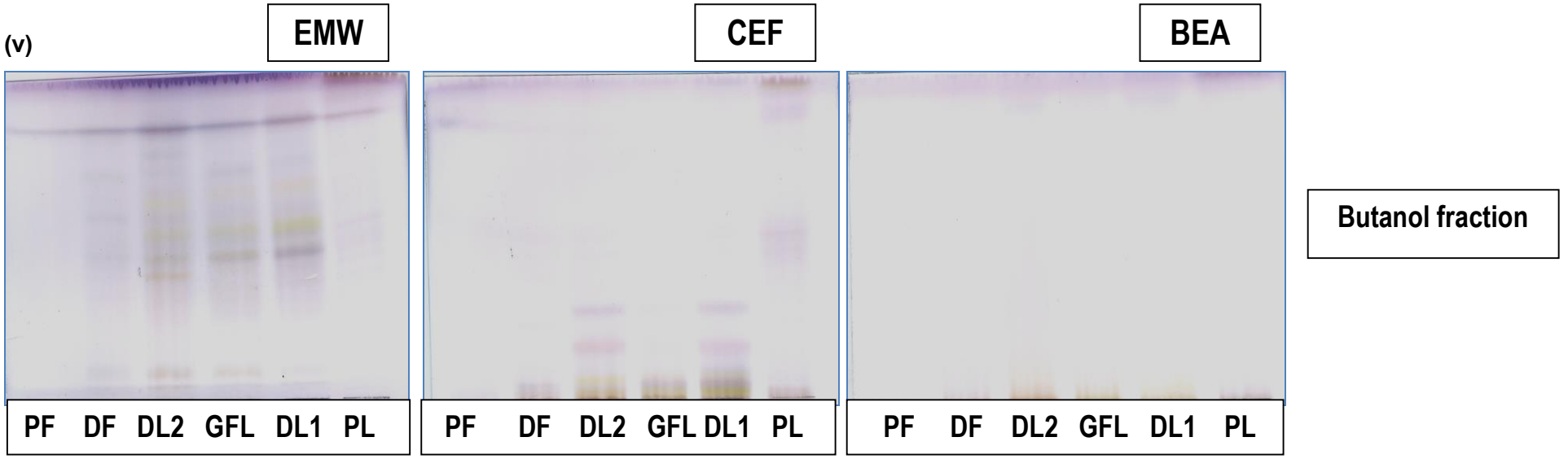
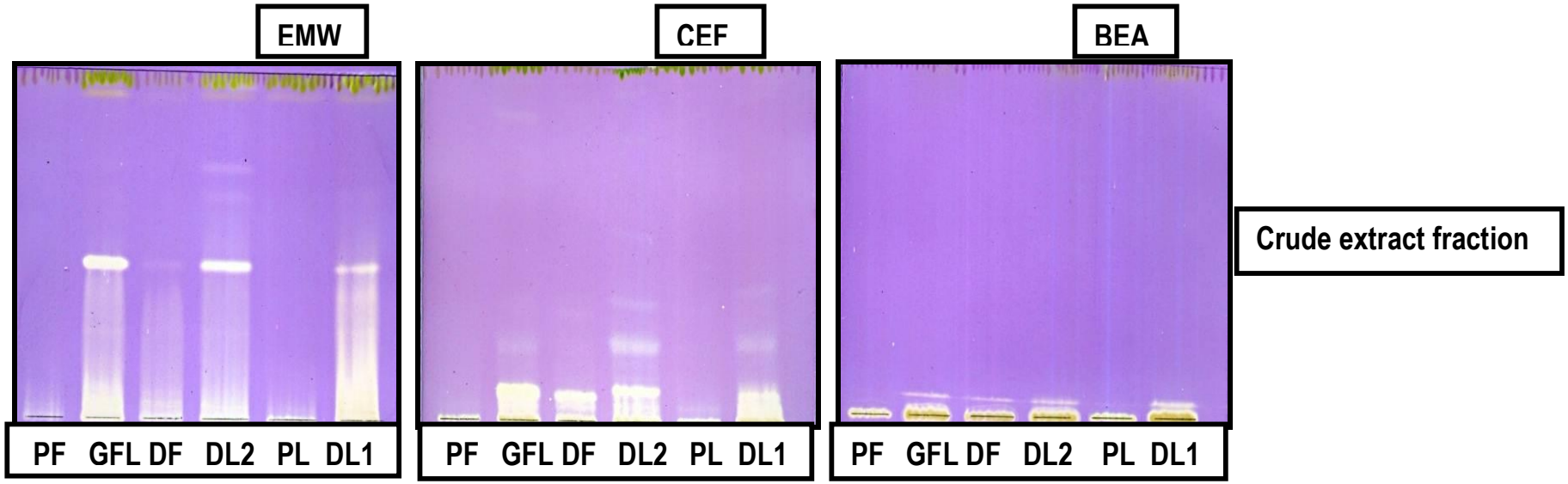
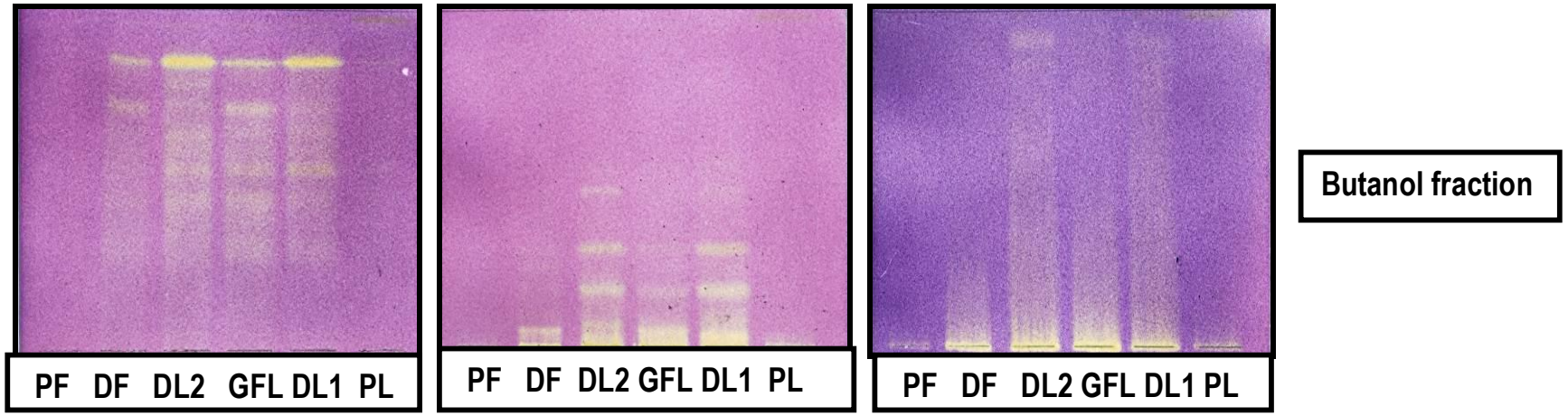


Figure 3.2: TLC chromatograms of *Pterocarya fraxinifolia* crude acetone extracts (i) and solvent-solvent derived fractions {(ii) chloroform, (iii) hexane, (iv) water/methanol and (v) butanol fractions} from trees growing in Denmark, Germany and Poland, developed using three mobile phases: ethyl acetate, methanol and water (EMW-left-40:5.4:4) chloroform, ethyl acetate, formic acid (CEF-middle-5:4:1) and butanol, ethanol, ammonia (BEA-right-18:2:0.2) viewed after spraying with vanillin sulphuric acid spray reagent. (PF-Fruit extract-Poland; DF-Fruit extract-Denmark; DL1-Leaf extract-Denmark; DL2- Leaf extract -Denmark; GFL-Leaf+fruit extracts-Germany; and PL-Leaf extract-Poland).

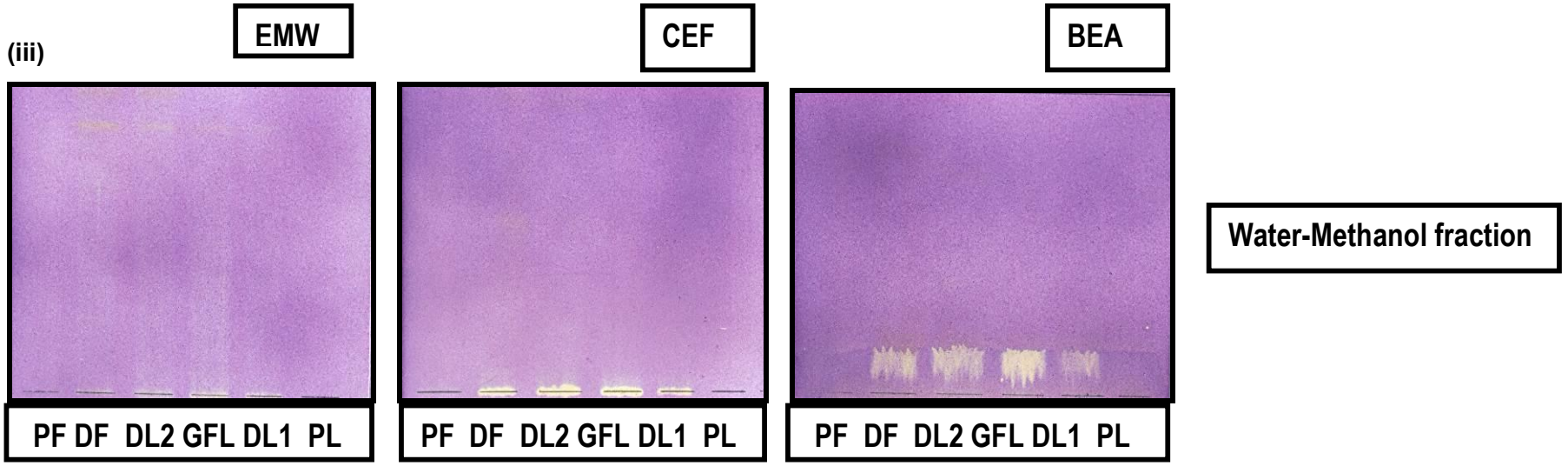
(i)



(ii)

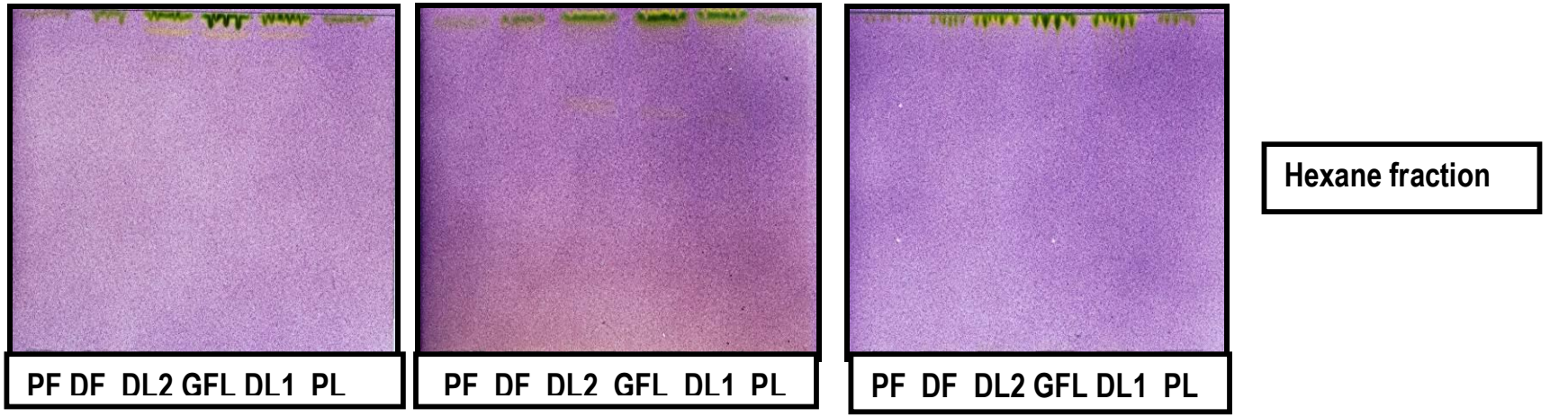


(iii)



Water-Methanol fraction

(iv)



Hexane fraction

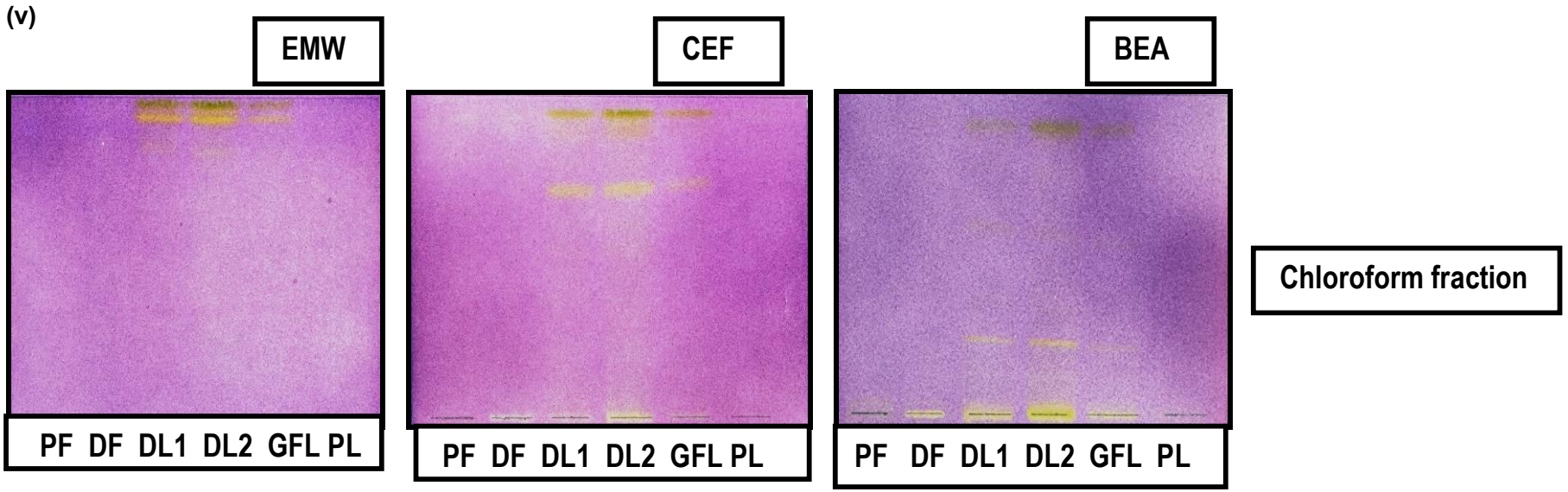


Figure 3.3: TLC chromatograms of *Pterocarya fraxinifolia* crude acetone extracts (i) and solvent-solvent fractions {(ii) Butanol, (iii) Water/methanol, (iv) hexane and (v) chloroform from trees growing in different areas developed using three mobile phases: ethyl acetate, methanol and water (EMW-left-40:5.4:4) chloroform, ethyl acetate, formic acid (CEF-middle-5:4:1) and butanol, ethanol, ammonia (BEA-right-18:2:0.2) viewed after spraying the DPPH spray reagent (PF-Fruit extract-Poland; DF-Fruit extract-Denmark; DL1-Leaf extract-Denmark; DL2- Leaf extract -Denmark; GFL-Leaf+fruit extracts-Germany; and PL-Leaf extract-Poland).

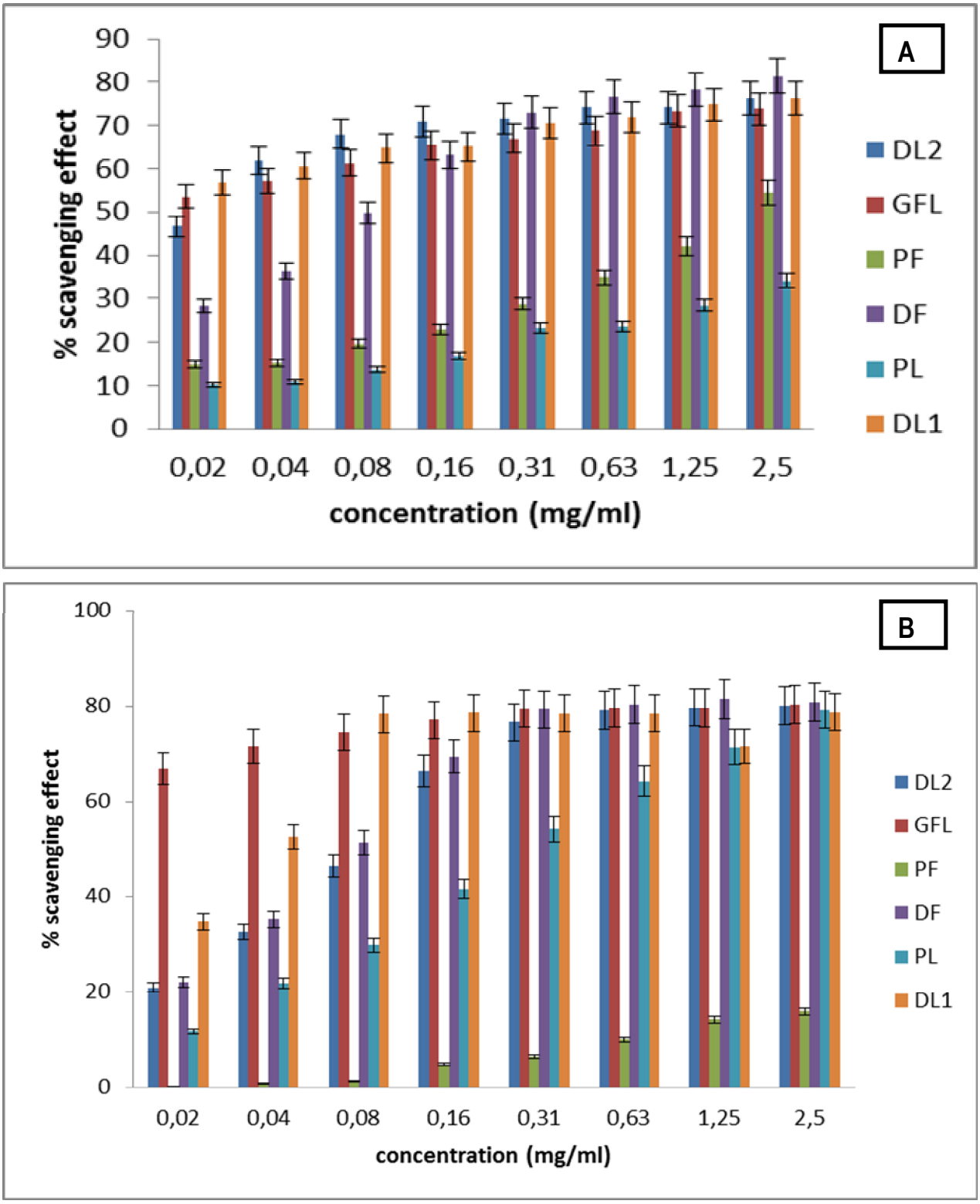
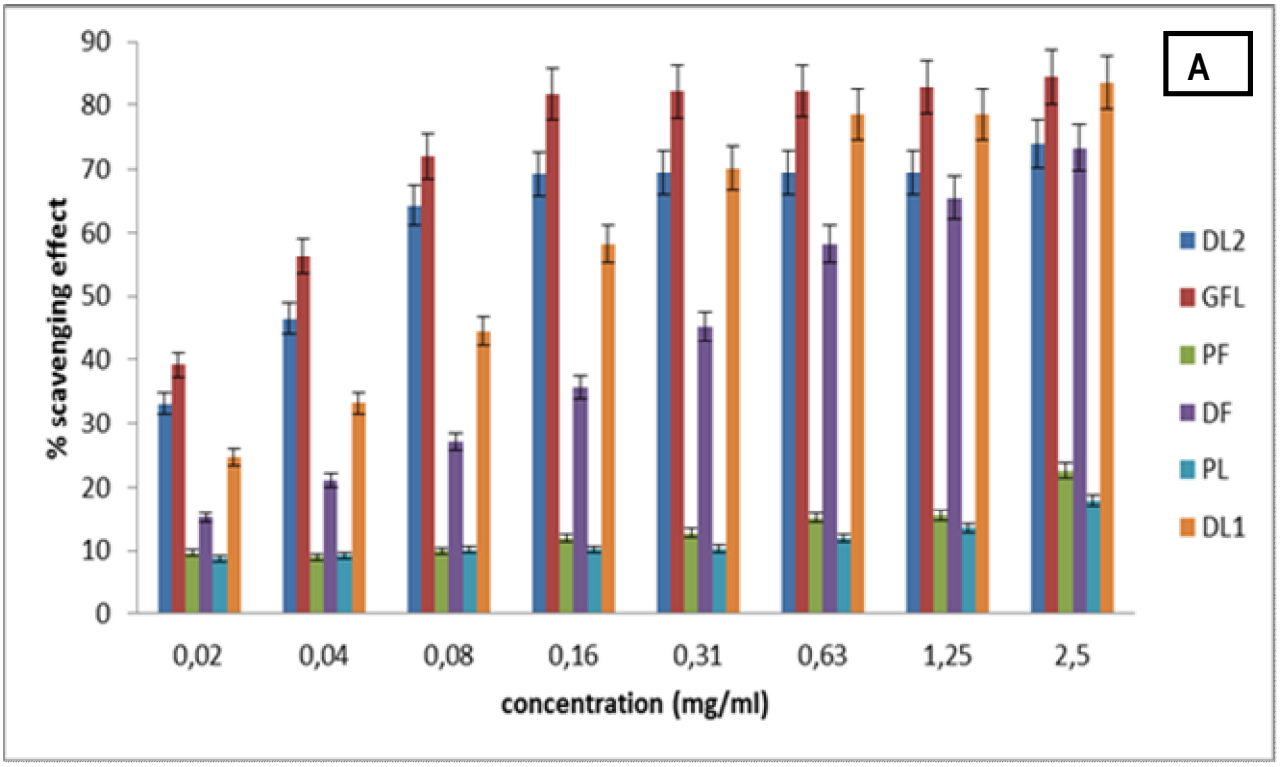


Figure 3.4: Radical scavenging effects of *P. fraxinifolia* crude acetone extract (A) and solvent-solvent derived butanol fraction (B) of trees growing in different areas in Poland, Germany and Denmark (PF-Fruit extract-Poland; DF-Fruit extract-Denmark; DL1-Leaf extract-Denmark; DL2- Leaf extract -Denmark; GFL-Leaf+fruit extracts-Germany; and PL-Leaf extract-Poland).



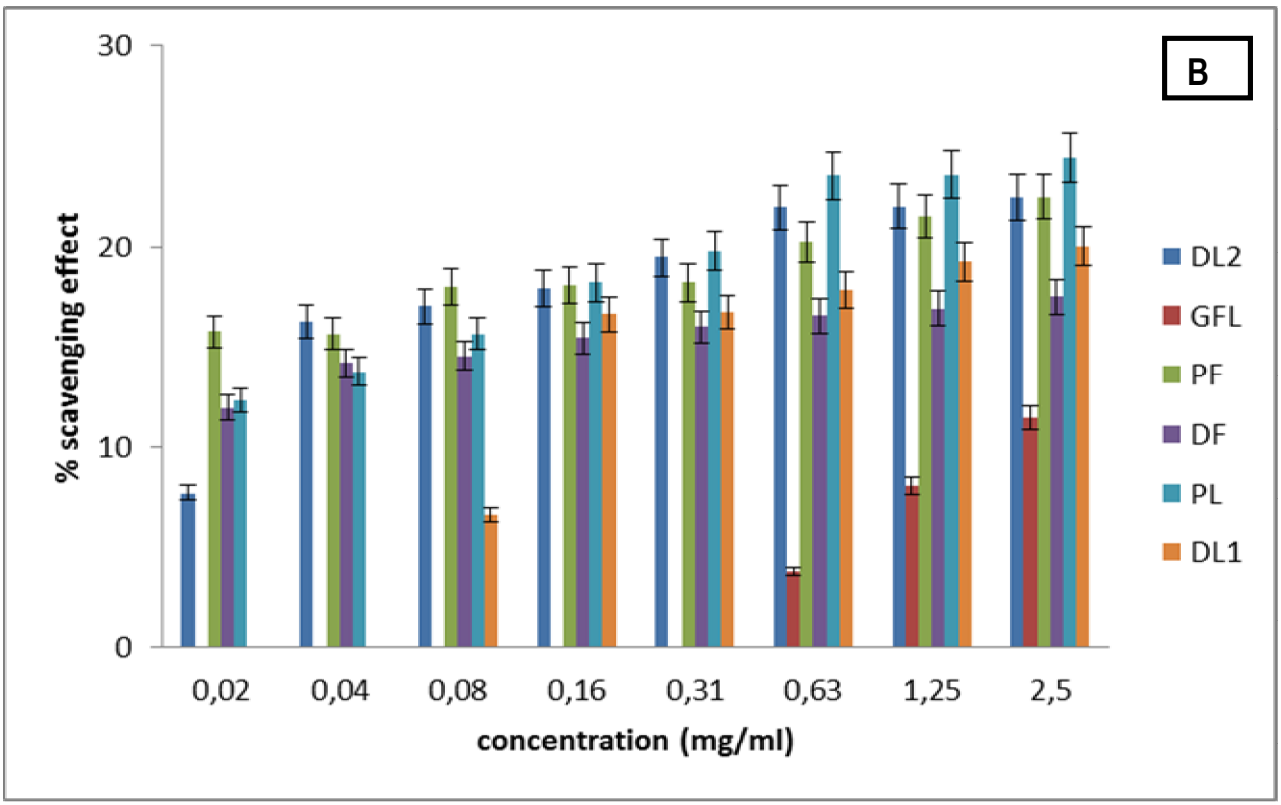


Figure 3.5: Radical scavenging effects of solvent-solvent derived water-methanol fraction (A) and hexane fraction (B) of *P. fraxinifolia* trees growing in different areas in Denmark, Germany and Poland. (PF-Fruit extract-Poland; DF-Fruit extract-Denmark; DL1-Leaf extract-Denmark; DL2- Leaf extract -Denmark; GFL-Leaf+fruit extracts-Germany; and PL-Leaf extract-Poland).

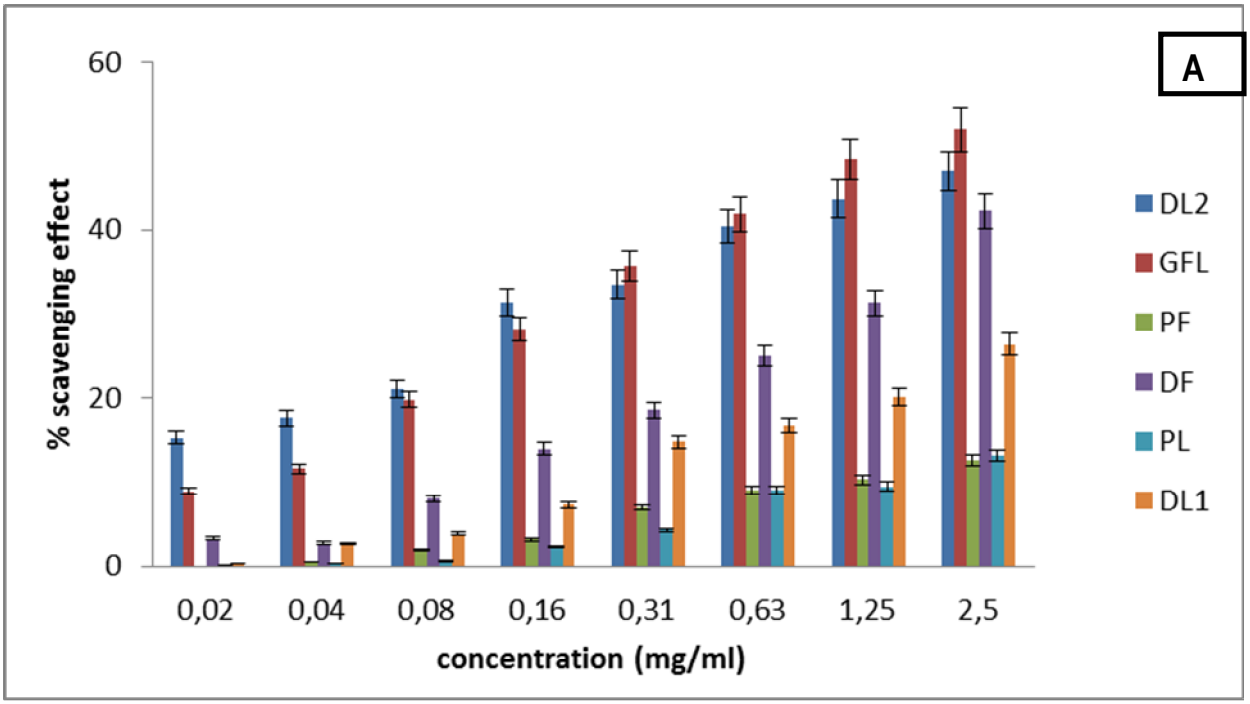
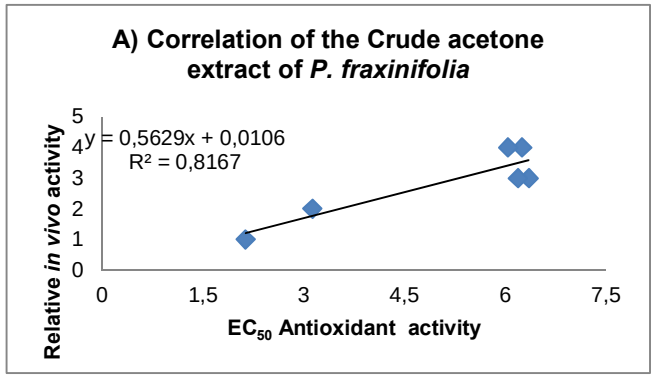
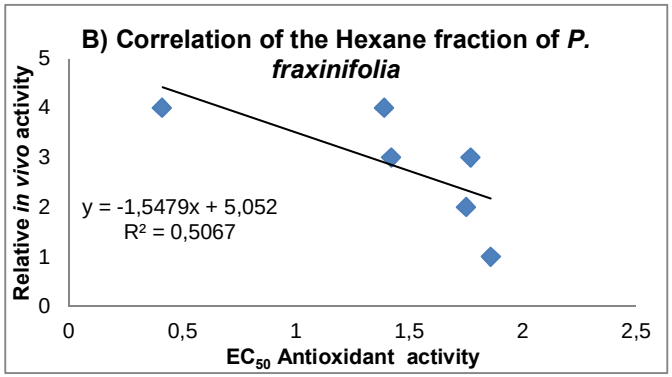
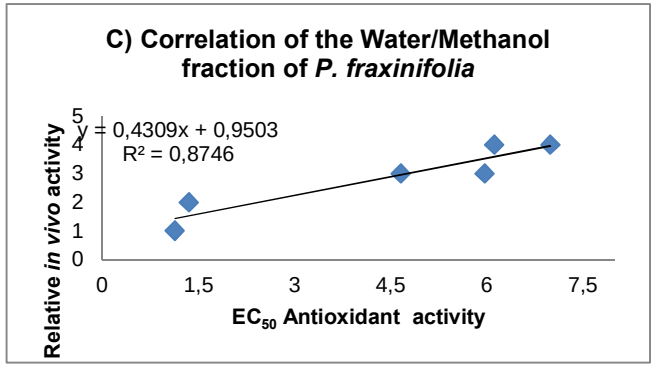
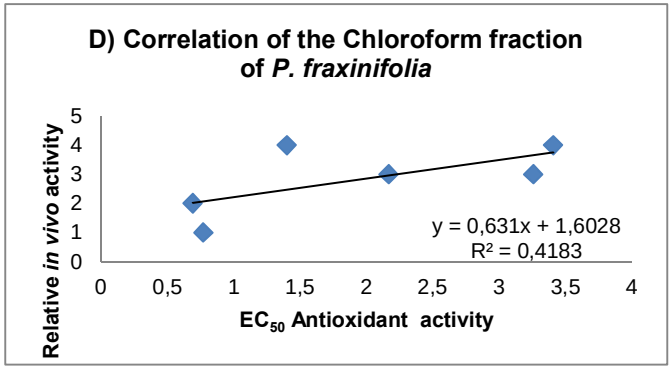


Figure 3.6: Radical scavenging activity of solvent-solvent derived chloroform fraction (A) of *P. fraxinifolia* trees growing in different areas in Denmark, Germany and Poland. (PF-Fruit extract-Poland; DF-Fruit extract-Denmark; DL1-Leaf extract-Denmark; DL2- Leaf extract -Denmark; GFL-Leaf+fruit extracts-Germany; and PL-Leaf extract-Poland).









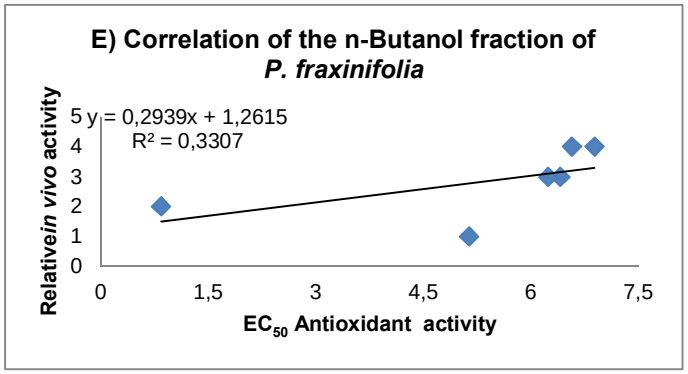


Figure 3.7: Correlation between relative *in vivo* activity and EC_{50} values of antioxidant activity of A) Crude acetone extract, B) Hexane fraction; C) Water/methanol fraction; D) Chloroform fraction; E) n-Butanol fraction of *P. fraxinifolia* from trees growing in different areas in Europe (Relative *in vivo* Activity Levels: High = 4; Medium = 3; Low = 2; Very low = 1).

Table 3.4: The EC_{50} (µg/ml) values of the Free Radical Scavenging activity of *P. fraxinifolia* samples from different regions of Europe (PF-Fruit extract-Poland; DF-Fruit extract-Denmark; DL1-Leaf extract-Denmark; DL2- Leaf extract -Denmark; GFL-Leaf+fruit extracts-Germany; and PL-Leaf extract-Poland).

Samples	**DL 1**	**DL2**	**GFL**	**PF**	**DF**	**PL**
Crude	6.25 ± 0.90	6.35 ± 0.11	6.04 ± 0.84	3.13 ± 0.91	6.19 ± 0.57	2.13 ± 0.91
Hexane fraction	1.39 ± 0.83	1.77 ± 0.25	0.41 ± 0.53	1.75 ± 0.74	1.42 ± 0.36	1.86 ± 0.19
Water/Methanol Fraction	6.12 ± 0.79	5.97 ± 1.08	6.99 ± 1.17	1.35 ± 0.91	4.66 ± 0.98	1.13 ± 0.37
Chloroform fraction	1.40 ±0 0.88	3.26 ± 0.84	3.41 ± 0.98	0.69 ± 0.87	2.17 ± 0.89	0.77 ± 0.63
Butanol fraction	6.57 ± 1.41	6.24 ± 0.71	6.89 ± 0.60	0.84 ± 0.83	6.41 ± 0.62	5.14 ± 0.99

3.4 Discussion and conclusion

Acetone is known and proven to extract a broader range of compounds than other common extractants (Eloff, 1998a) and that was the reason the initial extraction was done using acetone prior to the solvent-solvent fractionation. It should be kept in mind that intact plant material was fed to the animals and the compounds solubilised in the animal gut does not necessarily equate to that of an extract. It is therefore important not to use a selective extractant. The compounds in the crude acetone extracts did not move far from the origin in the TLC chromatograms indicating a relatively high polarity.

The compounds separated by solvent-solvent fractionation had a variety of polarities, from highly polar to non-polar compounds as indicated on the chromatograms developed under the three solvent systems used. The water-methanol and butanol fractions had the lowest diversity of compounds. The leaf samples had more compounds than the fruit samples of *P. fraxinifolia* from trees growing in the different areas in Denmark, Germany and Poland. The extracts from trees growing in Germany contained a mixture of compounds that were similar to the extracts from trees growing in Poland and Denmark.

The compounds present in *P. fraxinifolia* trees growing in different areas in Denmark, Poland and Germany had some free radical scavenging activity in two separate antioxidant assays. DPPH is a free radical that is very stable at room temperature and has a violet colour. It is reduced to a light yellow compound in the presence of oxidant molecules and used as an easy and rapid method for evaluating antioxidants. The crude acetone extracts had good antioxidant activity for the leaf samples from trees growing in Denmark and Poland.

The solvent-solvent derived fractions of both the leaf and fruit samples from trees growing in Denmark, Poland and Germany had equally good scavenging activity. The lowest activity was observed in the solvent-solvent derived chloroform, hexane and water-methanol fractions for trees growing in all three countries. This is not surprising because the more polar extracts should have the highest antioxidant activity. The presence of compounds that did not move far from the origin may indicate the presence of polyphenolics due to their high polarity and tight binding to normal phase silica (Davidson, 1964). . 2000).

It has been suggested and well established that more than one method should be used to determine antioxidant activity to obtained detailed information and knowledge of the activity of the test substance, below I discuss the two antioxidant assays used in this study. The TEAC assay despite its limitation is one of the most used methods in screening compounds, extracts and food

products for antioxidant activity (van der Berg *et al*, 1999). The TEAC antioxidant activity was carried out using the ABTS assay which involves the direct production of the blue/green ABTS chromophore through the reaction between potassium sulphate and ABTS (Miller *et al,* 1993). The ABTS radical cation has been used to screen the relative radical-scavenging abilities of flavonoids and phenols. Trolox and L-ascorbic acid were used to quantify the antioxidant activity (Re *et al,* 1999; Fukomoto & Mazza, 2000).

The antioxidant activity based on EC_{50} values of the extracts were good when compared with that of the standard used with the exception of the fruit sample from trees growing in Poland. TEAC values greater than one indicate higher antioxidant activity than trolox the positive control. The extracts tested from trees growing in Denmark, Poland and Germany had TEAC values of less than one as which is usually found for plant extracts.

DPPH has been used to determine the free radical scavenging activity of various plant extracts and pure compounds. The antioxidant molecules scavenge the free radical through hydrogen donation changing the colour of the solution to light yellow. This phenomenon was observed in the plant sample of *P. fraxinifolia* trees growing in different areas when all the crude extracts from each region/country was combined and assayed.

