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Evaluation of a phytogenic product from two western herbal medicines to replace an antimicrobial growth promoter in poultry production

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Declaration

The experimental work described in this thesis was conducted in the department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, Phytomedicine Programme under the supervision of Professor J.N. Eloff, Dr J. Picard and Dr S.P.R. Bisschop.

These studies are the result of my own investigations, except where the input of others is acknowledged and have not been submitted in any other form to another University.

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Abstract

Evaluation of a phytogenic product from two western herbal medicines to replace an antimicrobial growth promoter in poultry production

Ilse van Heerden

Promoter: Prof. J.N. Eloff

Co-promoter: Dr. J. Picard

Co-promoter: Dr. S.P.R. Bisschop

Antimicrobial growth promoters (AGPs) are substances that are added to feed in sub-therapeutic levels in intensive animal production to improve weight gain and conversion of feed (FCR) into body mass. AGPs have been used widely as growth promoters in broiler and pig production under high-density growth conditions. Despite the observed efficacy, the use of AGPs has been criticized due to its possible role in the development of antibiotic resistance in human pathogens. Directive 183/2003 of the European Parliament, issued in 2003, banned of the use of all antibiotic agents as growth promoters in the European Union from 2006. The new context caused an increase in the search for alternative growth promoters.

The aim of this study was to produce a commercially viable prophylactic antibacterial phytogenic product from *Ginkgo biloba* and *Hypericum perforatum* with a low potential to develop resistance, as an alternative to AGPs in poultry production.

The first objective of this study based on earlier results of the Phytomedicine Programme, was to evaluate the activity and potentize extracts from *Ginkgo biloba* and *Hypericum perforatum* for optimal activity against relevant bacterial pathogens. Extracts of ethyl acetate (EA), hexane, dichloromethane (DCM) and acetone (in order of activity) from a direct extraction procedure of powdered *G. biloba* leaves were active against *Enterococcus faecalis, Staphylococcus aureus* and *Clostridium perfringens*. The EA, hexane and DCM extracts were 2 to 3 times more active than the acetone extract (average total activity 1728 ml/g dry extract for the 3 pathogens). The DCM-, EA-, acetone- and hexane extracts (in order of activity) from the direct extraction procedure from *H. perforatum* were only active against *C. perfringens* with the first three extracts having a total activity of between 1026 and 1333 ml/g dry material and the hexane extract a total activity of 333 ml/g dry material. The spectrum of activity of *G. biloba* corresponds to that of Zn-bacitracin, which is commonly used an antibiotic growth promoter in the poultry industry.

The second objective in this study was to combine extracts or fractions of extracts of *G. biloba* and *H. perforatum* to optimise activity against selected bacterial pathogens. A synergistic effect could be observed when combining

a ratio of 1:5 of *G. biloba: H. perforatum* (hexane extracts) or 1:15 (acetone extracts) against *E. faecalis* while only an indifferent (neutral) effect was observed against *C. perfringens.*

After elucidation of the quantitative and qualitative aspects involved in the antimicrobial activity, the major antibacterial compound from *G. biloba* was isolated and characterized as ginkgolic acid ($C_{17:1}$). It was also determined whether activity against *E. faecalis* and *C. perfringens* in an extract or fraction of and extract of *G. biloba* can be attributed only to ginkgolic acid or whether synergism or other interactions also play a role in the antibacterial activity. It was shown that synergistic interactions are at play between constituents in the hexane and EA fraction, with the last mentioned fraction not containing any ginkgolic acid. These results support the use of the whole extract as opposed to isolated compounds as antimicrobial agents against pathogenic organisms.

Two important pharmacodynamic parameters were investigated i.e. resistance development to a hexane extract and the isolated ginkgolic acid from *G. biloba* against *E. faecalis* and secondly the time-kill dynamics of this hexane extract over 24 h against *E. faecalis*. The bactericidal nature of the hexane extract from *G. biloba* as well the absence of decreased susceptibility to this extract (and the isolated ginkgolic acid) in the resistance studies against *E. faecalis* indicate that this extract has potential to be exploited as a alternative to AGPs in the poultry industry.

The final objective was to determine the effect of extracts of *G. biloba* alone or in combination with *H. perforatum* extracts on the performance of broiler chickens over a 35 day period. The effect of these extracts on *C. perfringens* in the intestine of broilers was also investigated. No significant differences were found with relation to any of the production parameters studied (FCR, live weight or % survival) although a trend towards more favourable European Performance Efficiency Factor index values were observed for treatments containing *G. biloba* (5% improvement) or a combination of *G. biloba* and *H. perforatum* (2.1% improvement) compared to the untreated control. Similarly, Zn-Bacitracin resulted in a 5.5% improvement compared to the untreated control. There was a general trend (not statistically significant, P=0.05) towards a reduction in *C. perfringens* scores in the feed supplemented with *G. biloba*- in combination with *H. perforatum* extract which can probably be ascribed to the direct antimicrobial effect. The rate of colonization was however too low to cause infection probably due to lack of virulence of the *C. perfringens* challenge and the absence of predisposing factors due to the hygienic growth conditions used. It is necessary for an effective disease model to be developed in order for the efficacy of any new treatment method to be properly evaluated. Such a model will require a much higher incidence of disease and reproducibility than was achieved in this project.

The safety of using extracts of *G. biloba* with ginkgolic acid as the prime antibacterial compound was considered. The active dose was at least 42 times lower than safe dosage recommended in the literature. The combination of extracts of *G. biloba* and *H. perforatum* holds promise as a potential growth promoter in poultry production.

Better results may be achieved if potentized extracts are used and compared with Zn-Bacitracin and a negative control under industrial growth conditions where the birds are stressed and natural infections would take place.

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List of Abbreviations

AGP	Antibiotic growth promoter
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
AUCC	Animal Use and Care Committee
BCA	Blood Columbia Agar
BEA	Benzene/ ethanol/ ammonium hydroxide
BHI	Brain-heart-infusion
BTA	Blood Tryptose Agar
CEF	Chloroform/ ethyl acetate/ formic acid
DCM	Dichloromethane
EA	Ethyl acetate
EMW	Ethyl acetate/ methanol/ water
EPEF	European Performance Efficacy Index
FCR	Feed conversion ratio
FIC	Fractional inhibitory concentration
FOS	Fructo-oligosaccharides
GA	Ginkgolic acid
GIT	Gastro-intestinal tract
GOS	Gluco-oligosaccharides
GRAS	Generally regarded as safe
GRE	Glucopeptide resistant enterococci
HEN	Hen's egg test
INT	*p*-iodonitrotetrazolium violet
MEDUNSA	Medical University of Southern Africa
MH	Müeller Hinton
MIC	Minimum inhibitory concentration
MOS	Manno-oligosaccharides
MRSA	Methicillin resistant *Staphylococcus aureus*
NE	Necrotic enteritis
NMR	Nuclear Magnetic resonance
OD	Optical Density
P	Passage
PAF	Platelet activity factor
SANS	South African National Standards

SEM	Standard error of Mean
SJW	St. Johns wort
TA	Total activity
TLC	Thin Layer Chromatography
UV	Ultra Violet light
VRE	Vancomycin resistant *Enterococcus*
Zn-Bacitracin	Zinc-Bacitracin

1 General introduction

1.1 History of Antibiotic growth promoters (AGPs)

Antibiotic growth promoters (AGPs) are substances that are added to feed in sub-therapeutic levels in intensive poultry and pig animal rearing to improve weight gain and conversion of feed (FCR) into body mass (Dibner and Richards, 2005). Moore *et al* (1946) first reported increased weight gain after inclusion of antibiotics in chicken feed before commercialization in 1949. The advantages of antibiotics in animal feed were officially recognised by the US Food and Drug Administration (FDA) and in 1951 the FDA approved the use of antibiotics in animal feed without veterinary prescription (Jones and Ricke, 2003). In a review of over 12 000 studies, Rosen (1995) concluded that antibiotics will improve growth and feed conversion ratio (FCR) by 2-3%, 72% of the time.

1.2 Mode of action

Despite decades of use, the exact mode of action of AGPs still remains unclear. Early demonstrations that oral antibiotics do not have growth-promoting effects in germ-free animals (Coates *et al.,* 1955; Coates *et al.,* 1963) led to the focus on interactions between the antibiotic and the enteric microbiota. Thus, direct effects of AGP on the microflora can be used to explain decreased competition for nutrients and reduction in microbial metabolites that depress growth (Anderson *et al.,* 1999; Visek, 1978). Furthermore, the reduction in the mucosal thickening thought to be induced by a high microbial load allows for the better absorption of nutrients. It has also been found that AGPs are inhibitory to opportunistic pathogens such as *Clostridium perfringens*. Additional effects of AGPs in terms of physiological, nutritional and metabolic effects are shown in Table 1.1 (Anderson *et al.,* 1999; Visek, 1978).

1.3 The problem with AGPs

The main concern with respect to the use of AGPs is the occurrence of resistance to these AGPs as well as the occurrence of resistance to antibiotics used to treat Gram-positive infections in humans (Phillips *et al.,* 2004). A well-documented case of resistance build-up is that of avoparcin, a glycopeptide feed additive approved for use in broiler chickens and slaughter turkeys in Norway in 1986. By the middle 1990's, environmental reservoirs of vancomycin-resistant enterococci (VRE) in Europe were documented (Bates *et al.,* 1993). An association was found between an agricultural VRE reservoir and the use of avoparcin as growth promoter in food production (Aerestrup, 1995). Avoparcin confers cross-resistance to vancomycin. It was banned from 31 May 1995 for the use as an AGP due to the reported association between its use and VRE (Grave *et al.,* 2004).

In addition to resistance build-up, waste materials from animals might contain antibiotic residues, resulting in their wider dissemination in the environment. As a result of increased pressure from consumer groups, and scientific reports recommending a ban on antimicrobial use in food animals as a precautionary measure, Sweden was the first country to implement a partial ban on the use of AGPs in farm animals in 1986 (Aarestrup, 2003). Sweden was joined by the European Union (EU), which introduced a moratorium on the use of a number of AGPs in 1997 (Aarestrup, 2003). In 2006, the partial ban was replaced by a general ban on the use of all AGPs (including ionophore anti-coccidials) in animal feed. On a global level, the World Health Organization's global principles are recommended which include withdrawal of AGPs that are in classes also used to treat human disease, from food animal production until risk assessment is carried out (World Health Organization, 2001).

Table 1.1. Some physical, nutritional and metabolic effects ascribed to AGPs (modified from Rosen, 1995)

Physiological	Nutritional	Metabolic
Increases	Increases	Increases
Nutrient absorption	Plasma nutrients	Protein synthesis in the liver
Feed intake	Energy retention	Intestinal alkaline phosphatase
	Absorption of glucose and fatty acid	
	Nitrogen retention	
	Absorption of amino acids	
	Vitamin absorption	
	Calcium absorption	
	Trace mineral absorption	
Decreases	Decreases	Decreases
Feed transit time	Intestinal energy loss	Ammonia production
Intestinal wall diameter, length and weight	Vitamin synthesis	Toxic amine production
Faecal moisture		Aromatic phenols
Mucosal cell turnover		Mitochondrial fatty acid oxidation
		Faecal fat excretion
		Intestinal urease

In South Africa, producers continue to use AGPs such as Zinc Bacitracin in animal production (personal communication: Dr. S.P.R. Bisschop, 2009) but with increasing pressure from consumers, who are wary of the indiscriminate use of antibiotics, it may be inevitable that we too will face increasing restrictions on the use of AGPs in future. The use of AGPs also excludes South Africa from exporting meat and other animal products for consumption to the EU. Consequently, a need to develop alternative strategies to substitute AGPs or replace

them with antimicrobial agents not in use in humans has arisen in order to maintain current standards of animal production, health and welfare.

1.4 Consequences of banning AGPs for Animal Productivity and Health

The absence of growth promoters is known to result in reduced feed conversion efficiencies (Feighner and Dashkevicz, 1987). However, this effect in a well-managed farming system is minimal. Of greater concern is the emergence of some animal diseases that were suppressed by the use of AGPs. One of the most notable emerging diseases is necrotic enteritis (NE) of broiler chickens, which is caused by *C. perfringens* (van Immerseel *et al.,* 2008). This trend can also be seen in the increased consumption of the ionophore anticoccidial, which has activity against *C. perfringens* (Grave *et al.,* 2004; Martel *et al.,* 2004; Watkins *et al.,* 1997) in Norway and Denmark after the initial ban of AGPs. The full impact of the reduction in AGPs on the incidence of NE is not yet known, because many countries are still in the process of implementing strategies to phase out prophylactic antibiotic use in animal production but it has been estimated that NE cost the international poultry industry approximately two billion US dollars annually (van der Sluis, 2000).

1.5 Alternatives to AGPs

Several strategies have been proposed as possible alternative to AGPs. The mode of action of these replacements can be divided into four basic groups with distinct strategies: 1) improvement of nutrient utilisation by the host (exogenous feed enzymes); 2) stimulation/modulation of the immune system (cytokines, vaccines, gluco-(GOS) and manno-oligosaccharides (MOS)); 3) stimulation or introduction of beneficial bacteria (probiotics, fructo-oligosaccharides (FOS)) and 4) direct reduction of pathogens (MOS, organic acids, botanicals and herbs, bacteriocins, antimicrobial peptides, bacteriophages) (Kocher and Choct, 2008).

1.6 Characteristics of acceptable alternatives

The potential value of the alternative needs to be assessed based on the following criteria proposed by Collett and Dawson (2002):

1.6.1 Efficacy

An acceptable alternative must have a significantly beneficial effect on production and animal health at least as effective as the replaced pronutrient antibiotic. This can be reflected in improved FCR, decreased mortality and improved production in terms of weight gain and decreased disease incidence.

1.6.2 Safety and acceptability to regulatory agencies

An acceptable alternative must be proven safe for both animal and human use and not cause environmental degradation. Natural products derived from materials already on the market for human consumption have a great advantage for they are generally regarded as safe (GRAS). Depending on the country, regulatory statutes and specific toxicity testing may also dictate the use of certain alternatives.

1.6.3 Ease of use

Alternatives must be easy to apply and store. Low inclusion rates allow for uncomplicated diet formulation and balancing of rations. Application methods must allow for uniform distribution of the additives at low application rates. Stability during feed processing and a long shelf life are important considerations.

1.6.4 Economic considerations

Alternatives must be cost-effective and provide a substantial return on investment. The use of an alternative growth promoter may create a unique market and enable the product to enter the more profitable "natural" or "organic" niche markets. It may also have an application in niche markets such as exotic birds, racing pigeons or pets.

1.7 Plant extracts: A viable option for replacing AGPs?

Herbs and spices are well known to have *in vitro* antimicrobial action against important pathogens (Dorman and Deans, 2000; Hammer *et al.,* 1999 and Smith-Palmer *et al.,* 1998). The phytochemicals responsible for these actions can be divided into several categories: phenolics and polyphenols (simple phenols and phenolic acids, flavonoids, tannins and coumarins), terpenoids and essential oils, alkaloids, lectins and polypeptides (Cowan, 1999). Examples include cinnamaldehyde, eugenol and carvacrol in cinnamon extract which inhibits *Helicobacter pylori* (Tabak *et al.,* 1999) and which is also active against *Escherichia coli, Pseudomonas aeruginosa, Enterococcus faecalis, Staphylococcus aureus, S. epidermidis, Klebsiella pneumoniae, Salmonella* spp. and *Vibrio parahemolyticus* (Chang *et al.,* 2001) and also has inhibitory properties against *Aspergillus flavus* (Montes-Belmont and Carvajal, 1998). The essential oil of the aerial parts of *Lippia javanica* has antimicrobial activity against *K. pneumoniae*, *Bacillus cereus* and the yeast *Cryptococcus neoformans* (Viljoen *et al.,* 2005). Purothionins, basic polypeptides present in the endosperm of wheat and other cereal species, inhibits *Pseudomonas solanacearum*, *Xanthomonas campestris*, *Erwinina amylovora* and several *Corynebacterium* spp. (Fernandez de Caleya *et al.,* 1972).

Plant-derived additives used in animal feeding to improve performance have been called "phytogenic feed additives" (Windisch *et al.,* 2008). This class of feed additives has recently gained interest for use in swine and poultry with increasing numbers of scientific publications since the ban of AGPs in 1999. The primary mode of action of these growth-promoting feed additives can be attributed mainly to the stabilization of feed hygiene and also from the beneficial effect on the gastrointestinal microbiota through controlling pathogens (Roth and Kirchgessner, 1998). Mitsch *et al*. (2004) found that components in blends of essential oils i.e. thymol, eugenol, curcumin and piperin reduced *C. perfringens* concentrations in the intestinal tract and faeces of broilers through the entire growing period with no case of clinical NE in these experimental groups compared to the control, where necrotic lesions were evident. The results from the majority of literature cited (Table 1.2) indicate reduced feed intake at largely unchanged body weight gain or final body weight, leading to an improved feed conversion ratio when feeding phytogenic compounds (Windisch *et al.,* 2008).

Table 1.2. Effect of phytobiotic feed additives on production performance in poultry (adapted from Windisch *et al.* 2008)

Phytobiotic feed additive	Dietary dose (g/kg)	Treatment effect, % difference from untreated control				Reference
		Feed intake	Body weight	ADG	Feed conversion ratio	
Broilers						
Plant extracts						
Oregano	0.15	-6		-2	-4	Basmacioglu *et al*., 2004
Oregano	0.3	-3		+1	-2	Basmacioglu *et al*., 2004
Rosemary	0.15	0		-1	-1	Basmacioglu *et al*., 2004
Rosemary	0.3	-2		+1	-4	Basmacioglu *et al*., 2004
Thymol	0.1	+1		+1	-1	Lee *et al*., 2003
Cinnamaldehyde	0.1	-2		-3	0	Lee *et al*., 2003
Thymol	0.2	-5		-3	-3	Lee *et al*., 2003
Carvacol	0.2	+2		+2	-1	Lee *et al*., 2003
Yucca extract	2.0	-1		+1	-6	Yeo and Kim, 1997
Essential oil blend[a]	0.024	-4	-0		-4	Cabuk *et al.,* 2006
Essential oil blend[a]	0.048	-5	0		-6	Cabuk *et al.,* 2006
Plant extracts[b1]	0.2		-2	0	-2	Hernandez, *et al*., 2004
Plant extracts[c1]	5.0		+2	+3	-4	Hernandez, *et al*., 2004
Plant extracts[d1]	0.5	0	-2	-2	+2	Botsoglou *et al*., 2004
Plant extracts[d1]	1.0	+2	-1	0	+2	Botsoglou *et al*., 2004
Essential oil blend[e]	0.075	-7		-3	-4	Basmacioglu *et al*., 2004
Essential oil blend[e]	0.15	-7		-1	-1	Basmacioglu *et al*., 2004
Essential oil blend[a]	0.036	+3	-8		-5	Alcicek *et al*., 2004
Essential oil blend[a]	0.048	+2	-8		-4	Alcicek *et al*., 2004
Plant extracts [1]	0.1	+1		+1	0	Lee *et al*., 2003
Essential oil blend[a]	0.024	-2	0		-2	Alcicek *et al*., 2003
Essential oil blend[a]	0.048	0	+14		-12	Alcicek *et al*., 2003
Essential oil blend[a]	0.072	-2	+8		-9	Alcicek *et al*., 2003

[a]Mixtures of oregano-, laurel leaf-, sage leaf-, myrtle leaf-, fennel seed-, and citrus peel oil; [b]Mixtures of oregano, cinnamon and pepper; [c]Mixture of sage, thyme and rosemary; [d]Mixtures of *Agrinomia eutoria, Echinacea angustifolia, Ribes nigrum* and *Cinchona succirubra*; [e]Mixture of 14 Chinese herbs (identity unknown); [1]Entire product

Table 1.2 continued. Effect of phytobiotic feed additives on production performance in poultry (adapted from Windisch *et al.,* 2008)

Phytobiotic feed additive	Dietary dose (g/kg)	Treatment effect, % difference from untreated control				Reference
		Feed intake	Body weight	ADG	Feed conversion ratio	
Herbs and spices						
Oregano	5.0	+5		+7	-2	Florou-Paneri *et al*., 2006
Thyme	1.0	+1	+2		-1	Sarica *et al*., 2005
Garlic	1.0	-5	-5		0	Sarica *et al*., 2005
Herb mix[e]	0.25	0		+2	-2	Guo *et al*., 2005
Herb mix [e]	0.5	+5		+2	+3	Guo *et al*., 2005
Herb mix [e]	1.0	+2		+1	+1	Guo *et al*., 2005
Herb mix [e]	2.0	+1		+1	0	Guo *et al*., 2005
Turkeys						
Herbs and spices						
Oregano	1.25	-5	+2			Bampidis *et al*., 2005
Oregano	2.5	-6	+1			Bampidis *et al*., 2005
Oregano	3.75	-9	+1			Bampidis *et al*., 2005
Quail						
Essential oils						
Thyme	0.06	0		+6		Denli *et al*., 2004
Black seed	0.06	+1		+2		Denli *et al*., 2004
Herbs and spices						
Coriander	5.0	+3		+1	+1	Güler *et al*., 2005
Coriander	10.0	+3		+5	-1	Güler *et al*., 2005
Coriander	20.0	+4		+8	-4	Güler *et al*., 2005
Coriander	40.0	+5		+4	+1	Güler *et al*., 2005