The results suggest that acetone crude extracts and solvent-solvent derived n-butanol fraction of *P. fraxinifolia* have moderate to high antioxidant activity and that activity may be in part attributed to the presence of flavonoid compounds in the plant samples rather than phenols. This will also lend credence to the fact that the conditions in which the trees grow in different areas where the samples were collected aid the plant in the production of flavonoids rather than phenols. This is not an uncommon phenomenon in plants where the secondary metabolites produced in the plant can be affected by differing growth conditions in the areas the plant is found.

With this in mind a correlation was done using activity levels determined from *in vivo* results and antioxidant activity. The crude extracts from trees growing in different areas in Denmark, Germany and Poland scavenge free radicals in the DPPH assay to a moderate degree. All samples with medium-high relative activity levels *in vivo*, had good (medium-high) activity *in vitro* and the inactive sample from trees growing in Poland with very low relative activity of 1,had good antioxidant activity *in vitro*. Souri *et al* (2004) conducted antioxidant studies on sixty plants from Iran with *P. fraxinifolia* as one of the plants and he indicated that this plant had good dose related antioxidant activity. . From the overall results of this study *P. fraxinifolia* appears to be a good potential source of antioxidants.

The most important result found in this section is that there was an excellent correlation (R^2 of 0.8137-Figure 3) between the *in vivo* activity found when plant material was introduced into the feed of animals and the antioxidant activity of the crude acetone extract. These results indicate that antioxidant activity may be a very important factor in determining the *in vivo* activity of the plants. This not only presents an explanation for the mechanism of activity, but could also be a very useful parameter to select tree populations to be used as potential growth feed additives

Post script

In addition to the antioxidant activity, inflammation may also play a role in animal productivity if infections or other stress conditions cause inflammation. In the next chapter this will be discussed.

Chapter 4

Anti-inflammatory activity and cytotoxicity of crude extract and two fractions of *P. fraxinifolia*

Preface

There were substantial differences in the chemical composition, antioxidant and anti-inflammatory activity between extracts of trees grown in different areas. In this chapter the anti-inflammatory activity of extracts of these different plants will be investigated. Safety of plant extracts are also very important if these are to be used as feed additives. Consequently the cytotoxicity of extracts of the different plants was also determined.

4.1 Introduction

Inflammation is a pathophysiological response of living tissues to external or internal damage and leads to local or systemic accumulation of plasmatic fluid and blood cells (Sosa, *et al*, 2002). In the bodyÕs defence to inflammation there are several complex events and mediators that are involved in the inflammatory reaction that can induce, maintain and in certain cases may aggravate diseases (Sosa, *et al*, 2002). The symptoms of inflammation are generally characterised by pain followed by swelling, heat, reddening of the inflamed area and loss of function that results in the dilation of blood vessels leading to increased blood flow to the afflicted areas (Iwalewa, *et al*, 2007). The increase in blood flow causes the accumulation and movement of leukocytes, proteins and fluids into the inflamed regions (Iwalewa, *et al*, 2007).

There are several chemical mediators that are formed and produced in response to the inflammatory process. These chemical mediators are substances that are released as plasma proteins triggered by the allergic or chemical irritation, external or internal injuries or infections. The severity of the inflammatory activity is dependent on and determined by the duration of the injury. Chemical mediators produced during the inflammation process bind to specific target receptors on the cells. This binding may lead to an increase in the vascular permeability, promotion of neutrophil chemotaxis and stimulate the smooth muscle contractions. There is also a direct increase in enzymatic activity pain and oxidative damage (Coleman, 2002).

The inflammatory process is complex (Fig. 4.1) and is mainly divided into two categories or parts, the acute inflammation, which is characterised by the rapid onset and occurs for a shorter period of time compared to the second and longer lasting chronic inflammation (Dalgleish & OÕByrne, 2001). The acute phase produces fluids, plasma proteins and the migration of leukocytes. This response is believed to be the bodyÕs mechanism of defence targeting bacterial and viral infections. Chronic

inflammation on the other hand is manifested histologically by the secretion of lymphocytes and macrophages which leads to fibrosis and tissue necrosis of the inflamed area. Persistent chronic inflammation leads to the development of degenerative diseases (Dalgleish & O'Byrne, 2001).

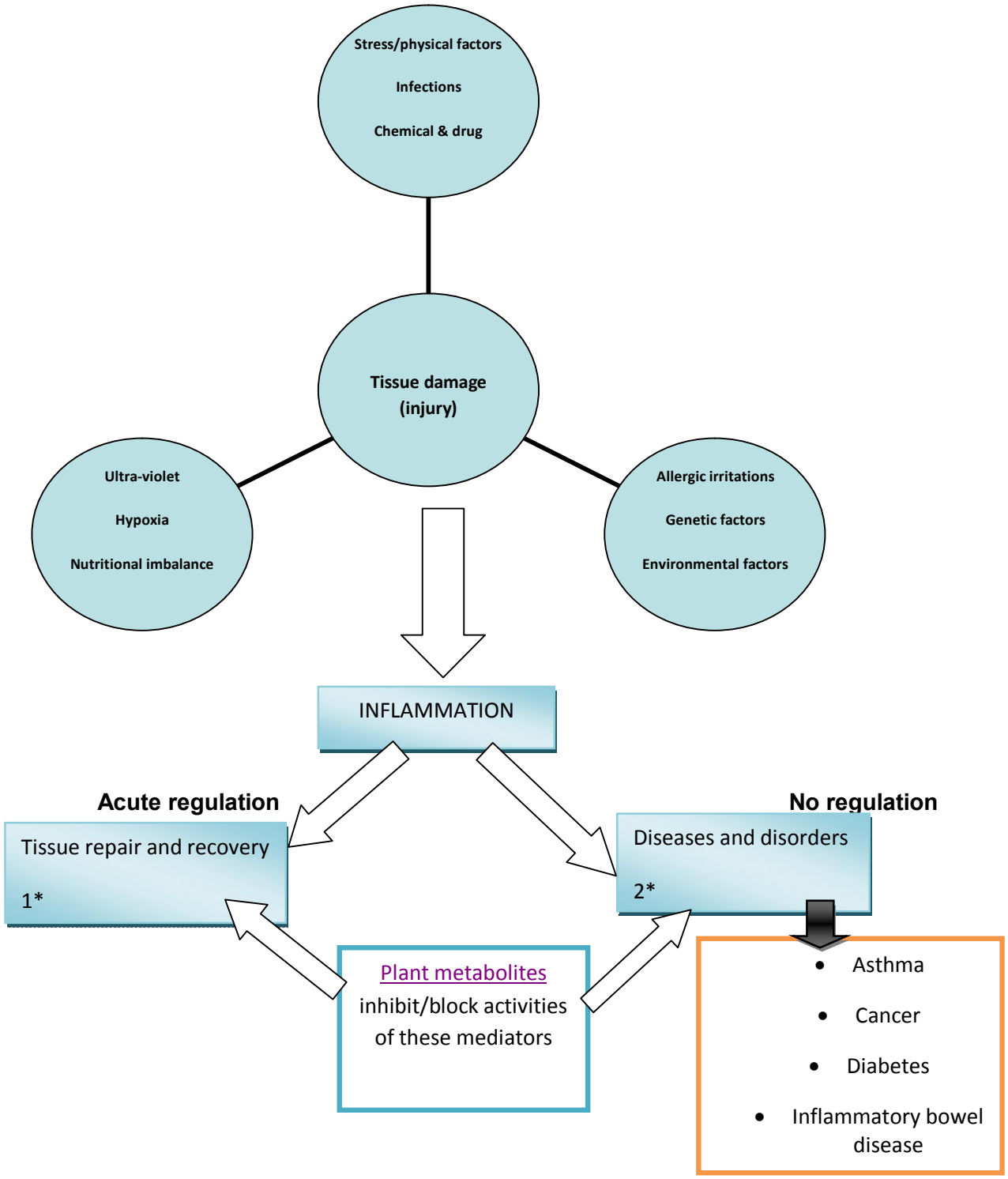


Figure 4.1: The sketch diagram shows the pathways of tissue damage and inflammatory diseases and disorders. The tissue repair and recovery happens in site 1* while site 2* shows disease and disorders in chronic inflammatory stage (Adapted and modified slightly from Iwalewa *at al*, 2007).

The inflammatory response is mediated by the migration of leukocytes, nitric oxide and arachidonic acid metabolism, reactive oxygen species, NF kappa B and pro-inflammatory cytokines. All these chemical mediates are triggered in a series of cascades as a result of tissue injury. Tissue damage induces the release of inflammatory mediators such as cytokines and tumour necrosis factor (TNF-Ŭ), interleukin-1 (IL-1) from leukocytes, monocytes and macrophages.

The release of all these chemical mediators further trigger the regulation of pro-inflammatory cytokines, chemokineŝ, immunoglobulinŝ and other cellular adhesion molecules. Eventually the activation of the transcription factor NFkB (nuclear factor kappa B) is triggered and this cascade regulates the production of inducible enzymes, inflammatory cytokines and other chemical mediators that enhance or initiate the inflammatory process (Colin & Monteil, 2003; Paterson, 2003; Nakamura *et al*, 2003, & Okamoto *et al*, 2004)

4.1.1 15-lipoxygenase inhibition

The enzyme lipoxygenase is one of the enzymes in the arachidonic acid pathway which catalyses the peroxidation of polyunsaturated fatty acids in a selective way. There are several lipoxygenases found in mammals and their metabolites contain many substances with high biological activity (Steinberg, 1999). The mechanism of action is based on the 15-lipoxygenase (15-LO) catalysed reaction between oxygen and a polyunsaturated fatty acid with a 1, 4-diene-type structure. A suitable structure for this is linoleic acid. During a reaction the formation of a conjugated double bond system leads to an increase in its absorbance at 234 nm. This increase is used to quantify the reaction product (Lyckander & Malterud, 1992).

4.1.2 Cytotoxicity

The use of herbal remedies in the treatment and control of disease in animals is well documented and there is a potential threat of toxicity. Compared to synthetic drug treatments the information on the safety and use of herbal remedies is limited (Taylor *et al*, 2001). Very little documentation on the long term toxicity of herbal remedies exists. Many natural products lack defined dose and potency information; the benefit in their use may lie in the fact that some or most contain compounds in the presence of other compounds that may influence its activity and safety. These other compounds may have a variety of influences on the physiology compared to the purified synthetic drugs based on a single molecular substance derived from the natural product (Taylor *et al*, 2001).

Allergic and toxic reaction to herbal remedies as a result of their pharmacological action and other possible effects, including mistaken identification of the plant species has been reported. In many of

the cases the side-effect from the use of a combination of herbal remedies is not known and this cause difficulty in their evaluation (Taylor *et al*, 2001). Although there were no apparent negative effects when non-extracted plant material was feed to animals as an additive, determining the cytotoxicity of extracts and fractions may indicate hidden or potential problems.

4.2 Materials and methods

4.2.1 Anti-inflammatory activity

4.2.1.1 15 - Lipoxygenase inhibitory activity

The lipoxygenase (LO) activity was determined using the method of Lyckander and Maltreud (1992). This method is based on the enzymatic oxidation of linoleic acid to a hydroperoxide. Soyabean 15-LO was incubated with linoleic acid to determine the formation of hydroperoxide. Soybean 15-LO (200 U) was incubated with linoleic acid (50 µM) in sodium borate buffer for 5 minutes at 25 ºC. The inhibition was determined for the crude acetone extracts and chloroform and hexane fractions over defined concentrations (0.02 mg/ml to 2.5 mg/ml).

All solutions were kept at room temperature prior to commencing the assay. The absorbance at 234 nm was measured on a microplate reader spectrophotometer (Epoch, Biotek). DMSO was used as solvent control and quercetin (10 mM) was used as a positive control. The anti-inflammatory activity was determined by calculating percentage inhibition of hydroperoxide production from changes in the optical density values of the plant samples at 234 nm after 5 min. The concentration causing 50% inhibition of the hydroperoxide release (IC_{50}) was calculated from the concentration-inhibition curve using the best fit linear regression analysis.

4.2.2 Cytotoxicity of crude acetone extracts against Vero cells

The cytotoxicity of the crude acetone extracts was determined by the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide] assay using Vero monkey kidney cells (Mosman, 1983 & McGaw *et al*, 2007). The cells were obtained from the department of Veterinary Tropical Diseases (University of Pretoria) and cultured in minimal essential medium (MEM) Earleûs Base, supplemented with 20 mM L-glutamine, 0.1% gentamicin and 5% foetal bovine serum. Confluent monolayer cell culture suspension were seeded into 96-well tissue culture plates at a density of 2 x 10^3 cells per well and incubated overnight at 37 ºC in a 5% CO_2 incubator. The sub-confluent cells in the microtitre plate were used in this assay. The extracts re-dissolved in DMSO to a concentration of 100 mg/ml. Serial 10-fold dilutions of each extract were prepared in growth medium (1-1000 µg/ml). The cells were viewed under an inverted microscope to see viable cells and the cytopathic effects of the extracts.

After the incubation the extract containing medium was removed from the wells and the remaining cells washed with phosphate buffer solution (PBS) (150 µl) before the addition of fresh MEM (200 µl). Cell proliferation and viability were determined using the Mosman (1983) method. A 30 µl solution of MTT (5 mg/ml) in PBS was added to each well and incubated for 4 hours at 37 ºC. The solution was removed after the incubation and the MTT concentrate was washed with PBS and DMSO was used to dissolve the crystallised MTT formazan. The amount of reduced MTT was quantified as the absorbance at 570 nm and the result expressed as a percentage of cell viability of the control cells and IC_{50} values were calculated.

4.3 Results

4.3.1 Cytotoxicity

The cytotoxicity the acetone crude extracts of *P. fraxinifolia* from trees growing in different areas on the Vero monkey cell line assay are provided in figure 4.2. Acetone extracts of *P. fraxinifolia* were all relatively toxic with EC_{50} values (Table 4.1) ranging from 0.77 ± 0.21 µg/ml to 1.72 ± 0.23 µg/ml and caused death to the Vero cells. More than 80% of the Vero cells treated with berberine were not viable at the concentration of 10 µg/ml.

Table 4.1: The cytotoxicity activity of *P. fraxinifolia* trees growing in Denmark, Germany and Poland based on EC_{50} values of the crude acetone extracts (PF-Fruit extract-Poland; DF-Fruit extract-Denmark; DL1-Leaf extract-Denmark; DL2- Leaf extract -Denmark; GFL-Leaf+fruit extracts-Germany; and PL-Leaf extract-Poland)..

Plant ID	EC_{50} (µg/ml)
GFL	0.79 ± 0.21
PL	0.77 ± 0.32
DL1	0.83 ± 0.37
DF	1.72 ± 0.39
PF	1.35 ± 0.23
DL2	0.81 ± 0.29

4.3.2 15-lipoxygenase inhibitory assay

The lipoxygenase inhibitory assay of the extracts tested is expressed as percentage inhibition at different concentrations (0.02 mg/ml to 2.5 mg/ml) for the acetone crude extracts, solvent-solvent derived chloroform and hexane fractions of *P. fraxinifolia* from trees growing in different areas in figures 4.2, 4.3 & 4.4. The choice to use only the acetone, chloroform and hexane extracts was because of their moderate to high activity in the antimicrobial assays (see results in the next chapter). In all cases, as expected dose related responses were found. The crude acetone extract

and solvent-solvent derived fractions all had moderate activity for trees growing in Denmark, Germany and Poland with EC_{50} ranging from 4.47 ± 0.15 to 7.19 ± 0.05 mg/ml (Table 4.2) but not as high as the quercetin positive control agent with an EC_{50} of 1.19 ± 0.92 mg/ml.

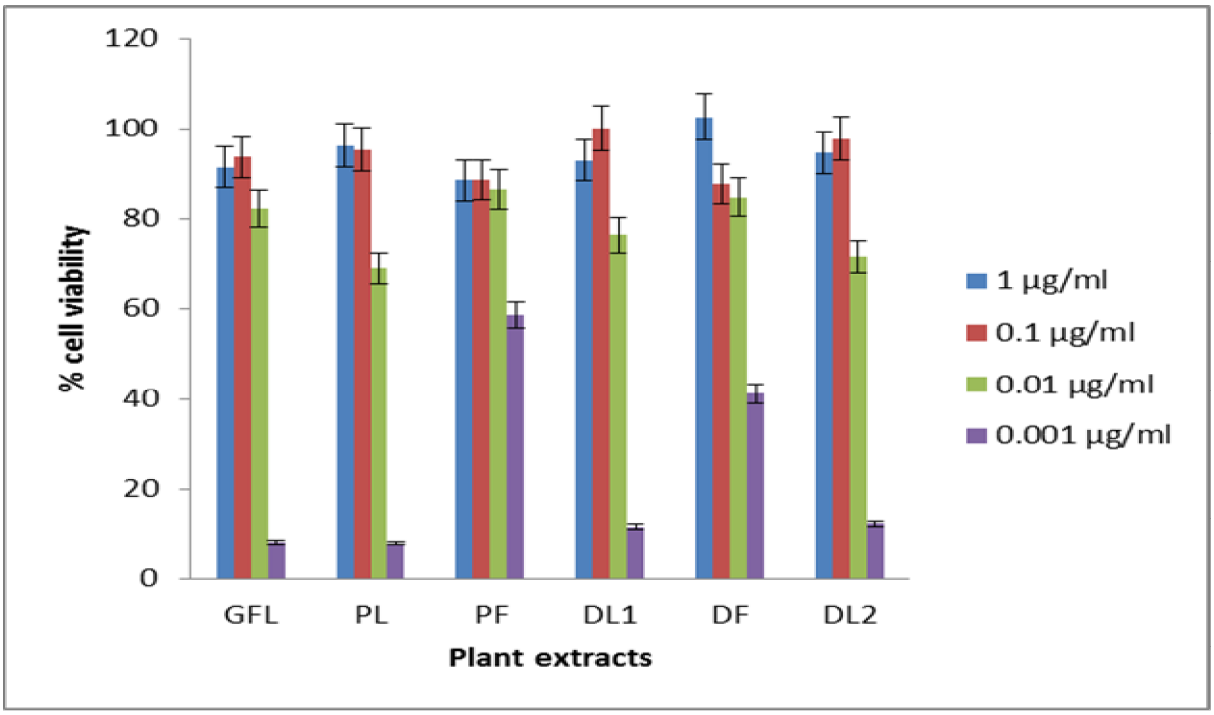


Figure 4.2: The percentage cell viability of the acetone crude extracts of *P. fraxinifolia* trees growing in different areas in Denmark, Germany and Poland on Vero Monkey kidney cells at predetermined concentrations (PF-Fruit extract-Poland; DF-Fruit extract-Denmark; DL1-Leaf extract-Denmark; DL2- Leaf extract -Denmark; GFL-Leaf+fruit extracts-Germany; and PL-Leaf extract-Poland).

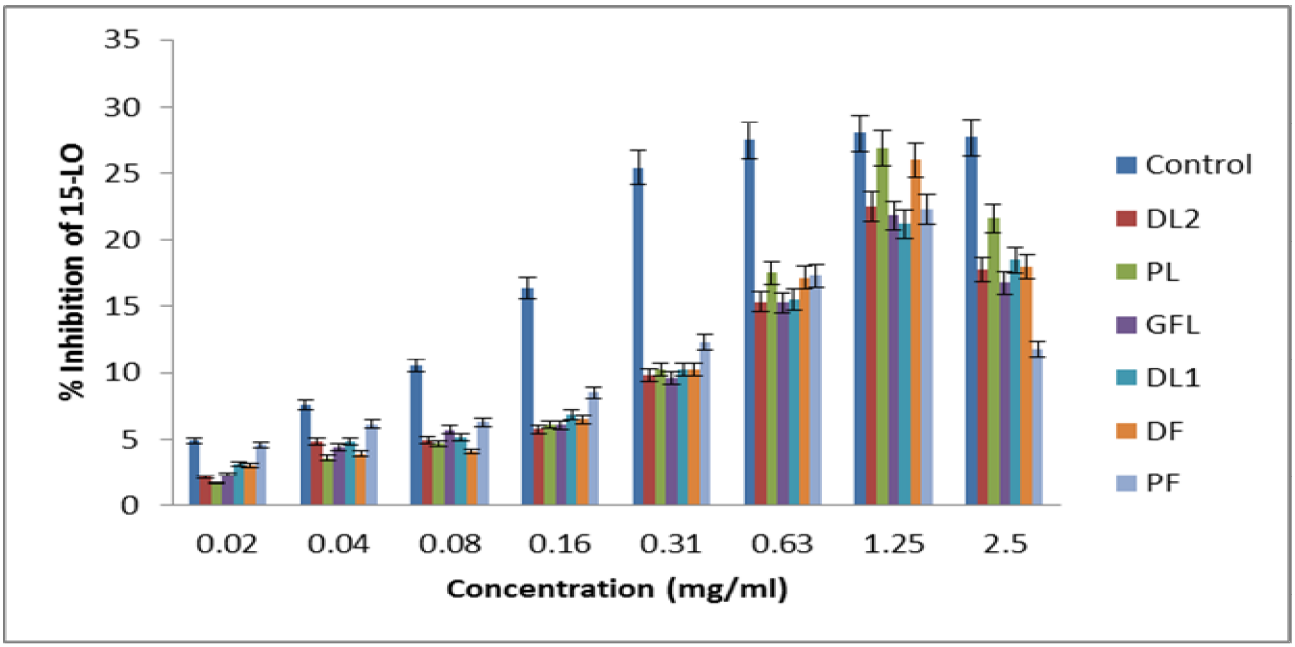


Figure 4.3: The concentration-inhibitory activity of the Soybean 15-LO enzyme of *Pterocarya fraxinifolia* solvent-solvent derived hexane fraction from trees growing in Denmark, Germany and Poland (PF-Fruit extract-Poland; DF-Fruit extract-Denmark; DL1-Leaf extract-Denmark; DL2- Leaf extract -Denmark; GFL-Leaf+fruit extracts-Germany; and PL-Leaf extract-Poland).Positive control was quercetin

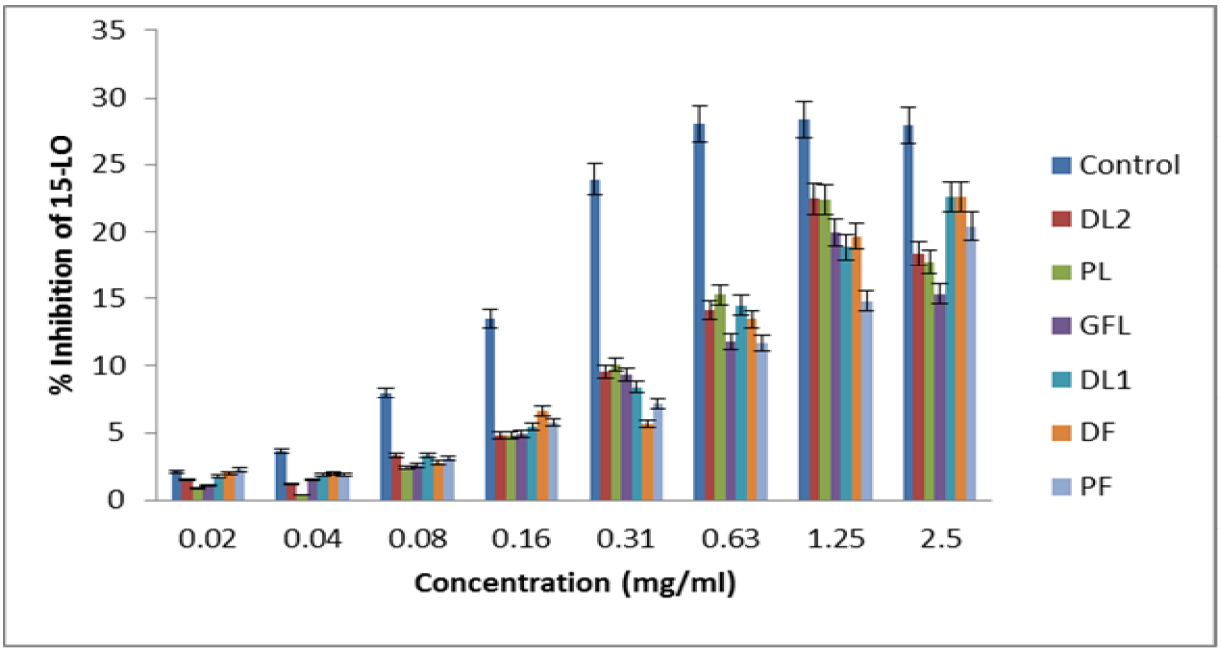


Figure 4.4: The concentration-inhibitory activity of Soybean 15-LO enzyme of *Pterocarya fraxinifolia* crude acetone extracts from trees growing in Denmark, Germany and Poland (PF-Fruit extract-Poland; DF-Fruit extract-Denmark; DL1-Leaf extract-Denmark; DL2- Leaf extract -Denmark; GFL-Leaf+fruit extracts-Germany; and PL-Leaf extract-Poland). Positive control was quercetin.

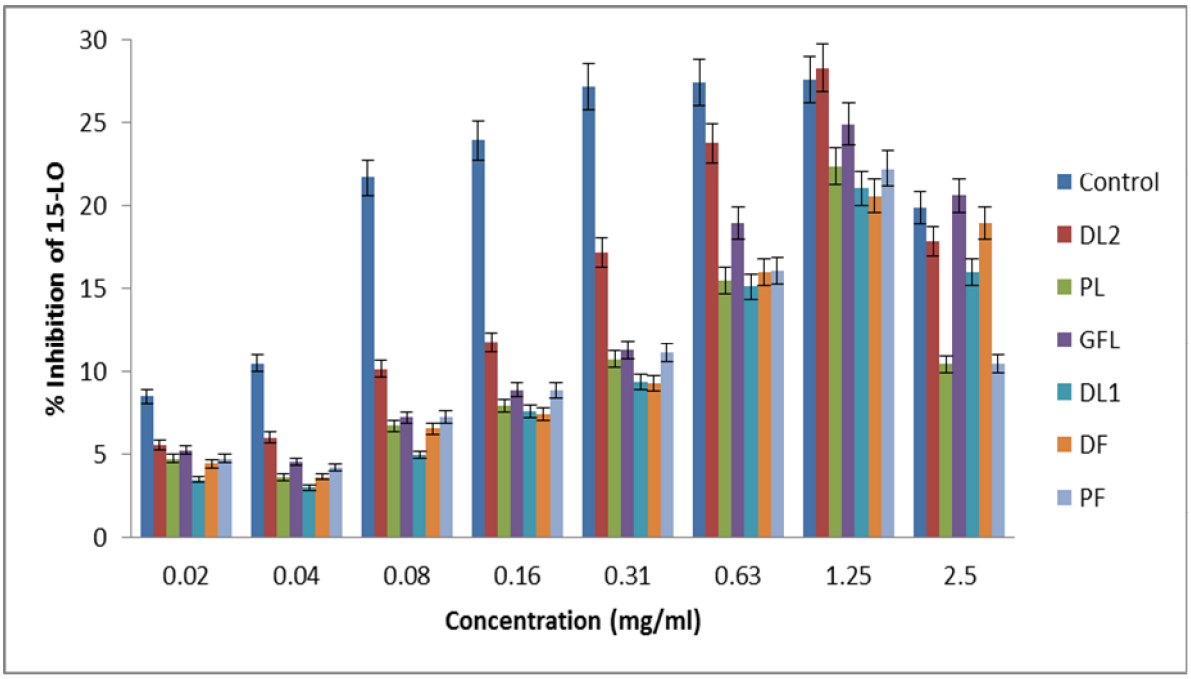
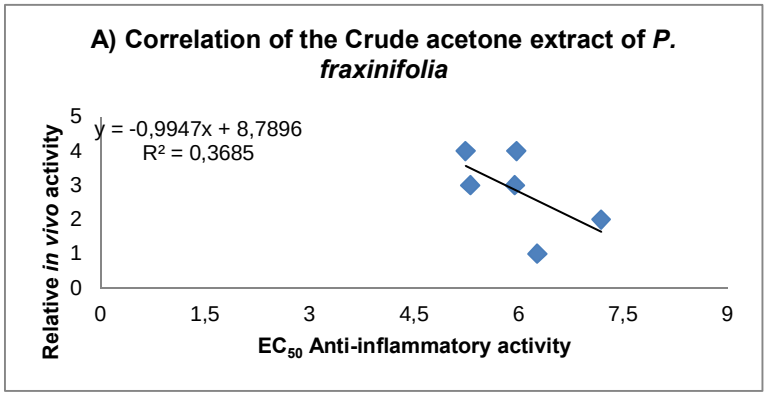


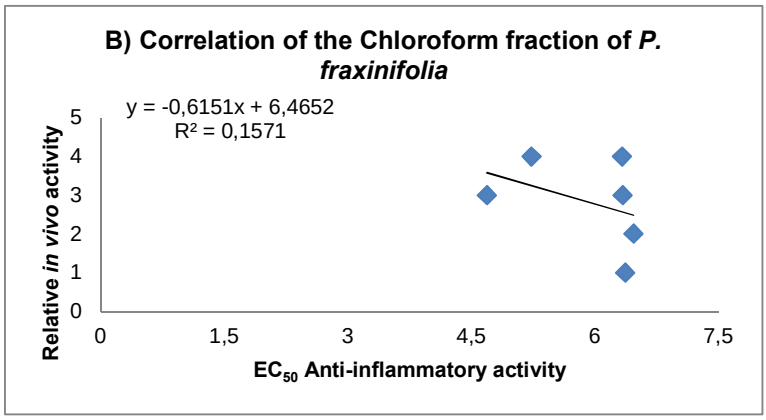
Figure 4.5: The concentration-inhibitory activity of Soybean 15-LO enzyme of *Pterocarya fraxinifolia* solvent-solvent derived chloroform fraction from trees growing in Denmark, Germany and Poland (PF-Fruit extract-Poland; DF-Fruit extract-Denmark; DL1-Leaf extract-Denmark; DL2- Leaf extract - Denmark; GFL-Leaf+fruit extracts-Germany; and PL-Leaf extract-Poland). Positive control was quercetin.

It is difficult to make comparisons between the different fractions presented in the Figures above. In no case was there more than 50% inhibition at the highest concentration tested. The EC_{50} was calculated by extrapolation from the results obtained (Table 4.2).

Table 4.2: 15-lipoxygenase activity of the crude acetone extract and solvent-solvent derived chloroform and hexane fractions of *P. fraxinifolia* trees growing in different areas in Denmark, Germany and Poland (PF-Fruit extract-Poland; DF-Fruit extract-Denmark; DL1-Leaf extract-Denmark; DL2- Leaf extract -Denmark; GFL-Leaf+fruit extracts-Germany; and PL-Leaf extract-Poland).

Plant	**EC_{50} (µg/ml)**			**% inhibition (2.5 mg/ml)**		
Region	**Crude**	**Chloroform**	**Hexane**	**Crude**	**Chloroform**	**Hexane**
DL2	5.95 ± 0.03	4.69 ± 0.17	5.68 ± 0.04	22 ± 1.83	28 ± 2.04	23 ± 0.21
PL	6.27 ± 0.10	6.37 ± 0.23	6.09 ± 0.03	22 ± 1.48	22 ± 1.54	21 ± 1.84
GFL	5.97 ± 0.14	5.23 ± 0.06	4.47 ± 0.15	22 ± 1.77	25 ± 1.89	27 ± 0.16
DL1	5.24 ± 0.11	6.33 ± 0.15	4.90 ± 0.16	23 ± 1.96	21 ± 1.51	26 ± 1.02
DF	5.31 ± 0.05	6.34 ± 0.11	6.37 ± 0.04	23 ± 1.88	21 ± 1.59	22 ± 4.58
PF	7.19 ± 0.05	6.47 ± 0.14	5.99 ± 0.04	20 ± 1.53	22 ± 1.61	22 ± 0.96





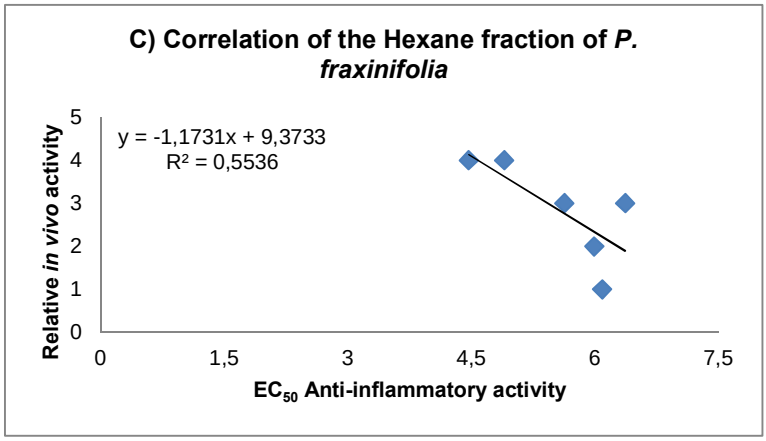


Figure 4.6: The correlation graphs of relative *in vivo* activity and EC_{50} values of anti-inflammatory activity of A) Crude acetone extract; B) Chloroform fraction and C) Hexane fraction of *P. fraxinifolia* from trees growing in different areas of Europe (Relative *in vivo* Activity levels: High = 4; Medium = 3; Low = 2; Very low = 1)

4.4 Discussion and conclusion

Anti-inflammatory activity

The prevention and mitigation of inflammation by phytomedicines have been investigated by many researchers over the past few years (Iwalewa et al, 2007). Several plant secondary metabolites have been discovered that help modify the effects e of inflammation. These secondary metabolites include flavonoids, sterols, triterpenoids, tannins, alkaloids and many others (Iwalewa et al, 2007). The anti-inflammatory activity in this study was concentration dependent as expected and indicated that the crude plant extracts of *P. fraxinifolia* trees growing in different areas in Denmark, Germany and Poland had good to moderate activity compared to quercetin.

Correlation between anti-inflammatory activity and in vivo activity

There was a very good correlation between antioxidant activity of the crude acetone extracts and *in vivo* activity as has been shown in the previous section. This indicates that antioxidant activity may play an important role in the effect of plants on animal growth. To determine if anti-inflammatory activity could also be involved, the correlation between relative *in vivo* activity levels and anti-inflammatory activity of the crude extract and fractions were determined. There was some correlation between the relative *in vivo* activity and the anti-inflammatory activity based on the EC_{50} values.

The higher the anti-inflammatory activity was (lower EC_{50}), the better the relative *in vivo* activity was with a poor correlation for the crude acetone extract (R^2 = 0.369). There was a better correlation between the hexane fraction and *in vivo* activity (R^2 = 0.553) but the correlation between the chloroform fraction and *in vivo* activity was very low (R^2 = 0.157). This may mean that non-polar compounds in the hexane are involved with anti-inflammatory activity rather than intermediate activity compounds present in the chloroform fraction. For antimicrobial activities the situation was usually the other way around with most activity in the intermediate polarity compounds (Kotze and Eloff, 2002).

Wangensteen *et al* (2006) investigated the DPPH scavenging activity and lipoxygenase enzyme inhibition and found that even though their plant had excellent lipoxygenase enzyme inhibition, the free radical scavenging effect was very low. On the other hand recently Cuello *et al* (2011) found a correlation between antioxidant activity and lipoxygenase inhibition. Our results are in agreement with those of Maiga *et al* (2006) who found that lipophilic extracts had higher activity as lipoxygenase inhibitors than radical scavengers but the opposite is true in the case of extracts that are highly polar in nature. Quercetin is a well-known inhibitor of 15-lipoxygenase and under experimental conditions it is known to have an EC_{50} value of 38 ± 2 µM corresponding to 1.19 ± 0.92

mg/ml in this study and other studies (Maiga *et al,* 2006). The results indicate that *P. fraxinifolia* crude extracts and solvent-solvent derived fractions from trees growing in different areas in Denmark, Germany and Poland are moderate inhibitors of 15-lipoxygenase and anti-inflammatory activity may not be a major factor involved in the *in vivo* activity of plants

Cytotoxicity

Compared to synthetic drugs the information available on the safety or relative toxicity of plant extracts or plant derived products is very limited and very little information exists on the long term toxicity of medicinal plants (Taylor *et al*, 2001). The cytotoxic activity of *P. fraxinifolia* trees have been previously studied on chicken embryo at a concentration dependent assay using 0.1, 1 and 10 mg/ml of the plant extract (Sadighara *et al*, 2009). Their results indicated that at the highest concentration there was 100% mortality of the embryos while at low concentrations (0.1 mg/ml) there was 50% mortality.

The results obtained by Sadighara *et al* (2009) lend credence to the highly toxic nature of *P. fraxinifolia* trees growing in different areas in Denmark, Germany and Poland as was the case in this study when Vero monkey cells where used at similar concentration. The known compound juglone that has been isolated from the plant is also known to be toxic, in toxicity assays conducted by Inbaraj and Chignell (2004) using keratinocytes, they exposed the cells to various concentration of the compound juglone (1-20 µM) and found 50% of the cells were not viable after 30 minutes and the activity continued to decrease with time further lending credence to the toxic nature of *P. fraxinifolia* extracts. No adverse effects were however found when plant material was included in the feed of animals.

In conclusion the anti-inflammatory activity may not play a large role in the usefulness of *P. fraxinifolia* as a potential growth promoter.

Post script

In the next chapter attention will focused on the antimicrobial activity of the acetone crude extracts and the solvent-solvent derived fractions of *Pterocarya fraxinifolia* trees growing in different areas.

Chapter 5

Antimicrobial activity of crude acetone extracts and different fractions of *P. fraxinifolia*

Preface

The antioxidant and anti-inflammatory activity to a lesser degree adds to the usefulness of *P. fraxinifolia* as a potential growth promoter with regard to its immune stimulating effects. In this chapter the antimicrobial activity of the extracts from the different regions will be investigated and compared with the antioxidant and anti-inflammatory activity to assess the potential role that antimicrobial activity can play in using the plant as a growth promoter.

5.1 Introduction

The mammalian gastrointestinal tract contains a complicated microflora population; the microbial colonization of the intestine drives the development of a healthy immune system (Garnsworthy & Wiseman, 2009). The entire microflora that colonize the gut, communicate with each other through signal transductions for the careful regulation of the immune system to allow for their rapid establishment in the gut of the host (Lalles *et al*, 2007).

The pig is highly susceptible to mortality during the post-weaning period which has been estimated to be about 17% of the pig population lending to huge losses in production. Post-weaning diarrhoea is known to be caused by the enterotoxigenic *Escherichia coli* K88, but other diseases caused by Rotavirus, salmonella, swine dysentery and haemophilia also have a huge impact on pig health during this period (Garnsworthy & Wiseman, 2009).

There has been renewed interest in use of plants as alternatives to synthetic drugs and the pharmaceutical industry considers plants as a viable option for the discovery of new leads (Soejarto, 1996). In an effort to discover new lead compounds, many research groups screen plant extracts to detect secondary metabolites with relevant biological activities that target various ailments. In this regard, several bioassays were developed for screening purposes (Hostettmann 1991). It is also estimated that natural products are implicated in the development of some new drugs and out of the 120 known compounds used in drug development 90 were originally isolated from plants (Hostettmann *et al*., 2000 and 2001).

Due mainly to the improper use of conventional antimicrobial agents, resistant or multi-resistant strains of pathogenic microorganisms are continuously appearing, leading to a thorough search for

new drugs (Silver and Bostian, 1993). Fungi and bacteria cause important human and animal diseases that lead loss of millions of dollars and euros.
The screening of plant species for active antimicrobials has shown that a great number of plant species contain active compounds. The activity has been demonstrated by various researchers for their antibacterial, antifungal and other biological activities (Shai *et al*, 2008). Plants produce a diverse number and range of bioactive molecules making them a rich source for different types of medicines (Sukanya *et al,* 2009).

The use of phytochemicals with known antimicrobial properties is of great importance and there are several research initiatives all over the world to prove the efficacy of these antimicrobials from plants (Sukanya *et al*, 2009). The active compounds from plants are mainly products of the secondary metabolic pathways.

5.1.1 *p*-iodonitrotetrazolium violet (INT) reaction

The activity is determined by adding INT to overnight incubated cultures on microtitre plates or spraying a solution of INT onto TLC plates with bacteria. The INT reacts with the bacteria and produces a purple-red colour which is indicative of bacterial growth. The INT reaction is based on the transfer of electrons from NADH to the tetrazolium dye.

Threonine dehydrogenase from bacteria catalyses the NAD-dependent oxidation of threonine to form a 2-amino-3-ketobutyrate and NADH cascade. During active bacterial growth, an electron is transferred from the NADH molecule to INT forming a purple-red colour. The clear areas on the chromatograms indicate areas of inhibition (Begue & Kline, 1972). The purpose of this study was to determine the antimicrobial activity of acetone extracts and fractions of *Pterocarya fraxinifolia* leaves and fruit mixture.

5.2 Materials and methods

5.2.1 Plant collection and extraction

As part of the EC funded research projects Rumen (QLK5-CT-2001-00992) and REPLACE (FOOD-CT-2004-506487), a collection of plant samples have been established by collecting plant material around six geographic locations in Europe; Aberdeen (UK), Hohenheim (DK), Leon, Reading and Dublin and Viborg (DK). The plants were all selected based on literature studies, communication with ethnobotanist and botanical gardens. Only the leaves and fruits of the plants were collected for this study but the general collection included parts/fractions of the whole plant to leaves, roots, flowers and fruits. The plant materials were collected at different stages of maturity, dried and milled into fine powder and stored in the dark at room temperature. Detailed information on the selected

plant can be obtained from the Rumen-Up and REPLACE collection list (www.rowett.ac.uk/rumen_up).
Finely ground leaf (Specimen number, D058R, D052R, and D054R), fruit (Specimen number, D056R and D053R) and leaf and fruit mixture (Specimen number, D021R) plant materials were obtained from Dr Ole Højberg, Aarhus University, Faculty of Agricultural Sciences, Department of Animal Health, Welfare and Nutrition, Denmark.

A standard procedure of extraction, previously described by Eloff (1998a), was employed in the preparation of the plant extracts. 3 g of plant material samples of fruit and leaves were weighed into 40 ml plastic centrifuge tubes. 30 ml of acetone, n-hexane, dichloromethane, or methanol (respectively) were added into the tubes. The tubes were vigorously shaken by hand and then on a linear shaker for at least 30 minutes. The tubes were centrifuged for 10 minutes at approximately 6000 rpm. The supernatant was filtered using premoistened Whatman No.1 filter paper. The extraction was repeated three times. The filtrate was placed into pre-weighed glass sample bottles. The solvent was allowed to evaporate under a stream of air in a fume hood at room temperature, leaving dry plant extract residues in the glass containers.

5.2.2 Antimicrobial assay

Minimum inhibitory concentration (MIC) is defined as the lowest concentration that the extract can inhibit visible growth of the micro-organism. The MIC was determined using a serial micro-dilution assay for the different microbial species with INT as growth indicator (Eloff, 1998b).

5.2.2.1 Bacterial test species

In this study five bacterial test microorganisms were used, namely: *Staphylococcus aureus* (Gram-positive) [American Type Culture Collection [ATCC] number 29213), *Pseudomonas aeruginosa* (Gram-negative) [ATCC 27853], *Escherichia coli* K88 (Gram-negative), *Clostridium perfringens* (Gram-positive) and *Campylobacter jejuni* (Gram-negative). These bacterial strains are important nosocomial pathogens, which are recommended as test strains for comparing the activity of antibiotics (NCCLS, 1992). Bacterial test cultures were kept on Müller-Hilton (MH) agar at a temperature of 4 °C. The bacterial cells were inoculated into fresh MH broth and incubated at 37 °C for 14 h, prior to the antibacterial assay. The densities of bacterial cultures after incubation overnight were as follows: *Staphylococcus aureus*, 2.6×10^{12} cfu/ml and *Escherichia coli*, 3.0×10^{11} cfu/ml.

Campylobacter jejuni and *Clostridium perfringens* were cultured according to anaerobic procedures. The cultures were obtained from the microbiology division of Department of Tropical and veterinary disease, Faculty of Veterinary Science, University of Pretoria. Both bacteria cultures were kept on

Blood tryptose agar, supplemented with 5% horse blood serum (Selecta media 510110) and incubated for 2-3 days in an microaerophillic atmosphere containing 6% O_2, 10% CO_2 and 84% N_2 (Afrox) at 37 ºC in a walk-in incubator. The cells were inoculated into fresh tryptose broth and incubated again under the above mentioned conditions overnight. The densities of the bacterial cultures were; $2.6x10^{12}$ cfu/ml for both bacterial species.

5.2.2.2 Fungal test species

The fungal organisms used in this study were moulds (*Aspergillus fumigatus*) and yeast (*Candida albicans*). Both fungal organisms were isolated from clinical cases that were not treated prior to sampling in the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria. *A. fumigatus* was isolated from a chicken and *C. albicans* from a Gordian finch. These fungi represent the most common and important disease-causing fungi of animals (Masoko *et al.*, 2005). Sabouraud dextrose (SD) agar (Oxoid, Basingstoke, UK) was used as the maintenance growth medium for all the fungal strains used, and the fungi were cultured in SD broth.

5.2.2.3 Minimum Inhibitory Concentration (MIC) assay

In this study the MIC was determined using the microplate serial dilution method (Eloff, 1998a). The MIC was determined by a 2-fold serial dilution of extracts beyond the level where no inhibition of microbial growth was observed. Plant extracts were individually reconstituted in acetone to the concentration of 10 mg/ml. One hundred microliter of each extract was serially diluted with distilled water in a 96-well microtitre plates. 100 µl of overnight microbial cultures (bacteria and fungi respectively) was added to each well and Gentamicin (Virbac®) was used as a reference antibiotic.

The microplates were sealed and incubated overnight at 37 ºC. After the incubation 40 µl of 0.2 mg/ml of INT solution was added to each well and further incubated at 37 ºC for a few hours for purple-red colour to develop which was used as an indicator of bacterial growth. The MIC was recorded as the lowest concentration of plant extract/fraction at which bacterial growth was inhibited and this was indicated by INT formazan formation. The bacterial and fungal assay were done separately but using the same method and repeated three times.

5.2.3 Total activity

The total activity is the measure of the quantity extracted from 1 gram in milligram of plant sample in relation to the MIC value of the extract, fraction or compound isolated. This is indicative of the volume to which the active extract from one gram of plant material can be diluted and still manage to inhibit the growth of the test microorganisms (Eloff, 2000). Total activity was determined by

dividing the mass in mg extracted from 1 gram of plant material by the MIC in mg/ml. The results in ml/g can be used to compare different plants.

5.3 Results

5.3.1 Minimum inhibitory concentration of crude extracts and fractions

5.3.1.1 Antibacterial assay

The antimicrobial activity of the plant extracts was evaluated based on the following criteria; Excellent activity = 0.02 - 0.04 mg/ml, Good-low activity = 0.07 - 0.08 mg/ml, moderate activity = 0.16 - 0.64 mg/ml and poor activity = > 1 mg/ml (Pauw and Eloff, 2014). The crude acetone extracts of leaf samples from trees growing in Denmark and Poland and leaf and fruit mixture sample from trees growing in Germany generally had moderate activity on all the microorganisms tested (Table 5.1).

Antimicrobial activity against E. coli (K88)

The activity against *E.coli* (K88) was good for all extracts tested with MIC values ranging from 0.07 to 0.16 mg/ml. Good antimicrobial activity of *Pterocarya fraxinifolia* samples against *E.coli* (K88) was observed in extracts of trees growing in Denmark (0.07 mg/ml). The activity of extract from trees growing in Germany and Poland (0.16 mg/ml) was moderate. The antimicrobial activity of the solvent-solvent derived fractions was moderate. The solvent-solvent derived chloroform and hexane fractions had better activity then the solvent-solvent derived butanol and water-methanol fractions. Good antimicrobial activity was observed in the solvent-solvent derived chloroform fraction (MIC = 0.08 mg/ml) of extracts from trees growing in Denmark. The extract from trees growing in Germany (MIC = 0.08 mg/ml) had good activity. The rest of the samples from trees growing in different areas had moderate antimicrobial activity (MIC values ranging between 0.16 to 0.31 mg/ml). The other solvent-solvent derived fractions all had moderate to poor activity with MIC values ranging from 0.31 to 2.5 mg/ml (Table 5.2) for *P. fraxinifolia* extracts from trees growing in different areas.

Antimicrobial activity against S. aureus

Antimicrobial activity of the extracts against *S. aureus* was good for the acetone crude extracts and the highest antimicrobial activity observed in the crude acetone extracts was extracts from trees growing in Denmark (MIC = 0.08 mg/ml). Excellent activity was observed in the extract of trees growing in Germany and extracts of trees growing in the second region (DL2) of Denmark (0.02 mg/ml) (Table 5.1). The solvent-solvent derived chloroform fraction had moderate activity with MIC values ranging between 0.16 to 1.25 mg/ml for trees growing in Denmark and Germany. The rest of the extracts from trees growing in Poland had moderate to poor activity with MIC ranging between

0.16 to 0.31 mg/ml. The solvent-solvent derived butanol and water-methanol fractions had moderate to poor antimicrobial activity with MIC values ranging from 0.31 to 2.5 mg/ml (Table 5.3).

Antimicrobial activity against P. aeruginosa

Antimicrobial activity of the crude acetone extracts against *P. aeruginosa* was excellent for the extracts of trees growing in Denmark (MIC = 0.04 mg/ml) and moderate for extracts of trees growing in Germany (MIC = 0.16) and Poland (MIC = 0.16 mg/ml) (Table 5.1). The antimicrobial activity of solvent-solvent derived chloroform fractions of trees growing in Denmark was excellent (MIC = 0.04 mg/ml) and activity of extracts from trees growing in Germany was good (MIC = 0.08 mg/ml). The overall antimicrobial activity of the solvent-solvent derived fractions against *P. aeruginosa* was good to moderate (Table 5.4).

Antimicrobial activity against C. perfringens

Antimicrobial activity against *C. perfringens* was excellent for the acetone crude extract of trees growing in Poland and activity was good for extracts from trees growing in Denmark (0.02 mg/ml and 0.08 mg/ml respectively) (Table 5.1). The rest had MIC values of 0.16 mg/ml which is indicative of moderate activity. The solvent-solvent derived chloroform fraction had moderate antimicrobial activity compared to the other fractions for trees growing in all the different areas. Moderate activity (MIC value of 0.31 mg/ml) was observed in extracts of trees growing in Poland, fruit extract of trees growing in Denmark and leaf extract of trees growing in Denmark. Solvent-solvent derived Hexane, butanol and water-methanol fractions all had poor antimicrobial activity with MIC values in the range of 1.25 to 2.5 mg/ml (Table 5.5).

Antimicrobial activity against C. jejuni

Antimicrobial activity against *C. jejuni* was excellent for crude acetone fruit extracts of trees growing in Poland (MIC = 0.02 mg/ml) and good activity was observed in extracts from Germany. The activity of extracts from trees growing in Denmark and extracts of trees growing in Poland (0.08 mg/ml) was good (Table 5.1). The solvent-solvent derived chloroform fraction of fruit extracts from trees growing in Poland had good activity with MIC value of 0.08 mg/ml. Activity was moderate for solvent-solvent chloroform and hexane fractions with MIC values in the range of 0.31 to 0.63 mg/ml.. The solvent-solvent derived hexane fractions from trees growing in all three countries had moderate activity with MIC values of 0.61 mg/ml while the n-butanol and water-methanol fractions all had poor activity with MIC values of 2.5 mg/ml (Table 5.6).

The samples of *Pterocarya fraxinifolia* trees growing in the different areas had varying degrees of antimicrobial activity but the leaf samples from *P. fraxinifolia* trees growing in the different areas dominated in activity on the all microorganisms tested.

Table 5.1: Minimum inhibitory concentration (MIC in mg/ml) and total activity (TA) in ml of acetone crude extracts of *Pterocarya fraxinifolia* trees growing in different areas in Denmark, Germany and Poland against five of the five microorganisms used in this study. Excellent activity = 0.02 - 0.04 mg/ml, Good-low activity = 0.07 - 0.08 mg/ml, moderate activity = 0.16 - 0.64 mg/ml and poor activity = > 1 mg/ml.

Plant ID*	**Mass (mg)**	***E.coli (k88)***	**TA (ml)**	***S. aureus***	**TA (ml)**	***P. aeruginosa***	**TA (ml)**	***C. jejuni***	**TA (ml)**	***C. perfringens***	**TA (ml)**
PF	230	2.5	92	0.63	365	0.63	365	**0.02**	11500	**0.02**	11500
DF	220	1.25	176	0.63	349	1.25	176	0.61	361	0.61	361
DL2	280	**0.07**	4000	**0.08**	3500	0.16	1750	0.61	459	**0.08**	3500
GFL	260	0.16	1625	**0.02**	13000	0.16	1625	**0.08**	3250	0.61	426
DL1	300	0.43	698	**0.02**	15000	**0.04**	7500	**0.08**	3750	0.61	492
PL	240	0.16	1500	0.16	1500	0.16	1500	**0.08**	3000	0.61	393

*(PF-Fruit extract-Poland; DF-Fruit extract-Denmark; DL1-Leaf extract-Denmark; DL2- Leaf extract -Denmark; GFL-Leaf+fruit extracts-Germany; and PL-Leaf extract-Poland).

Table 5.2: The minimum inhibitory concentration (MIC-mg/ml) and total activity (TA) in ml of all the solvent-solvent derived fractions of *Pterocarya fraxinifolia* trees growing in different areas against *E. coli* (K88) after 24 hours of incubation period (PF-Fruit extract-Poland; DF-Fruit extract-Denmark; DL1-Leaf extract-Denmark; DL2- Leaf extract -Denmark; GFL-Leaf+fruit extracts-Germany; and PL-Leaf extract-Poland). Excellent activity = 0.02 - 0.04 mg/ml, Good-low activity = 0.07 - 0.08 mg/ml, moderate activity = 0.16 - 0.64 mg/ml and poor activity = > 1 mg/ml.

Samples	**PF**			**DF**			**DL2**		
	Mass (mg)	MIC	TA	Mass (mg)	MIC	TA	Mass (mg)	MIC	TA
Fractions									
Hexane	220	0.31	710	200	0.31	645	230	0.16	1438
CHCL3	120	1.25	96	110	1.25	88	220	0.08	2750
Butanol	110	2.5	44	120	1.25	96	120	1.25	96
Aq. MeOH	80	2.5	32	90	0.63	143	110	0.63	175
Gentamicin		0.02			0.02			0.02	

Samples	**GFL**			**DL1**			**PL**		
	Mass (mg)	MIC	TA	Mass (mg)	MIC	TA	Mass (mg)	MIC	TA
Fractions									
Hexane	360	0.08	4500	330	0.63	524	140	0.63	222
CHCL3	220	0.31	710	140	0.31	452	160	2.5	64
Butanol	290	0.31	935	80	0.63	127	170	0.63	270
Aq. MeOH	120	0.31	387	120	2.5	48	130	2.5	52
Gentamicin		0.02			0.02			0.02	

Table 5.3: The minimum inhibitory concentrations (MIC) and total activity (TA) in ml of all the solvent-solvent derived fractions of *Pterocarya fraxinifolia* trees growing in different areas against *S. aureus* after 24 hour incubation period (PF-Fruit extract-Poland; DF-Fruit extract-Denmark; DL1-Leaf extract-Denmark; DL2- Leaf extract -Denmark; GFL-Leaf+fruit extracts-Germany; and PL-Leaf extract-Poland). Excellent activity = 0.02 - 0.04 mg/ml, Good-low activity = 0.07 - 0.08 mg/ml, moderate activity = 0.16 - 0.64 mg/ml and poor activity = > 1 mg/ml.

Samples	**PF**			**DF**			**DL2**		
	Mass (mg)	MIC	TA	Mass (mg)	MIC	TA	Mass (mg)	MIC	TA
Fractions									
Hexane	220	0.16	1375	200	0.31	645	230	0.08	2875
CHCL3	120	1.25	96	110	1.25	88	220	0.31	710
Butanol	110	2.5	44	120	1.25	96	120	1.25	96
Aq. MeOH	80	2.5	32	90	0.31	290	110	0.31	355
Gentamicin		0.02			0.02			0.02	

Samples	**GFL**			**DL1**			**PL**		
	Mass (mg)	MIC	TA	Mass (mg)	MIC	TA	Mass (mg)	MIC	TA
Fractions									
Hexane	360	0.08	4500	330	0.16	2063	140	0.31	452
CHCL3	220	0.16	1375	140	0.16	875	160	1.25	128
Butanol	290	0.63	460	80	1.25	64	170	0.63	270
Aq. MeOH	120	0.31	387	120	0.31	387	130	2.5	52
Gentamicin		0.02			0.02			0.02	

Table 5.4: The minimum inhibitory concentrations (MIC- mg/ml) and total activity (TA) in ml of all the solvent-solvent derived fractions of *Pterocarya fraxinifolia* trees growing in different areas against *P. aeruginosa* after 24 hour incubation period (PF-Fruit extract-Poland; DF-Fruit extract-Denmark; DL1-Leaf extract-Denmark; DL2- Leaf extract -Denmark; GFL-Leaf+fruit extracts-Germany; and PL-Leaf extract-Poland). Excellent activity = 0.02 - 0.04 mg/ml, Good-low activity = 0.07 - 0.08 mg/ml, moderate activity = 0.16 - 0.64 mg/ml and poor activity = > 1 mg/ml.

Samples	**PF**			**DF**			**DL2**		
	Mass (mg)	MIC	TA	Mass (mg)	MIC	TA	Mass (mg)	MIC	TA
Fractions									
Hexane	220	0.31	710	200	0.08	2500	230	0.08	2875
CHCL3	120	0.31	387	110	0.16	688	220	0.04	5500
Butanol	110	2.5	44	120	0.63	190	120	2.5	48
Aq. MeOH	80	2.5	32	90	0.31	290	110	1.25	88
Gentamicin		0.02			0.02			0.02	

Samples	**GFL**			**DL1**			**PL**		
	Mass (mg)	MIC	TA	Mass (mg)	MIC	TA	Mass (mg)	MIC	TA
Fractions									
Hexane	360	0.08	4500	330	0.08	4125	140	0.63	222
CHCL3	220	0.08	2750	140	0.16	875	160	0.63	254
Butanol	290	0.31	935	80	1.25	64	170	0.63	270
Aq. MeOH	120	0.31	387	120	1.25	96	130	1.25	104
Gentamicin		0.02			0.02			0.02	

Table 5.5: The minimum inhibitory concentrations (MIC - mg/ml) and total activity (TA) in ml of all the solvent-solvent derived fractions of *Pterocarya fraxinifolia* trees growing in different areas against *C. perfringens* after 24 hour incubation period (PF-Fruit extract-Poland; DF-Fruit extract-Denmark; DL1-Leaf extract-Denmark; DL2- Leaf extract -Denmark; GFL-Leaf+fruit extracts-Germany; and PL-Leaf extract-Poland). Excellent activity = 0.02 - 0.04 mg/ml, Good-low activity = 0.07 - 0.08 mg/ml, moderate activity = 0.16 - 0.64 mg/ml and poor activity = > 1 mg/ml.

Samples	**PF**			**DF**			**DL2**		
	Mass (mg)	MIC	TA	Mass (mg)	MIC	TA	Mass (mg)	MIC	TA
Fractions									
Hexane	220	2.5	88	200	2.5	80	230	2.5	92
CHCL3	120	0.31	387	110	0.31	355	220	0.31	710
Butanol	110	2.5	44	120	2.5	48	120	2.5	48
Aq. MeOH	80	2.5	32	90	2.5	36	110	2.5	44
Gentamicin		0.02			0.02			0.02	

Samples	**GFL**			**DL1**			**PL**		
	Mass (mg)	MIC	TA	Mass (mg)	MIC	TA	Mass (mg)	MIC	TA
Fractions									
Hexane	360	1.25	288	330	1.25	264	140	2.5	56
CHCL3	220	2.5	88	140	2.5	56	160	2.5	64
Butanol	290	2.5	116	80	2.5	32	170	2.5	68
Aq. MeOH	120	2.5	48	120	2.5	48	130	2.5	52
Gentamicin		0.02			0.02			0.02	

Table 5.6: The minimum inhibitory concentrations (MIC ï mg/ml) and total activity (TA) in ml of all the solvent-solvent derived fractions of *Pterocarya fraxinifolia* trees growing in different areas against *C. jejuni* after 24 hour incubation period (PF-Fruit extract-Poland; DF-Fruit extract-Denmark; DL1-Leaf extract-Denmark; DL2- Leaf extract -Denmark; GFL-Leaf+fruit extracts-Germany; and PL-Leaf extract-Poland).

Samples	**PF**			**DF**			**DL2**		
	Mass (mg)	MIC	TA	Mass (mg)	MIC	TA	Mass (mg)	MIC	TA
Fractions									
Hexane	220	0.61	361	200	0.61	328	230	0.61	377
CHCL3	120	0.08	1500	110	0.61	180	220	0.625	352
Butanol	110	2.5	44	120	2.5	48	120	2.5	48
Aq. MeOH	80	2.5	32	90	2.5	36	110	2.5	44
Gentamicin		0.02			0.02			0.02	

Samples	**GFL**			**DL1**			**PL**		
	Mass (mg)	MIC	TA	Mass (mg)	MIC	TA	Mass (mg)	MIC	TA
Fractions									
Hexane	360	0.61	590	330	0.61	541	140	0.61	230
CHCL3	220	0.31	710	140	0.31	452	160	0.31	516
Butanol	290	2.5	116	80	2.5	32	170	2.5	68
Aq. MeOH	120	2.5	48	120	2.5	48	130	2.5	52
Gentamicin		0.02			0.02			0.02	

5.3.1.2 Antifungal activity

Antifungal activity against A. fumigatus

In this chapter the plant samples tested had activity against the microorganisms used. The crude acetone extracts (Table 5.7) of trees growing in Denmark and Germany had moderate activity against *A. fumigatus* with MIC values of 0.16 mg/ml. . The activity of the remaining samples from trees growing in Denmark, Germany and Poland also had moderate activity with MIC in the range of 0.63 mg/ml. The solvent-solvent derived chloroform fractions had moderate activity with MIC values of 0.31 mg/ml and 0.63 mg/ml for extracts of trees growing in Germany, leaf extracts from trees growing in Denmark and fruit extract from trees growing in Poland respectively. The solvent-solvent derived butanol fraction also had moderate activity for fruit extracts of trees growing in Denmark and Leaf and fruit extract of trees growing in Germany (0.63 mg/ml). Solvent-solvent derived hexane and water-methanol fractions had MIC values of 2.5 mg/ml and greater indicating poor activity against *A. fumigatus* (Table 10).

Antifungal activity against C. albicans

Antifungal activity against *C. albicans* was excellent for crude acetone extracts (Table 5.7) of trees growing in Germany and leaf extracts of trees growing in Denmark (MIC of 0.02 mg/ml). The leaf extract from trees from the other region in Denmark had moderate activity (0.63 mg/ml). The

solvent-solvent derived chloroform fractions of trees growing in Denmark and Germany had moderate antifungal activity with MIC of 0.31. The rest of the solvent-solvent derived fractions all had poor activity with MIC values well above 1.25 mg/ml (Table 11). The activities of the samples tested were not as effective as that of the reference product amphotericin B but still indicative of moderate antimicrobial activity.

Table 5.7: The minimum inhibitory concentration (mg/ml) and total activity (TA) in ml of *Pterocarya fraxinifolia* trees growing in Denmark, Poland and Germany against *A. fumigatus* and *C. albicans* after 24 hour incubations period (PF-Fruit extract-Poland; DF-Fruit extract-Denmark; DL1-Leaf extract-Denmark; DL2- Leaf extract -Denmark; GFL-Leaf+fruit extracts-Germany; and PL-Leaf extract-Poland).

Plant ID	**Mass (mg)**	***A. fumigatus*(MIC)**	**TA (ml)**	***C. albicans*(MIC)**	**TA (ml)**
PF	230	0.63	365	2.5	92
DF	220	1.25	176	2.5	88
DL2	280	**0.16**	1750	0.63	444
GFL	260	**0.16**	1625	**0.02**	13000
DL1	300	0.63	476	**0.02**	15000
PL	240	0.63	1750	0.63	381

Table 5.8: The minimum inhibitory concentration (MIC-mg/ml) and total activity (TA) in ml of all the solvent-solvent derived fractions of *Pterocarya fraxinifolia* trees growing in different areas against A. fumigatus after 24 hour incubation period (PF-Fruit extract-Poland; DF-Fruit extract-Denmark; DL1-Leaf extract-Denmark; DL2- Leaf extract -Denmark; GFL-Leaf+fruit extracts-Germany; and PL-Leaf extract-Poland).

Samples	**PF**			**DF**			**DL2**		
	Mass (mg)	MIC	TA	Mass (mg)	MIC	TA	Mass (mg)	MIC	TA
Fractions									
Hexane	220	2.5	88	200	2.5	80	230	2.5	92
CHCL3	120	0.63	190	110	2.5	44	220	1.25	176
Butanol	110	2.5	44	120	0.63	190	120	2.5	48
Aq.MeOH	80	2.5	32	90	2.5	36	110	2.5	44
Samples	**GFL**			**DL1**			**PL**		
	Mass (mg)	MIC	TA	Mass (mg)	MIC	TA	Mass (mg)	MIC	TA
Fractions									
Hexane	360	2.5	144	330	2.5	132	140	2.5	56
CHCL3	220	0.31	710	140	0.31	452	160	2.5	64
Butanol	290	0.63	460	80	1.25	64	170	2.5	68
Aq.MeOH	120	2.5	48	120	2.5	48	130	2.5	52

Table 5.9: The minimum inhibitory concentration (MIC-mg/ml) and total activity (TA) in ml of all the solvent-solvent derived fractions of *Pterocarya fraxinifolia* trees growing in different areas against *C. albicans* after 24 hour incubation period (PF-Fruit extract-Poland; DF-Fruit extract-Denmark; DL1-Leaf extract-Denmark; DL2- Leaf extract -Denmark; GFL-Leaf+fruit extracts-Germany; and PL-Leaf extract-Poland).

Samples	**PF**			**DF**			**DL2**		
	Mass (mg)	MIC	TA	Mass (mg)	MIC	TA	Mass (mg)	MIC	TA
Fractions									
Hexane	220	2.5	88	200	2.5	80	230	2.5	92
CHCL3	120	2.5	48	110	2.5	44	220	0.31	710
Butanol	110	2.5	44	120	2.5	48	120	2.5	48
Aq.MeOH	80	2.5	32	90	2.5	36	110	2.5	44

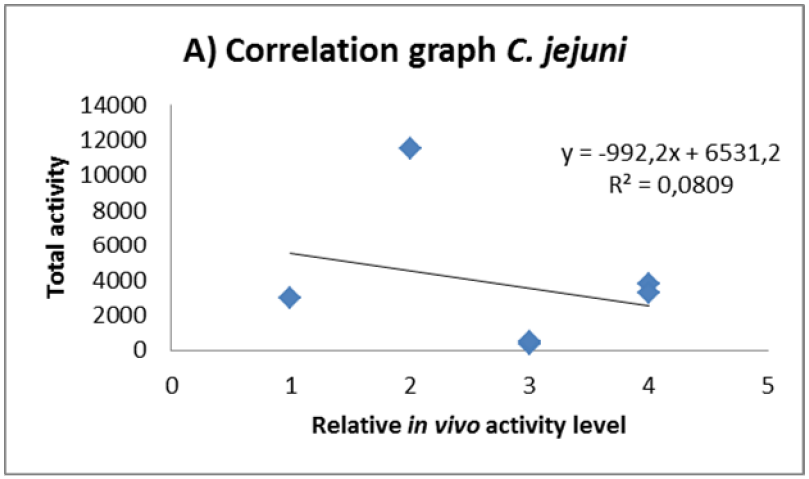
Samples	**GFL**			**DL1**			**PL**		
	Mass (mg)	MIC	TA	Mass (mg)	MIC	TA	Mass (mg)	MIC	TA
Fractions									
Hexane	360	2.5	144	330	2.5	132	140	2.5	56
CHCL3	220	0.31	710	140	0.63	222	160	2.5	64
Butanol	290	1.25	232	80	2.5	32	170	1.25	136
Aq.MeOH	120	2.5	48	120	2.5	48	130	2.5	52

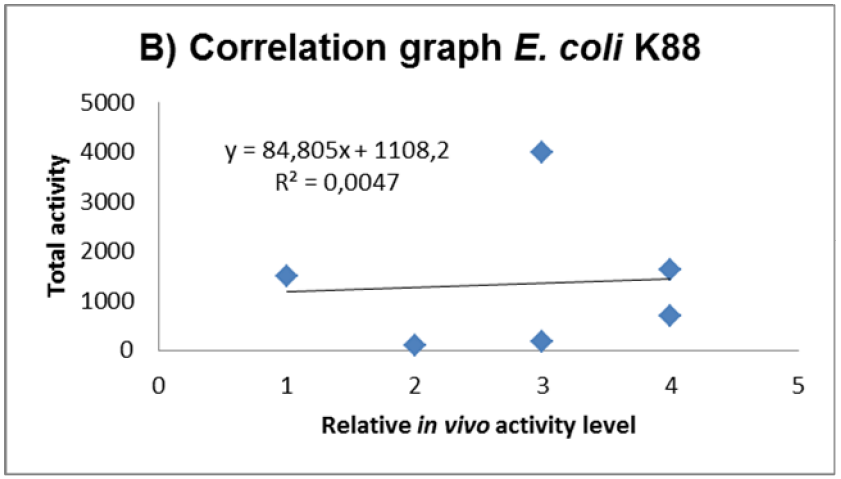
5.3.2 Total activity

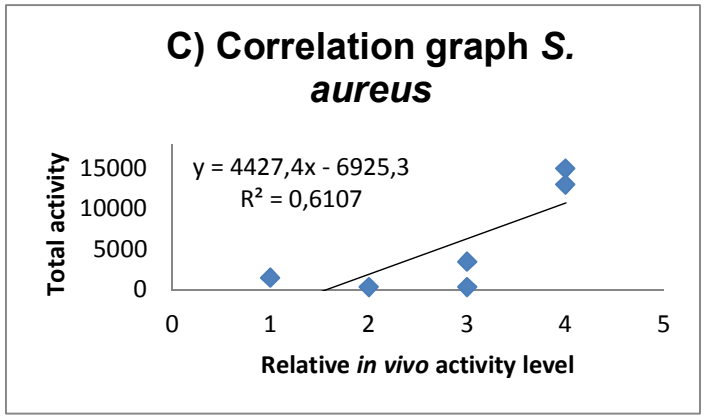
The total activity (TA) was calculated and presented in Table 5.10.There was good correlation between the total activity and the activity levels determined from the relative *in vivo* results, in the case of *C. jejuni* (R^2 = 0.0809) (Figure 5.1A). The correlation between total activity and the relative *in vivo* activity levels for the other organisms was good, *S. aureus* (R^2 = 0.6107), *E.coli* (k88) R^2 = 0.0047; *P. aeruginosa* R^2 = 0.2506. The crude extract of *P. fraxinifolia* trees growing in Denmark, Germany and Poland correlation on *C. perfringens* (R^2 = 0.1092) was good. The correlation coefficient of each of the microorganisms is presented in table 5.11. The total activity ranged from 32 to 15000 mg/ml. The highest total activity of 15000 ml/g was observed in the acetone crude extracts of leaf samples from trees growing in Denmark and leaf and fruit mixture samples from trees growing in Germany.

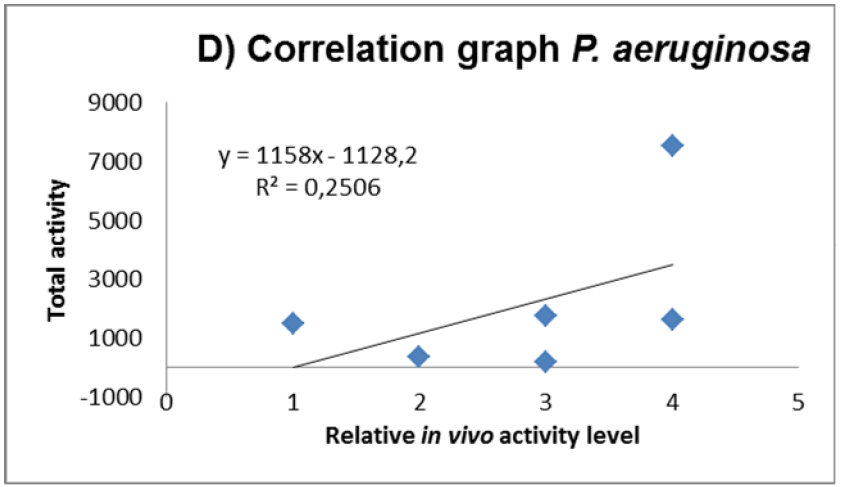
Table 5.10: The summary of the relative *in vivo* activity and total activity (ml) values of all pathogens used in this study. The sample *P. fraxinifolia* trees growing in Denmark and Germany show very promising results against bacterial and fungal pathogens. Total activity values show how many times a sample can be diluted and still have an effect on a pathogen

Plant	Relative *in vivo* activity	Total Activity (Bacteria)					Total Activity (Fungi)	
		***E. coli* K88**	***S. aureus***	***P. aeruginosa***	***C. jejuni***	***C. perfringens***	***A. fumigatus***	***C. albicans***
PF	2	92	365	365	11500	11500	365	92
DF	3	176	349	176	361	361	176	88
DL2	3	4000	3500	1750	459	3500	1750	444
GFL	4	1625	13000	1625	3250	426	1625	13000
DL1	4	698	15000	7500	3750	492	476	15000
PL	1	1500	1500	1500	3000	393	1750	381









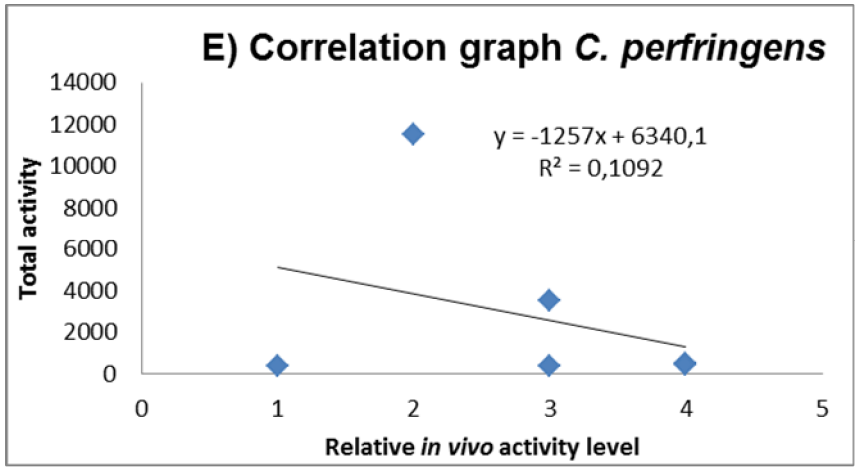
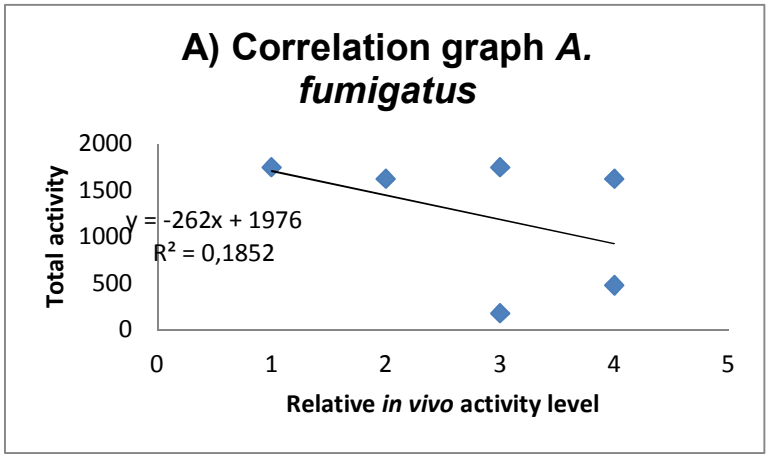


Figure 5.1: The correlation graphs of relative *in vivo* activity levels and total activity of the crude acetone extract of *P. fraxinifolia* from trees growing in different areas against A) *C. jejuni*; B) *E. coli* K88; C) *S. aureus*; D) *P. aeruginosa*; and E) *C. perfringens*. The extracts were active against all pathogens with *E. coli* K88, *C. perfringens* and *C. jejuni* having the most pronounced activity.