[a]Mixtures of oregano-, laurel leaf-, sage leaf-, myrtle leaf-, fennel seed-, and citrus peel oil; [b]Mixtures of oregano, cinnamon and pepper; [c]Mixture of sage, thyme and rosemary; [d]Mixtures of *Agrinomia eutoria, Echinacea angustifolia, Ribes nigrum* and *Cinchona succirubra*; [e]Mixture of 14 Chinese herbs (identity unknown); [1]Entire product

1.8 Do *Ginkgo biloba and Hypericum perforatum* leaf extracts have potential to be developed in AGPs?

The Phytomedicine Programme at the University of Pretoria has compiled a database of plant extracts with known antimicrobial activity that could be considered for inclusion in into poultry feed as growth promoters. Using this database, it was found that the dried leaves of *Ginkgo biloba* and *Hypericum perforatum* had good antibacterial activity (Ntloedibe 2000, Ntloedibe, 2005). Furthermore, these plants have proven to be safe for consumption and are readily available.

1.8.1 *Ginkgo biloba*

Ginko biloba (also known as Maidenhair Tree) is one of the oldest of living plants. Fossil records demonstrate that the group of trees known as Ginkgoales, of which today *G. biloba* is the sole living member, date back over 200 million years. It is believed that their unusual resistance to pests such as insects, bacteria, viruses and fungi, accounts in part for the longevity of the trees and also, in turn, for the longevity of the species (Major, 1967). *G. biloba* trees are cultivated in large plantations all over the world including China, France and USA with an annual output of 8000 tonnes of leaves (Nakanishi, 2005). Raw materials can therefore be sustainably acquired.

This plant has been used therapeutically for centuries in Chinese medicine and also, more recently, in Western medicine. The proprietary extract, generally named EGb761® (Schawbe) is obtained from dried leaves using acetone-water as a solvent and is standardised on the basis of its content of flavonol glycosides (24%) and terpenoids (6%) (Mazzanti *et al.,* 2000).

Ginkgo biloba is generally regarded as safe and has displayed no substantial drug interactions when ingested (Hoffenberth, 1989; Le Bars *et al.,* 1997). In rare cases, patients have exhibited skin reactions, headaches and mild gastro-intestinal upsets (Kanowski, 1996). In his review of the *G. biloba* extract, Diamond *et al* (2000) concludes that given the history of the use and the number of studies that have administered *G. biloba* in different dosages and durations to patients of different ailments and ages, *G. biloba* has a notable record of safety.

The major therapeutic indications of the standard *G. biloba* extracts are peripheral vascular diseases, particularly “cerebral insufficiency” in the elderly (Kleijnen and Knipschild, 1992). The main activities on the arterial, venous and capillary components of the vascular tree consist of “vaso-protection”, “tissue-protection” and ”cognition enhancement” (De Feudis, 1991). Furthermore, it is also of high interest that ginkgolides possess anti-platelet activating factor (PAF) activity (Kleinen and Knipschild, 1992; Shen *et al.,* 1995).

Several studies have shown the antimicrobial effect of *G. biloba* extracts. Adawadkar and El Sohly (1981) reported the activity of anacardic acids against *Mycobacterium smegmatis* and Itokawa (1987) observed a weak antimicrobial activity of bilobol and cardanol on *S. aureus* and *E. faecalis*. Mazzanti *et al.* (1999) found the non-polar fraction of a solvent-solvent fractionated *G. biloba* extract to have strong inhibitory action against *E. faecalis* (<0.019 mg/ml). In a study by Lee and Kim (2002) using the agar diffusion method, components of *G. biloba* leaf extracts had good antimicrobial activity against *C. perfringens* and *E. coli* but not against human intestinal bacteria such as *Bifidobacterium bifidum, Bifidobacterium longum, Bifidobacterium adolescentis,* or *Lactobacillus acidophilus.* The acetone extract of *G. biloba* had activity of 0.08 mg/ml against *C. perfringens* (Ntloedibe, 2005).

1.8.2 Hypericum perforatum

The genus *Hypericum* is part of a large family, Guttiferae which comprises more than 1 000 species (Dall' Agnol *et al.,* 2003). St. John's wort (*Hypericum perforatum* L.) (SJW) is an herbaceous plant that has been used as a medicinal plant for centuries to fight against infections, and for the treatment of respiratory and inflammatory diseases, peptic ulcers and skin wounds (Medina *et al.,* 2005). Several clinical studies provide evidence that SJW is as effective as conventional synthetic antidepressants (Brenner *et al.,* 2000; Phillip *et al.,* 1999; Volz, 1997; Woelk, 2000).

A series of bioactive compounds has been detected in the crude material namely, phenylpropanes, flavonol derivatives, biflavones, proanthocyanidins, xanthones, phloroglucinols, amino acids, naphthodianthrones and essential oil constituents (Bombardelli and Morazzoni, 1995). In the last few years, the phloroglucinol derivative, hyperforin has attracted attention as the main component with antidepressant effects (Laakmann *et al.,* 1998). Other bioactivities for hyperforin with potential pharmacological or biomedical interest were also found. These include anti-inflammatory (Schempp *et al.,* 1999) and proinflammatory effects (Feisst and Werz, 2004), antitumoral effects (Schemmp *et al.,* 2002), antiangiogenic (Martinez-Poveda *et al.,* 2005) and antimicrobial effects (Gurevich *et al.,* 1971). Schempp *et al* (1999) observed growth inhibition of purified hyperforin from *H. perforatum* against multiresistant *S. aureus* strains (MIC=1.0 µg/ml), *Streptococcus pyogenes* (MIC=1.0 µg/ml), *Streptococcus agalactiae* (MIC=1.0 µg/ml), *Corynebacterium diphtheriae* (MIC=0.1 µg/ml) but not *E. coli*, *P. aeruginosa* or *Candida albicans.* Ntloedibe (2005) observed activity of the acetone extract *of H. perforatum* against *S. aureus* (0.02 mg/ml), *E. coli* (0.024 mg/ml), *S. enterica.* Enteriditis (0.096 mg/ml), *P. aeruginosa* (0.186) and *C. perfringens* (0.096mg/ml).

1.9 Background on development of extracts of *G. biloba* and *H. perforatum*

Different methods were used to extract *G. biloba* and *H. perforatum* plant material and the extracts obtained were tested against a host of bacterial species (Ntloedibe 2000, Ntloedibe, 2005). The leaf material was either cold, reflux or serially extracted. In the cold extraction, the dried plant material was treated with different concentrations of ethanol (100%, 80%, 60% 40% and 20%) and 100% acetone. In the reflux extraction, the leaves were extracted using different concentrations of ethanol under reflux with each set of plant material being extracted once at a temperature of 70°C for 30min. In the serial extraction, the plant material was either first extracted with hexane and then extracted with ethanol or the ethanol extraction came first followed by the extraction with hexane. The acetone extracts of *H. perforatum* were shown to have the highest antimicrobial activity with MICs ranging between 0.02 and 0.75 mg/ml for susceptible strains of bacterial pathogens such as *S. aureus, E. faecalis, E. coli, S. enterica.* Enteriditis, *P. aeruginosa* and *C. perfringens* (Ntloedibe, 2005). The

acetone extracts of *G. biloba* had activity of 0.16 mg/ml, 1.26 mg/ml, 0.32 mg/ml, 0.62 mg/ml, and 0.08 mg/ml for *S. aureus, E. faecalis, E. coli, S. enterica* Enteriditis and *C. perfringens* respectively (Ntloedibe, 2005).

Chikoto (2006) continued work on these extracts in combination with grape seed extract (as an antioxidant component) by evaluating the *in vivo* tolerance of broiler chickens at 1, 2 and 5 times the recommended human dose over a 21 day period (Zn-bacitracin was used as positive control). It was found that the extracts did not influence the taste of the feed and that the broilers could tolerate the extracts, since they resulted mostly in identical masses. Results indicated that the lowest dose used for *G. biloba* and *H. perforatum* was the most effective and these extracts were subsequently included alone or in combination into the feed at 14 mg/kg (14ppm) combined with grape-seed-extract in a *C. perfringens* challenged *in vivo* experiment with broiler chickens over a 42 day period. In the challenged environment of this experiment, the grape seed extract in combination with the *G. biloba* extract was statistically the best feed additive, followed by the combination of grapeseed, *G. biloba* and *H. perforatum* extracts (Table 1.3). However, treatment with grapeseed-extract alone had a negative impact on production, probably due to the presence of proanthocyanidins that have the potential to bind proteins. This could have led to low digestibility and absorption as a result of binding nutritive amino acids and digestive enzymes (Chikoto, 2006).

Table 1.3. Mean live body mass (g) (each pen analysed as an experimental unit) of *C. perfringens* challenged broiler chickens fed different combinations of feed additives (Chikoto, 2006)

	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
Zn-bacitracin	48.5^{a}	136.3^{a}	331.3^{a}	633.0^{ab}	855.9^{a}	1253^{ab}	1840^{ab}
Negative control	47.4^{a}	129.7^{a}	321.7^{a}	655.9^{a}	836.3^{a}	1258^{ab}	1814^{ab}
G:B:H	48.3^{a}	131.1^{a}	326.2^{ab}	647.0^{ab}	874.6^{a}	1237^{b}	1867^{ab}
G:B	48.1^{a}	125.4^{a}	332.2^{a}	619.6^{ab}	862.2^{a}	1301^{a}	1902^{a}
G:H	46.6^{a}	130.2^{a}	313.0^{bc}	616.9^{b}	842.5^{a}	1203^{bc}	1799^{b}
G	48.2^{a}	126.9^{a}	297.1^{c}	573.3^{c}	796.4^{a}	1149^{c}	1693^{c}
P-value	0.457	0.578	0.006	0.004	0.101	0.002	0.004

G - Grapeseed extract, B - *G. biloba* extract, H - *H. perforatum* extract

N=4 for each treatment. Means followed by a different letter within columns are significantly different at the 5 % level (ANOVA).

1.10 Expansion of the work of Ntloedibe (2005) and Chikoto (2006)

Ntloedibe (2001) screened a variety of extracts obtained from different extraction procedures but did not use methods later developed in the Phytomedicine Programme to increase (potentize) the antimicrobial activity of

extracts. It is clear that most of the activity in the *G. biloba* extract against relevant spp. (*E. faecalis, S. aureus, and C. perfringens*) resides in the non-polar fraction (Lee and Kim, 2002, Mazzanti *et al.*, 2000). The qualitative and quantitative aspects involved in antibacterial activity of the *G. biloba* extract still need to be elucidated.

Information on the development of resistance in pathogenic bacteria against herbal extracts is not readily available. The potential of *Enterococcus* spp. to develop resistance to *G. biloba* extracts needs to be investigated, as resistance and cross-resistance to antibiotics used in human medicine could lead to similar problems to those experienced with AGPs such as avoparcin in the past. *Enterococcus* spp. are widespread and, as typical lactic acid bacteria, they are part of the intestinal flora of humans and production animals and consequently also part of the microflora of fermented food and feed. The transfer of resistance from enterocci in food animals to those in the human intestine is a risk. Therefore, *Enterococcus faecalis* was selected as a test organism in order to evaluate the potential of resistance development against *G. biloba*.

As mentioned in section 1.9., Chikoto (2006) measured growth responses in poultry due to *G. biloba* and *H. perforatum* in combination with grapeseed extract which had a deleterious effect on growth promotion. Results also indicated that *G. biloba*, even in combination with the grapeseed extract, yielded the best results in terms of broiler weight and feed conversion. Therefore, it is important for the active antimicrobial extract of *G. biloba* to be tested alone to establish its efficacy. In the *in vivo* tolerance studies it was concluded that the *H. perforatum* extract can be tolerated at doses of up to 10 mg/kg. In spite of this finding, *H. perforatum* extract was only included at 14ppm in the feed which is equivalent to 2 mg/kg i.e. five times lower than proven safe dose. This extract needs to be included as treatment at a higher dose.

In spite of the fact that phytochemicals act directly on the gastrointestinal microbiota by inhibiting pathogens (Roth and Kirchgessner, 1998), the effect of treatment with the different extracts on *C. perfingens* colonization in the GIT of broilers was not investigated even though necrotic enteritis was successfully induced. The effect of *G. biloba* and/or *H. perforatum* on the colonization of *C. perfringens* in the intestines of broiler chickens should be investigated.

1.11 Aim of this study

The aim of the study is to produce an antibacterial phytogenic product from *G. biloba* and *H. perforatum* with a low potential to develop resistance, as an alternative to AGPs in poultry production.

1.12 Objectives

The objectives to attain this aim are to:

a) Evaluate the activity and potentize extracts from *G. biloba* and *H. perforatum* for optimal activity against selected bacterial pathogens (Chapter 2).
b) Combine extracts or fractions of extracts of *G. biloba* and *H. perforatum* to optimise activity against selected bacterial pathogens (Chapter 2).
c) Isolate and identify the major antibacterial compound in *G. biloba* (Chapter 3).
d) Evaluate pharmacodynamic aspects of *G. biloba* extracts i.e. to evaluate the potential of *E. faecalis* to develop *in vitro* resistance against fractions/compounds of *G. biloba* extracts and study the time-kill kinetics of the same fraction of *G. biloba* extract using the same pathogen over a 24 h period (Chapter 4).
e) Determine the effect of extracts of *G. biloba* and/or *H. perforatum* on growth promotion and *C. perfringens* colonization in the intestines of broiler chickens challenged with *C. perfringens* (Chapter 5).

1.13 References

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2 Activity of extracts and fractions of extracts of *G. biloba* and *H. perforatum* alone or in combination against bacterial pathogens

2.1 Introduction

The devastating effects of bacterial pathogens on human and animal health are well known. Currently scientists are investigating the use of plant-derived products as inhibitors of harmful bacteria because plants constitute a rich source of bioactive chemicals and many of them are largely free from harmful effects (Rios and Recio, 2005). *Ginkgo biloba* and *Hypericum perforatum* are among these plant species currently under investigation for their antimicrobial properties.

Ginkgo biloba is an ancient Chinese tree, which has been cultivated for its health-promoting properties, which includes treating symptoms of various forms of cerebrovascular insufficiency and dementia (including memory loss, disturbed concentration, dizziness, sleep disturbances, mood swings, decreased stamina and debilitation, morbus Alzheimer) (Kleinen and Knipschild, 1992). It has also specifically been used to treat patients with peripheral arterial occlusive disease (improvement in pain-free walking distance) (van Wyk and Wink, 2004). The antimicrobial activity has also been investigated. Ahn, *et al* (1994) reported activity against *Clostridium perfringens* which was confirmed by Lee and Kim (2002). Activity against *Escherichia coli* has been reported by several authors (Lee and Kim, 2002; Mazzanti *et al*., 2000). Adawadkar and El Sohly (1981) found activity against *Mycobacterium smegmatis* and Itokawa *et al*. (1987) observed weak activity against *Staphylococcus aureus* and *Enterococcus faecalis*. This is in contrast with the findings of Mazzanti *et al.* (2000) who found that the ethyl acetate extract had a strong inhibitory action to *E. faecalis* (MIC≤0.019 mg/ml). A chloroform fraction prepared from the sarcotesta showed potent inhibitory activity against vancomycin-resistant *Enterococcus* (VRE) (Choi *et al.,* 2008).

Hypericum perforatum has been used for centuries to fight infections and for the treatment of respiratory and inflammatory diseases, septic ulcers and skin wounds (Medina *et al.,* 2006). It has been used for the last 450 years in traditional European medicine to treat neuralgia, anxiety, neurosis and depression (Bilia, *et al.,* 2002). The antibacterial properties of *H. perforatum* extracts were reported in 1959 and 1971 (Gaind and Ganjoo, 1959; Gurevich *et al.,* 1971) and the active compound was found to be hyperforin (Gurevich *et al.,* 1971), an acylphloroglucinol-type compound that consists of a phloroglucinol skeleton substituted with lipophilic isopren-chains (Bystrov *et al.,* 1975). Schempp *et al*. (1999) found hyperforin to be active against methicillin-resistant *S. aureus* (MRSA), *Streptococcus pyogenes*, *Streptococcus agalactiae* and *Corynebacterium diphtheriae*.

Many of the most effective phytomedicines are on the drug market as whole extracts of plants, and it is believed that synergistic interactions between the components of the individual or mixtures of herbs are a essential part of their therapeutic efficacy (Boucher and Tam, 2006; Williamson, 2001). If the effect of the combination exceeds the expected effect of the individual active compound, the combination is said to be synergistic (Boucher and Tam, 2006). Although the effects of combinations of whole plant extracts in a wide range of therapeutic fields are well-known (Williamson, 2001), few studies have investigated the effect of combining antimicrobial plant extracts to see if activity can be improved (van Vuuren and Viljoen, 2008; Brown *et al.,* 2009).

Based on earlier successes obtained in the Phytomedicine Programme (Chikoto, 2006) this study investigates the quantitative- and qualitative activity of different extracts of *G. biloba* and *H. perforatum* against several Gram-positive and Gram-negative bacteria with the aim of selecting the best extraction fractionation system. The effect of combining extracts or fractions of extracts of these two plant species in order to detect possible interaction (synergy, antagonism) was also investigated.

2.2 Materials and methods

2.2.1 Plant collection

Leaf powders of *G. biloba* and *H. perforatum* were obtained from Biomox Pharmaceuticals (Pty) Ltd., South Africa.

2.2.2 Plant extraction procedure

Different extraction methods were tested in an attempt to select the procedure that would yield an extract with high antimicrobial activity with corresponding high concentration/s of active ingredient/s.

2.2.2.1 Serial extraction

Dried material was serially extracted with 100% concentrations of hexane, dichloromethane (DCM) and ethyl acetate (EA). A ratio of 1:10 dried material: extractant was used in all cases. Plant-solvent suspensions were shaken for 10 min in a Labotec 20.2 shaking machine at high speed. The extracts were centrifuged at 1322 *x g* for 10 min before decanting into labelled containers. The process was repeated three times on the same material and the extracts were combined. Solvents were evaporated from the extracts at room temperature under a continuous stream of air.

2.2.2.2 Direct extraction

Dried leaf material from *G. biloba* and *H. perforatum* was extracted using 100% concentrations of acetone, hexane, DCM or EA as well as 60% acqueous acetone and 80% ethanol. Additionally methanol was also used to extract *H. perforatum*. A ration of 1:10 material: extractant was used in all cases. Mixtures were treated and dried as described for the serial extraction procedure.

2.2.2.3 Solvent-solvent fractionation

Solvent-solvent fractionation was carried out in accordance with the method described by Lee and Kim (2002). Dried material was extracted twice at room temperature with 60% aqueous acetone (method 1) (*G. biloba* and *H. perforatum*) and/or 80% aqueous ethanol (method 2) (for *H. perforatum*) at a ratio of 1:10 dried material vs. extractant after which it was filtered. The extract was concentrated by using rotary evaporation at 45°C after which the extract was sequentially partitioned into hexane, EA, butanol and H_2O portions. Each step was carried out three times to ensure adequate extraction. All solvents were saturated with distilled H_2O before use to ensure adequate separation. The solvent portions were concentrated by rotary evaporation at 45°C and further dried at room temperature under a continuous stream of air.

2.2.3 Phytochemical analysis

Chemical constituents of the extracts were analysed by Thin Layer Chromatography (TLC) using aluminium-backed Silica Gel 60 plates (MERCK). TLC plates were loaded with 100 µg of each of the extracts (10 µl of 10 mg/ml solution) with a graduated micropipette. The prepared plates were each developed with one of three eluent systems:

EA/methanol/water: 40 / 5.4 / 4 (EMW) (polar/neutral);
Chloroform/EA/formic acid: 5 / 4 / 1 (CEF) (intermediate polarity/acidic);
Benzene/ethanol/ammonia (aq): 90 / 10 / 1 (BEA) (non polar/basic).