Table 5.11: The correlation coefficient between the relative *in vivo* activity and total activity. The extracts were very active against *E. coli* K88 and *C. jejuni*. The extracts had moderate activity against the rest of the pathogens used in this study (* values are the square root of the R2 values)

Pathogens	R2 value	Correlation coefficient*
***E. coli* K88**	0.0047	0.069
S. aureus	0.6107	0.781
P. aeruginosa	0.2506	0.501
C. jejuni	0.0809	0.284
C. perfringens	0.1092	0.331
A. fumigatus	0.1852	0.430
C. albicans	0.5889	0.767



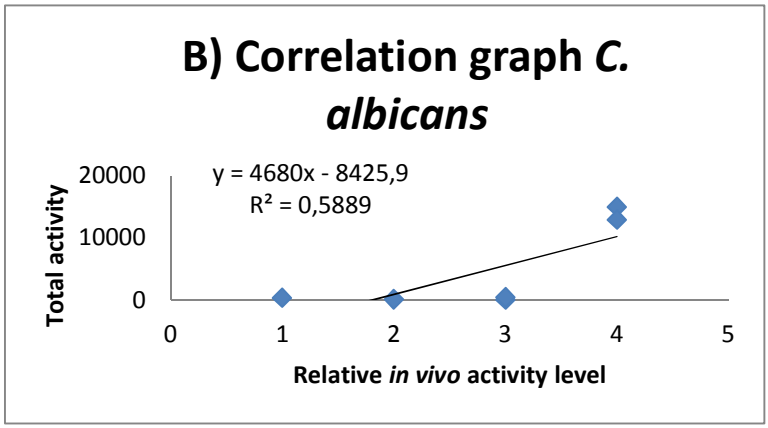


Figure 5.2: The correlation graphs of relative *in vivo* activity level and total activity of the crude acetone extract of *P. fraxinifolia* from trees growing in different areas against A) *A. fumigatus* and B) *C. albicans*. The fungal pathogen *A. fumigatus* had better activity compared to *C. albicans* but overall activity was moderate.

5.4 Discussion and conclusion

The microtitre plate assay was used to quantify the levels of activity of the plant extract from the different areas by determining their minimum inhibitory concentration (MIC) values. Previously there were limited validated criteria for MIC end point values for *in vitro* testing of plant extracts. Eloff, (2001), Rios *et* al, (1988) and Holetz *et al* (2002) have all proposed a system to try and validate MIC values. They proposed that MIC less than 0.1 mg/ml is excellent to good antimicrobial activity, good activity was said to be between 0.1 and 0.6 mg/ml and moderate antimicrobial activity was between 0.6 and 1 mg/ml. MIC value greater than 1 mg/ml was considered poor activity.

Recently Pauw and Eloff (2014) investigated the MICs of extracts of more than 530 plants and proposed the following 8 groups: < 0.03 mg/ml; 0.03 to 0.04 mg/ml; 0.05 to 0.08 mg/ml; 0.09 to 0.16 mg/ml; 0.17 to 0.31 mg/ml; 0.32 to 0.63 mg/ml; 0.64 to 1.25 mg/ml and >1.26 mg/ml. MICs higher than 0.16 mg/ml were considered inactive. There was generally moderate or poor antimicrobial activity of extracts of *P. fraxinifolia* trees growing in different areas on Gram-positive bacteria compared to better activity observed on Gram-negative bacteria (Table 5.10).

The crude acetone plant extracts from trees growing in Denmark and Germany had good to moderate activity against the Gram-positive and Gram-negative bacteria. The solvent-solvent derived hexane and chloroform fraction from trees growing in Denmark and Germany varied in activity against both Gram- negative and Gram-positive bacteria.

Correlation between relative *in vivo* activity and total antimicrobial activity

To determine if the total antimicrobial activity of extracts could be responsible for the *in vivo* activity the correlations between activity against different microorganisms and *in vivo* activity was determined (Figures 5.1.and 5.2). In some cases (*C. jejuni, E.coli* K88, *C. perfringens* and *A.fumigatus*) there was in inverse relationship i.e. higher total antimicrobial activity was associated with lower *in vivo* activity.

This clearly indicates that activity against these pathogens could not have been responsible for the *in vivo* activity. In two cases, activity against *S. aureus* (R2 = 0.611) and *C. albicans* (R2 = 0.589) there was a reasonable correlation between total antimicrobial activity and *in vivo* activity. It is possible that activity against one of these pathogens could explain the in vivo activity found, but it is unlikely because these pathogens are not generally considered as being responsible for negative effects that could be countered by the addition of antibiotic feed additives.

Post script

The antimicrobial screening indicated that the plant extracts from the different regions have a generally moderate activity in the crude and solvent-solvent fractions. Only with two pathogens *S. aureus* and *C. albicans* were there good correlations between total antimicrobial activity and in *vivo activity*. Before an attempt to isolate the antimicrobial compounds present in the extracts or fractions it is important to determine the number of compounds present and also the R_f values to facilitate the

Chapter 6

Bioautography of crude acetone and different fractions of *P. fraxinifolia*

Preface

In addition to the preliminary screening, antioxidant, anti-inflammatory activity, the antimicrobial activity of the plant extract from different regions in Europe had generally moderate activity as an antimicrobial agent. It is important to identify the number of compounds present in the extracts that indicated promising activity against the pathogens evaluated. In some cases the activity is due to the presence of a mixture of compounds and these compounds must be separated and evaluated by bioautography. Bioautography is there an important step in selecting the extract with the best activity and also makes it easier to isolate the active compounds.

6.1 Introduction

The use of medicinal plants as a source of relief from aliments can traced back to written documents of the early civilizations in China, India and Asia (Hamburger & Hostettmann, 1991). The industrial revolution and the development of organic chemistry resulted in the use and preference of synthetic products for pharmacological treatments (Rates, 2001). There were many reasons why synthetic compounds where chosen during the industrial revolution, one been that they were easily available or obtained and structural modifications to produce potentially more potent, active and safe drugs could be easily performed (Rates, 2001).

The quality control of synthetic compounds is much easier and they may be produced at a lower cost that herbal medicines in some cases. In industrialised countries medicinal plants have had its ups and downs during the past few decades. Centuries of empirical use of herbal preparations have gained momentum and this was solidified by the early discoveries and isolation of active principles such as alkaloids, morphine and quinine amongst others in the early 19th century (Hamburger & Hostettmann, 1991).

Over the last decade interest in drugs of plant origin has been growing steadily. It has been established that about 25% of the drugs prescribed worldwide come from plants. According to the World Health Organization (WHO) 11% of the 252 drugs considered as basic and essential are exclusively of plant origin (Hamburger & Hostettmann, 1991 & Rates, 2001). Despite the enormity of plants as potential source of drugs it is estimated that of the 250 000 plus plant species only a small group has been investigated properly in terms of their pharmacological properties (Payne et al, 1991).

The research into the therapeutic materials from plant origin is difficult and expensive and it has been estimated that each new drug requires investments hundreds of millions of dollars and a minimum of ten years of work (Rates, 2001). There is basic science involved and these cover fields in botany, chemistry and pharmacology but other paths of knowledge are also involved (Rates, 2001). The approach of drug development from plant resources depends on the aim and different strategies will result in herbal medicine or isolated active compounds and thus the selection of a suitable plant for pharmacological study is important (Soejarto, 1996).

When screening for biologically active plant constituents the selection of the plant species is crucial. The discovery of promising plant extracts and the subsequent activity guided isolation put specific requirements on the bioassay to be used for this purpose (Hamburger & Hostettmann, 1991). There are certain consideration that need to be taken into account when setting up bioassays to evaluate plant extracts and obtaining the active compounds, these include target, sample throughput, selectivity and sensitivity and cost amongst other things (Hamburger & Hostettmann, 1991).

To obtain the active compound, the plant extracts were subjected to qualitative analysis on TLC, using bioautograpgy and screened to determine the biological activity or to obtain a general evaluation of biological activity (Rates, 2001). The biological activity is determined through various bioassay guided steps using microorganisms, cell cultures and other ways (Hamburger & Hostettmann, 1991). All these methods have their advantages and disadvantages and the appropriate method and steps must be chosen at each point for any biological study aimed at the development of a drug (Rates, 2001).

Bioautography is a method that combines TLC with a bioassay in situ and allows for the localization of active constituents in a complex matrix. It has been said that it is the most efficient method for activity guided isolation. Bioautography can be considered as a highly efficacious assay for the detection of antimicrobial compounds because it permits a target-directed isolation of the active constituents (Rahalison *et al.*, 1991). Bioautography has enabled rapid progress for quick detection of new antimicrobial compounds from plants and other natural products. This technique allows the localization of antimicrobial activity directly on a chromatographic plate where the organism is applied (Navarro *et al* 1998).

A number of bioautographic assays have been developed, which can be divided into three groups (Rios *et al.*, 1988). These include direct bioautography; where the microorganisms grow directly on thin-layer chromatography (TLC) plates, contact bioautography; where the antimicrobial compounds

are transferred from the TLC plate to an inoculated agar plate through direct contact, and agar overlay or immersion bioautography; where a seeded agar medium is applied onto the TLC plate. Since then bioautography has been developed to such a level that only direct bioautography has been used by many authors (Masoko *et al*, 2005).

The INT reaction is based on the transfer of electrons from NADH to the tetrazolium dye. Threonine dehydrogenase from bacteria catalyses the NAD-dependent oxidation of threonine to form a 2-amino-3-ketobutyrate and NADH cascade. During active bacterial growth, an electron is transferred from the NADH molecule to INT forming a purple-red colour. The clear areas on the chromatograms indicate areas of inhibition (Begue & Kline, 1972).

6.2 Materials and methods

6.2.1 Plant collection and preparation

See chapter three and four for detailed outline of the plant collection and preparation

6.2.2 Bioautography

Bioautography is a rapid bioassay guided method for the determination of antimicrobial compounds from crude extracts and fractions. The activity of the plant extract against microorganisms is assayed and determined on chromatograms. This method was developed by Begue and Kline (1972). Initially it only worked well for bacteria, but Masoko *et al*, (2005), has developed it for fungi as well. TLC plates were loaded with 10 µl of a 10 mg/ml extract and developed in three different mobile systems (BEA, CEF and EMW). The developed plates were left under a fan to dry and remove all traces of solvent on the plates. The dried plates were sprayed with a suspension of actively growing cells of microorganisms, expect for the *C. jejuni* and *C. perfringens* (due to potential contamination of the laboratory and safety reasons). The plates were incubated in a sealed sterile plastic container at 37 ºC for 18 hours at 100% relative humidity. The plates were then sprayed with a solution of p-iodonitrotetrazolium (INT) violet (2 mg/ml in water) and further incubated overnight for bacteria and longer for fungi for the colour to develop. White spots or areas on the purple background were indicative of INT reduction due to the presence of compounds that inhibited the growth of tested organisms.

6.2.3 Bacterial test species

In this study three bacterial test microorganisms were used, namely: *Staphylococcus aureus* (Gram-positive) [American Type Culture Collection [ATCC] number 29213), [ATCC 27853], Escherichia *coli* (Gram-negative, ATCC 25922) and *Escherichia coli* (K88). These bacterial strains are important nosocomial pathogens, which are recommended as test strains for comparing the activity of

antibiotics (NCCLS, 1992). Bacterial test cultures were kept on Müller-Hilton (MH) agar at a temperature of 4 °C. The bacterial cells were inoculated into fresh MH broth and incubated at 37 °C for 14 h, prior to the antibacterial assay. The densities of bacterial cultures after incubation overnight were as follows: *Staphylococcus aureus*, 2.6×10^{12} cfu/ml and *Escherichia coli*, 3.0×10^{11} cfu/ml.

6.2.4 Fungal test species

The fungal organisms used in this study were moulds (*Aspergillus fumigatus*) and yeast (*Candida albicans*). Both fungal organisms were isolated from clinical cases that were not treated prior to sampling in the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria. *A. fumigatus* was isolated from a chicken and *C. albicans* from a Gordian finch. These fungi represent the most common and important disease-causing fungi of animals (Masoko *et al*., 2005). Sabouraud dextrose (SD) agar (Oxoid, Basingstoke, UK) was used as the maintenance growth medium for all the fungal strains used, and the fungi were cultured in SD broth.

6.3 Results

6.3.1 Bacterial and fungal bioautography

The appearance of white areas against a purple-red background on the chromatograms denotes inhibition of growth of the bacteria or fungi due to the presence of compounds that inhibit their growth. Actively growing microorganisms have the ability to reduce INT to a purple-red colour. The bioautography of the crude acetone extracts and solvent-solvent derived fractions are shown in figures 6.1 to 6.5 for the crude acetone extracts and solvent-solvent derived fractions. The crude acetone extracts and solvent-solvent derived fractions all contained compounds that inhibit the growth of Gram-positive bacteria *S. aureus* as indicated on the TLC bioautograms (Fig. 6.1).

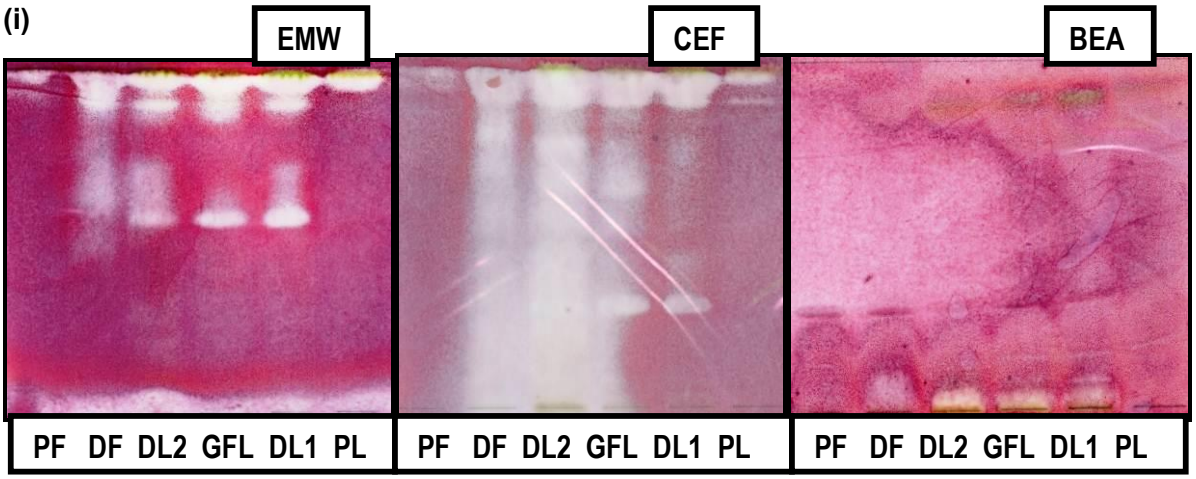
There were a number of separated compounds active against Gram-negative *E.coli* isolated from chickens. The activity of separated compounds in the crude acetone extract and solvent-solvent derived fractions where not as clear and appeared very faint (Fig. 6.2). The extracts from trees growing in Germany (GFL) and Denmark (DF and DL1) contained many antibacterial compounds. . The TLC bioautograms also indicated the Rf values of compounds that where not present in extracts from trees growing in other areas when compared with extracts from trees growing in Denmark and Germany (Fig. 6.3).

Anti-fungal compounds were observed on the TLC bioautograms in extracts from trees growing in Denmark and Germany and one of the extracts from trees growing in Poland (PF) in the solvent-solvent derived butanol and chloroform fractions (Figs. 6.4 & 6.5). In some cases inhibition of

bacterial and fungal growth was not observed for extracts of trees growing in Denmark, Germany and Poland.

The relative front (R_f) values were calculated for each active spot and the results tabulated in tables 6.1, 6.2, 6.3, 6.4 and 6.5. The R_f value is the ratio of the distance moved by the compound from its origin in response to the movement of the solvent system from the bottom of the plate and is constant under similar TLC conditions.

(i)



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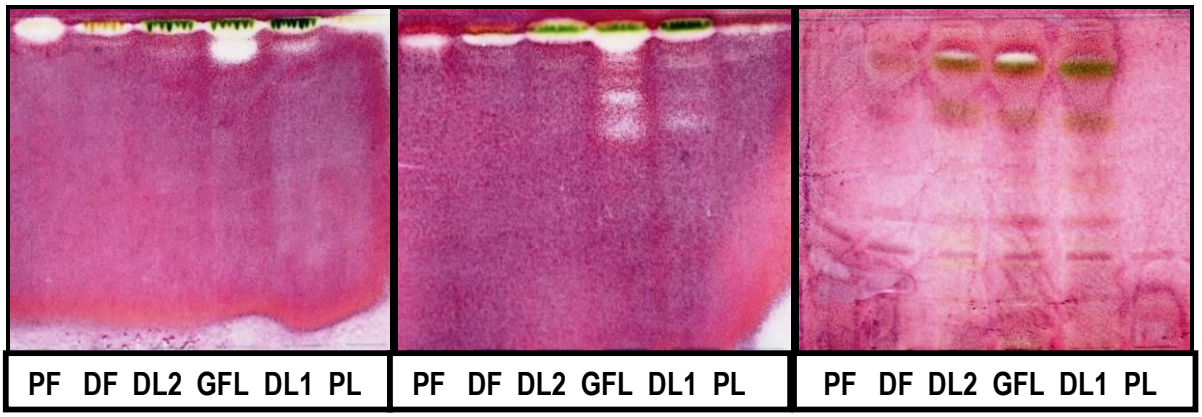

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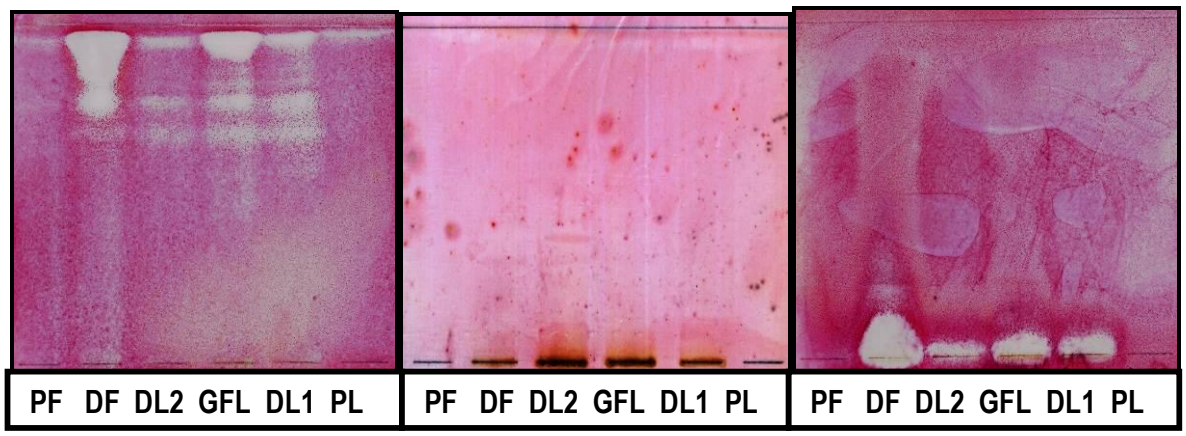

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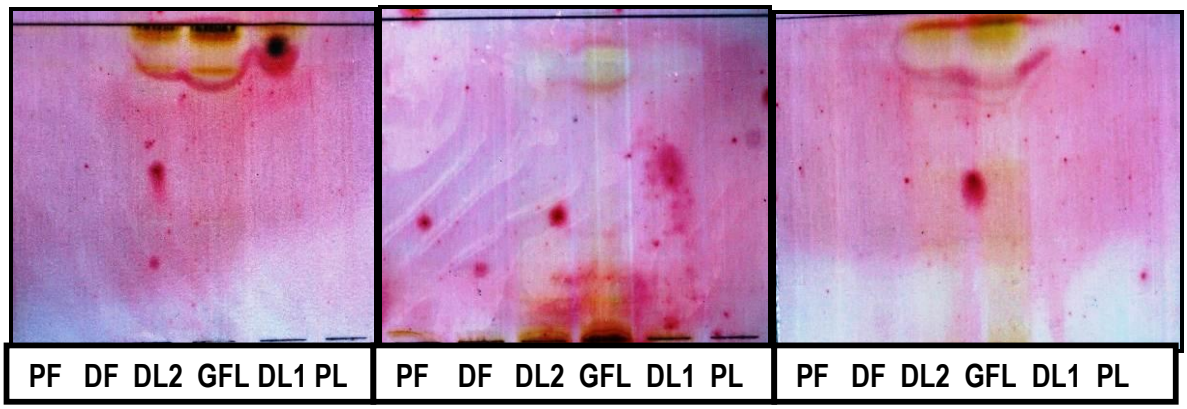

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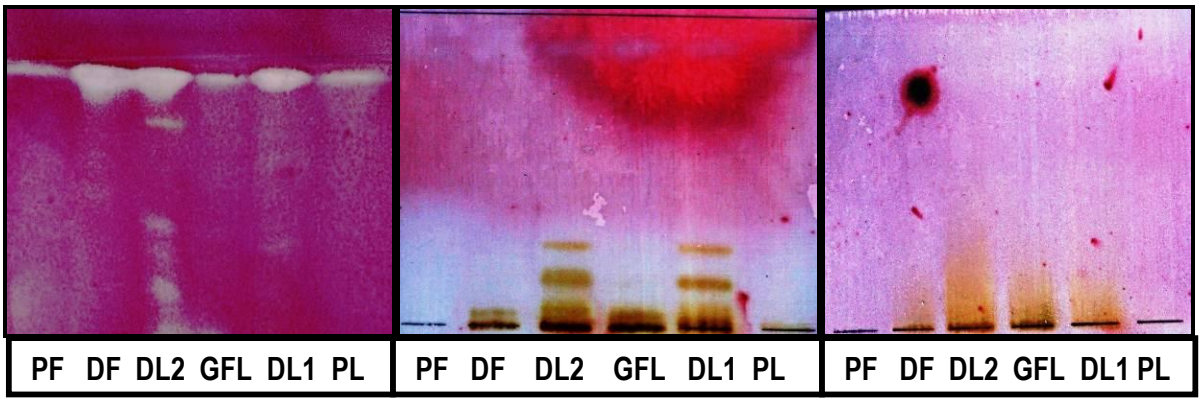


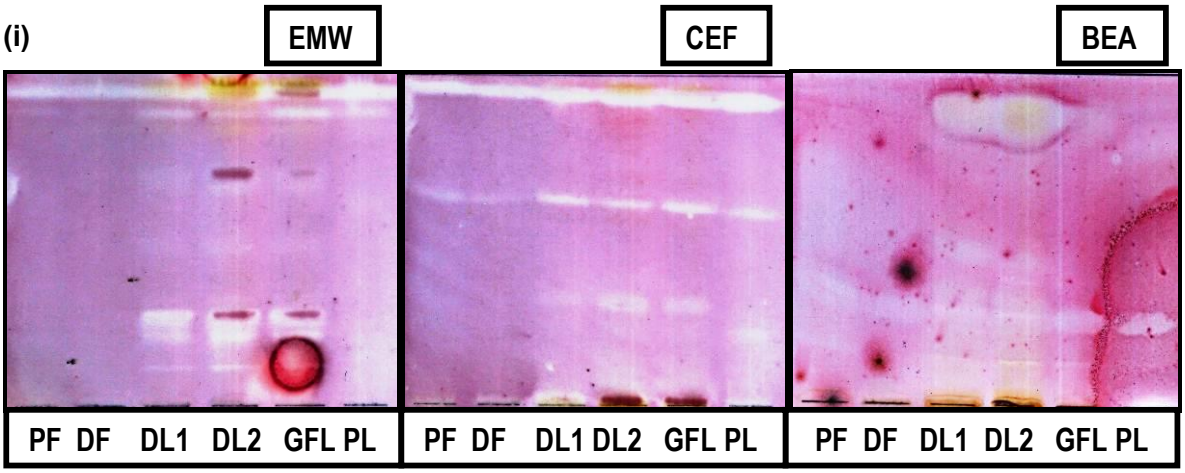
Figure 6.1: Bioautography chromatograms of *P. fraxinifolia* trees growing in different areas in Denmark, Germany and Poland. (i) Acetone crude extracts and solvent-solvent derived fractions{ (ii) Hexane, (iii) Water-methanol, (iv) chloroform and (v) butanol} treated with *S. aureus* culture incubated overnight and sprayed with INT. Growth inhibition is indicated by colourless (white) zones. TLC plates where eluted in EMW, CEF & BEA solvent systems. (PF-Fruit extract-Poland; DF-Fruit extract-Denmark; DL1-Leaf extract-Denmark; DL2- Leaf extract -Denmark; GFL-Leaf+fruit extracts-Germany; and PL-Leaf extract-Poland).

Table 6.1: Inhibition of growth on bioautography TLC plates of plant extracts of *P. fraxinifolia* trees growing in different in areas against *S. aureus*

Plant	Solvent system	Solvent	Rf values	Active bands
PF	CEF	acetone	0.97, 0.97	1
	BEA	hexane	0.97, 0.97	1
	EMW	chloroform	0.97	1
		butanol	0.97	1
		water-methanol		0
		Total number of different active bands according to Rf values		**1**
DF	CEF	acetone	0.97, 0.88, 0.75	3
	BEA	hexane	0.97, 0.88,	2
	EMW	chloroform	0.97, 0.88, 0.80	3
		butanol	0.97, 0.88	2
		water-methanol	0.97. 0.80, 0.74, 0.05,	4
		Total number of different active bands according to Rf values		**5**
DL1	CEF	acetone	0.97, 0.80, 0.75, 0.56, 0.05	5
	BEA	hexane	0.97, 0.80	2
	EMW	chloroform	0.97, 0.88, 0.74	3
		butanol	0.97, 0.80, 0.74	3
		water-methanol	0.97. 0.80, 0.74, 0.05	4
		Total number of different active bands according to Rf values		**6**
DL2	CEF	acetone	0.97, 0.74, 0.88, 0.56	4
	BEA	hexane	0.56, 0.75, 0.97, 0.80	4
	EMW	chloroform	0.74, 0.97, 0.88, 0.80	4
		butanol	0.97	1
		water-methanol	0.97. 0.80, 0.05, 0.74	4
		Total number of different active bands according to Rf values		**5**
GFL	CEF	acetone	0.97, 0.88, 0.05, 0.26, 0.56	5
	BEA	hexane	0.05, 0.97, 0.88	3
	EMW	chloroform	0.97, 0.88, 0.80	3
		butanol	0.97	1
		water-methanol	0.97. 0.80, 0.05, 0.74	4
		Total number of different active bands according to Rf values		**6**
PL	CEF	acetone	0.97, 0.88	2
	BEA	hexane		0
	EMW	chloroform	0.97	1
		butanol	0.97	1

		water-methanol	0.97	1
		Total number of different active bands according to Rf values		**2**

(i)



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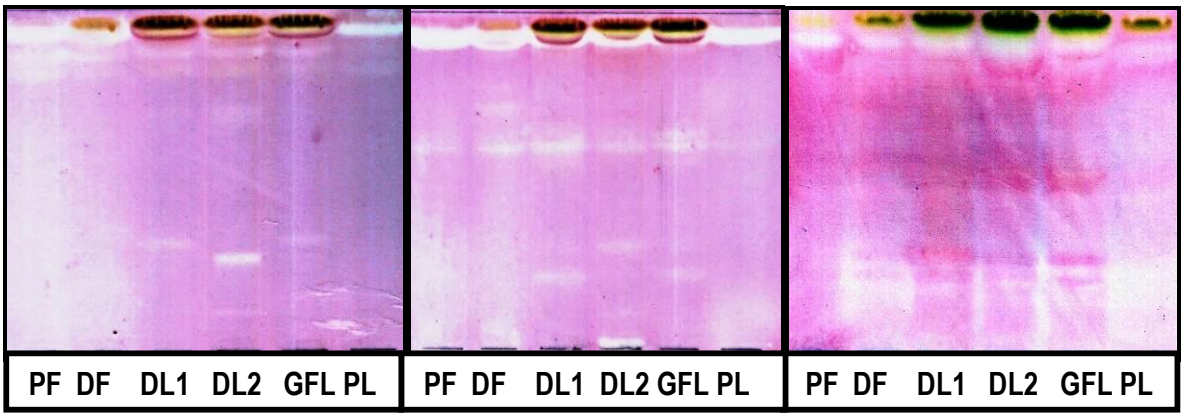

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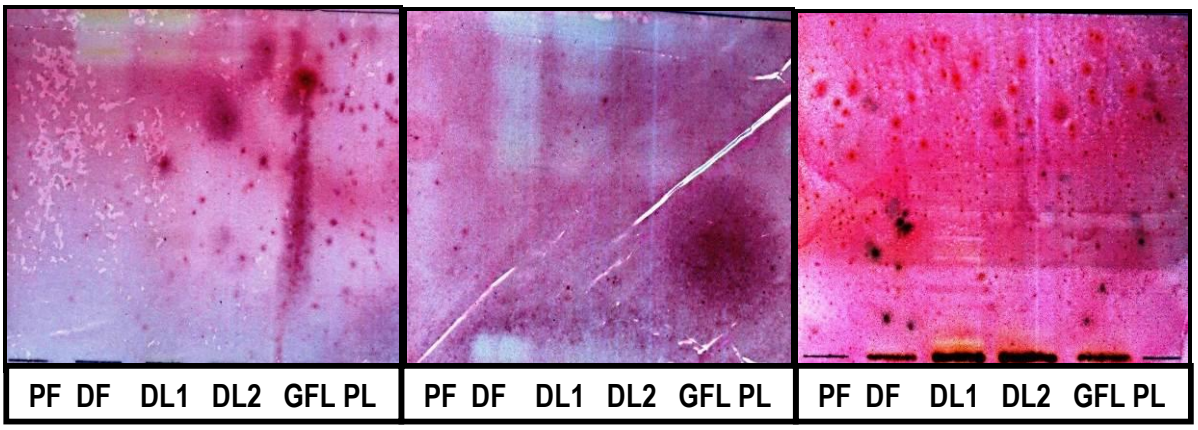

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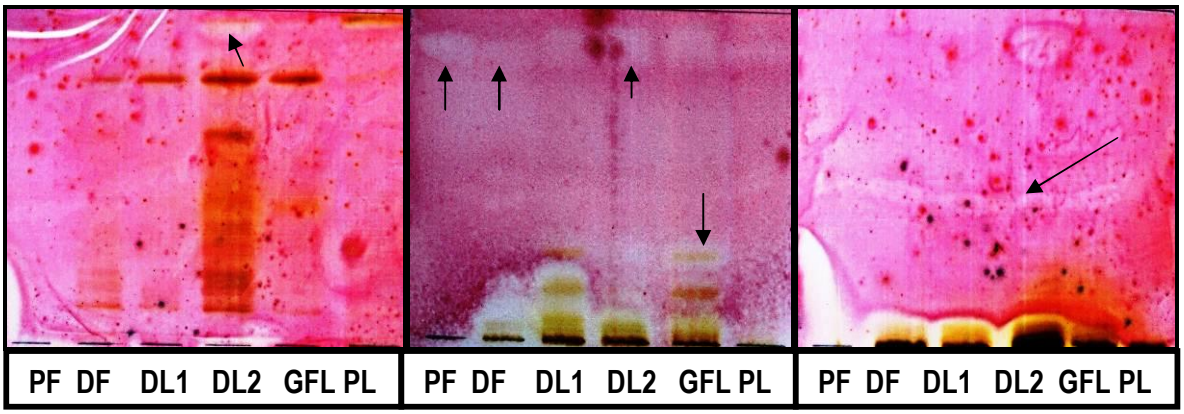

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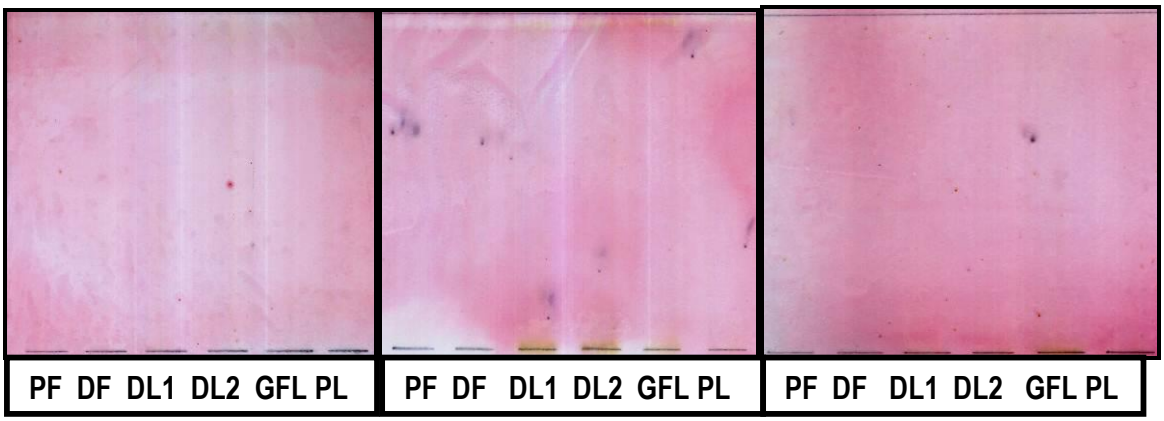
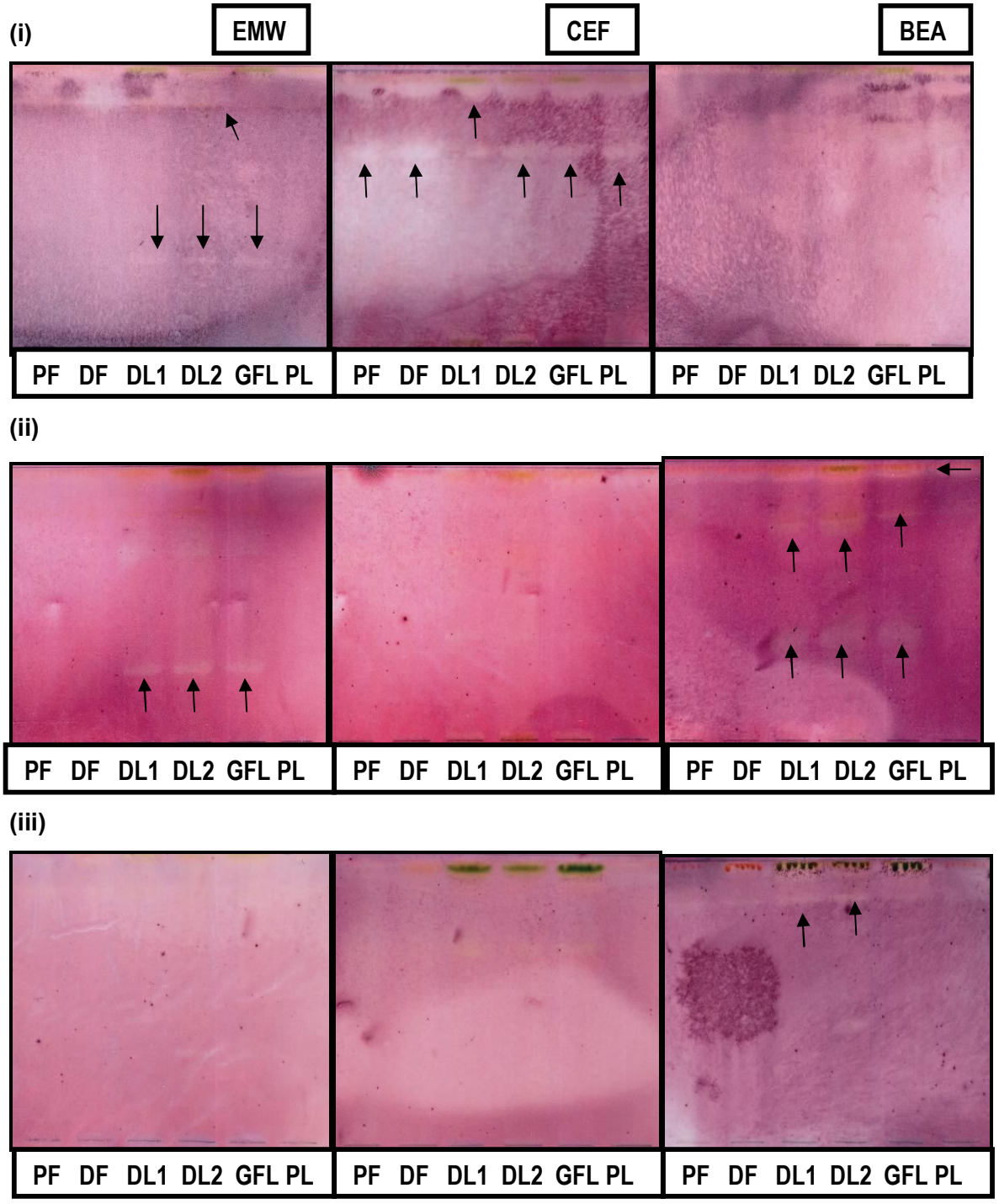


Figure 6.2: Bioautography chromatograms of *P. fraxinifolia* trees growing in different areas in Denmark, Germany and Poland. The solvent-solvent derived fractions{ (i) chloroform, (ii) hexane, (iii) water-methanol, (iv) butanol} and (v) acetone crude extract treated with *E.coli* (chicken isolate-ATCC 25922) culture incubated overnight and sprayed with INT. Growth inhibition is indicated by colourless (white) zones. TLC plates where eluted in EMW, CEF & BEA solvent systems (PF-Fruit extract-Poland; DF-Fruit extract-Denmark; DL1-Leaf extract-Denmark; DL2- Leaf extract -Denmark; GFL-Leaf+fruit extracts-Germany; and PL-Leaf extract-Poland).

Table 6.2: Inhibition of growth on bioautography TLC plates of plant extracts of *P. fraxinifolia* trees growing in different areas against *E.coli* (ATCC 25922)

Plant	Solvent system	Solvent	Rf values	Active bands
PF	CEF	acetone		0
	BEA	hexane	0.97	1
	EMW	chloroform	0.97	1
		butanol		0
		water-methanol		0
		Total number of different active bands according to Rf values		**1**
DF	CEF	acetone		0
	BEA	hexane	0.97	1
	EMW	chloroform	0.97, 0.61	2
		butanol		0
		water-methanol	0.97, 0.61, 0.31	3
		Total number of different active bands according to Rf values		**3**
DL1	CEF	acetone		0
	BEA	hexane	0.97, 0.61, 0.31	3
	EMW	chloroform	0.97, 0.61, 0.31, 0.22	4
		butanol		0
		water-methanol	0.97, 0.61, 0.31	3
		Total number of different active bands according to Rf values		**4**
DL2	CEF	acetone		0
	BEA	hexane	0.97, 0.61, 0.31	3
	EMW	chloroform	0.97, 0.61, 0.22, 0.31	4
		butanol	0.97, 0.61	2
		water-methanol	0.97, 0.61	2
		Total number of different active bands according to Rf values		**4**
GFL	CEF	acetone		0
	BEA	hexane	0.97, 0.61, 0.31	3
	EMW	chloroform	0.97, 0.61, 0.22, 0.31	4
		butanol	0.97, 0.22	2
		water-methanol		0
		Total number of different active bands according to Rf values		**4**
PL	CEF	acetone		0
	BEA	hexane	0.97	1
	EMW	chloroform	0.97, 0.61	2
		butanol		0

		water-methanol		0
		Total number of different active bands according to Rf values		**2**



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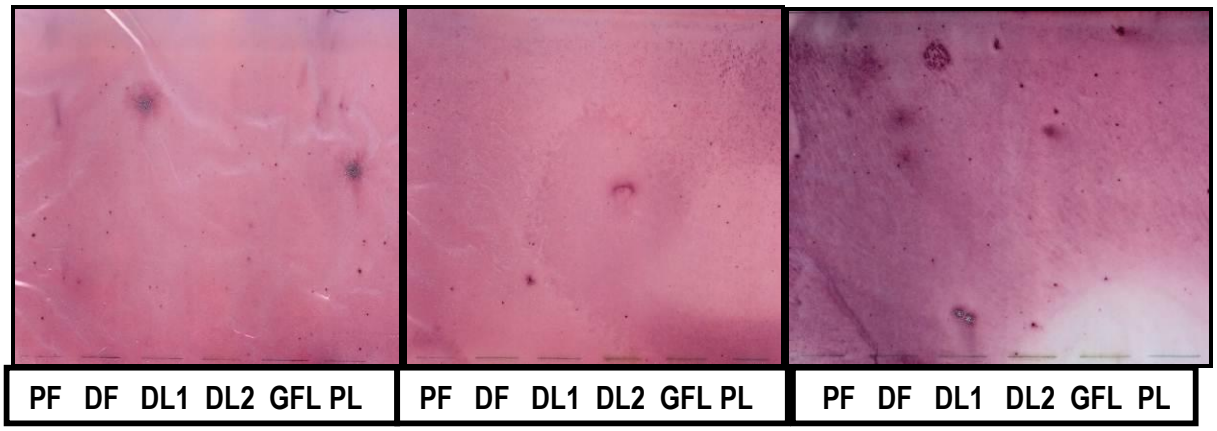

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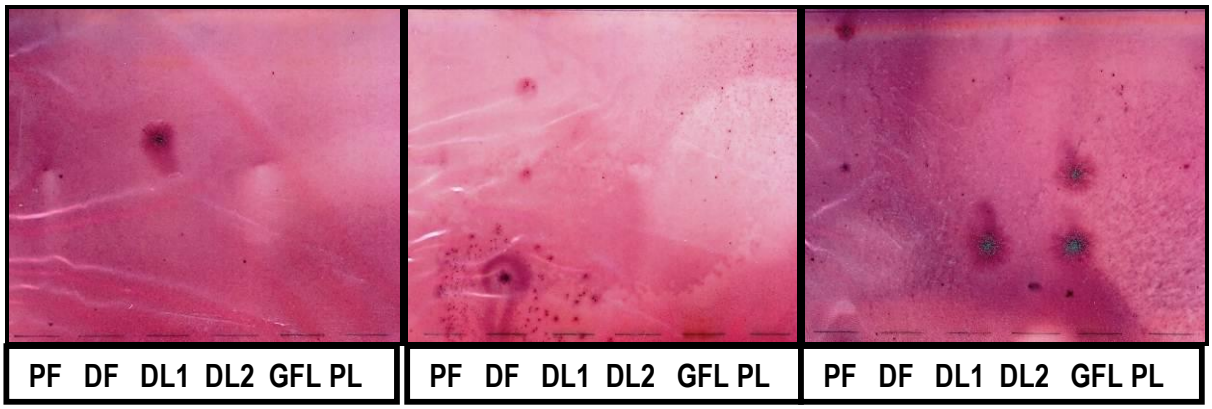


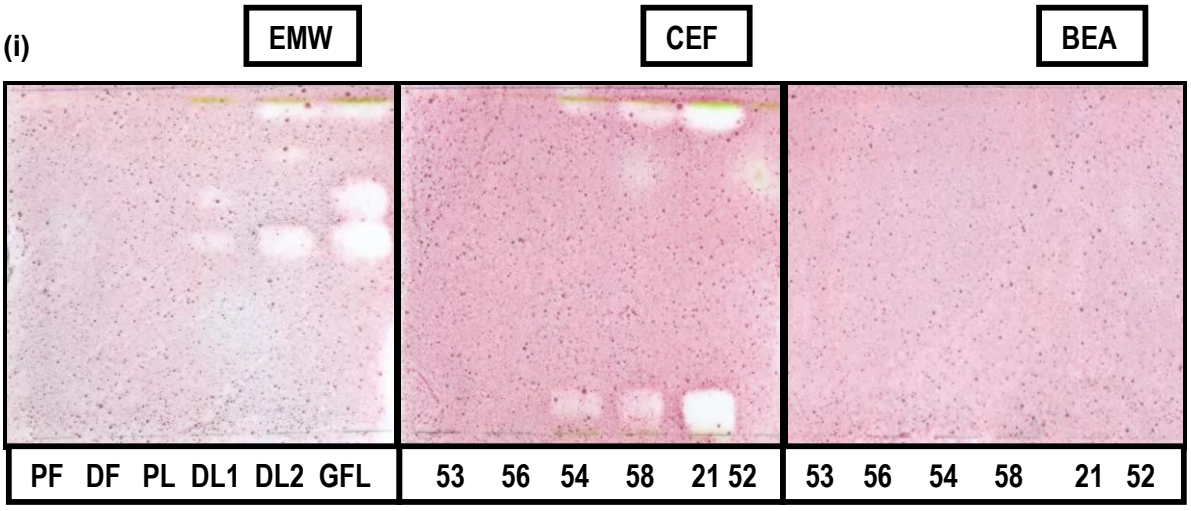
Figure 6.3: Bioautography chromatograms of *P. fraxinifolia* trees growing in different areas in Denmark, Germany and Poland. (i) Acetone crude extract and solvent-solvent derived fractions{ (ii) chloroform, (iii) hexane, (iv) water-methanol, (v) butanol} treated with *E.coli* (k88 isolate) culture incubated overnight and sprayed with INT. Growth inhibition is indicated by colourless (white) zones indicated by arrows. TLC plates where eluted in EMW, CEF & BEA solvent systems (PF-Fruit extract-Poland; DF-Fruit extract-Denmark; DL1-Leaf extract-Denmark; DL2- Leaf extract -Denmark; GFL-Leaf+fruit extracts-Germany; and PL-Leaf extract-Poland).

Table 6.3: Inhibition of growth on bioautography TLC plates of plant extracts of *P. fraxinifolia* trees growing in different areas against *E.coli* (K88)

Plant	Solvent System	Solvent	Rf values	Active bands
PF	CEF	acetone	0.97	1
	BEA	hexane		0
	EMW	chloroform	0.97	1
		butanol		0
		water-methanol		0
		Total number of different active bands according to Rf values		**1**
DF	CEF	acetone	0.97, 0.88	2
	BEA	hexane		0
	EMW	chloroform	0.97	1
		butanol		0
		water-methanol		0
		Total number of different active bands according to Rf values		**2**
DL1	CEF	acetone	0.97, 0.88, 0.22	3
	BEA	hexane	0.88	1
	EMW	chloroform	0.97, 0.77, 0.22	3
		butanol		0
		water-methanol		0
		Total number of different active bands according to Rf values		**4**
DL2	CEF	acetone	0.97, 0.88, 0.22	3
	BEA	hexane	0.88	1
	EMW	chloroform	0.97, 0.77, 0.22	3
		butanol		0
		water-methanol		0
		Total number of different active bands according to Rf values		**4**
GFL	CEF	acetone	0.97, 0.80, 0.22	3
	BEA	hexane		0
	EMW	chloroform	0.97, 0.88, 0.22	3
		butanol		0
		water-methanol		0
		Total number of differnt active bands according to Rf values		**3**
PL	CEF	acetone	0.97, 0.88	2
	BEA	hexane		0

	EMW	chloroform		0
		butanol		0
		water-methanol		0
		Total number of different active bands according to Rf values		**2**

(i)



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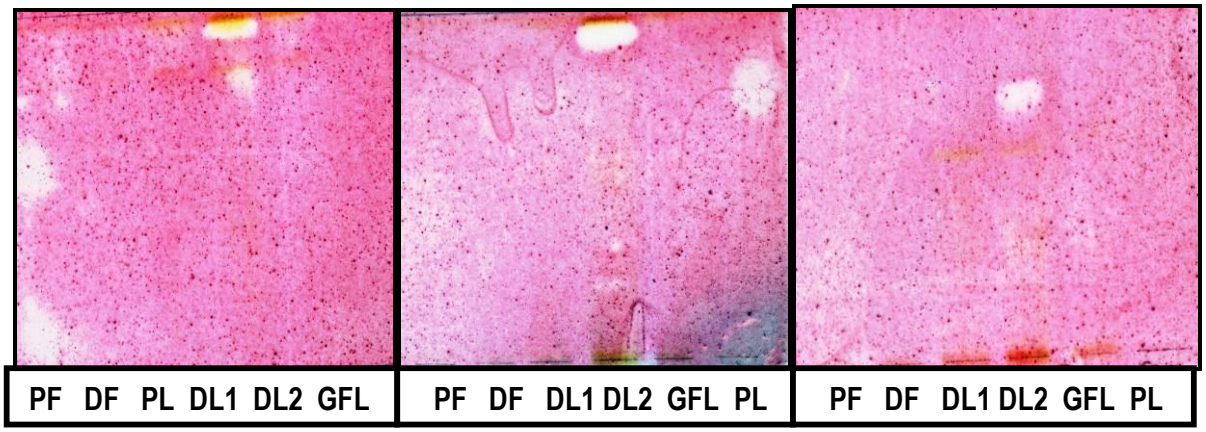

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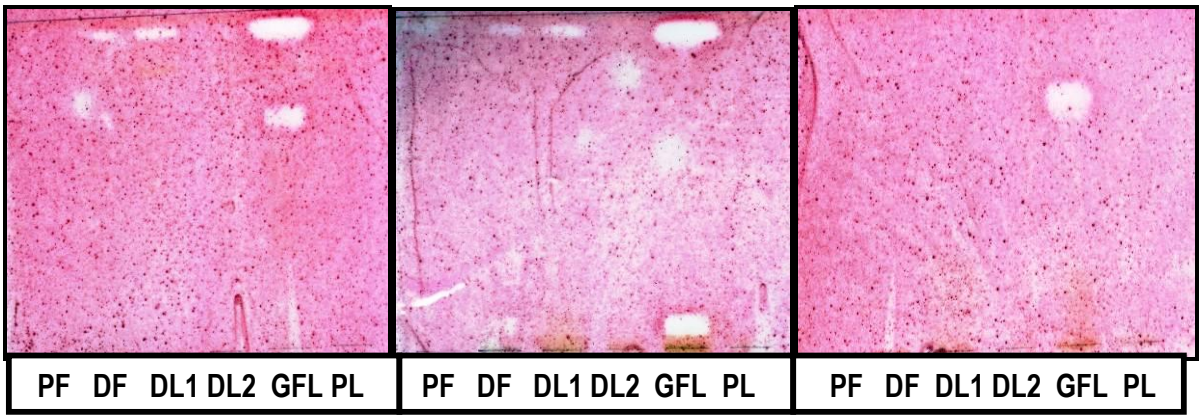

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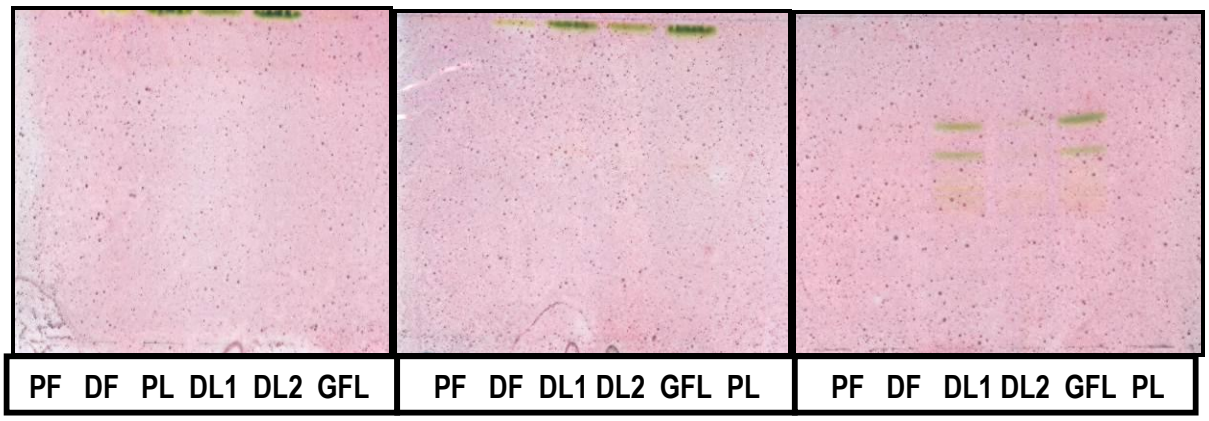

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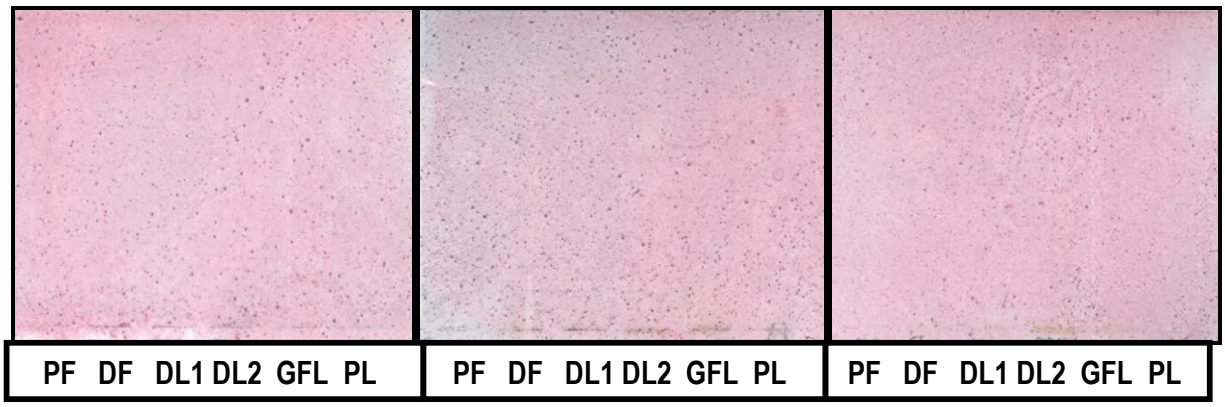


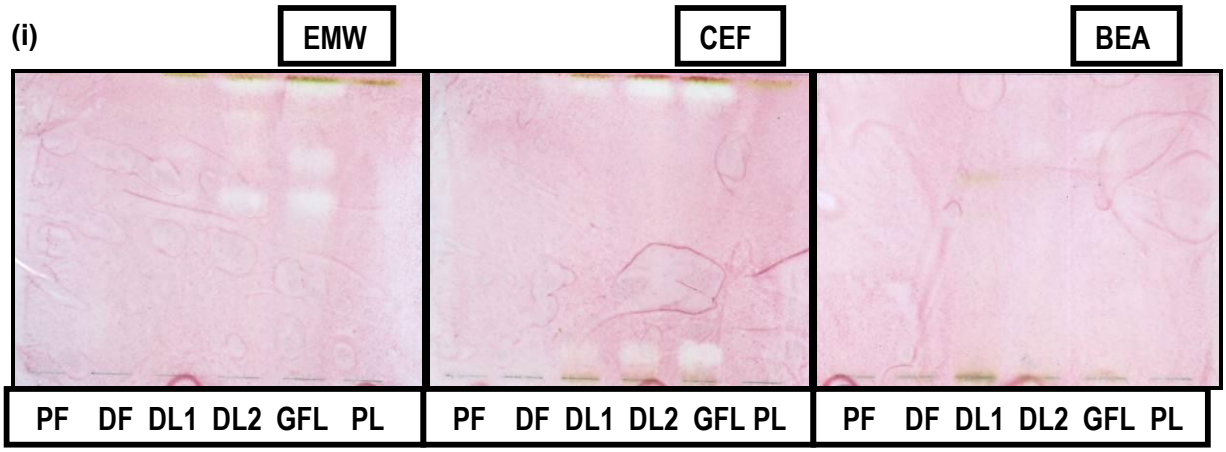
Figure 6.4: Bioautography chromatograms of *P. fraxinifolia* trees growing in different areas in Denmark, Germany and Poland. (i) Acetone crude extract and solvent-solvent derived fractions{ (ii) chloroform, (iii) butanol, (iv) hexane, (v) water-methanol} treated with *A. fumigatus* culture incubated overnight and sprayed with INT. Growth inhibition is indicated by colourless (white) zones indicated by arrows. TLC plates where eluted in EMW, CEF & BEA solvent systems (PF-Fruit extract-Poland; DF-Fruit extract-Denmark; DL1-Leaf extract-Denmark; DL2- Leaf extract -Denmark; GFL-Leaf+fruit extracts-Germany; and PL-Leaf extract-Poland).

Table 6.4: Inhibition of growth on bioautography TLC plates of plant extracts of *P. fraxinifolia* trees growing in different areas against *A. fumigatus*

Plant	Solvent system	Solvent	Rf values	Active bands
PF	CEF	acetone		0
	BEA	hexane		0
	EMW	chloroform		0
		butanol		0
		water-methanol		0
		Total number of different active bands according to Rf values		**0**
DF	CEF	acetone		0
	BEA	hexane		0
	EMW	chloroform		0
		butanol	0.97	1
		water-methanol		0
		Total number of different active bands according to Rf values		**1**
DL1	CEF	acetone	0.97. 0.80, 0.61	3
	BEA	hexane		0
	EMW	chloroform	0.97	1
		butanol	0.97	1
		water-methanol		0
		Total number of different active bands according to Rf values		**1**
DL2	CEF	acetone	0.97, 0.80, 0.61, 0.05	4
	BEA	hexane		0
	EMW	chloroform	0.97, 0.80	2
		butanol		0
		water-methanol		0
		Total number of different active bands according to Rf values		**2**
GFL	CEF	acetone	0.97, 0.80, 0.61, 0.05	4
	BEA	hexane		0
	EMW	chloroform		0
		butanol	0.97, 0.80, 0.61	3
		water-methanol		0
		Total number of different active bands according to Rf values		**4**
PL	CEF	Acetone		0
	BEA	Hexane		0
	EMW	Chloroform		0

		Butanol		0
		water-methanol		0
		Total number of different active bands according to Rf values		**0**

(i)



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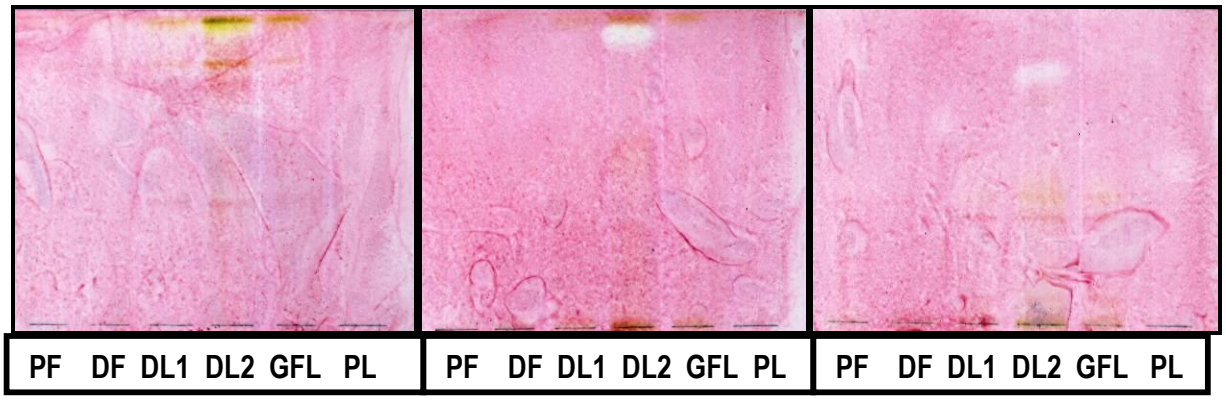

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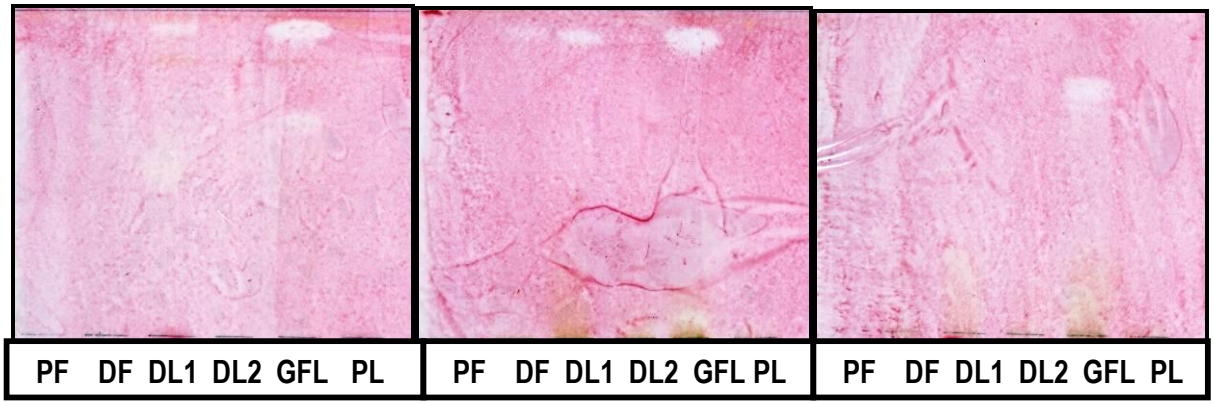

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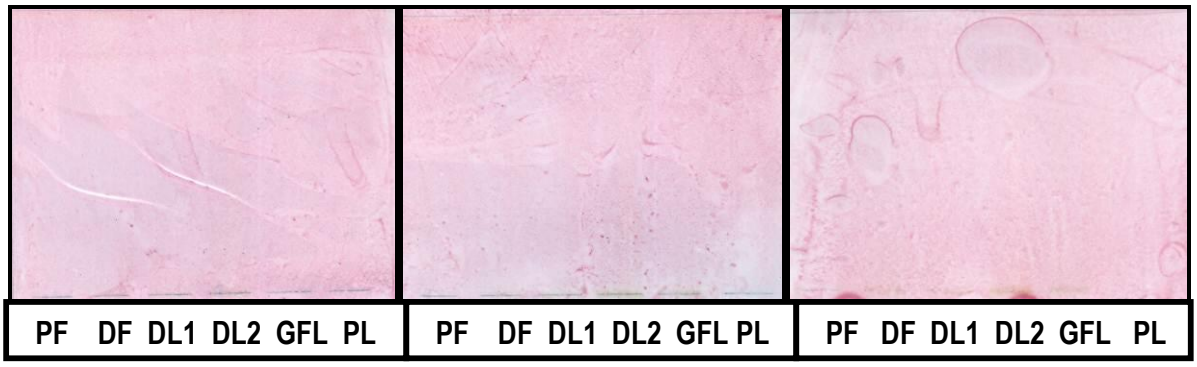

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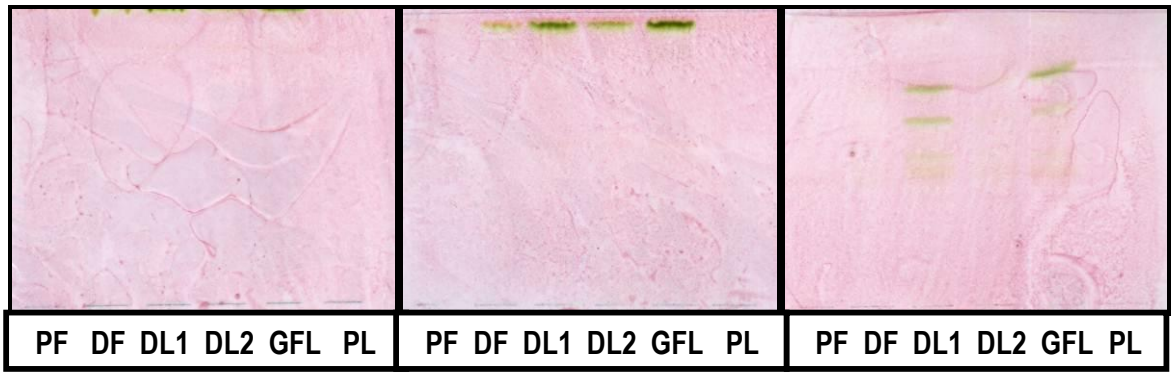

Figure 6.5: Bioautography chromatograms of *P. fraxinifolia* trees growing in different areas in Denmark, Germany and Poland. (i) Acetone crude extract and solvent-solvent derived fractions{ (ii) chloroform, (iii) butanol, (iv) water-methanol, (v) hexane} treated with *C. albicans* culture incubated overnight and sprayed with INT. Growth inhibition is indicated by colourless (white) zones indicated by arrows. TLC plates where eluted in EMW, CEF & BEA solvent systems (PF-Fruit extract-Poland; DF-Fruit extract-Denmark; DL1-Leaf extract-Denmark; DL2- Leaf extract -Denmark; GFL-Leaf+fruit extracts-Germany; and PL-Leaf extract-Poland).

Table 6.5: Inhibition of growth on bioautography TLC plates of plant extracts of *P. fraxinifolia* trees growing in different areas against *C. albicans*

Plant	Solvent system	Solvent	Rf values	Active bands
PF	CEF	acetone		0
	BEA	hexane		0
	EMW	chloroform		0
		butanol		0
		water-methanol		0
		Total number of different active bands according to Rf values		**0**
DF	CEF	acetone		0
	BEA	hexane		0
	EMW	chloroform		0
		butanol	0.97	1
		water-methanol		0
		Total number of different active bands according to Rf values		**1**
DL1	CEF	acetone	0.97, 0.80, 0.61	3
	BEA	hexane		0
	EMW	chloroform		0
		butanol	0.97	1
		water-methanol		0
		Total number of different active bands according to Rf values		**3**
DL2	CEF	acetone	0.22. 0.97, 0.80, 0.61	4
	BEA	hexane		0
	EMW	chloroform	0.97, 0.80	2
		butanol		0
		water-methanol		0
		Total number of different active bands according to Rf values		**2**
GFL	CEF	acetone	0.97, 0.22, 0.80, 0.61	4
	BEA	hexane		0
	EMW	chloroform		0
		butanol	0.97, 0.80	2
		water-methanol		0
		Total number of different active bands according to Rf values		**4**
PL	CEF	acetone		0
	BEA	hexane		0
	EMW	chloroform		0
		butanol		0

		water-methanol		0
		Total number of different active bands according to Rf values		**0**

6.4 Discussion and conclusion

To obtain information on active components of the plant crude acetone extracts and solvent-solvent derived fractions of *P. fraxinifolia* were analysed using TLC bioautography against different pathogens. A total of thirty six (36) compounds were counted based on the R_f values in the crude acetone extracts and solvent-solvent derived hexane, chloroform, butanol and water/methanol fractions for all extracts from the different areas. The solvent-solvent derived fractions water/methanol and butanol also contained similar compounds that inhibit microbial growth but their numbers were lower. Indicating again that in many plants the intermediate polarity compounds are the most active (Kotze and Eloff, 2002).

There were a considerable difference in the number of separated compounds that had antimicrobial activity between crude acetone extracts and the solvent-solvent fractions of *P. fraxinifolia* trees growing in different areas in Europe. This may be explained by a concentration of compounds in the solvent-solvent fractions that were not present in a high enough concentration to give a positive effect in the crude extracts. The leaf extracts from trees growing in Denmark and Poland had better activity and contained more active compounds than the fruit extracts from the same areas even though there are similar active compounds present in both the leaf and fruit samples.

When counting the number of different compounds it is very important to note the R_f values. The total number of different compounds present in acetone crude extract and fractions extracts was 36. The crude acetone extract, and hexane and chloroform fractions all had eight (8) prominent compounds based on R_f values. The butanol and water/methanol fractions had six (6) prominent compounds each based on different R_f values. The most active and prominent compounds in all the extracts were active against both bacteria and fungi (R_f value = 0.97, 0.88, 0.80, 0.74, and 0.61).

The inactivity or the absence of inhibition may be due to absence of active compounds but also be attributed to factors such as the evaporation of the compound during the removal of the solvent or the photo-oxidation of the compound to an inactive form during drying. In cases where active extracts do not lead to on the identification of active bands on the bioautograms, may be due to the disruption of synergistic effects between different compounds on the TLC plate.

Based on the number of compounds counted and present in the crude acetone and solvent-solvent derived fractions hexane and chloroform, the crude acetone extract was selected for further analysis to isolate the active compounds with particular focus on the chloroform and hexane solvent-solvent derived fractions of extracts from trees growing in Denmark and Germany. Because there were not

sufficient quantities of the different trees available, all the samples were combined in an attempt to isolate the antimicrobial compounds.

Post script

In the next chapter the isolation and characterisation of the active compounds identified from *Pterocarya fraxinifolia* trees growing in different areas in Denmark, Germany and Poland was carried out.

Chapter 7

Isolation and characterisation of bioactive compounds in *Pterocarya fraxinifolia*

Preface

To simplify the isolation of the active compounds from *P. fraxinifolia*, the extract was fractionated into different fractions based on polarity. Since acetone extracts both polar and intermediate polar compounds (Eloff, 1988), it was used as the extractant of choice. Bioassay guided procedures for antibacterial activity using open column chromatography with silica gel and Sephadex LH 20 was used. The compounds isolated were further characterised both chemically and biologically.

7.1 Introduction

The plant kingdom is now been screened for new and more effective chemotherapeutic agents. It is already well documented that higher plants can serve as potential antimicrobial drugs and a new source of new anti-infectious agents (Kamboj & Saluja, 2010). The most important objective in medicinal plant research and development is the identification, isolation and characterization of the bioactive components present in the plant extract. Plants produce a diverse array of chemical compounds. The isolation of active compounds and determination of biological activities may provide vital insight into the pharmacological properties of these plant compounds (Sun & Sheng, 1998). There are different separation techniques that can be employed in the separation and isolation of the complex compounds that occur in plants. The structure elucidation and characterisation of some of these natural compounds has created new leads for various agents in the control and treatment of various ailments.

The separation of the active components of a plant from its inactive components can be categorised into three phases; the extraction phase, group separation phase and chromatography phase. Each phase requires rapid and easy techniques in order to achieve the desired result. There are various chromatographic methods that have been used to isolate active compounds. There are several principles involved in the separation and isolation processes based on aspects such partition between mobile and stationery liquid phases, adsorption on the stationary phase, selective elution and molecular size.

There are different spectroscopic techniques used to gather information on isolated compounds, the most common is the nuclear magnetic resonance spectroscopy (NMR) and mass spectroscopy (MS). Column chromatography is simple, cheap and has widespread use. There are several disadvantages associated with this method particularly the fact that the method is slow and require large volumes of solvent. Open column chromatography is carried out under gravitational force

using silica gel or any other stationary phase. The separation of compounds from the extract is based on polarity of the gradient elution. Increasing the polarity of the mobile solvent phase allows the target compound to separate and elute in sequence (Simpson *et al*, 2011).

MS is an important tool applied in structure elucidation of compounds from natural products. According to Rijke et al (2006) MS is based on the fundamental principle for the use of different physical means for sample ionization and separation of the ions generated based on their mass and charge ratio. MS has high sensitivity compared to NMR (Simpson *et al*, 2011).

Pterocarya fraxinifolia is a plant indigenous to Northern Iran and the local people use the leaves as an anaesthetic agent for catching fish, as a dye and also as an anti-fungal agent and also it contains compounds that inhibit seed germination. Juglone, a naphthoquinone compound has already been isolated from the leaves and hulls. The antimicrobial activities of 1, 4-naphthoquinone derivatives have been reported (Hadjmohammadi & Kamel, 2006).

In this study *P. fraxinifolia* leave and fruit samples were extracted with acetone, separated into groups based on solvent-solvent fractionation and fractionated by open column chromatography procedures for the separation and isolation of the active compounds. Separation and isolation was carried out according to the diagram in figure 7.1. The fractions collected were tested against cultures of *Staphylococcus aureus* and *Escherichia coli* K88 as this was the primary microorganism concerned in this isolation process and study.

7.2 Materials and Methods

7.2.1 Plant collection

Finely ground leaf (Specimen number, D058R, D052R, and D054R), fruit (Specimen number, D056R and D053R) and leaf and fruit mixture (Specimen number, D021R) plant materials were obtained from Dr Ole Højberg, Aarhus University, Faculty of Agricultural Sciences, Department of Animal Health, Welfare and Nutrition, Denmark. He also provided an estimate of the relative efficacy of different populations in in vivo animal experiments. A standard procedure of extraction, previously described by Eloff (1998a), was employed in the preparation of the plant acetone extracts. Finely ground plant material (200 g) from each region was extracted with 2000 ml of acetone; the solvent was allowed to shake on a shaker for a few hours and left overnight. The resulting solvent was filtered and dried in a rotary evaporator under reduced pressure. The procedure was repeated three times.

7.2.2 Solvent-solvent fractionation

The solvent-solvent fractionation procedure simplifies extracts by separating the components of the extracts based on their polarity. In this study the method developed by the US National Cancer Institute (Suffness & Douros, 1979)was used with slight modification to suite this study (Figure 7.1). The dried acetone extract was dissolved in a 1:1 mixture of chloroform and water in a separating funnel. The resulting water fraction was mixed with equal volume of n-butanol in a separating funnel to yield water and butanol fractions. The chloroform fraction was dried in a rotavapour to remove all chloroform and equal volumes of hexane and 10% water/methanol mixture was mixed into a separating funnel. The hexane fraction was removed with a separatory funnel. The 10% water/methanol phase was then further diluted to 20% by adding water and then eventually diluted to 35%. The resulting mixture was then mixed with chloroform to yield the water/methanol fraction and the chloroform fraction. To determine the TLC chemical profile 10 mg of each extract was weighed into a vial and made up to a contraction of 10 mg/ml for each plant material. The antimicrobial activity of each fraction collected was test using bioautography method developed by Begue and Kline (1972).

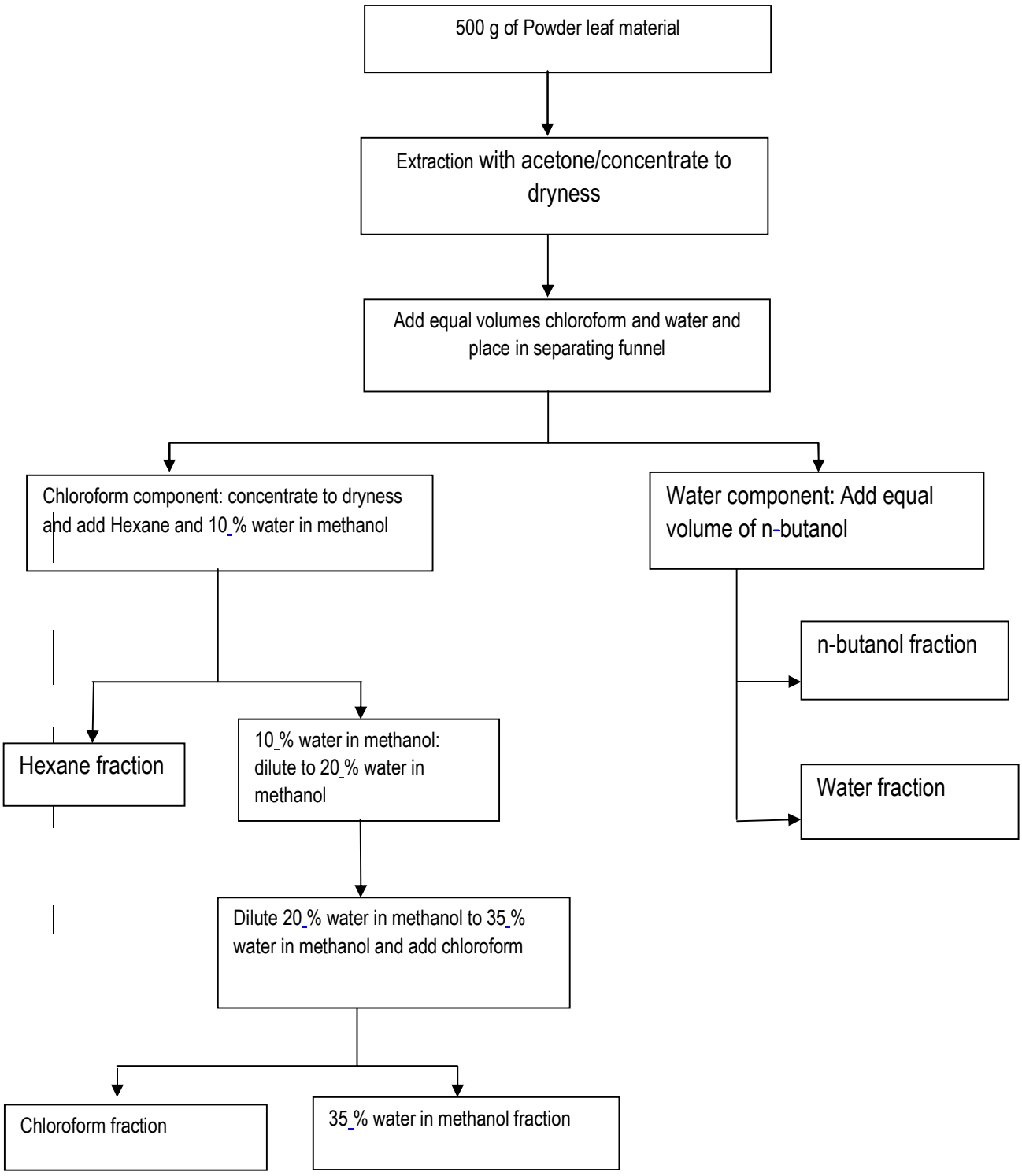

Figure 7.1: The protocol used for the solvent-solvent fractionation of the acetone crude extract of *P. fraxinifolia* for samples from trees growing in Denmark, Germany and Poland. The combined extraction and fractionation of all the samples of *P. fraxinifolia* samples (adapted from Suffness and Douros, 1979). Benzene was left out due to toxicity.