Development of the chromatograms took place in a closed glass tank in which the atmosphere was saturated with the eluent vapour using Whatman filter paper to line the TLC tank. The separated components were visualized under visible and ultraviolet light (254 and 360nm Camac Universal UV lamp TL-600) and sprayed with either 5% anisaldehyde in a 5% sulphuric acid in ethanol solution or with vanillin/sulphuric acid-methanol solution and heated at 100°C for 3-5 min.

2.2.4 Biological assays

2.2.4.1 Microorganisms used.

Table 2.1: Bacterial isolates used as test organisms

Bacterial isolate	ATTC/Isolate no.	Gram-positive/Gram-negative
Enterococcus faecalis	29212	+
Clostridium perfringens	44/03	+
Escherichia coli	25922	-
Pseudomonas aeruginosa	27853	-
Salmonella enterica. Typhimurium	13311	-
Staphylococcus aureus	29213	+

2.2.4.2 Microdilution assay

The microplate dilution assay of Eloff (1998) was used in accordance with the recommendations by the Clinical Laboratory Standards Institute, 2008 to determine the minimum inhibitory concentrations (MIC) of the extracts against the selected bacteria. Four nosocomial human pathogens, namely *E. faecalis* (ATCC 29212), *E. coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) and, *S. aureus* (ATCC 29213) (Table 2.1) that are used to quality control antimicrobial susceptibility testing were used (CLSI, 2008). Additionally *C. perfringens* (B44/03) obtained from a bovine lung and *Salmonella enterica.* Typhimurium (13311) isolated from poultry were obtained from Dr. J. Picard as part of the culture collection of the Bacteriology Laboratory, Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, UP (BL-VT).

All strains were sub-cultured from the original strains, stored at -70°C at BL-VT. Aerobic strains were transferred to Müeller Hinton (MH) agar and *C. perfringens* to on deoxygenated Blood tryptose (BTA) agar (Onderstepoort Biological Products). Aerobic bacteria were incubated at 37°C in an incubator and *C. perfringens* in an anaerobic cabinet (6% oxygen; 10% carbon dioxide and 85% nitrogen) (Steldon Manufacturing Inc.).

Three to 5 colonies of the test bacteria from an 18 – 24 h agar plate culture were inoculated into 2 ml sterile distilled water with 0.02% Tween 80 (BDH). After thorough mixing, 1-10 µl were transferred to 10 ml MH broth (brain heart infusion broth for *C. perfringens*) to give a final concentration of approximately 5 x 10^5 CFU/ml.

Test solutions were prepared by mixing dried extract (10 mg/ml) with a 1:1 acetone and water mixture. Prepared extracts were serially diluted two-fold in 96 well microplates followed by addition of an equal volume of a 10^5 bacterial suspension in all the wells and an overnight incubation at 37°C. MIC were then determined visually at

the lowest concentration that led to growth inhibition after the addition of 40 µl of 0.2 mg/ml *p*-iodonitrotetrazolium violet (Sigma®) (INT) and further incubation for 2 h. All determinations were done in triplicate. Total activity (TA) was determined as an indication of the volume to which compounds present in 1 g of dried plant material could be diluted and still retain activity (measured in ml/g dried material) (Eloff, 2000, 2004). Zn-Bacitracin was used as a positive control to confirm the sensitivity of the system.

2.2.4.3 Bioautography

A bioautography procedure was used based on the method developed by Begue and Kline (1972). Briefly, TLC plates (10X20 cm) were loaded with 100 µg (10 µl of 10 mg/ml) of each of the extracts. The plates were developed in the three mobile systems as described above. Chromatograms were dried for 24 h at room temperature to remove the remaining solvent. Cultures were grown on MH agar and incubated at 37°C overnight. Broth cultures were prepared by transferring 2-3 bacterial colonies with a sterile swab from agar into 250 ml Erlenmeyer flasks containing 100 ml MH broth. Broth cultures were incubated for 24 h at 37°C. Developed TLC plates were inoculated with a fine spray of the bacterial suspension containing approximately 10^8 cells/ml of actively growing bacteria in a Biosafety Class II cabinet (Labotec, SA). The plates were sprayed until they were just wet and incubated at 37°C overnight in a chamber at 100% relative humidity in the dark. The plates were subsequently sprayed with a 2 mg/ml solution of INT and incubated for 2-3 h in the same chamber. White areas indicated where reduction of INT to the coloured formazan did not take place due to the presence of compound/s that inhibited the growth of the test bacteria. Bioautograms were sealed in clear plastic envelopes and scanned for a permanent digital record.

2.2.5 Synergy / antagonistic interactions between extracts of *G. biloba* and *H. perforatum*

Once the independent MIC was determined for the different extracts of the two plant species, the interaction between extracts of the two plant species was investigated. The hexane extracts from the solvent-solvent separation technique and the acetone extracts from the direct extraction technique of the two plant species were combined in duplicate and tested against *E. faecalis* and *C. perfringens* to evaluate if there was a possibility that synergistic interactions were at play. The fractional inhibitory concentration (FIC) was calculated. The FIC is expressed as the interaction of two agents where the concentration of each test agent in combination is expressed as a fraction of the concentration that would produce the same effect when used independently (Berenbaum, 1978).

The FIC was then calculated for each test sample independently as specified in the following equations:

$$FIC^{(a^*)} = \frac{\text{MIC (a*) in combination with (b*)}}{\text{MIC (a) independently}}$$

$$FIC^{(b^*)} = \frac{\text{MIC (b) in combination with (a)}}{\text{MIC (b) independently}}$$

(a*) and (b*) = in this study represent the above mentioned hexane- or acetone extract of *G. biloba* or *H. perforatum*.

The sum of the FICs, known as the FIC index is thus calculated as

$\sum FIC = FIC^{(a)} + FIC^{(b)}$ i.e.

$$\sum FIC = \frac{\text{Actual [MIC (a) + MIC (b)]}}{\text{Expected [MIC (a) + MIC (b)]}}$$

The FIC index (Schelz *et al*., 2006), is determined as the correlation between the two combined test substances and may be classified as either synergistic (≤0.5), additive (>0.5-1), indifferent (neutral) (>1 to 4) or antagonistic (≥4). If a combination of fractions leads to a lower level of activity it is a cancelling effect (indifferent / neutral) and not necessarily an antagonistic effect.

Determination of % Stimulation:

In the Phytomedicine Programme, a less biased and more simplistic method, % Stimulation, has been developed. Percentage Stimulation is determined by using the following equation keeping in mind that MIC is inversely related to antibacterial activity:

$$\% \text{ Stimulation} = \frac{\text{Expected MIC} - \text{Actual MIC}}{\text{Actual MIC}} \times 100$$

Expected MIC = MIC of (a) + (b) determined separately and calculated in the ratio present in the mixture, Actual MIC = MIC of (a) and (b) determined in combination. Values ≥ 50% indicate stimulation, > -50% and < 50% an indifferent effect and ≤ -50% denote antagonism. A 100% stimulation means that the activity is doubled and with a 0% stimulation there was no synergistic or antagonistic activity.

2.3 Results and Discussion

2.3.1 Extraction and phytochemical analysis

Due to the differences in the nature of the chemical constituents found in a plant, a single solvent is unable to extract all the components. Acetone has a very wide spectrum of solubility and is the best for wide screening of biological activities of plant extracts (Eloff, 1998; Kotze and Eloff, 2002). The solvent systems used separated many compounds in the *G. biloba* and *H. perforatum* extracts that reacted with the vanillin and *p*-anisaldehyde spray reagents (Figure 2.1 – Figure 2.4). The chemical components in both plants were best separated using the solvent-solvent separation technique described by Lee and Kim (2002) using CEF or EMW as mobile phases (Figure 2.2 for *G. biloba* and Figure 2.4 for *H. perforatum*), which separates compounds in terms of varying polarity into a hexane, EA and butanol fraction. Fractionation of the aqueous ethanol extract (method 2) of *H. perforatum* resulted in higher concentrations of certain compounds compared to fractionation of the aqueous acetone extract (method 1) as evident after spraying with vanillin (Figure 2.4).

Although the serial extraction technique also separated the compounds of *G. biloba* and *H. perforatum* into the different fractions in terms of polarity (Figure 2.1 and Figure 2.3), it was found to be labour-intensive, time consuming and uneconomical in terms of amounts of solvents used. The direct extraction procedure is an easy and economical method but does not give adequate information about the quantitative and qualitative nature of the compounds. When comparing the hexane extracts from the direct extraction- and solvent-solvent fractionation techniques (using a DCM: methanol (9:1) in an additional experiment) it was clear that the compounds from the solvent-solvent separation technique were more concentrated (Figure 2.5). The difference in concentration of active compounds will be discussed in more detail in chapter 3.

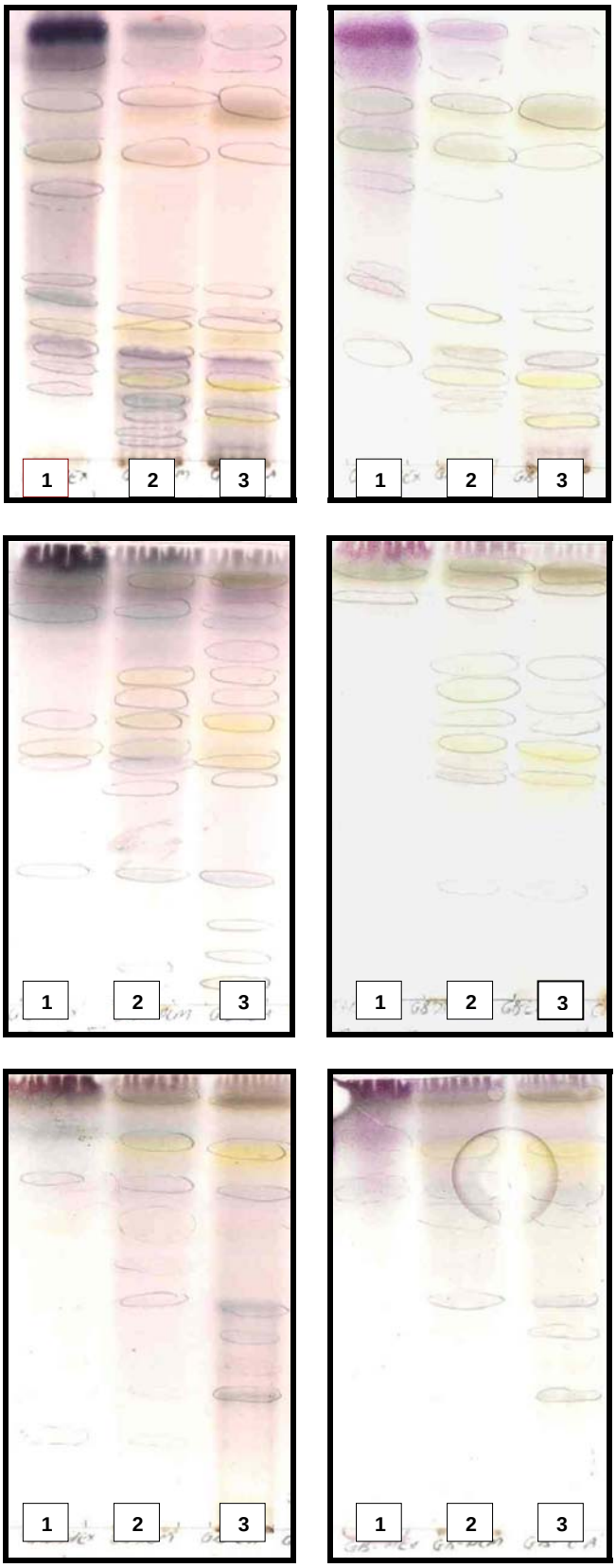


Figure 2. 1. Serially extracted *G. biloba* extracts sprayed with p-anisaldehyde (left) and vanillin (right) with mobile phase BEA (top), CEF (middle) and EMW (bottom)