7.2.3 Column chromatography; isolation of bioactive compounds

Column chromatography of the crude acetone extracts of the combined regions were performed based on the results of the antimicrobial activity and bioautograms. A hexane/acetone step gradient was followed in the running the column. Initially 100 % hexane was used and reduced by 0.5% after collection of every ten fractions until the column was clear of most of the extract.
Two columns were set-up, one with the chloroform fraction and the other with the crude acetone extract. The eluent from the columns were collected into test tubes and Compounds present in different test tubes were analysed by TLC.

The fractions containing compounds of the same R_f values were combined after testing for antimicrobial activity using *E.coli* K88 and the bioautography method of Begue and Kline (1972) was used to access the activity of the compounds/bands on the TLC plate. The combined fractions of compounds with the same R_f values were subject to another column chromatogram this time containing Sephadex and eluted with different ratios of DCM and Methanol starting with 100 % DCM, 98:2, 96:4, 92:8 and finally 90:10 as mobile phase.

The column was eluted and 40 drops were collected per test tube. TLC plates were developed for the contents of each test tube to determine which of the test tubes contained pure compound. Column chromatography was performed on MN silica gel 60 (0.063-0.2 mm / 70-230) mesh. Preparative TLC was performed using high-purity grade powder silica gel (60 A, 2-25 µm) Sigma-Aldrich, Germany. Pre-coated plates of TLC silica gel 60 F_{254} (Merck, Germany) were used for monitoring fractions and spots were detected with UV light (254 and 365 nm) and then sprayed with vanillin-H_2SO_4 followed by heating to 110 °C for *c*. 4 min.

The characterization of the compounds was performed by one dimensional (1H and 13C) and two dimensional NMR spectra. 1H NMR spectra were measured at 399 MHz and the 13C NMR were run at 100 MHz. All NMR experiments were conducted at a constant temperature of 26 ºC. The time for each compound was dependent on the amount of the samples loaded. ^{1}H- and ^{13}C-NMR spectra were recorded with a Varian spectrometer at 400 MHz. Chemical shifts (δ) were quoted in parts per million (ppm) from internal standard tetramethylsilane (TMS).

7.2.4 Antimicrobial activity and cytotoxicity of isolated compound(s)

7.2.5.1 Microbial cultures

In this study bacterial test microorganism used, were Gram-negative *Escherichia coli* (K88) and

Gram-positive *Staphylococcus aureus*. The choice of these two bacterial species was based on the plant samples ability to inhibit growth of both bacteria. The bacterial strain is important nosocomial pathogens, which are recommended as test strains for comparing the activity of antibiotics.

7.2.5.2 Bioautography

Bioautography is a rapid bioassay guided method for the determination of antimicrobial compounds from crude extracts and fractions. The activity of the plant extract against microorganisms is assayed and determined on chromatograms. This method was developed by Begue and Kline (1972). TLC plates were loaded with 10 µl of a 10 mg/ml fraction and developed in an acetone and hexane solvent system (ratio = 3:7) plates were left under a fan the dry and remove all trace of solvent on the plates. The dried plates were sprayed with a suspension of actively growing cells of E.coli k88 and *Staphylococcus aureus*. The plates were incubated in a sealed sterile plastic container at 37 ºC for 18 hours at 100% relative humidity. The plates were then sprayed with a solution of p-iodonitrotetrazolium (INT) violet (2 mg/ml) and further incubated for a few hours for the colour to develop. White spots or areas on the purple background were indicative of INT reduction due to the presence of compounds that inhibited the growth of tested organisms.

7.2.5.3 Minimum inhibitory concentration of isolated compounds (MIC)

Minimum inhibitory concentration (MIC) is defined as the lowest concentration that the extract can inhibit visible growth of the micro-organism. The MIC was determined using the serial micro dilution assay for the different microbial species (Eloff, 1998b). The microorganisms used in this assay were *E. coli* (k88) and *Staphylococcus aureus*.

7.2.5.3 Cytotoxicity of isolated compounds

The cytotoxicity of the compounds was determined by the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide] assay using Vero monkey kidney cells (Mosman, 1983 & McGaw ***et al***, 2007). The sub-confluent cells in the microtitre plate were used in this assay. The compounds were re-dissolved in DMSO to a concentration of 100 mg/ml. Serial 10-fold dilutions of each compound were prepared in growth medium (1-1000 µg/ml). The cells were viewed under an inverted microscope to see viable cells and the cytopathic effects of the compounds.

The compound containing medium was removed from the wells and the remaining cells washed with phosphate buffer solution (PBS) (150 µl) before the addition of fresh MEM (200 µl). A 30 µl solution of MTT (5 mg/ml) in PBS was added to each well and incubated for 4 hours at 37 ºC. MTT concentrate was washed with PBS and DMSO was used to dissolve the crystallised MTT formazan. The amount of reduced MTT was quantified as the absorbance at 570 nm and EC_{50} values were calculated.

7.3 Results

The crude acetone extract of the leaves of *P. fraxinifolia* trees growing in Denmark, Germany and Poland was subjected to repeated silica gel column chromatography followed by Sephadex LH-20 and preparative thin layer chromatography - as described at the general experimental procedure part- to afford five compounds coded NPF1 to NPF5. The structures of two compounds were elucidated by interpretation of their spectroscopic (NMR) data and also by comparison with previous literature reports.

One had colourless white powder, one was pure colourless oil and the other three had a yellowish oily appearance. The colourless oil elucidated and found to be a small compound called pentacosanol (compound 2) and the white powder was found to be *β*-sitosterol (compound 1). The structures of other three compounds could not be elucidated because insufficient material was isolated.

Antimicrobial activity

All the compounds isolated had activity on the bioautograms but the MIC values were all greater than of 2.5 mg/ml. The activity on the microorganism was poor except in the case of *S. aureus*, which was used as the standard to isolate the compounds that had activity (Fig 7.2).

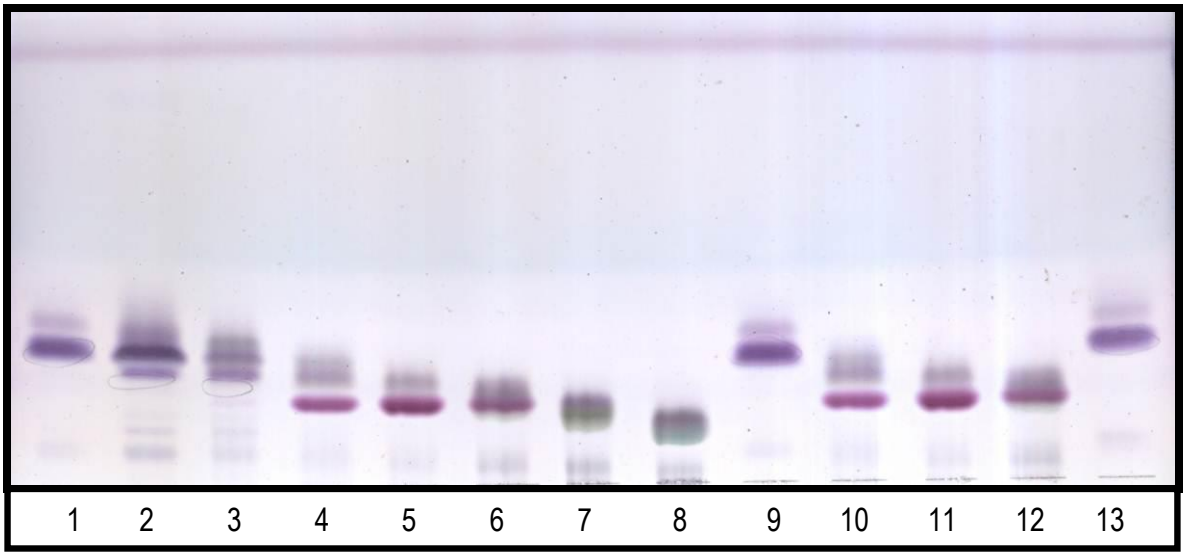

Figure 7.2: TLC chromatogram of fractions collected and developed in an acetone hexane solvent system (8:2). Fractions that had similar R_f values were later combined and purified in a column containing sephadex.

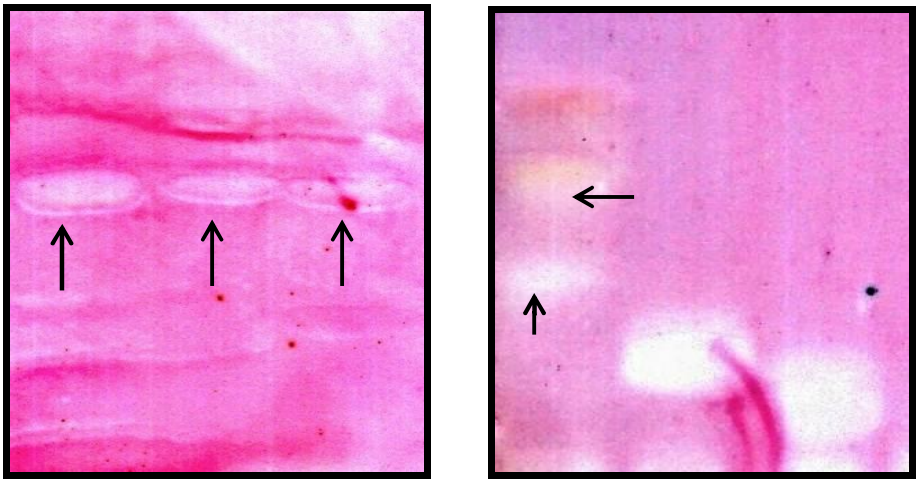

Figure 7.3: TLC chromatograms sprayed with bacterial suspension of *S. aureus* for A) fractions that had similar R_f values and B) the compounds that were obtained from the *P. fraxinifolia* sample. The active zone appears as clear white areas on the purple background. The solvent system used was acetone and hexane (8:2).

7.3.1 Structure Elucidation of Compounds

7.3.1.1 Compound 1 β-sitosterol

Compound 1 (PF1) was obtained as white powder and gave a dark green coloration to the Liebermann-Buchard's test characteristic of sterol (Kumar *et al*.; 2007). The presence of total number of 30 carbons on its ^{13}C NMR spectrum (Appendix A Fig 10.2) including signals due to six angular methyl groups was relevant for this compound to be a phytosterol. The ^{13}C NMR spectrum and DEPT (Appendix A Fig.10.3) displayed signals at *δ* 140.7 (C), 121.7 (CH) and 71.8 (CH) ppm corresponding respectively to carbons C-5, C-6 and C-3 of *β*-sitosterol structure. The ^{1}H NMR spectrum (Appendix A Fig 10.4) exhibited signals at *δ* 5.35 (1H, br*d*, 4.0 Hz, H-6), 3.53 (1H, *m*, H-3) corresponding to protons H-6 and H-3 respectively. Furthermore, the ^{1}H NMR spectrum exhibited signals at *δ* 1.01 (3H, *s*, H-19), 0.93 (3H, *d*, 8.0 Hz, H-21), 0.85 (3H, *d*, 8.0 Hz, H-26), 0.83 (3H, *d*, 8.0 Hz, H-27), 0.81 (3H, *d*, 8.0 Hz, H-29) and 0.68 (3H, *s*, H-18) assignable to six methyl groups. The ^{1}H and ^{13}C NMR and DEPT data were in agreement with those of previously reported *β*-sitosterol (**1**) (Al-Oqail *et al*.; 2012). This compound (**1**) which is very common in plant kingdom is isolated for the first time from this species.

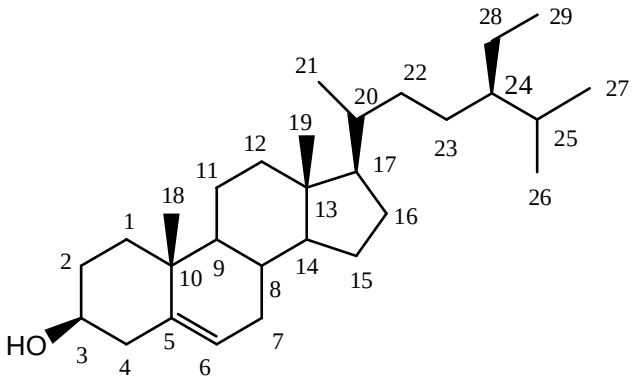

Figure 7.4: Structure of Compound 1 ß-sitosterol

7.3.1.2 Compound 2 Pentacosanol

Compound 2 (PF 2) was obtained as colourless oil. The ^{1}H NMR spectrum (Appendix A Fig 10.5) had four signals at *δ* 3.65 (2H, *t*, 8.0 Hz, H-1), 1.58 (2H, br*s*, H-2), 1.26 (22H, br*s*, H-3 to H-22) and 0.89 (3H, *t*, 8.0 Hz, H-25) characteristic for a fatty acid derivative and corresponding to protons H-1, H-2, H-3 to H-24 and H-25, respectively. The ^{13}C NMR spectrum (Appendix A Fig 10.6) and DEPT (Appendix A Fig 10.7) exhibited signals at *δ* 63.1 (CH_2, C-1), 32.8 (CH_2, C-2), 31.9 ï 22.7 (22 CH_2, C-3 to C-24) and 14.1 (CH_3, C-25). These data were similar to those reported for pentacosanol (**2**) previously isolated from the seed of *Artemisia halodendron* (Wang *et al*.; 2007). This compound (**2**) is isolated for the first time from this species.

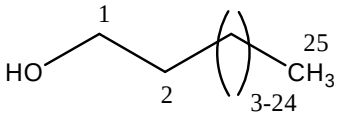

Figure 7.5: Structure of Compound 2 Pentacosanol

7.3.1.3 Compound 3

Compound three was not identified due to high levels of impurities even after cleaning of the compound using a Sephadex column. The NMR spectrum indicates that the class of compound could be a fatty acid derivative. (Appendix A figure 10.8 and 10.9)

7.3.1.4 Compound 4 and 5

These compounds could not be identified on NMR due the low level (less than 1 mg/) that was isolated and the impurities that could not be cleaned using sephadex. The NMR spectra showed that the class of compound could be a fatty acid derivative (Appendix A figure 10.10 and 10.11).

Not any of the five compounds inhibited the growth of E.coli (K88), *S.aureus, C. jejuni*, and *C. perfringens* at the highest concentration tested 2.5 mg/ml. The isolated compounds had varying degrees of toxicity to the Vero cell lines with LC_{50} ranging from 6.55 ±1.22 to 72.78 ±0.99 µg/ml. The most cytotoxic compounds were *β-sitosterol* (NPF1 = 6.55 ± 1.22 µg/ml), NPF3 (17.54 ± 1.32 µg/ml) and NPF5 (9.34 ± 1.09 µg/ml). The least cytotoxic compound was pentacosanol (NPF2 or compound 2 = 72.78 ±0.99 µg/ml) (Table 7.1.)

Table 7.1: The EC_{50} of the cytotoxic assay of the compounds isolated from *P. fraxinifolia* trees growing in different areas in Denmark, Germany and Poland.

Compounds	**EC50 (µg/ml)**
β-sitosterol	6.55 ±1.22
Pentacosanol	72.78 ±0.99
Compound 3	17.54 ±1.32
Compound 4	33.66 ±1.11
Compound 5	9.34 ±1.09

7.4 Discussion and Conclusion

Bioassay guided investigation of *Pterocarya fraxinifolia* resulted in the isolation of *β*-sitosterol, pentacosanol and three other unknown compounds (C3-C5) from an acetone extract of the aerial parts of *Pterocarya fraxinifolia* trees growing in different areas in Denmark, Germany and Poland.

β ï sitosterol is the most abundant phytosterol and probably occurs in all plants. It has anti-inflammatory, angiogenic and immune-modulating properties. This compound is also known for its antioxidant activity and free radical scavenging effects. *β* ï sitosterol is a good example of a compound which is able to incorporate into animal membranes and also manipulate sterol levels when ingested. This compound is known to cause changes in cancer cells by causing the depolarisation of mitochondrial membrane potential (Vundru, et al., 2013 and Hac-Wydro, 2013)

Pentacosanol is a fatty acid alcohol derivative; this is the first record of its isolation from *P. fraxinifolia*. Fatty alcohols are high-molecular weight straight chain molecules derived from natural fats and oils. They are colourless oily liquids, although impure samples may appear yellow. Many of the fatty alcohols are produced by bacteria, plants and animals for varying purposes as sources of energy, thermal insulation and metabolic purposes. Fatty alcohols have been reported to lower plasma cholesterol and can be found in many unrefined natural products and plant derived foods (Noweck and Grafahrend, 2006).

Fatty alcohols are relatively benign materials and produce fewer health effects. Repeated exposure may lead to low levels of toxicity and there is no evidence that fatty alcohols are carcinogenic or cause any reproductive toxicity. They are easily eliminated from the body when exposed which limits the possibility of retention or bioaccumulation. This compound may have contributed greatly to the activity observed in the anti-inflammatory and antioxidant assays in this study due its cholesterol reduction ability which in-turn may boost immune response thus helping in immune modulation effects of *P. fraxinifolia* (Sanderson *et al,* 2009).

It was an immense disappointment that the MICs of the isolated compounds were all higher than 2.5 mg/ml. The R_f values of the isolated compounds were similar to the R_f values of the compounds that had antimicrobial activity in the bioautography (Figure 7.5). It is possible that the real antimicrobial compounds were not isolated or that the isolated compounds were inactivated by photooxidation during the final isolation. Another possibility is that the excellent antimicrobial activity found in some cases, may have been due to synergism between compounds with differing antimicrobial activity once isolated. If a compound with no antimicrobial activity *per se* influenced

the uptake or decreased the extrusion or inactivation of an antimicrobial compound the combined compounds could have had much higher activity.

It has been stated that for an extract or compound to be considered toxic the EC_{50} value must be 20 µg/ml and below (Kuete *et al,* 2011). The results of the toxicity assay of compounds isolated from *P. fraxinifolia* trees growing in different areas show that even though the crude extract was highly toxic the compounds isolated had varying degrees of toxicity with EC_{50} values of 6.55, 9.34, 17.54, 33.66 and 72,78 ug/ml. An aspect not stressed earlier is that the *in vivo* activity was determined by including powdered plant material in the animal feed. The assumption that the acetone extract would have similar activity and safety as plant material fed to animals may be incorrect.

Postscript

It is clear that the concentration of *β*-sitosterol or pentacosanol cannot be used to determine which tree populations would have a high likelihood of having *in vivo* activity. It seemed to be such a logical assumption that the presence of antimicrobial compounds would be the best predictor of *in vivo* activity at the start of the study. In the next section all the results will be discussed and proposals for future work made

Chapter 8

8.1 General discussion and conclusion

The original aim of this project, following the success achieved in the *in vivo* trials using un-extracted plant material, was to isolate and characterize the antimicrobial compounds from *P. fraxinifolia* trees growing in different areas, active against relevant pathogens in order to facilitate the selection of tree populations with good activity without having to carry out animal experiments. A secondary aim was to investigate alternative mechanism of activity to replace antibiotic feed additives in the REPLACE programme. A supplementary aim was to find out what *in vitro* method can be used to predict the *in vivo* activity of different tree populations.

The objectives identified to attain this aim were as follows:

- To evaluate *in vitro* antimicrobial activity of *P. fraxinifolia* trees growing in different regions of Europe against selected Gram-positive (*Staphylococcus aureus* and *Clostridium perfringens*) and Gram-negative bacteria (*Campylobacter jejuni* and *E. coli strains* K88 and fungal yeast (*Candida albicans*) and mould (*Aspergillus fumigatus*).
- To determine the antioxidant and anti-inflammatory activity as possible alternative mechanisms of action and also the cytotoxicity of *P. fraxinifolia* extracts.
- To isolate the bioactive compound from the plant and determine the structure and antimicrobial activity of the isolated compounds.
- To determine the correlation between *in vivo* activity and *in vitro* activity of extracts from different tree populations against *E.coli* K88, *Clostridium perfringens* and *Campylobacter jejuni.*
- To discuss the possible mode of actions and variation of activity between extracts of tree populations from different areas.

The degree to which the above mentioned objectives were attained is discussed below.

8.1.1 To evaluate *in vitro* antimicrobial activity of *P. fraxinifolia* tree extracts growing in different regions of Europe against selected bacteria and fungi

Some of the crude extracts had very good antimicrobial activity against some pathogens, but other crude extracts had relatively low antibacterial activity. The important aspect is that there were poor correlations between the antimicrobial activity of extracts from different populations and the *in vivo* activity of plants from different populations against the different pathogens. This indicates that antimicrobial activity may not be the mechanism of activity of plants included in the feed of the

animals. In general the antimicrobial activities of the solvent-solvent derived fractions were lower that of the crude extracts pointing to synergistic activities between different compounds in the crude extract. In considering the antimicrobial activity of the crude extracts the pathogens that are usually considered to play an important role had very low R^2 values, (*E. coli* K88 0.0047, *C. jejuni* 0.0809, *C. perfringens* 0.1092). There were better correlations between pathogens that are not considered that important (R^2 *S. aureus* 0.6107 and *C. albicans* 0.5889). This may be a chance effect

From the bioautography results it was clear that there are many antimicrobial compounds present in the extracts. It is not clear if these compounds would have become bioavailable when ground plant material is included in the animal feed.

8.1.2 To determine the antioxidant and anti-inflammatory activity as possible alternative mechanisms of action and also the cytotoxicity of *P. fraxinifolia* extracts

8.1.2.1 Antioxidant activity

The crude acetone extracts and hexane, chloroform and water-methanol solvent-solvent fractions of *P. fraxinifolia* had significant radical scavenging activity through proton and hydrogen donation activity. Antioxidants have been known to boost the immune system and by doing so also influence the ability of the immune system in combating infections and reversal of certain conditions associated with immune deficiencies.

The antioxidant activity of the plant samples of *P. fraxinifolia* trees growing in different areas in Denmark, Germany and Poland can be attributed to the high levels of phenolic and flavonoid compounds. The strong antioxidant activity indicates the presence of compounds with potentially important mechanisms of pharmacological relevance in the reduction of weaning and post weaning infections and degenerative effects of the oxidative stress during weaning.

Antioxidants have been studied and are known to disrupt bacterial cell membrane function and structure and this phenomenon also aids the inhibitory effects of antioxidants on microbes and this was true for *P. fraxinifolia* samples as they had moderate activity as extracted and un-extracted plant material was used.

There was an excellent correlation between the antioxidant activity of the crude acetone extract and the *in vivo* activity of plants from different populations (R^2= 0.8167). The correlation was even better in the case of the water/methanol fraction (R^2 = 0.8746). This is understandable because antioxidant compounds are usually polar and this was the most polar fraction. In the case of the

highly non-polar hexane fraction there was an inverse relationship. Because the content of the animal gastrointestinal track consists of an aqueous solution, it is understandable that polar compounds could be readily solubilized from the plant material included in the diet.

8.1.2.2 Anti-inflammatory activity

The aetiology of many diseases involves inflammation and one of the mediators in the pathway is leukotriene from lipoxygenase. Leukotrienes are produced in the lipoxygenase enzyme pathway and this enzyme is one of the target modulators in the inflammation process during weaning. The plant samples of *P. fraxinifolia* trees growing in different areas in Denmark, Germany and Poland all had relative inhibitory activity against 15-LO enzyme. Antioxidants are compounds known to limit the progression of inflammation in disease and boost immune system response through the scavenging of free radicals produced during inflammation.

The results indicate that the anti-inflammatory activity and scavenging effect may be in part due to a combination in the action of both flavonoid and phenol compounds present in the plant samples. The correlation between antioxidant and anti-inflammatory activity has been well documented and supported by other researchers. The combination of antioxidant and anti-inflammatory effects of *P. fraxinifolia* makes the plant a viable candidate as an antimicrobial agent and also helps in stimulating the immune system, with advantageous effects for the host during and post weaning.

There was not such a good correlation between anti-inflammatory activity and *in vivo* activity of the crude acetone extract as was the case for antioxidant activity (R2 =0.3685). It is interesting that there was a better correlation between *in vivo* activity and anti-inflammatory activity for the hexane fraction (R2 = 0.5536). This may indicate that non-polar compounds are involved with anti-inflammatory activity.

8.1.2.3 Cytotoxicity

The crude acetone extracts of *P. fraxinifolia* trees growing in Denmark, Germany and Poland were relatively toxic with an LD_{50} varying between 0.77 and 1.75 µg/ml. The lack of toxicity in the animal trials may possible be due to the lower solubility and/or lower bioavailability of toxic metabolites in the aqueous gastro intestinal fluid. Hence *in vitro* toxicity may not always equate to whole organism toxicity.

P. fraxinifolia is known to be toxic and its traditional use as fish poison is well established by the natives of Iran. Juglone is a naphthoquinone class of compounds which has been isolated from *P. fraxinifolia* with known toxicity against chick embryos *ex vivo* (Sadighara *et al*, 2009). Further work is

required to test the crude extracts of *P. fraxinifolia* trees growing in different areas against other cell lines to prove without a doubt that the plant is toxic. When *P. fraxinifolia* plant material was used in the animal trials no toxicity was observed in any of the treatments.

8.1.3 To isolate the bioactive compound from the plant and determine the structure and antimicrobial activity of the isolated compounds

The isolation and characterization of the compound(s) from *P. fraxinifolia* trees growing in different areas in Denmark, Germany and Poland was performed using bioassay-guided procedures for antibacterial activity using open column chromatography with silica gel and then Sephadex LH 20 as a stationary phase. The first compound (NPF1) was identified as *β*-sitosterol a phytosterol common in all plants isolated for the first time from this plant species. This compound has known antioxidant and anti-inflammatory activity.

The second compound (NPF2) was identified as pentacosanol a fatty acid derivative. The data obtained from analysis was similar to those reported for pentacosanol which has been previously isolated from the seed of *Artemisia halodendron.* This compound is also isolated for the first time from this plant species. Pentacosanol is known to lower cholesterol levels which in turn help boost immune functions in animals and humans. The other three compounds (NPF3, 4 and 5) could not be identified because there was not enough material isolated. The isolation did not yield any novel compound of importance. It was also very disappointing that the compounds isolated had such low antimicrobial activity. Although bioassay guided fractionation was used up to a certain point the assumption that compound present in high concentration id active fractions were the active compound may have been wrong.

8.1.4 To determine the correlation between *in vivo* activity and *in vitro* activity of extracts from different tree populations against *E.coli K88, C. perfringens* and *C. jejuni*.

To determine the correlation between *in vivo* activity and different parameters we used an indication on a five point scale of the activity of different populations provided by Dr Ole Hojberg found during the EU REPLACE programme. Unfortunately this remains a judgement estimate. If hard figures for in vivo activity were available to determine the correlations, better results may have been obtained.

As has already been stated there were good correlations between in vivo activity and antioxidant activity, intermediate correlations with anti-inflammatory activity and poor correlations with antimicrobial activity.

8.1.5 To discuss the possible mode of actions and variation of activity between extracts of tree populations from different areas.

In contrast to the expectations when the project was started it does not appear as if antimicrobial activity plays an important role in the *in vivo* activity of plants from different populations.

The mechanism of action for this plant may therefore be mainly attributed to its *in vivo* antioxidant and to a lesser degree anti-inflammatory activity. The effect of the antioxidant activity may be via stimulation of the immune system of the animals, thereby enabling the animal to combat microbial infections and inflammation. If these are the main factors responsible for activity, there may be decreased resistance development when using this plant as a potential growth promoter. The results of this study indicated that plants are a reservoir of bioactive compounds and the degree of activity vary and can be dependent on several factors. The variation in activity can be attributed to environmental factors in the areas where these trees grow.

8.2 Conclusion

One of the main outcomes of this study is that antioxidant activity can be used as a parameter to select tree populations to be used as feed additives. Another positive aspect is that it appears that direct killing of pathogens by the plant extract is not a major factor responsible for the *in vivo* activity.

This has an added advantage in that there is little chance of the development of resistance by pathogens against antimicrobial compounds present in the extracts.

The information gained in this study provides a meaningful baseline to facilitate the selection of tree populations with good activity and for other scientists to explore in depth, the possible mechanisms suggested for *P. fraxinifolia* extracts and/or un-extracted plant material as a commercially viable replacement to antibiotic growth promoters in pig production in Europe. This could be done through vigorous pre-screening procedures using the methods discussed in this study prior to commencing *in vivo* animal trials. An in depth study and clinical trials should be undertaken to access the cellular activity of *P. fraxinifolia* extracts from trees growing in different areas in Europe using other alternative assays and chemical screening processes to better understand the activity and help facilitate the selection of active tree populations for possible commercialisation.

References

Adam, K., Sivropoulou, A., Kokkini, S., Lanaras, T., Arsenakis, M., 1998. Antifungal activities of *Origanum vulgare* subsp. *Hirtum, Mentha spicata, Lavandula ongustifolia* and *Salvia fruticosa* essential oils against human pathogenic fungi. Journal of Agricultural Food Chemistry. 46: 1739-1745.

Afolayan, A.J., 2003. Extracts from the shooy=ts of *Arctotis arctotoides* inhibit the growth of bacteria and fungi. Pharmaceutical Biology. 41(1): 22-25.

Al-Oqail, M., Hassan, W.H.B., Ahmad, M.S., Al-Rehaily, A. J., 2012. Phytochemical and biological studies of *Solanum schimperianum* Hochst. *Saudi Pharmaceutical Journal* **20**(4), 371-379.

Akhani, H., Salimian, M., 2003. An extant disjunct stand of *Pterocarya fraxinifolia* (Juglandaceae) in Central Zagros (Iran). *Willdenowia* 33: 113ï 120.

Alexander, T.J.L., 1994. Neonatal diarrhoea in pigs: In *Escherichia coli* In Domestic Animals and Humans. Gyles, C.L (Ed.). CAB International, Wallingford, UK. Pp151-170.

Allen, N.E., Nicas, T.I., 2003. Mechanism of action of oritavancin and related glycopeptide antibiotics. FEMS Microbiology Reviews 26: 511-532.

Apajalathi, J., 2005. Comparative gut microflora, metabolic challenges and potential opportunities. Journal of Applied Poultry Resources. 14: 444-453.

Aynehchi, Y., Mahoodian M., 1973. Chemical examination of Zizyphus spina-christi (L) Willd. Acta Pharma Suecica 10: 515-517.

Benchaar, C., Calsamiglia, S., Chaves, A.V., Fraser, G.R., Colombatto, D., McAllister, T.A., Beauchemin, K.A., 2008. A review of plant-derived essential oils in ruminant nutrition and production. *Animal Feed Science and Technology* 145: 209ï 228.

Browicz, K., 1982. Chorology of trees and shrubs in South-West Asia and adjacent region, Vol. 1. Polish Scientific Publishers, PoznaŒ

Barton, M.D., 2000. Antibiotic use in animal feed and its impact on human health. Nutrition Research Reviews 13: 279-299.

Bodas, R., Lopez, S., Fernandez, M., Garcia-Gonzalez, R., Rodriguez, A.B., Wallace, R.J., Butaye, P., Devriese, L.A., Haesebrouck, F., 2003. Antimicrobial growth promoters used in animal feed: effects of less well known antibiotics on Gram-positive bacteria. Clinical Microbiology Reviews. Pp.175-188.

Begue, W.J., Klein, R.M., 1972. The use of tetrazolium salts in bioautographic procedure. Journal of Chromatography 88 182-184.

Bhat, P.N., Mohan, N.H., Such, D., 2010. Pig production. Stadium Press Publishers Pty LTD, India.

Blomberg, L., Henriksson, A., Conway, P.L., 1993. Inhibition of adhesion of Escherichia coliK88 to piglet Ileal mucus by Lactobacillus spp. Applied and Environmental Microbiology (January 1993) p. 34-39.

Buchanan, N.P., Hott, J., Cutlip, S., Rack, A., Asamer, A., Moritz, J., 2008. The effects of a natural antibiotic alternative and a natural growth promoter feed additive on broiler performance and carcass quality. Journal of Applied Poultry Resource 17: 202-210.

Buzby, J.C., Roberts, T., 1997. Economic costs and trade impacts of microbial foodborne illness. World Health Statistics Quarterly Rapport 50(1-2): 57-66.

Caturla, N., Ver-samper, E., Villalani, J., Mateo, CR., Micol, V., 2003. The relationship between the antioxidant and the antibacterial properties of Galloylated catevhins and the structure of phospholipid model membrane. Free Radical Biology and Medicine. 34(6): 648-662

Cocito, C., Di-Giambattista, M., Nyssan, E., Vannuffel, S., 1997. Inhibition of protein synthesis by Streptogramins and related antibiotics. Journal of Antimicrobial Chemotherapy 39(1): 7-13.

Coleman, J., 2002. Nitric oxide: a regulator of most cell activation and most cell-mediated inflammation. Clinincal and Experimental Immunology 129(1): 4-10.

Cowan, M.M., 1999. Plant products as antimicrobial agents. Clinical Microbiology Review 12(4): 564-582

Chowdhury, R., Haque, M.N., Islam, K.M.S., Khaleduzzaman, A.B.M., 2009. A review on antibiotics in an animal feed. Bangladesh Journal of Animal Science 38(1&2): 22-32.

Cuello, S., Alberto, M.R., Zampini, I.C., Ordonez, R.M., Isla, M.I., 2011. Comparative study of antioxidant and anti-inflammatory activities and genotoxicity of alcoholic and aqueous extracts of four Fabiana species that grow in mountainous areas of Argentina. Journal of Ethnopharmacology 137: 512-522.

Colin, D.A., Monteil, H., 2003. Control of the oxidative burst of human neutrophils by Staphylococcal Leukotoxins. Infection and Immunity, July 2003, 3724-3729.

Crosby, N.T., 1991. Determination of veterinary residues in food. Ellis Horwood Series in Food Science and Technology. Ellis Harwood Limitted.

Cowart, R.P., Casteel, S.W., 2001. An outline of swine diseases; A handbook, 2nd Edition. Iowa state University Press, Blackwell Science Company.

Conway, P.L., Blomberg, L., 1994. An ecological perspective of fimbriae function in the porcine digestive tract: In P. Klemm (Ed.). Fimbriae, CRC Press, Inc., New York. 227 -238.

Dalglish, A.G., O'Byrne, K.J., 2001. Chronic immune activation and inflammation as the cause of malignancy. British Journal of Cancer 85(4): 473-483

Davidson, R.L., 1964. An experimental study of succession in the Transvaal Highveld. In: Davis, D.H.S., (Eds.), Ecological studies in Southern Africa, Junk, The Haque. pp. 113-125.

De Groot, H., 1994. Reactive oxygen species in tissue injury. Hepato-Gastroenterology 41: 328-332.

Dibner, J.J., Richards, J.D., 2004. Antibiotic growth promoters in Agriculture: History and mode of action. Poultry Science 84: 634-643.

Dritz, S.S., Qwen, K.Q., Nelssen, J.L., Goodband, R.D., Tokach, M.D., 1996b. Influence of weaning age and nursery diet complexity on growth performance and carcass characteristics and composition of high-health status pigs from weaning to 109 kilograms. Journal of Animal Science 74: 2975-2984.

Eloff, J.N., 2000. Antibacterial activity of Marula (*Sclerocarya birrea* (A.Rich) Hochst. Subsp. *Caffra* (Sond) Kokwaro) Anacardiaceae) bark and leaves. Journal of Ethnopharmacology 76(3): 305-308.

Eloff, J.N., Jager, A.K., van Staden, J., 2001. The stability and the relationship between anti-inflammatory activity and antibacterial properties of southern African *Combretum* species: research in action. South African Journal of Science 97: 291-294.

Eloff, J.N., 1998a. A sensitive and quick method to determine the minimal inhibitory concentration of plant extracts for bacteria. Planta Medica, 64: 711ï 713.

Eloff, J.N., 1998b. Which extractant should be used for the screening and isolation of antimicrobial components from plants? Journal of Ethnopharmacology 60: 1ï 8.

Eloff JN., McGaw LJ., 2014. Using African plant biodiversity to combat microbial infections pp 163-173 in GURIB-FAKIM A (Ed) Novel Plant Bioresources: Applications in Food Medicine and Cosmetics. John Wiley DOI: 10.1002/9781118460566.ch12.

EFSA., 2006. http://ec.europa.eu/food/food/animalnutrition/feedaddtives/index_en.html. (Last accessed January 2012).

Engberg, R.M., Jensen, B.B., Hojberg, O., 2008. Plant of the *Juglandaceae* family as alternative to antibiotic growth promoters in broiler production. 16th European Symposium on Poultry Nutrition. 293-296

Farnsworth, N.R., 1993. Ethnopharmacology and drug development in: Prance G.T. (ed), Ethnobotany and the search for new drugs. Wiley, Chichester (Ciba Foundation Symposium 185) pp. 42-59.

Farnsworth, N.R., 1984. The role of medicinal plants in drug development. In: Krogsgard-Larsen, P., Brogger Christensen, L., Kofold, H., (Eds.), Natural products and drug development, Munksgaard: Copenhagen, 17-28.

Fessenden, R.J., Fessenden, J.S., 1982. Organic chemistry, Second edition. Willard Grant Press, Boston, Mass.

Fernandez-Lopez, J., Zhi, N., Aleson-Carbonell, L., Perez-Alvarez, J.A., Kuri, V., 2005. Antioxidant and antibacterial activities of natural extract: application in beef meatballs. Meat Science 69: 371-380.