1) Hexane extract; 2) DCM 3); EA extract

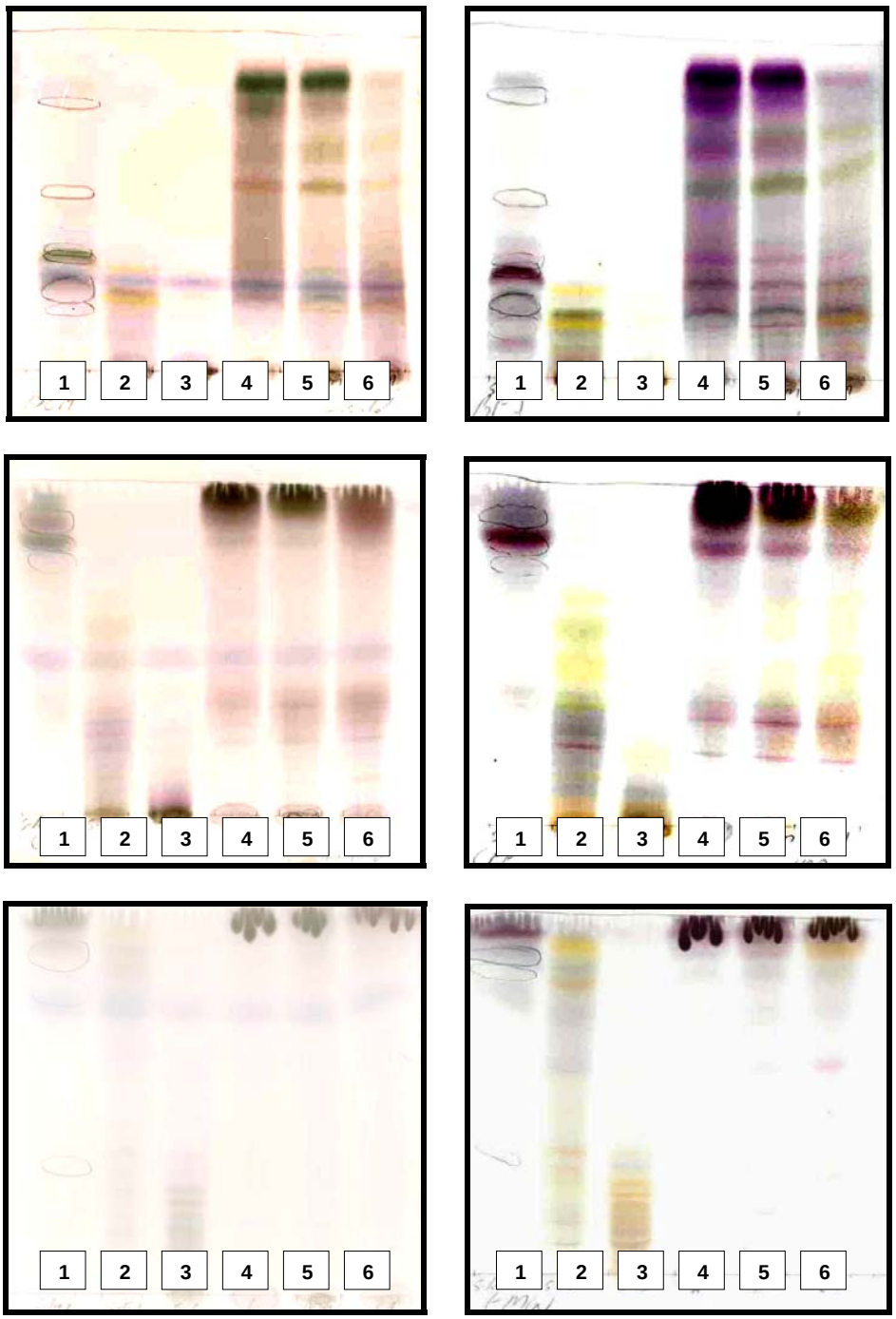


Figure 2.2. *G. biloba* extracts/fractions from the direct- and solvent-solvent extraction procedures sprayed with p-anisaldehyde (left) and vanillin (right) with mobile phase BEA (top), CEF (middle) and EMW (bottom)

1) Hexane fraction (solvent-solvent separation), 2) EA fraction (solvent-solvent separation), 3) Butanol fraction (solvent-solvent separation), 4) Hexane extract (direct extraction), 5) DCM extract (direct extraction) 6) EA extract (direct extraction)

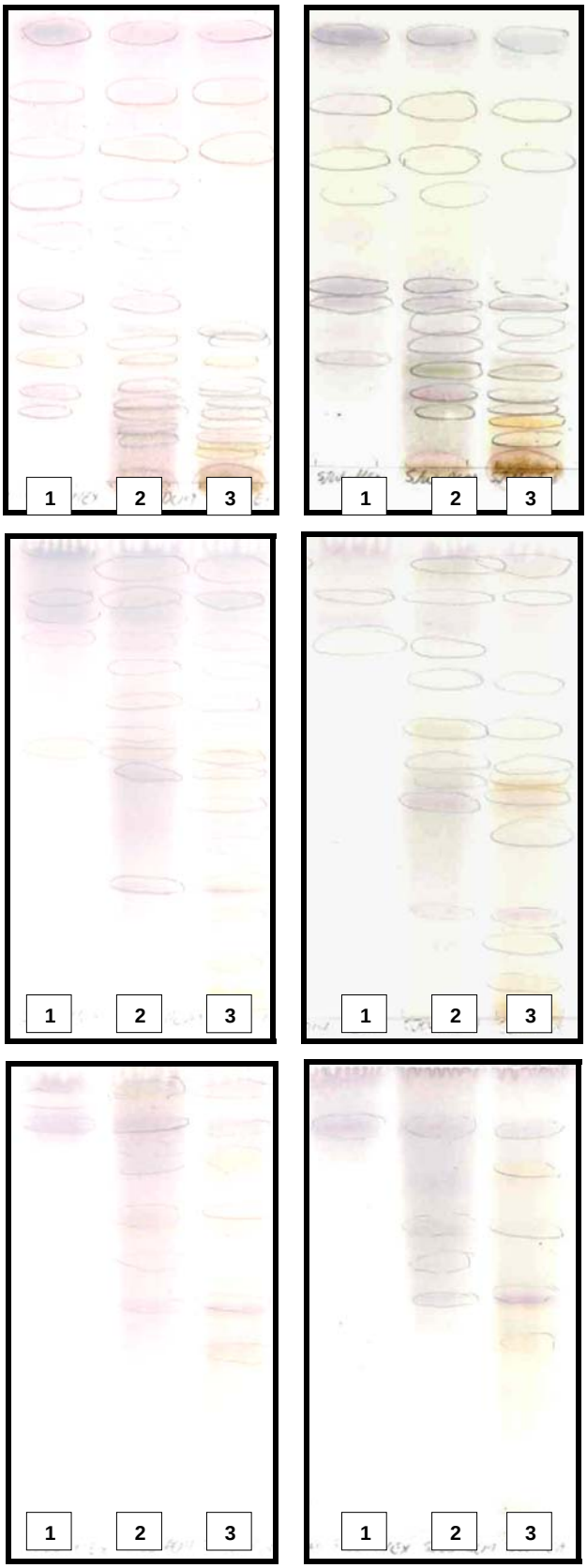


Figure 2.3. Serially extracted *H. perforatum* extracts sprayed with p-anisaldehyde (left) and vanillin (right) with mobile phase BEA (top), CEF (middle) and EMW (bottom)

1) Hexane extract, 2) DCM 3) EA extract

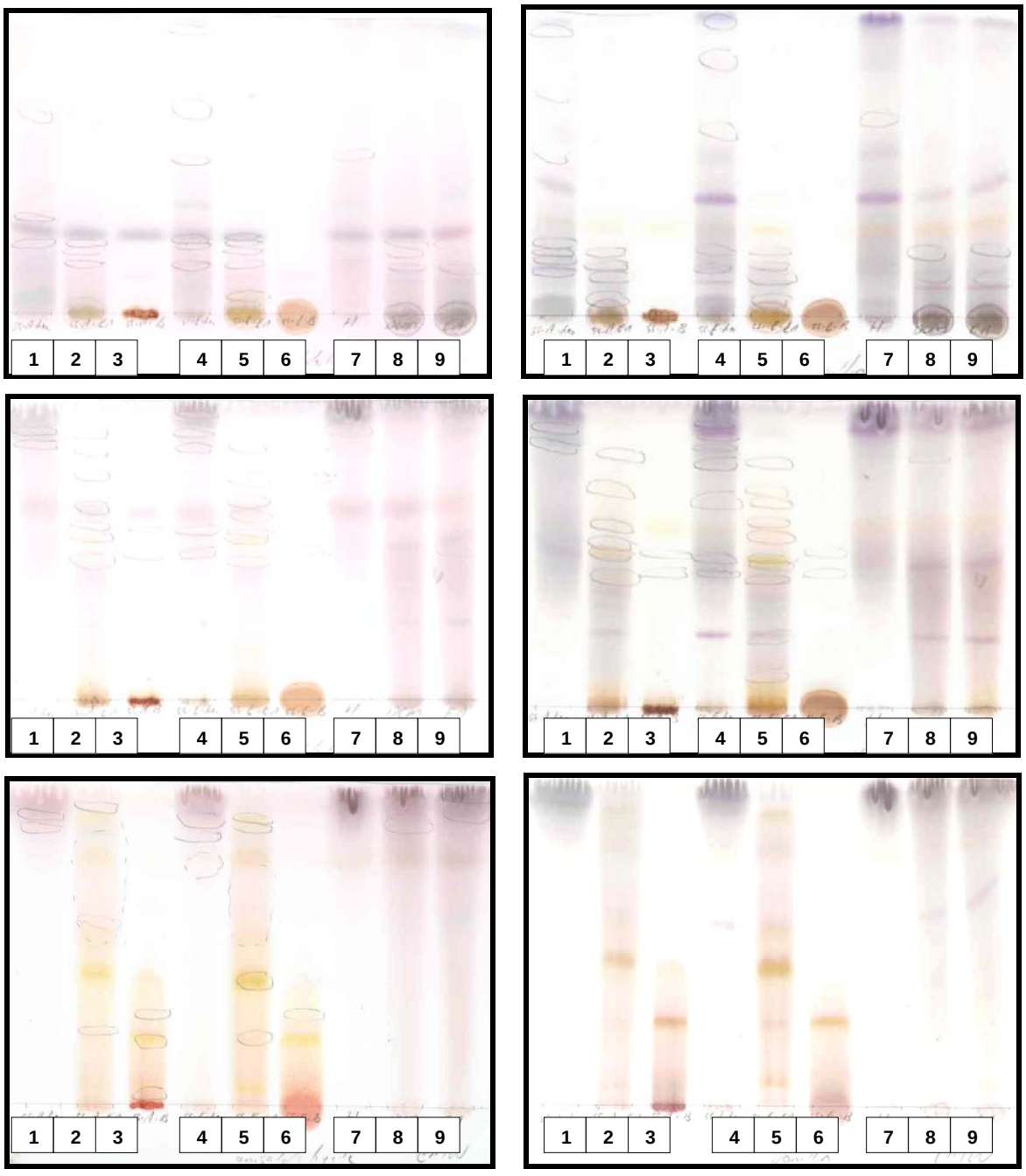


Figure 2.4. *H. perforatum* extracts/fractions from direct- and solvent-solvent separation procedures sprayed with p-anisaldehyde (left) and vanillin (right) with mobile phase BEA (top), CEF (middle) and EMW (bottom)

1) Hexane fraction (solvent-solvent separation - method 1), 2) EA fraction (solvent-solvent separation - method 1), 3) Butanol fraction (solvent-solvent separation - method 1) , 4) Hexane fraction (solvent-solvent separation - method 2), 5) EA fraction (solvent-solvent separation - method 2), 6) Butanol fraction (solvent-solvent separation - method 2), 7) Hexane extract (direct extraction), 8) DCM extract (direct extraction) 9) EA (direct extraction)

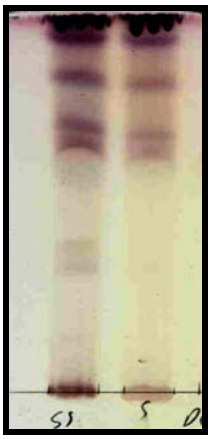


Figure 2.5. Hexane fraction of *G. biloba* from the solvent-solvent extraction procedure (left) and from the direct extraction procedure (right) sprayed with vanillin with mobile phase DCM: methanol (9:1)

2.3.2 Biological assays

The objective of extraction and fractionation of plant material are two-fold: firstly to determine the number and polarity of antibacterial compounds by bioautography and to quantify the antimicrobial activity in the plant material and secondly to find the extract/fraction with the most antibacterial potential to be developed into a herbal product. In the current study, fractionation gave the best results in terms of the first objective. Determination of the total activity gave an indication of the best extraction method and solvent to be used for the benefit of developing an herbal product with high antimicrobial activity (Eloff, 2004). The hexane, DCM and EA extracts of powdered *G. biloba* leaves from the direct extraction procedure had the highest total activity against *C. perfringens* and *E. faecalis* with a total activity ranging between 11 667 and 12 950 ml/g dry mass and 291-647 ml/g dry mass respectively (Table 2.2). The acetone extract also has potential with values of 4320 and 432 ml/g dry mass against *C. perfringens* and *E. faecalis* respectively. With regards to *H. perforatum*, acetone-, DCM- and EA extracts from the direct extraction procedure also had the highest total activity against *C. perfringens* (1 026, 1 333 and 1 179 ml/g dry mass respectively).

According to Eloff (2000) *in vitro* antimicrobial activities of a plant extract with MIC's below 0.1 mg/ml are of value. Extracts and fractions from *G. biloba* were most active against *C. perfringens* with values as low as 0.008 mg/ml in extracts from the direct extraction procedure as well as the hexane extract from the solvent-solvent fractionation procedure (Table 2.2). Activity, although only weak to moderate, against *E. faecalis* was observed in all extracts and fractions except in the polar butanol fraction. Weak to moderate activity (0.1 - 0.31 mg/ml) was observed against *S. aureus*. Zn-Bacitracin gave similar results in that it was highly active against *C. perfringens* and moderately active against *S. aureus* and *E. faecalis.* As expected for a drug with a predominantly Gram-positive activity, it had a low activity to the Gram-negative *E. coli*, P*. aeruginosa* and *S enterica*.Typhimurium.

In spite of the fact that fractionation of the aqueous ethanol extract (method 2) of *H. perforatum* yielded higher concentrations of compounds compared to fractionation of the aqueous acetone extract (method 1), no difference in antimicrobial activity was observed (Table 2.2).

One bio-inhibitory compound (R_f 0.68) was consistently present in all three *G. biloba* extracts from the direct extraction technique when sprayed with *S. aureus* (Figure 2.7), *E. faecalis*, *E. coli* and *P. aeruginosa* and using EMW as mobile phase (Table 2.3). When the 60% acetone extract was fractionated with the solvent-solvent fractionation technique, this compound only appeared in the hexane fraction. This hexane fraction also contained two other compounds (R_f 0.77 and 0.55) with only one of these (R_f 0.55) also appearing in the EA extract from the direct extraction technique when sprayed with *S. aureus.* As shown in Figure 2.6 these 3 compounds in the hexane fraction were more clearly separated using DCM:methanol (19:1) as the mobile phase. When comparing the hexane fractions from the solvent-solvent technique with respect to the test organisms, the compound with the R_f value of 0.55 appeared when sprayed with the Gram-positive test organisms while the compound with the R_f value of 0.77 were associated with activities versus *S. aureus* and the Gram-negative test organisms.

Activity against *E. coli* and *P. aeruginosa* was low in all direct extracts or fractions of extracts in spite of the fact that an active compound could be observed using bioautography. The reason for this could be that this active compound is present in too low concentrations or that antagonistic effects are present between components in the extracts/ fractions.

Figure 2.6. Hexane extract and fraction of *G. biloba* from the solvent-solvent extraction procedure (left) and from direct extraction procedure (right) sprayed with *S. aureus* and mobile phase DCM: methanol (19:1)

Mazzanti *et al* (2000) fractionated a methanol extract and obtained three fractions with different polarity i.e. EA, n-butanol and water. The non-polar EA fraction was active against different *S. aureus* isolates (MIC 0.312-1.2 mg/ml), *E. faecalis* (≤0.019 mg/ml) and other Gram-positive bacteria such as *Staphylococcus epidermidis* (0.625 mg/ml) and *Streptococcus sanguis* (0.019 mg/ml). The separation by reverse HPLC revealed that this fraction

was enriched by two substances and they concluded that most of the activity against *E. faecalis* was a result of these two substances. Lee and Kim (2002) used an agar diffusion method to determine activity of *G. biloba* leaf-derived fractions against several bacterial pathogens. They fractionated a 60% aqueous acetone extract into hexane, EA, butanol and water. They found strong activity (structures not determined) against *C. perfringens* in the acetone extract and EA and water fractions. In contrast to the findings in the current investigation, *E. coli* was also highly susceptible to the aqueous acetone extract and butanol fraction (Lee and Kim, 2002). The agar diffusion method used by Lee and Kim (2002) has severe limitations due to the inability of non-polar compounds to diffuse into the aqueous matrix of the agar. This may explain why there was no activity in the non-polar extracts (Eloff, 1998). The agar diffusion asay is however no longer acceptable as a good scientific method of determining MIC (Eloff, 1998). The EA fraction had weak activity against *E. coli*. The results obtained by determination of the MIC in the study of Mazzanti (2000) were compared with the data from this study.

As with *G. biloba*, the most pronounced activity of *H. perforatum* leaf extracts and fractions were observed against *C. perfringens* with activities as low as 0.02-0.04 mg/ml in extracts of acetone, hexane, DCM and the EA (direct extraction procedure) and hexane fraction from the solvent-solvent fractionation procedure. Only one compound (R_f 0.92) was present in the *H. perforatum* extracts from the direct extraction technique when using EMW as mobile phase and sprayed with the test organisms (Figure 2.8). As with *G. biloba*, the same compound only appeared in the hexane fraction from the solvent-solvent technique (method 1 and 2). This compound was probably present at insufficient concentrations to exhibit any marked inhibitory effect against *E. coli, S. aureus, P. aeruginosa* and *E. faecalis* (Table 2.2 and Figure 2.8).

Schempp *et al* (1999) reported activities with MICs as low as 1 ug/ml for different MRSA isolates for hyperforin, a known antibacterial compound isolated from *H. perforatum*. Hyperforin also has excellent activity against *S. agalactiae and S. pyogenes* (both at 1 ug/ml) as well as *Corynebacterium diphtheriae* at 0.1 ug/ml. Dall'Agnol *et al* (2003) investigated antibacterial activity of some *Hypericum* species and found activity of crude methanolic extract against *S aureus* (0.1 mg/ml), *Bacillus subtilis* (0.2 mg/ml) and *M. luteus* (0.2 mg/ml) but not against *S. epidermidis* and *E. coli*.