Fukumoto, L.R., Mazza, G., 2000. Assessing antioxidant and prooxidant activities of phenolic compounds. Journal of Agriculture and Food Chemistry 48, 3597-3604.
Feigher, S.D., Dashkervicz, M.P., 1987. Subtherapeutic levels of antibiotics in poultry feeds and their effects on weight gain, feed efficiency and bacterial cholytaurine hydrolase activity. Applied Environmental Microbiology 53: 331-336.

Gatesoupe, F.J., 1999. The use of Probiotics in Aquaculture. Aquaculture 180: 147-165

Gaskins, H.R., Collier, C.T., Anderson, D.B., 2002. Antibiotics as growth promotants: mode of action. Animal Biotechnology 13: 29-42.

Grace, P.A., 1994. Ischaemia-reperfusion injury. British Journal of Surgery 81: 637-647.

Garnsworthy, P.C., Wiseman, J., 2009. Recent advances in animal nutrition. Nottingham University Press. Nottingham.

George, S., Brat, P., Alter, P., Amiot, M.J., 2005. Rapid determination of polyphenols and vitamin C in plant-derived products. Journal of Agricultural Food Chemistry 53: 1370-1373.

Gyles, C.L., 1994. *Escherichia coli* Vero toxin and other cytotoxins. *Escherichia coli* in Domestic Animals and Humans. Gyles, C.L (Ed.). CAB International, Wallingford, UK. 151 -170.

Hac-Wydro, K., 2013. Studies on ϐsitosterol and ceramide-induced alterations in the properties of cholesterol/sphingomyelin/ganglioside monolayers. *Biochimica et Biophysica Acta* 1828. 2460-2469.

Hamburger, M., Hostettmann, K., 1991. Bioactivity in plants: The link between phytochemistry and medicine. Phytochemistry, Vol 30, 12: 3864-3874.

Hammer K.A., Carson, C.F., Riley, T.V., 1999. Antimicrobial activity of essential oils and other plant extracts. Journal of Applied Microbiology 86: 985-990.

Hatano, T., Kagawa, H., Yasuhara, T., Okuda, T., 1988. Two new flavonoids and other constituents of Licorice root: their relative astringency and radical scavenging effects. Chemical Pharmacology Bulletin 36: 2090-2097.

Hadjmohammadi, M.R., Kamel, K., 2006. Determination of Juglone (5-hydroxy 1, 4-naphthoquinone) in Pterocarya fraxinifolia by RP-HPLC. Iranian Journal of Chemistry and Chemical Engineering 25(4): 73-76.

Harborne, J.B., 1998. Phytochemical Methods A guide to modern techniques of plant analysis. Third Edition. Chapman and Hall, London, 302.

Hinton, M., Rixson, P.D., Allen, V., Linton, A.H., 1985. The persistence of drug resistant *Escherichia coli* strains in the majority faecal flora of calves. Journal of Hygiene. Cambridge. 93: 547-557.

Hoffman, C., Evans, A.C., 1911. The uses of spices as preservatives. Journal of Indian Engineering and Chemistry 3, 835-838.

Hojberg, O., Engberg, R.M., Jensen, B.B., Wallace, R.J., 2012. *In vitro* antimicrobial activity of materials from 450 European plant species towards *Clostridium perfringens*, enterotoxigenic *Escherichia coli* (ETEC) and *Campylobacter jejuni*. Alternatives to Antibiotics. The World Organization for Animal Helath 25-28 September 2012.

Holetz, F.B., Pessini, G.L., Sanchez, N.R., Cortez, D.A.G., Nakamura, C.V., Filho, B.P.D., 2002. Screening of some plants used in the Brazilian folk medicine for the treatment of infectious diseases. Memórias do Instituto Oswaldo Cruz 97, 1027-1031.

Hostettmann, K., 1991. Assays for bioactivity. Methods in Plant Biochemistry, vol. 6, Academic Press, San Diego, 360.

Hostettmann, K., Marston, A., Ndjoko, K., Wolfender, J., 2000. The potential of African plants as a source of drugs. Current Organic Chemistry 4, 973-1010.

Hostettmann, K., Wolfender, J., Terreaux, C., 2001. Modern screening techniques for plant extracts. Pharmaceutical Biology 39, Supplement, 18-32.

Hadjmohammadi, M.R., Kamel, K., 2006. Determination of Juglone (5-hydroxy 1, 4-naphthoquinone) in *Pterocarya fraxinifolia* by RP-HPLC. Iran Journal of Chemical Engineering, Vol 25 (4): 73-76.

Iwalewa, E.O., McGaw, L.J., Naidoo, V., Eloff, J.N., 2007. Inflammation: the foundation of disease and disorders. A review of phytomedicines of South African origin used to treat pain and inflammatory conditions. African Journal of Biotechnology. Vol. 6 2868-2885.

Jamaroz, D., Wertelecki, T., Houszka, M., Kamel, C., 2006. Influence of diet type on the inclusion of plant origin active substances on morphological and histochemical characteristics of the stomach and jejunum walls in chicken. Journal of Animal Physiology. Animal Nutrition (Berl.) 90: 255-268.

Jang, I.S., Ko, Y.H., Yang, H.Y., Ha, J.S., Kim, Y.J, Kang, S.Y., Yoo, D.H., Nam, D.S., Kim, D.H., Lee, C.Y., 2004. Influence of essential oil components on growth performance and the functional activity of the pancreas and small intestine in broiler chickens. Asian-australas. Journal Animal Science. 17: 394-400.

Jin, Z., Yang, Y.X., Choi, J.Y., Shinde, P.L., Yoon, S.Y., Hahn, T.W., Lim, H.T., Park, Y.K., Hahm, K.S., Joo, J.W., Chae, B.J., 2008. Effects of potato (*Solanum tuberosum* L. cv. Golden valley) protein having antimicrobial activity on the growth performance and intestinal microflora and morphology in weaning pigs. Animal Feed Science and Technology 140: 139-154.

Jouany, J.-P., Morgavi, D.P., 2007. Use of natural products as alternatives to antibiotic feed additives in ruminant production. Animal 1(10): 1443-1466.

Kamboj, A., Saluja, A.K., 2011. Isolation of stigmasterol and ɓ-sitosterol from petroleum ether extract of aerial parts of *Ageratum conyzoides* (Asteraceae). International Journal of Pharmacy and Pharmaceutical Sciences 3 (1): 94-96.

Kamel, C., 2001. Tracing modes of action and roles of plant extracts in non-ruminants In: Garnsworthy, P.C., Wiseman, J. (Eds). Recent advances in animal nutrition. Nottingham Press, Nottingham, UK. Pp135-150.

Katerere, D.R., Eloff, J.N., 2005. Antibacterial and antioxidant activity of *Sutherlandia frutescens* (Fabaceae), a reputed anti-HIV/AIDS Phytomedicine. Phytotherapy Research 19: 779-781.

Kumar, G.S., Jayaveera, K.N., Kumar, C.K.A., Sanjay, U.P, Swamy, B.M.V., Kumar, D.V.K., 2007. Antimicrobial effects of Indian medicinal plants against acne-inducing bacteria. *Tropical Journal of Pharmaceutical Research* **6**(2), 717-723.

Kuete, V., Krusche, B., Youns, M., Voukenga, I., Fankam, A. G., Tankeo, S., Lacmata, S., Efferth, T., 2011. Cytotoxicity of some Cameroonian species and selected medicinal plant extracts. Journal of Ethnopharmacology 134: 803-812

Kutbay, H.G., Merev, N., Ok, T., 1999. Anatomical phytosociological and ecological properties of *Pterocarya fraxinifolia* (Pioret) Spach. Turkish Journal of Agricultural Forestry 23 (5): 1189-1196.

Lynckander, I. M., Malterud, K.E., 1992. Lipophilic flavonoids from *Orthosiphan spicatus* as inhibitors of 15-liopoxygenase. Acta Pharmaceutica Nordica 4: 159-165.

Lalles, J.-P., Bosi, P., Smidt, H., Stokes, C.R., 2007. Weaning- A challenge to gut physiologists. Livestock Science 108: 82-93.

Lalles, J.-P., 2008. Nutrition and gut health of the young pig around weaning: what news? Archiva Zootechica 11(1): 5-15.

Laparra, J.M., Sanz, Y., 2009. Interactions of gut microbiota with functional food components and nutraceuticals. Pharmacological Research. Doi:10.1016/j.phrs.2009.11.001.

Lee, K.W., Everts, H., Kappert, H.J., Frehner, M., Losa, R., Beynen, A.C., 2003. Effects of dietary essential oil components on growth performance, digestive enzymes and lipid metabolism in female broiler chickens. Br. Poultry Science 44: 450-457.

Lin, J., Opoku, A.R., Geheeb-Keller, M., Hutchings, A.D., Terblanche, S.E., Jager, A.K., van Staden, J., 1999. Preliminary screening of some traditional Zulu medicinal plants for anti-inflammatory and anti-microbial activities. Journal of Ethnopharmacology 68: 267-274.

Luangtongkum, T., Morishita, T.Y., Ison, A.J., Huang, S., McDermott, P.F., Zhang, Q., 2006. Effects of conventional and organic production practices on the prevalence and antimicrobial resistance of *Campylobacter* spp in poultry. Applied and Environmental Microbiology. 72(5), 3600-3607.

Madec, F., Geers, R., Vesseur, P., Kjeldsen, N., Blaha, T., 2001. Traceability in the pig production chain. Rev. Sci. tech. Off. Int. Epiz. 20(2): 528-537.

Mahan, D.C., 1993. Effect of weight, split-weaning and nursery feeding programs on performance response of pigs to 105 kilograms body weight and subsequent effects on sow rebreeding interval. Journal of Animal Science 71: 1991-1995.

Mahan, D.C., Lepine, A.J., 1991. Effect of pig weaning weight and associated nursery feeding programs on subsequent performance to 105 kilograms body weight. Journal of Animal Science 69: 1370-1378.

Maiga, A., Malterud, K.E., Diallo, D., Paulsen, B.S., 2006. Antioxidant and *15-lipoxygenase* inhibitory activities of the Malian medicinal plants *Diospyros abyssinica* (Hiern) F. White (Ebenaceae), *Lannea velutina* A. Rich (Anacardiaceae) and *Crossopteryx febrifuga* (Afzel) Benth. (Rubiaceae). Journal of Ethnopharmacology 104: 132-137.

Masoko, P., Eloff, J.N., 2005. The diversity of antifungal compounds of six South African *Terminalia* species (combretaceae) determined by bioautography. African Journal of Biotechnology 4, 1425-1431.

Masoko, P., Picard, J., Eloff, J.N., 2005. Antifungal activities of six South African T*erminalia* species (Combretaceae). Journal of Ethnopharmacology 99, 301-308.

Masoko, P., Eloff, J.N., 2006. Bioautography indicated the multiplicity of antifungal compounds from twenty-four southern African *Combretum* species (Combretaceae). African Journal of Biotechnology, vol. 5 (18): 1625-1647.

McDevitt, R.M., Brooker, J.D., Acamovic, T., Sparks, N.H.C., 2006. Necrotic enteritis; A continuing challenge for the poultry industry. Worlds Poultry Science Journal 62, 221-247.

McGaw, L.J., Steenkamp, V., Eloff, J.N., 2007. Evaluation of *Athrixia* bush tea for cytoxicity, antioxidant activity, caffeine content and presence of pyrolizidine alkaloids. Journal of Ethnopharmacology 110: 16-22.

McGaw, L.J., Jager, A.K., van Staden, J., 1997. Prostaglandins synthesis inhibitory activity in Zulu, Xhosa and Sotho medicinal plants. Phytohter. Res. 11: 113-117.

Mead, P.S., Slutsker, L., Diets, V., McCraig, L. F., Bresee, J.S., Shapiro, C., Griffin, P.M., Tauxe, R. V., 1999. Food-related illness and death in the United States. Emerging Infectious Diseases 5: 607-625.

Metchnikoff, E., 1908. Optimistic studies New York: Putman's Sons, 161-183.

Meurer, B., Wray, V., Wiermann, R., Strack, D., 1998. Hydroxycinnamic acid-spermidine amides from pollen of *Alnus glutinosa, Betula verrucosa* and *Pterocarya fraxinifolia*. Phytochemistry 27(3): 839-843.

Michiels, J., Missotten, J.A.M., Fremaut, D., De Smet, S., Dierick N.A., 2009. *In vitro* characterisation of the antimicrobial activity of selected essential oil compounds and binary combinations against the pig gut flora. Animal Feed Science and Technology 151: 111-127.

Mitsch, P., Zitterl-Eglseer, K., Kohler, B., Gabler, C., Losa, R., Zimpernik, I., 2004. The effect of two different blends of essential oil components on the proliferation of *Clostridium perfringens* in intestines of broiler chickens. Poultry Science. 83: 669-675.

Miguel, J.C., Pettigrew, J.E., 2005. The emerging picture of diet effects on gastrointestinal microbial populations. Proceedings of Midwest Swine Nutrition Conference, Indianapolis, IN. pp45-59.

Miller, N.J., Rice-Evans, C.A., Davies, M.J., Gopinathan, V., Milner, A., 1993. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. Clinical Science 84, 407-412.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. Journal of Immunological Methods 65, 55-63.

Moughan, P.J., Verstegen, M.W.A., Visser-Reyneveld, M.I., 2000. Feed evaluation; principles and practice. Wageningen Press. Netherlands.

Moore, J., Madden, H., Kerr, J.R., Wilson, T.S., Murphy, P.G., 1996. Erythromycin resistant thermophilic *Campylobacter* species isolated from pigs. Vet. Rec. 138: 306-307.

Nakamura, Y., Kozuka, M., Naniwa, K., Takabayashi, S., Torikal, K., Hayashi, R., Sato, H., Osawa, T., 2003. Arachidonic acid cascade inhibitors modulate phorbol ester-induced oxidative stress in female ICR mouse skin: Differential roles of 5-lipoxygenase and cyclooxygenase-2 in leukocyte infiltration and activation. Free Radical Biology and Medicine, vol. 35. 9: 997-1007.

NCCLS., 1990. The National Committee for Clinical Laboratory Standards, Performance Standards for Antimicrobial Disk Susceptibility Tests. 4th Ed. Approved by Standard, NCCLS document M2-A4, Villanova, PA, USA.

Navarro, V., Rojas, G., Delgado, G., Lozoya, X., 1998. Antimicrobial compounds detected in *Bocconia arborea* extracts by direct bioautographic method. Archives Medical Research 29, 191-194.

Niewold, T.A., 2007. The nonantibiotic anti-inflammatory effects of antimicrobial growth promoters, the real mode of action? A hypothesis. Poultry Science 86: 605-609.

Novak, J.S., Juneja, V.K., 2002. *Clostridium perfringens*: hazards in new generation foods. Innovative Food Science and Emerging Technologies 3: 127-132.

Noweck, K.m,, Grafahrend, W., 2006. Fatty alcohols. In Ullmann's Encyclopaedia of Industrial Chemistry. Wiley-VCH, Weinheim.

Oliver, W. Leus, K. (2008). Sus scrofa. In: IUCN 2008. IUCN Red List of Threatened Species. Retrieved 6 March 2013. Database entry includes a brief justification of why this species is of least concern.

Okamoto, T., Gohil, K., Finkelstein, E.I., Bove, P., Akaike, T., van der Vliet, A., 2004. Multiple contributing roles for NOS2 in LPS-induced acute airway inflammation in mice. Am. J. Physiol. Lung Cell Mol. Physiol. 286:198-209.

Owusu-Asiedu, A., Nyachoti, C.M., Marquardt, R.R., 2003. Response of early-weaned pigs to an enterotoxigenic *Escherichia coli* K88 challenge when fed diets containing spray-dried porcine plasma or pea protein isolate plus egg yolk antibody, zinc oxide, fumaric acid, or antibiotic. Journal of Animal Science 81: 1790-1798.

Ono, K., Yamamoto, K., 1999. Contamination of meat with *Campylobacter jejuni* in Saitama, Japan. International Journal of Food Microbiology 47: 211-219.

Page, S., 2006. Current use of antimicrobial growth promoters in food animals; The benefits . P19-51 in Antimicrobial Growth Promoters: Where do we go from here? D. Barug, J. de Jong, A.K.Kies, and M. Verstegen, ed. Wageningen Academic Publishers, Wageningen, the Netherlands.

Paterson, H.M., Murphy, T.J., Purcell, E.J., Shelley, O., Kriynovich, S.J., Lien, E., Mannick, J.A., Lederer, J.A., 2003. Injury primes the innate immune system for enhanced toll-like receptor reactivity. Journal of Immunology 171: 1473-1483.

Pauw, E., Eloff, JN., 2014. Which tree orders in Southern Africa have the highest antimicrobial activity and selectivity against bacterial and fungal pathogens of animals? BMC Complementary and Alternative Medicine.14: 317

Payne, G., Bringi, V., Prince, C., Shuller, M., 1991. The quest for commercial production of chemicals from plant cell culture, plant cell and tissue culture in liquid systems, Oxford University Press, Oxford.

Parekh, J., Chanda, S., 2007. Antibacterial and phytochemical studies on twelve species of Indian medicinal plants. African Journal of Biomedical Research vol. 10: 175-181.

Perumal, R.S., Ignacimuthu, S., Raja, P.D., 1999. Preliminary screening of Ethnomedicianl plants from India. Journal of Ethnopharmacology 66(2): 235-240

Platel, K., Srinivasan, K., 2004. Digestive stimulant action of species: A myth or reality? Indian Journal Medicinal Research 119: 167-179.

Pluske, J.R., Le Dividich, J., Vestegen, M.W.A., 2003. Weaning the pig: concepts and consequences. Wageningen Academic Publishers.

Phillipson, J.D., Anderson, A., 1989. Ethnopharmacology and western medicine. Journal of Ethnopharmacology 25(1): 61-72.

Papatsiros, V.G., Tzika, E.D., Tassis, P.D., Kantas, D., Filippopoulos, L.C., Papaioannou, D.S., 2011. Greek experience of the use of phytogenic feed additives in organic pig farming. Journal of Cell and Animal Biology, Vol. 5 (16) pp.320-323.

Rao, R.R., Platel, K., Srinivasan, K., 2003. In vitro influence of spices and spice-active principals on digestive enzymes of rat pancreas and small intestines. Nahrung 47: 408-412.

Rastin, N., 1983. Vegetationskundliche Untersuchungen in Hochwaldresten der Kaspischen Ebene. Phytocoenologia 11: 245-289.

Rochfort, S., Parker, A.J., Dunshea, F.R., 2008. Plant bioactives for ruminant health and productivity. Phytochemistry 69: 299-322.

Rios, J.L., Recio, M.C., Villar, A., 1988. Screening methods for natural products with antimicrobial activity: A review of the literature. Journal of Ethnopharmacology 23, 127-149.

Rahalison, L., Hamburger, M., Hostettmann, K., Monod, M., Frenk, E., 1991. A bioautographic agar overlay method for the detection of antifungal compounds from higher plants. Phytochemical Analysis 2, 199-203.

Rates, S. M. K., 2001. Plants as source of drugs. Toxicon 39: 603-613.

Re, R., Pellegrini, N., Proteggente, A., Pannala A., Yang, M., Rice-Evans, C., 1999. Antioxidant activity applying an improved ABTS radical cation decolourization assay. Free radical Biology and Medicine, Vol 26: 1231-1237.

Rijke, E., Out, P., Niessen, W.M.A., Ariese, F., Gooijer, C., Brinkman, U.A., 2006. Analytical separation and detection methods for flavonoids. Journal of Chromatography A 1112: 31-63.

Sadighara, P., Ashrafihelan, J., Barin, A., Aliesfahani, T., 2009. Histopathology and cholinergic assessment of *Pterocarya fraxinifolia* on chicken embryo. Interdisciplinary Toxicology vol. 2 (4): 254-256.

Si, W., Gong, J., Tsao, R., Zhou, T., Yu, H., Poppe, C., Johnson, R., Du, Z., 2006. Antimicrobial activity of essential oils and structurally related synthetic food additives towards selected pathogenic and beneficial gut bacteria. Journal of applied Microbiology 100: 296-305.

Souri, E., Amin, G., Dehmobed-Sharifabadi, A., Nazifi, A., Farsam, H., 2004. Antioxidant activity of sixty plants from Iran. Iraian Journal of Pharmaceutical Research 3: 55-59.

Soejarto, D.D., 1996. Biodiversity prospecting and benefit-sharing: perspectives from the field. Journal of Ethnopharmacology 51 (1-3): 1-15.

Sosa, S., Balick, M.J., Arvigo, R., Esposito, R.G., Pizza, C., Altinier, G., Tubaro, A., 2002. Screening of the topical anti-inflammatory activity of some Central American plants. Journal of Ethnopharmacology 81: 211-215.

Srivastava, J., Lambert, J., and Vietmeyer, N. 1996. Medicinal Plants: An Expanding Role in Development. World Bank Technical Paper. No. 320.

Sukanya, S. L., Sudisha, J., Hariprasad, P., Niranjana, S.R., Prakash, H.S., Fathima, S.K., 2009. Antimicrobial activity of leaf extracts of Indian medicinal plants against clinical and phytopathogenic bacteria. African Journal of Biotechnology, vol. 8 (23): 6677-6682.

Shai, L.J., McGaw, L.J., Masoko, P., Eloff, J.N., 2008. Antifungal and antibacterial activity of seven traditionally used South African plant species active against *Candida albicans*. South African Journal of Botany 74: 677-684.

Steinberg, D., 1999. At last, direct evidence the lipoxygenase play a role on atherogenesis. Journal of Clinical Investigation 103(11): 1487-1488.

Steiner, T., 2006. Managing gut health: Natural growth promoters as key to animal performance. Nottingham University Press. Nottingham.

Steiner, T., 2009. Phytogenics in animal nutrition: natural concepts to optimize gut health and performance. Nottingham University Press, Nottingham.

Straw, B.E., Zimmerman, J.J., D'Allaire, S., Taylor, D.J., 2006. Diseases of swine, 9th Edition. Blackwell Publishing.

Suffness, M., Douros, J., 1979. Drugs of plant origin. Methods in Cancer Research 26, 73-126.
Sun, J.W., Sheng, J.F., 1998. A Handbook of bioactive compounds from plants. China medical-pharmacological science and Technology Publishing House, Beijing.

Silver, L.L., Bostian, K.A., 1993. Discovery and development of new antibiotics: the problem of antibiotic resistance. Antimicrobial Agents and Chemotherapy 37, 377-383.

Swann, N.M., 1969. Swann Report Joint Committee on the use of Antibiotics in Animal Husbandry and Veterinary Medicine. HMSO, London, Cmnd 4190.

Sorum, H., Sunde, M., 2001. Resistance to antibiotics in the normal flora of animals. Vet. Res. 32: 227-241.

Simon, O., 2005. Micro-organisms as feed additives-Probiotics. Advances in Pork Production 6: 161-167.

Simpson, A.J., McNally, D.J., Simpson, M.J., 2011. NMR spectroscopy in environmental research: from molecular interactions to global processes. Progress in Nuclear Magnetic Resonance Spectroscopy 58(3-4): 97-175.

Sheykholislami, A., Ahmadi, T., 2009. The study of Caucasian Walnut (*Pterocarya fraxinifolia* (Lam.) Spach.) in forests of mashelak (Noshahr, Iran). Botany Research Journal 2(2-4): 28-33.

Singleton, V.L., 1981. Phenolic substances as food toxicants In: Chichester, C.O. (Ed.), Advances in Food Research, vol. 27. Academic Press Inc., New York, pp. 191-208.

Taylor, J.L.S., Rabe, T., McGaw, L.J., Jager, A.K., van Staden, J., 2001. Towards the scientific validation of traditional medicinal plants. Plant Growth Regulation 34; 23-37.

Vazquez, F., Gonzallez, E.A., Garabal, J.I., Valderrama, S., Blanco, J., Baloda, S.B., 1996. Development and evaluation of an Elisa to detect *Escherichia coli* K88 (F4) fimbrial antibody levels. Journal of Medical Microbiology vol. 44: 453-463.

van den Berg, R., Haenen, G.R.M.M., van den Berg, H., Bast, A., 1999. Applicability of an improved Trolox equivalent antioxidant capacity (TEAC) assay for evaluation of antioxidant capacity measurements of mixtures. Food Chemistry 66, 511-517.

van den Bogaard, A.E., Stobberingh, E.E., 2000. Epidemiology of resistance to antibiotics links between animals and humans. International Journal of Antimicrobial Agents. 14: 327-335.

Vundru, S.S., Kale, R.K.,Singh, P., 2013. ßsitosterol induces G1 arrest and causes depolarization of mitochondrial membrane potential in breast carcinoma MDA-MB-231 Cells. *BMC Complementary and Alternative Medicine* 13: 280, 1-9.

Wang Y., Yin J., Qiao Y., Zhang H., Lu X., 2007. Studies on antioxidant activity and chemical constituents of *Artemisia halodendron*. *Asian Journal of Traditional Medicines* **2**(1), 30-33.

Wangensteen, H., Miron, A., Alamgir, M., Rajia, S., Samuelsen, A.B., Malterud, K.E., 2006. Antioxidant activity and *15-lipoxygenase* inhibitory activity of rotenoids, isoflavones and phenolic glycosides from *Sarcolobus globosus*. Fitoterapia (Elsevier) 77 290-295.

Westrell, T., Ciampa, N., Boelaert, F., Korsgaard, H., Chriel, M., Ammon, A., Makela, P., 2009. Zoonotic infections in Europe in 2007: A summary of the EFSA Euro surveillance 14: 1-3.

Wallace, R.J., Chesson, A., 1995. Biotechnology in animal feeds and animal feeding. Weinheim ISBN 3-527-30065-1 (VCH, WEINHEIM).

Wallace, R.J., 2004. Antimicrobial properties of plant secondary metabolites. Proceedings of the Nutrition Society 63, 621-629.

Wallace, R.J., 1994. Rumeinal microbiology, biotechnology and ruminant nutrition: progress and problems. Journal of Animal Science 72: 2992-3003.
Wegener, H.C., 2003. Antibiotics in animal feed and their role in resistance development. Current Opinion in Microbiology. 6: 439-445.

Wallace, R., 2005. REPLACE: A European project on plants, their extracts and other natural alternatives to antimicrobials in animal feeds. Euro Surveillance.http://www.eurosurveillance.org/ew/

Wallace, R.J., 2008. Enzymes, direct-fed microbials and plant extracts in ruminant nutrition. Animal Feed Science and Technology 145: 1-4.

Windisch, W., Schedle, K., Plitzner, C., Kroismayr, A., 2008. Use of phytogenic products as feed additives for swine and poultry. Journal of Animal Science 86: E140-E148.

Wijnands, D.O., 1989. *Pterocarya* ï P. 20 in: Walters, S.M., Alexander, J.C., Bradt, A., Brickell, C.D., Cullen, J., Green, P.F., Heywood, W.H., Matthews, V.A., Robson, N.K.B., Yeo, P.F. and Knees, S.J. (eds.). The European garden flora 3: Cambridge.

World Health Organisation., 2000. WHO global principals for the Containment of Antimicrobial Resistance in Animals Intended for Food. Pp1-23 in Document WHO/CDS/CSR/APH/2000.4. WHO, Geneva, Switzerland.

www.wikipedia.com/images

www.rowett.ac.uk/rumen-up

Zhang, L., Xu, Y.Q., Liu, H.Y., Lai, T., Ma, J.L, Wang, J.F., Zhu, Y.H., 2010. Evaluation of *Lactobacillus rhamnosus* GG using and *Escherichia coli* K88 model of piglet diarrhoea: Effects on diarrhoea incidence, faecal microflora and immune responses. Veterinary Microbiology 141: 142-148.

Appendix A

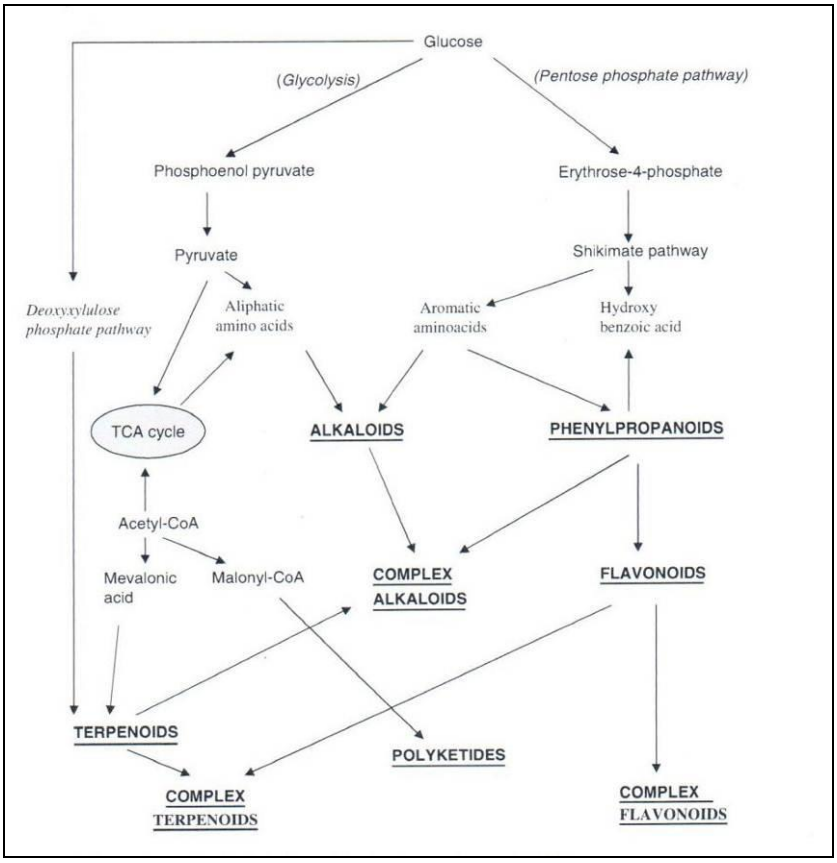

Figure 10.1: The process of plant metabolism and the class of compounds formed during the process (Jouany & Morgavi, 2007).

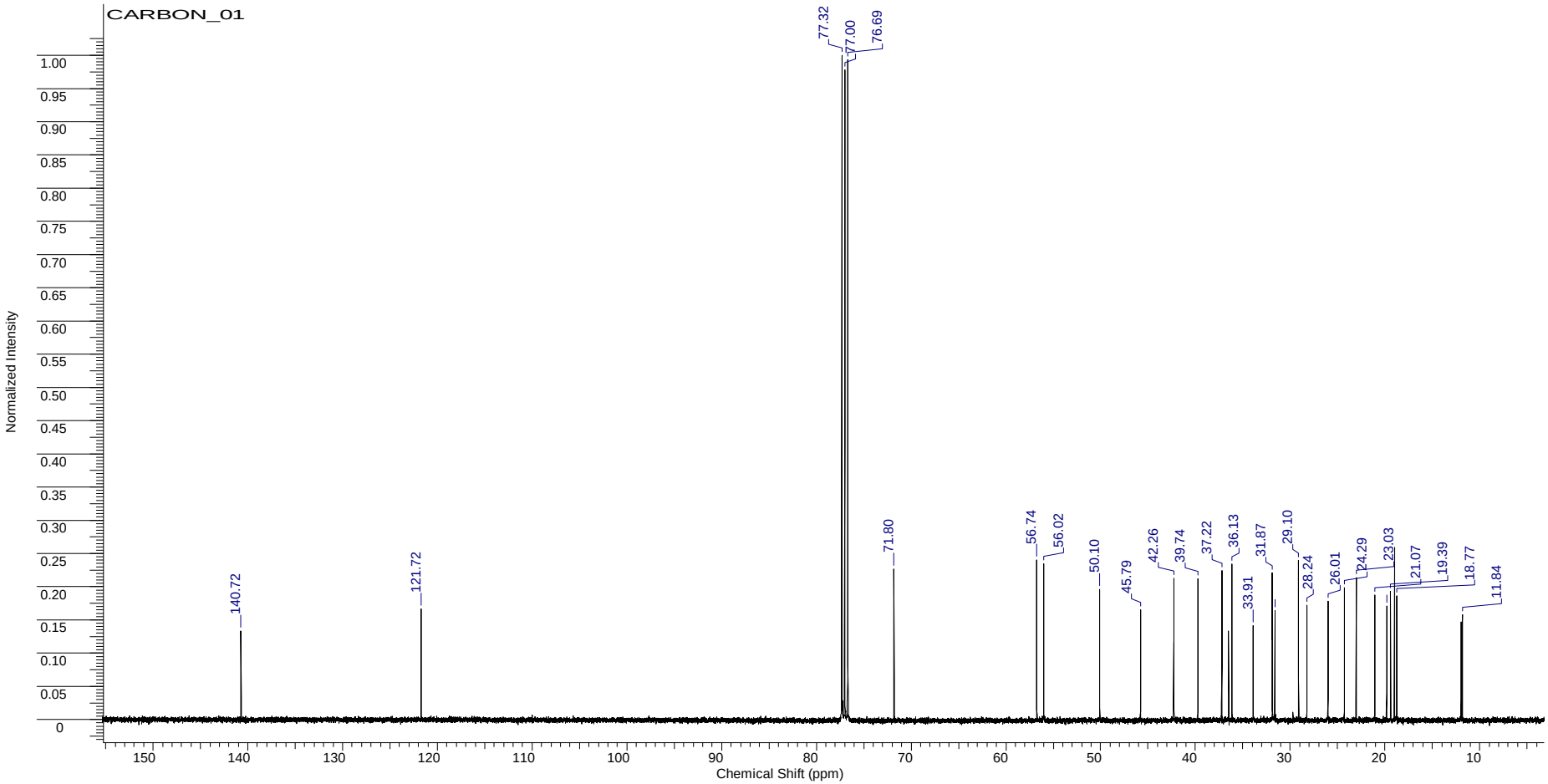

Figure 10.2: ^{13}C NMR (100 MHz, $CDCl_3$) spectrum of PF 1 or *β*-sitosterol (**1**)

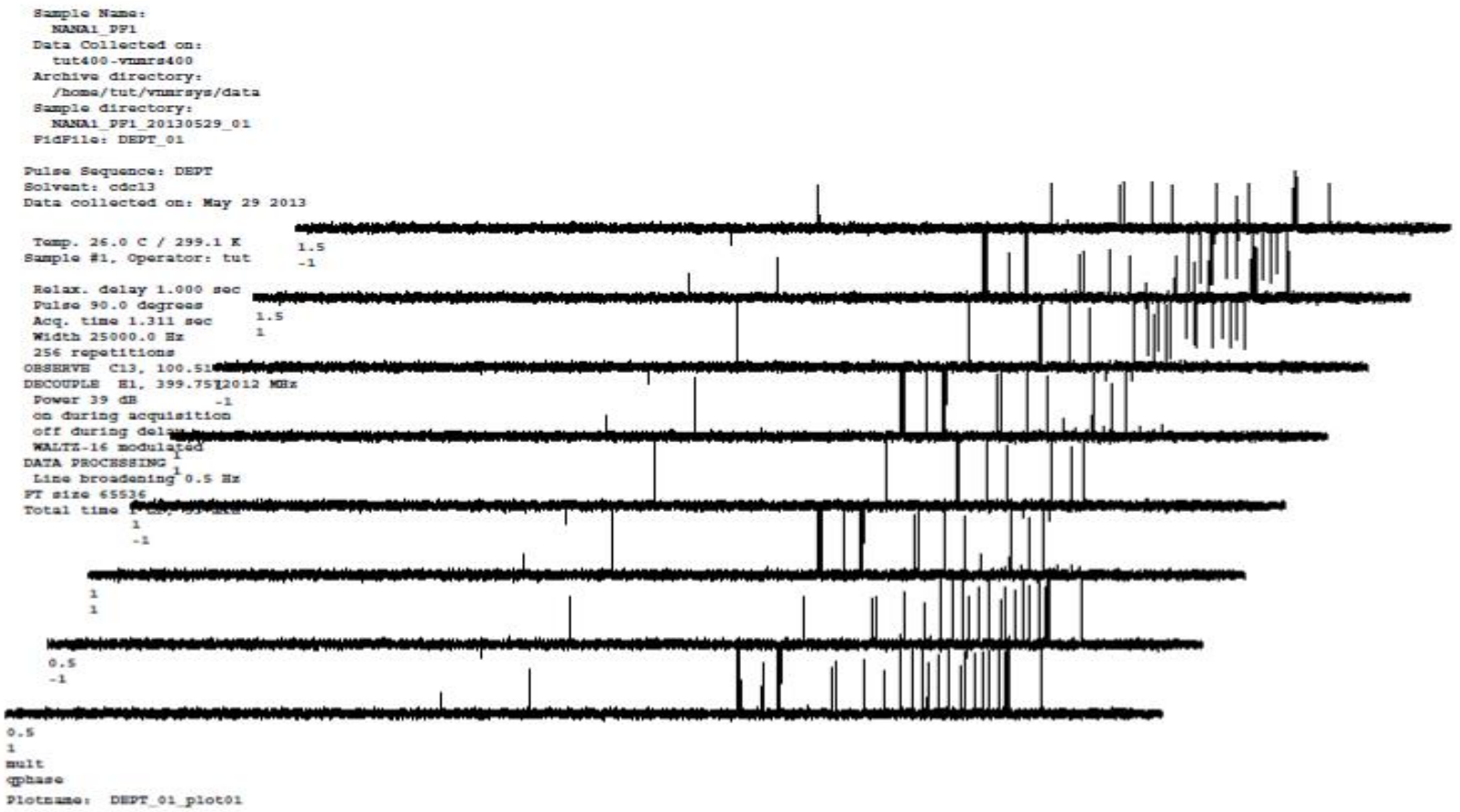

Figure 10.3: DEPT (100 MHz, $CDCl_3$) spectrum of PF1 or *β*-sitosterol (1)

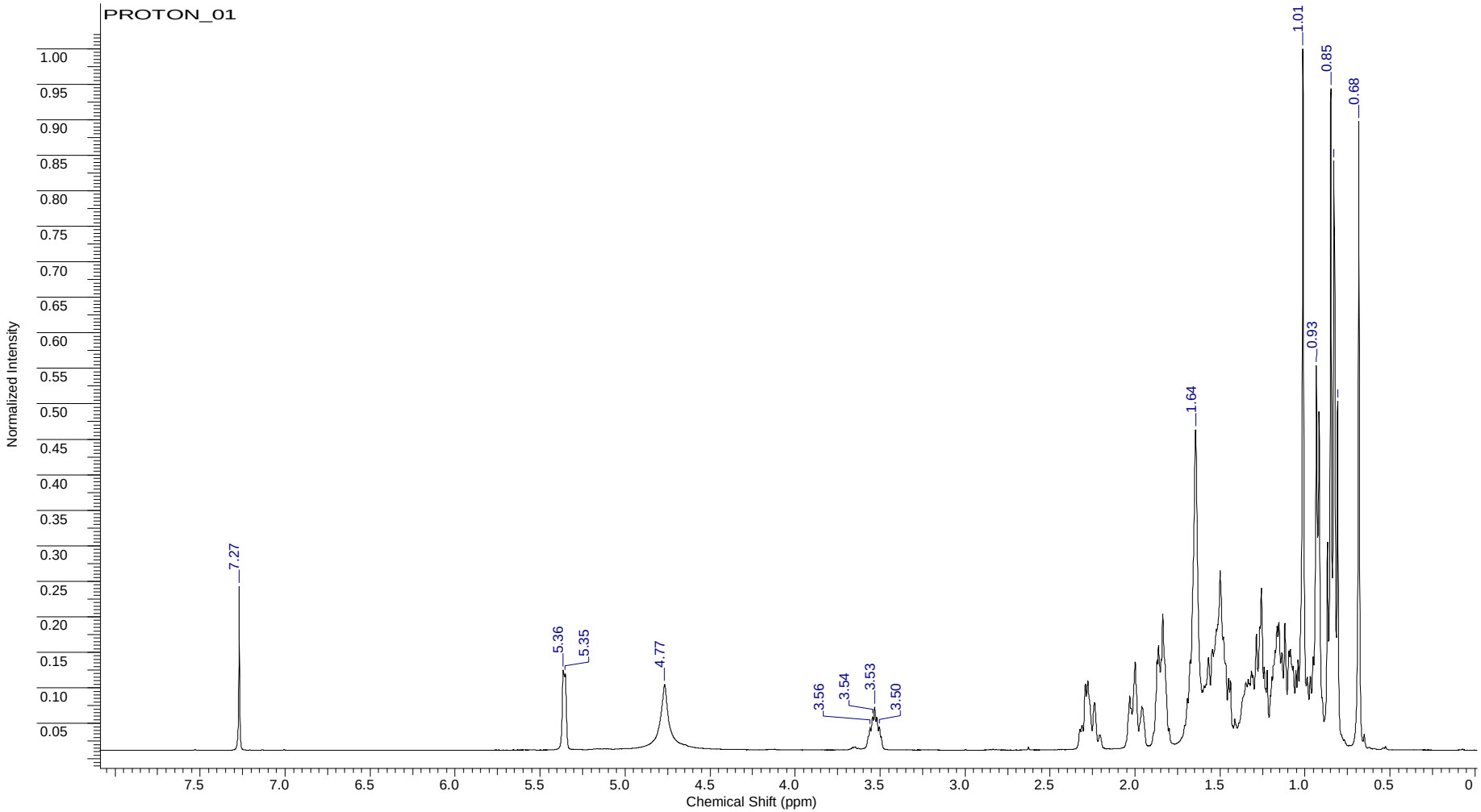

Figure 10.4: ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of PF 1 or *β*-sitosterol (1)

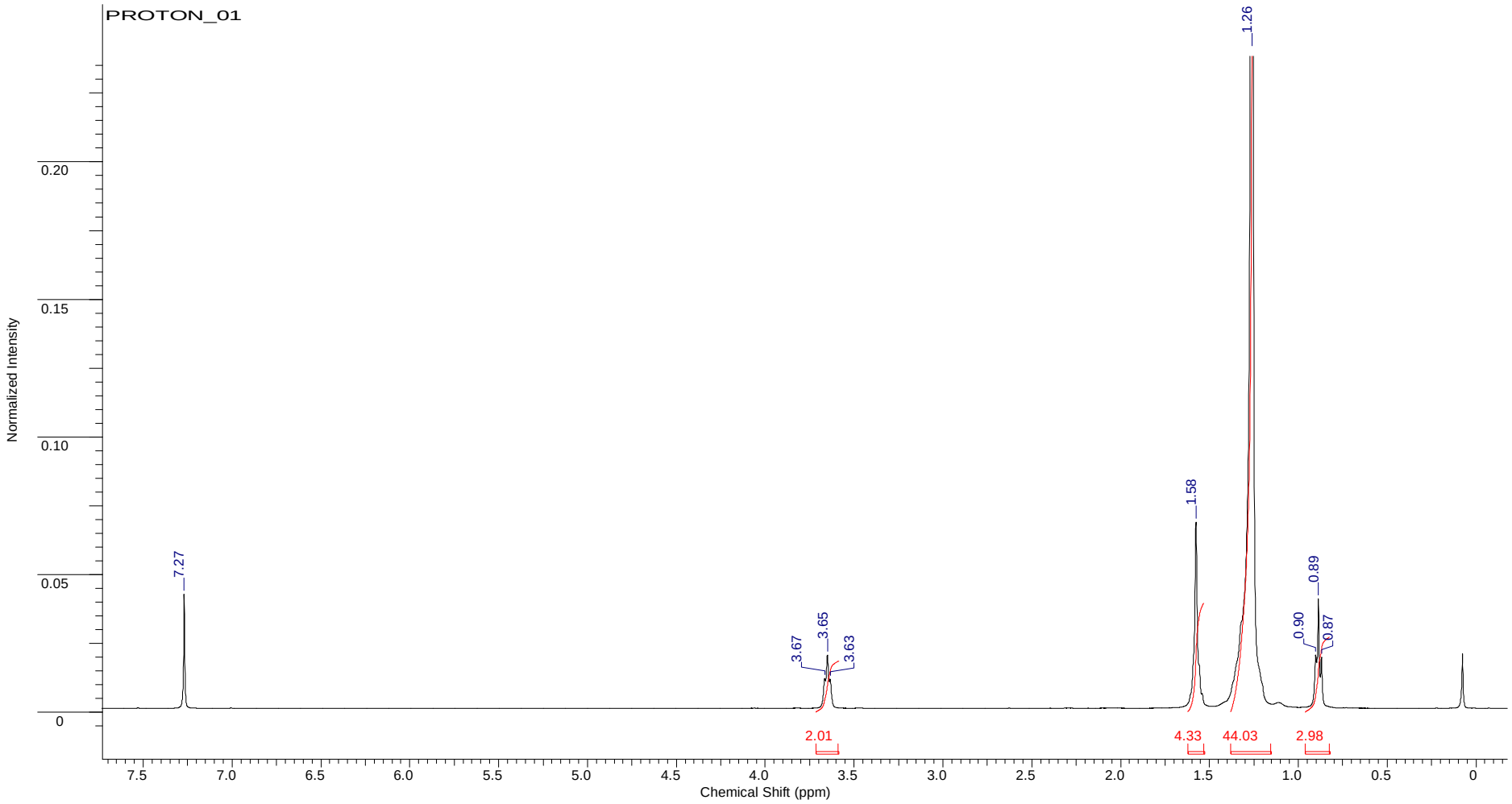

Figure 10.5: ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of PF 2 or pentacosanol (**2**)

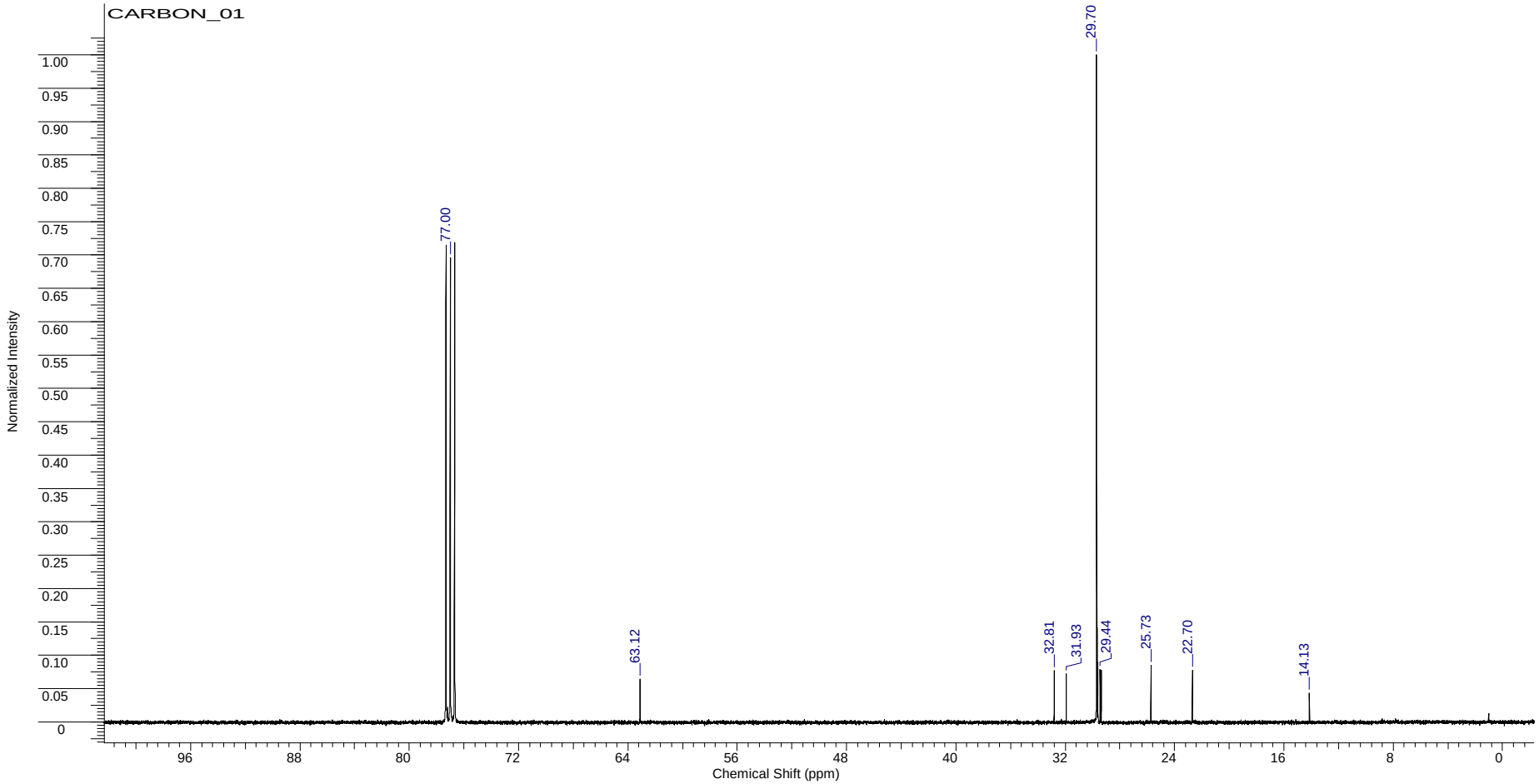

Figure 10.6: ^{13}C NMR (100 MHz, $CDCl_3$) spectrum of PF 2 or pentacosanol (**2**)

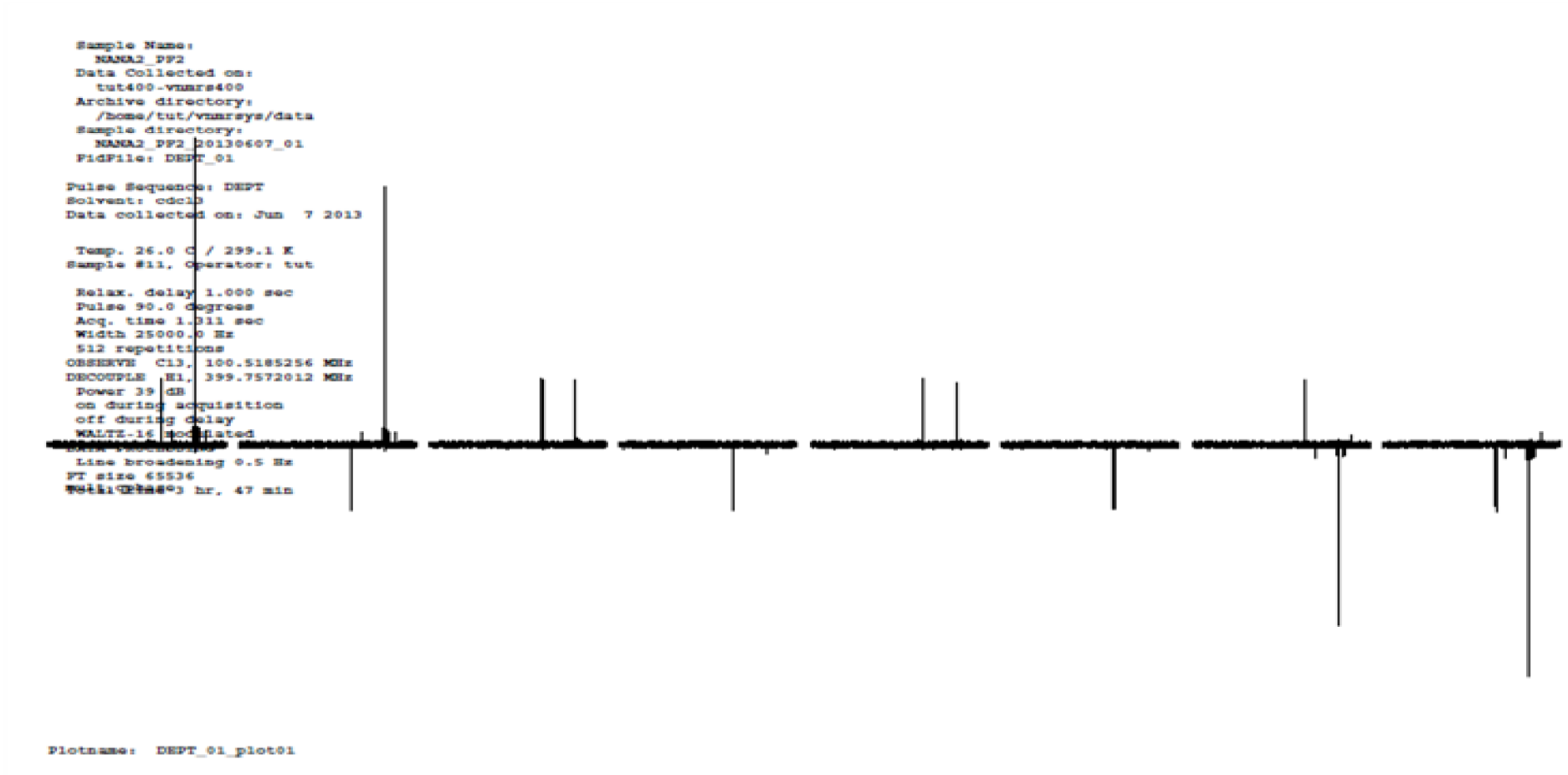

Figure 10.7: DEPT (100 MHz, $CDCl_3$) spectrum of NPF 2 or pentacosanol (**2**)

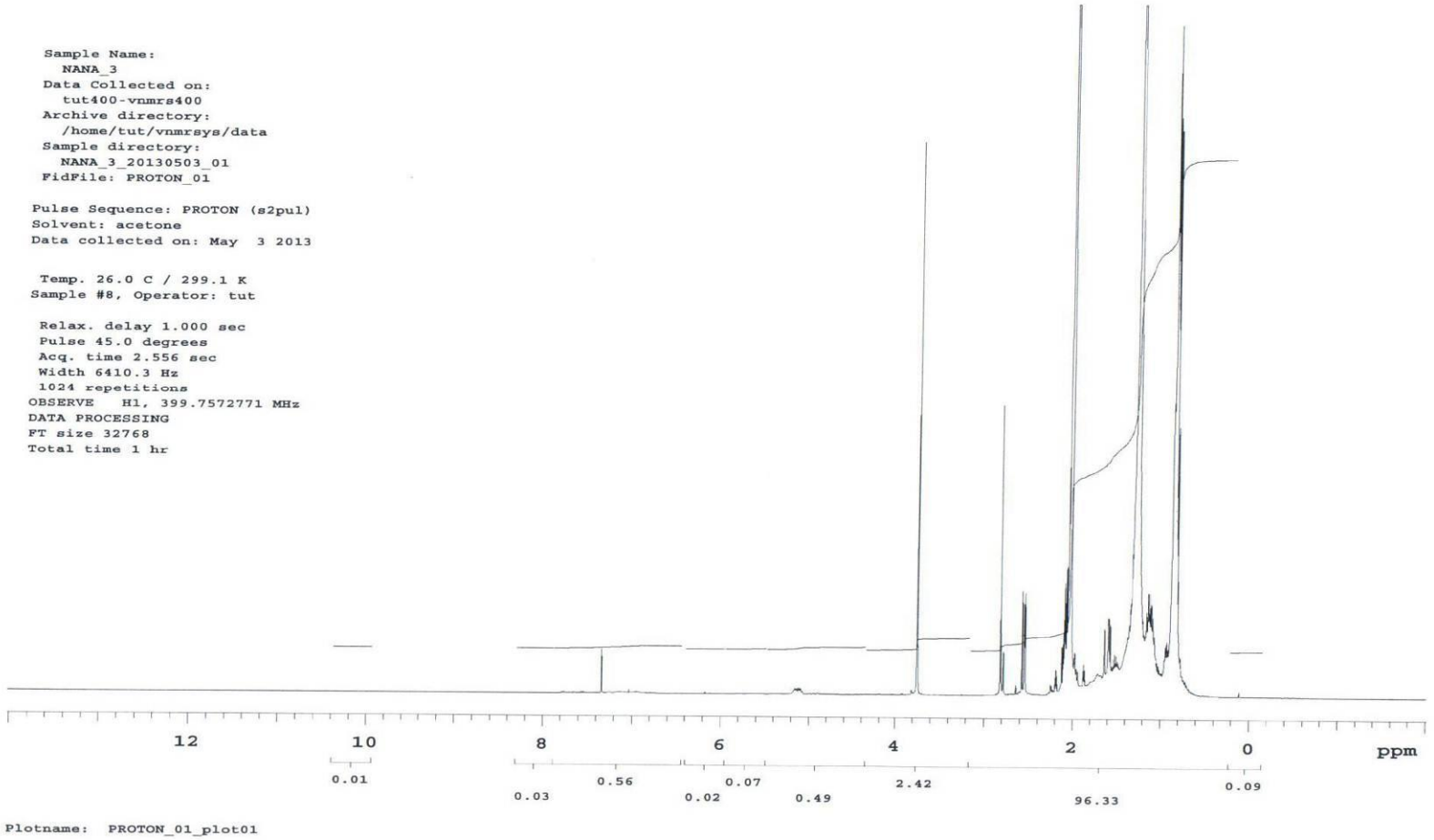

Figure 10.8: ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of unknown compound NPF3

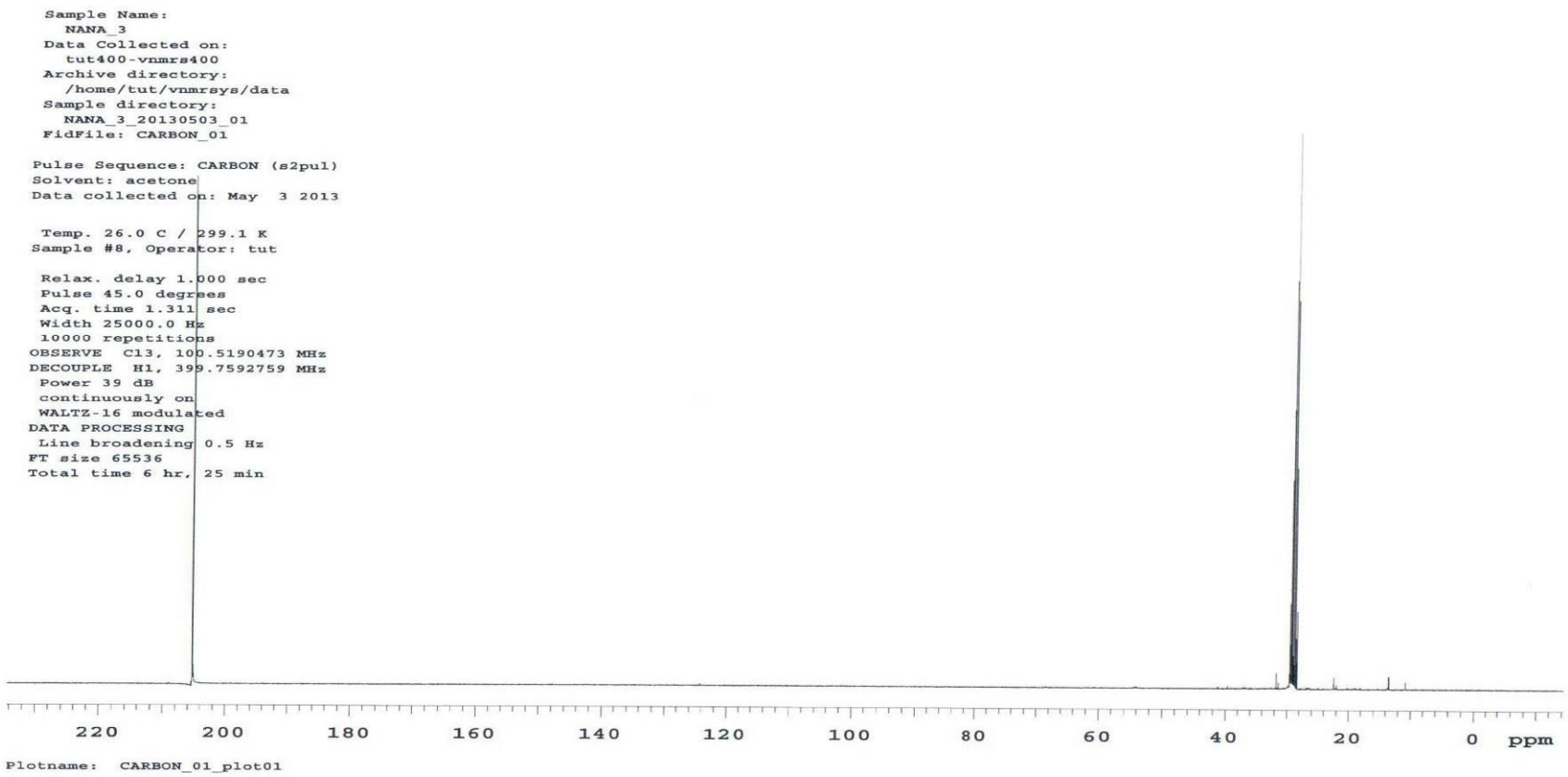

Figure 10.9: ^{13}C NMR (100 MHz, $CDCl_3$) spectrum of unknown compound NPF3

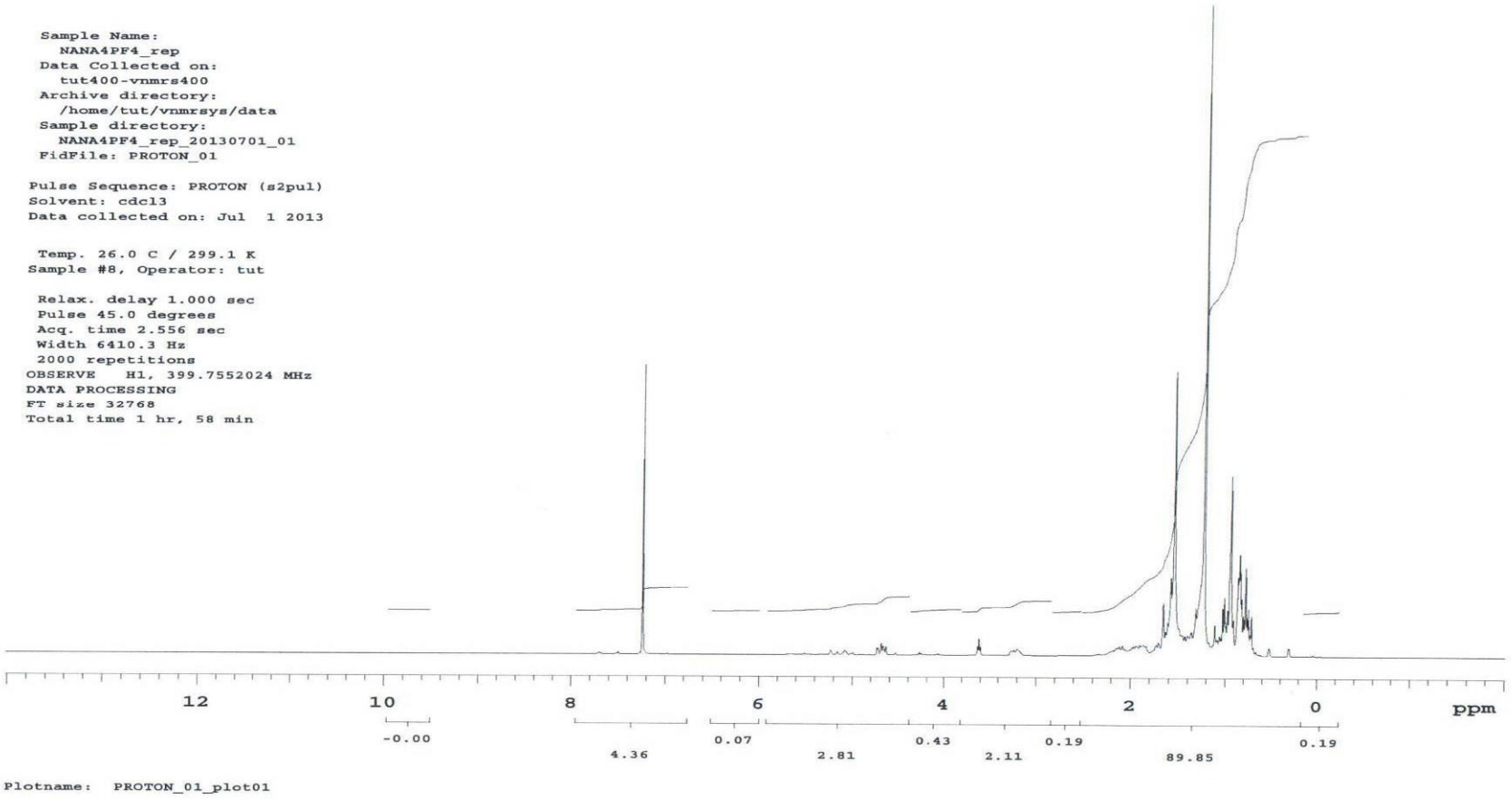

Figure 10.10: ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of unknown compound NPF4

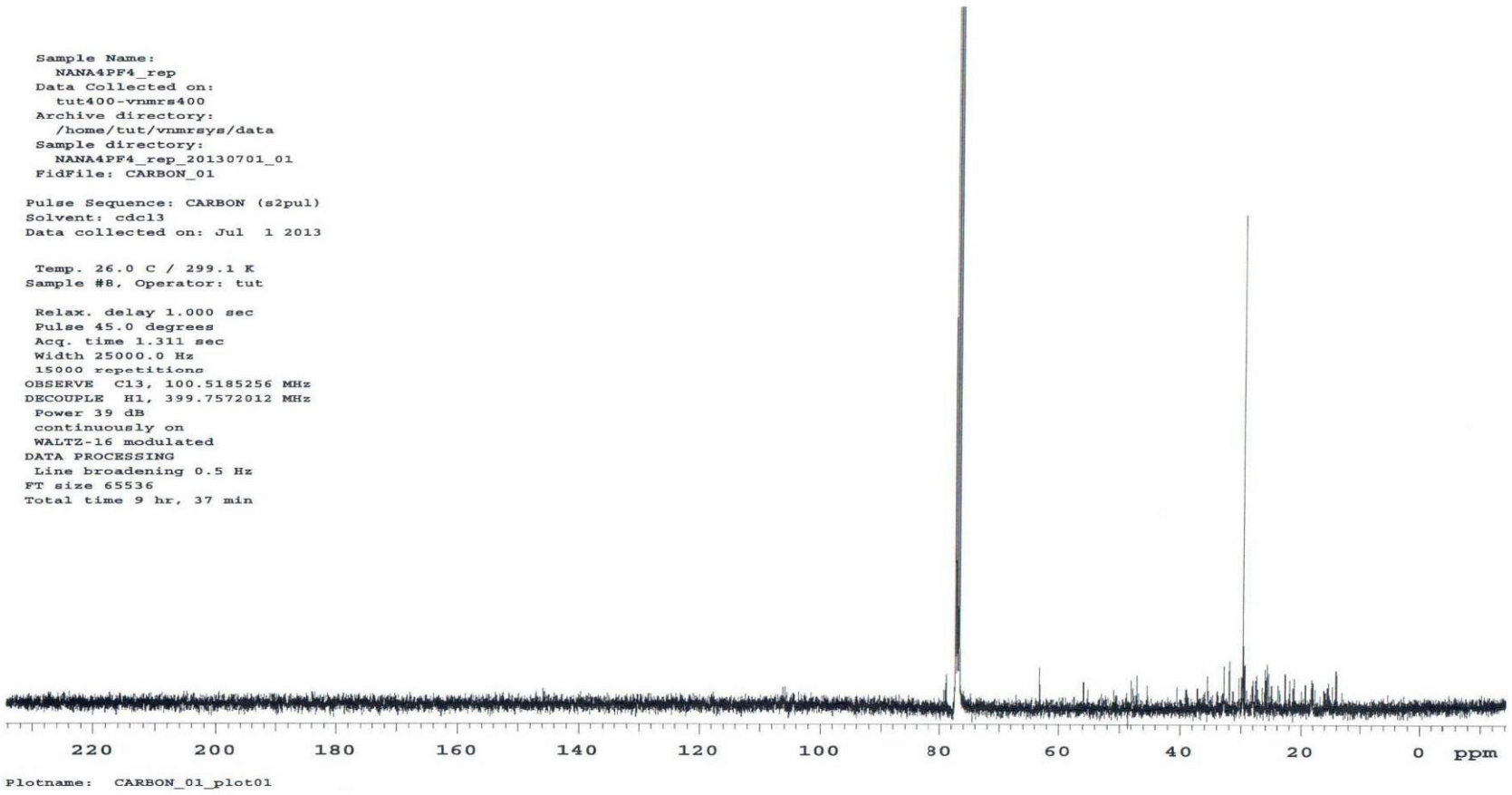

Figure 10.11: ^{13}C NMR (100 MHz, $CDCl_3$) spectrum of unknown compound NPF4

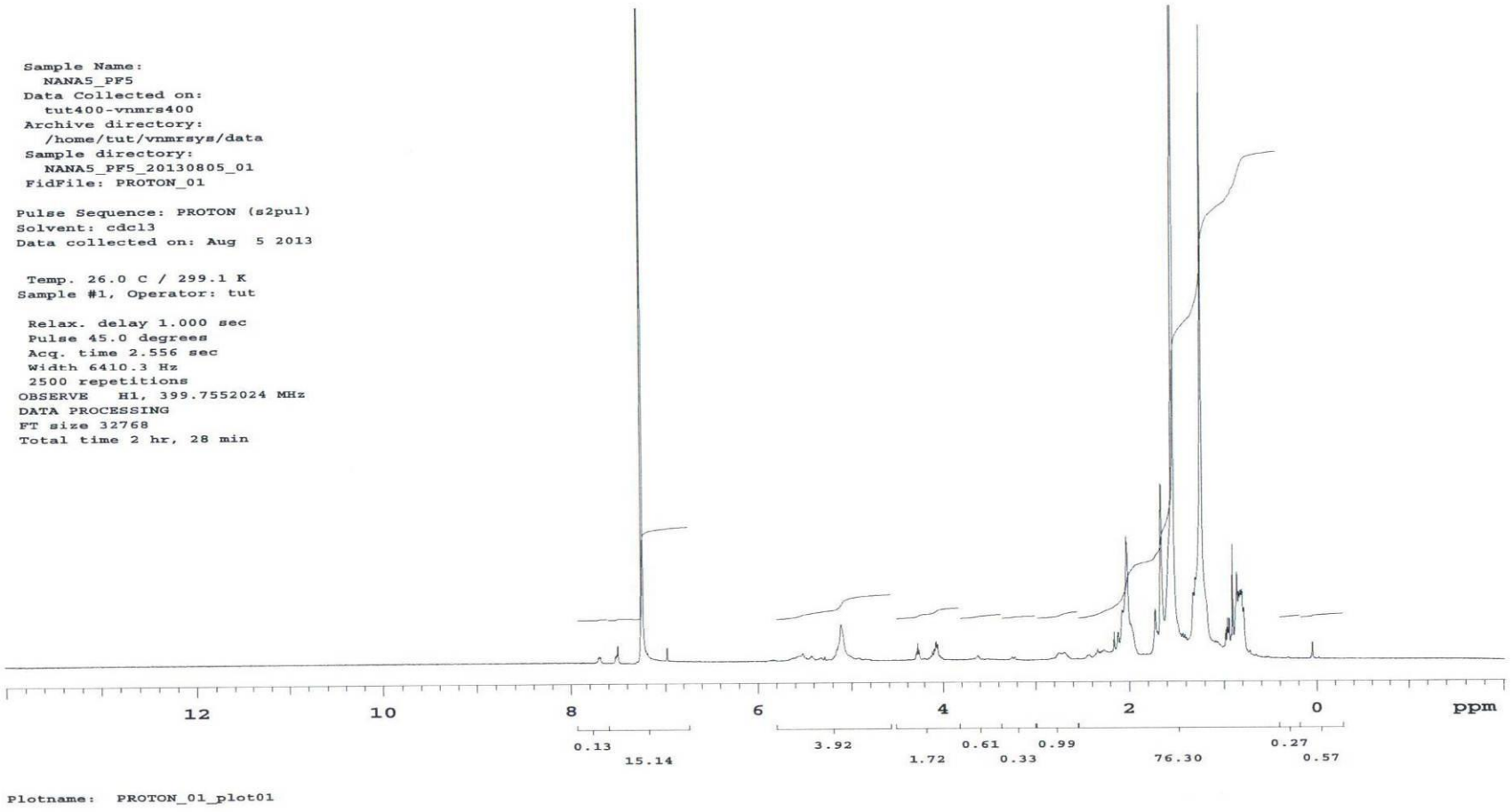

Figure 10.12: ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of unknown compound NPF5

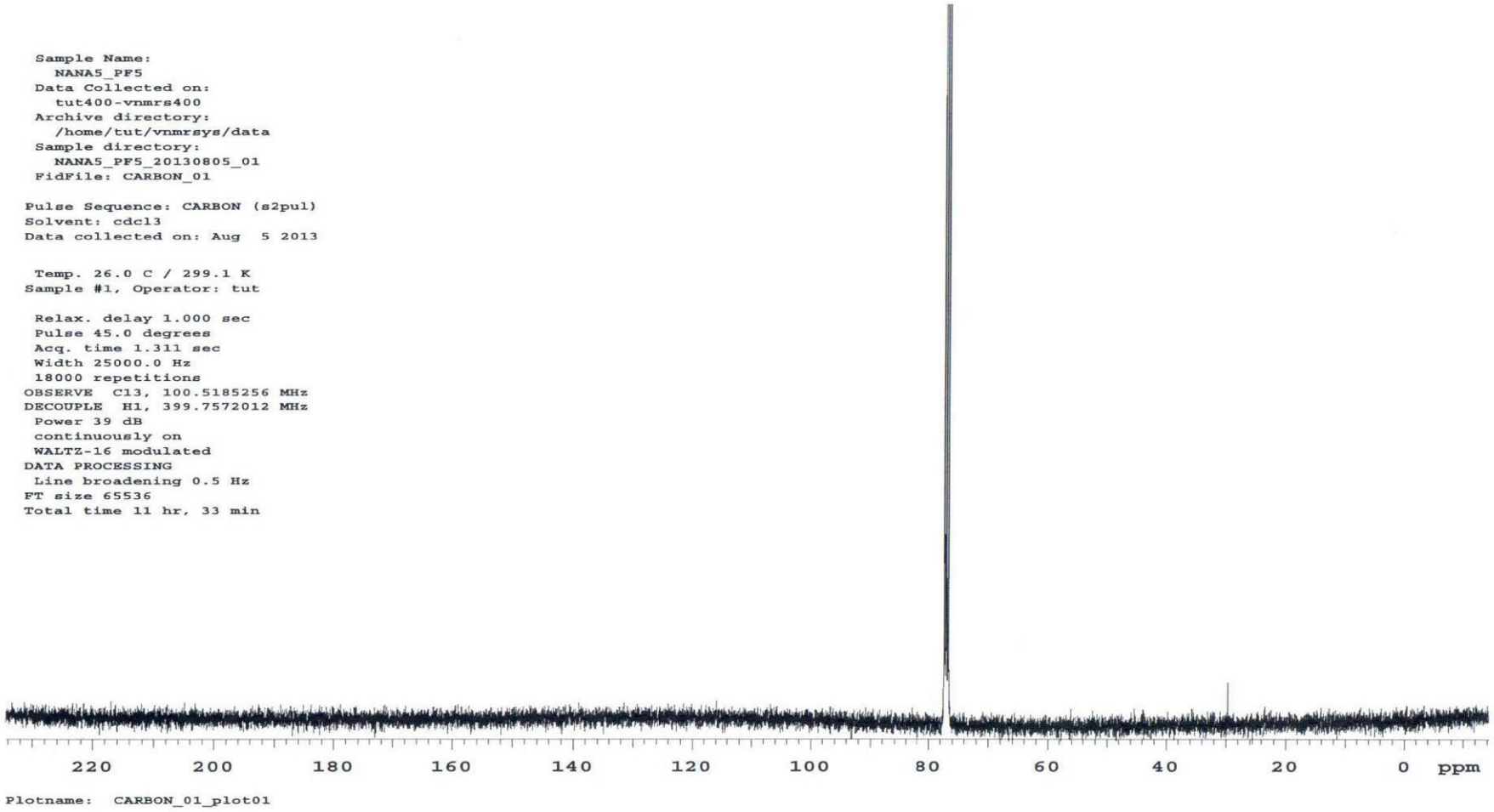

Figure 10.13: ^{13}C NMR (100 MHz, $CDCl_3$) spectrum of unknown compound NPF5