2.3.3 Synergistic / antagonistic interaction between extracts of *G. biloba* and *H. perforatum*

In this study, synergistic/additive effects were observed when combining either the hexane fractions (FIC = 0.44 or 123.5% stimulatory effect) (Table 2.4 and Figure 2.9) or the acetone extracts (FIC=0.59 or 68% stimulatory effect) (Table 2.5 and Figure 2.10) of *G. biloba* and *H. perforatum* against *E. faecalis*. This was found in spite of the hexane fraction and acetone extract of *H. perforatum* not exhibiting any activity against *E. faecalis*. These results are in agreement with a previous report where a synergistic effect was observed when combining extracts that did not show any activity by themselves (Nascimento *et al.,* 2000). This effect could be due to interaction

between constituents of the extracts/fractions of the two species. Another explanation is that this could be possibly due to a protective effect, for example antioxidants from extracts/fractions in the one plant species may protect the active constituents in the extracts/fractions of the other plant species from decomposition (Williamson, 2001). Only an indifferent was observed using the same combinations against *C. perfringens* (Table 2.6 and Table 2.7; Figure 2.11 and Figure 2.12).

A FIC of 0.4 was reported by van Vuuren and Viljoen (2008) when combining the root and leaf extracts of *Croton gratissimus* against the yeast *Cryptococcus neoformans,* and synergy was also observed when combining different plant parts against *B. cereus* and *Candida albicans*. The observed synergistic interactions indicate that the approach to finding a single chemical entity responsible for the effect, may be short sighted. Biological activity may thus be enhanced through the use of a combination of phytochemicals with different metabolic effects.

Table 2. 2. Minimum inhibitory concentrations (MIC's) (mg/ml) and total activity (TA*) of extracts (ml/g dry plant material) or fractions of extracts (ml/g dry extract) of *G. biloba* and *H. perforatum*

Extracts/ fractions	*E. coli*		*S. aureus*		*P. aeruginosa*		*E. faecalis*		*C. perfringens*		*S. enterica* Typhimurium		
	MIC	TA	MIC	*TA*	MIC	TA	MIC	TA	MIC	TA	MIC	TA	Yield (%)
G. biloba													
Serial extraction procedure													
Hexane	>2.50	<40	>2.50	<40	1.25	80	0.31	321	ND	ND	>2.50	<40.16	10.0
DCM	1.25	16	1.25	16	0.52	37	0.52	37	ND	ND	>2.50	<7.8	1.9
EA	1.04	5	1.25	4	0.31	18	0.31	18	ND	ND	>2.50	<2.2	0.6
Direct extraction procedure													
Acetone	2.50	54	0.31	432	2.50	54	0.31	432	0.031	4320	>2.50	<54.00	13.5
Hexane	2.50	40	0.31	321	>2.50	<40	0.31	321	0.008	12872	>2.50	<40.16	10.0
DCM	>2.50	<36	0.16	583	>2.50	<36	0.31	291	0.008	11667	>2.50	<36.4	9.1
EA	>2.50	<40	0.10	971	>2.50	<40	0.16	647	0.008	12950	>2.50	<40.4	10.1
Solvent –solvent fractionation procedure													
Hexane	1.04	24	0.16	158	1.72	14	0.13	195	0.008	3167	>2.50	<9.88	2.5
EA	1.67	54	1.25	72	0.63	144	0.31	289	0.039	2313	>2.50	<36.08	9.0
Butanol	>2.50	<24	>2.50	<24	>2.50	<24	>2.50	<24	0.417	144	>2.50	<24.00	6.0

Table 2.2. continued. Minimum inhibitory concentration (MIC's) (mg/ml) and total activity (TA*) of extracts (ml/g dry material) or fractions of extracts (ml/g dry extract) of *G. biloba* and *H. perforatum*

Extracts/ fractions	*E. coli*		*S. aureus*		*P. aeruginosa*		*E. faecalis*		*C. perfringens*		*S. enterica* Typhimurium		
	MIC	TA	MIC	*TA*	MIC	TA	MIC	TA	MIC	TA	MIC	TA	Yield (%)
H. perforatum													
Serial extraction procedure													
Hexane	>2.50	<7	>2.50	<7	1.04	17	>2.5		ND	ND	ND	ND	1.8
DCM	2.50	5	2.50	5	0.42	54	1.25	18	ND	ND	ND	ND	2.3
EA	1.25	6	2.50	3	0.52	13	0.63	11	ND	ND	ND	ND	0.7
Direct extraction procedure													
Acetone (100%)	2.50	16	2.50	16	2.50	16	2.50	16	0.04	1026	2.50	16	4.0
Acetone (60%)	ND	ND	ND	ND	ND	ND	ND	ND	0.63	238	ND	ND	14.9
Methanol	2.50	22	2.50	22	2.50	22	ND	ND	0.21	269	2.50	22	5.6
Ethanol (80%)	ND	ND	ND	ND	ND	ND	ND	ND	0.63	155	ND	ND	9.7
Hexane	2.50	5	2.50	5	2.50	5	2.50	5	0.04	333	2.50	5	1.3
DCM	2.50	10	1.25	21	2.50	10	2.50	10	0.02	1333	2.50	10	2.6
EA	2.50	9	0.63	37	2.50	9	2.50	9	0.02	1179	2.50	9	2.3
Solvent –solvent fractionation procedure (method 1 – Acetone (60%)													
Hexane	2.50	5	2.50	5	1.25	9	1.25	9	0.03	371	2.50	5	1.2
EA	1.25	27	1.25	27	0.63	55	1.25	27	0.47	73	2.50	14	3.4
Butanol	2.50	27	1.25	53	0.63	106	0.63	106	0.73	91	2.50	27	6.7
Solvent –solvent fractionation procedure (method 2 – Ethanol 80%)													
Hexane	1.25	ND	2.50	ND	1.04	ND	1.25	ND	ND	ND	2.50	ND	ND
EA	1.25	ND	1.25	ND	0.63	ND	1.25	ND	ND	ND	2.50	ND	ND
Butanol	2.50	ND	2.5	ND	2.50	ND	0.83	ND	ND	ND	2.50	ND	ND
Bacitracin	>0.5	NA	0.04	NA	>0.50	NA	0.02	NA	0.0003	NA	>1.00	NA	NA

ND - Not determined; NA - Not applicable

TA - Total activity is the volume to which compounds present in 1 g of dried plant material can be diluted and still retain activity (Eloff, 2000, 2004).

Table 2.3. The bioautographic qualitative inhibition of bacterial growth by extracts from different extraction procedures of *G. biloba* or *H. perforatum* separated by TLC with EMW as eluent: Rf values of active components

	R_f values			
Extract	*S. aureus*	*E. faecalis*	*E. coli*	*P. aeruginosa*
G. biloba				
Solvent-solvent separation technique				
Hexane	0.77		0.77	0.77
	0.68	0.68	0.68	0.68
	0.55	0.55		
EA	-*	-	-	-
Butanol	-	-	-	-
Direct extraction procedure				
Acetone	0.68	0.68	0.68	0.68
Hexane	0.68	0.68	0.68	0.68
DCM	0.68	0.68	0.68	0.68
EA	0.68	0.68	0.68	0.68
	0.55			
H. perforatum				
Solvent-solvent separation technique				
Hexane	0.92	0.92	0.92	0.92
EA	-	-	-	-
Butanol	-	-	-	-
Direct extraction procedure				
Acetone	0.92	0.92	0.92	0.92
Hexane	0.92	0.92	0.92	0.92
DCM	0.92	0.92	0.92	0.92
EA	0.92	0.92	0.92	0.92

* No active compounds observed

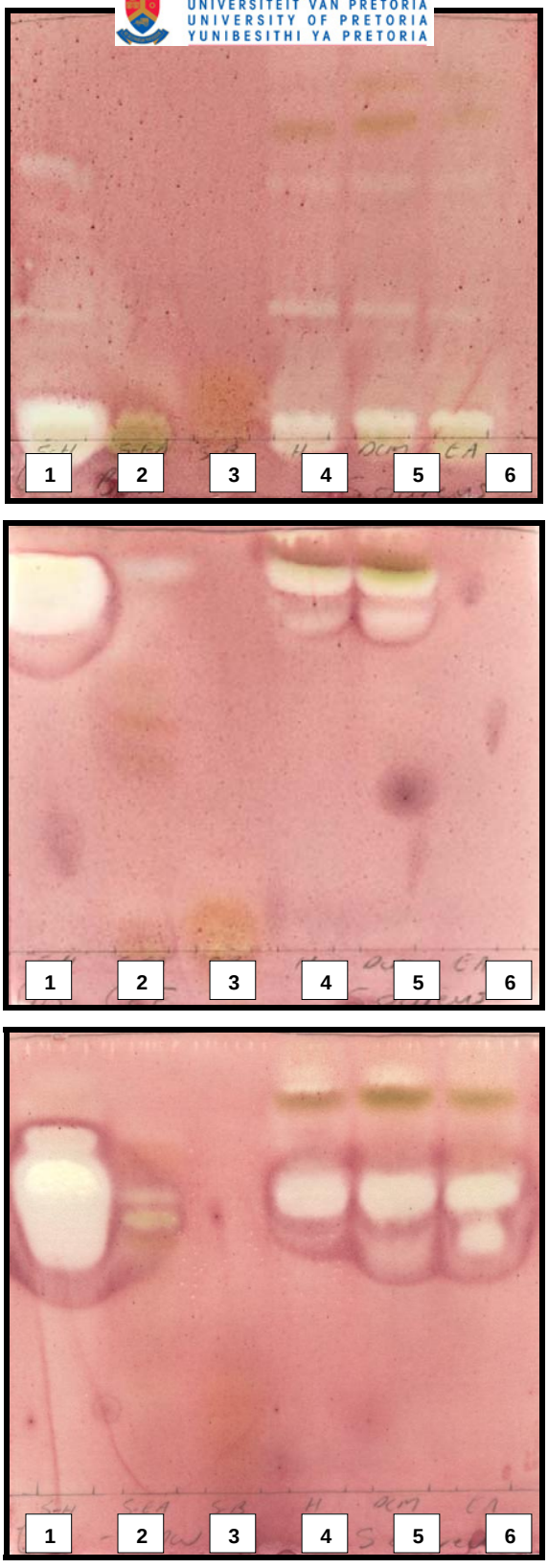


Figure 2.7. *G. biloba extracts* sprayed with *S. aureus* with mobile phases BEA (top), CEF (centre) and EMW (bottom)

1) Hexane fraction (solvent -solvent extraction – method 1), 2) EA fraction (solvent-solvent extraction – method 1), 3) Butanol fraction (solvent -solvent extraction – method 1), 4) Hexane extract (direct extraction), 5) DCM extract (direct extraction), 6) EA extract (direct extraction)

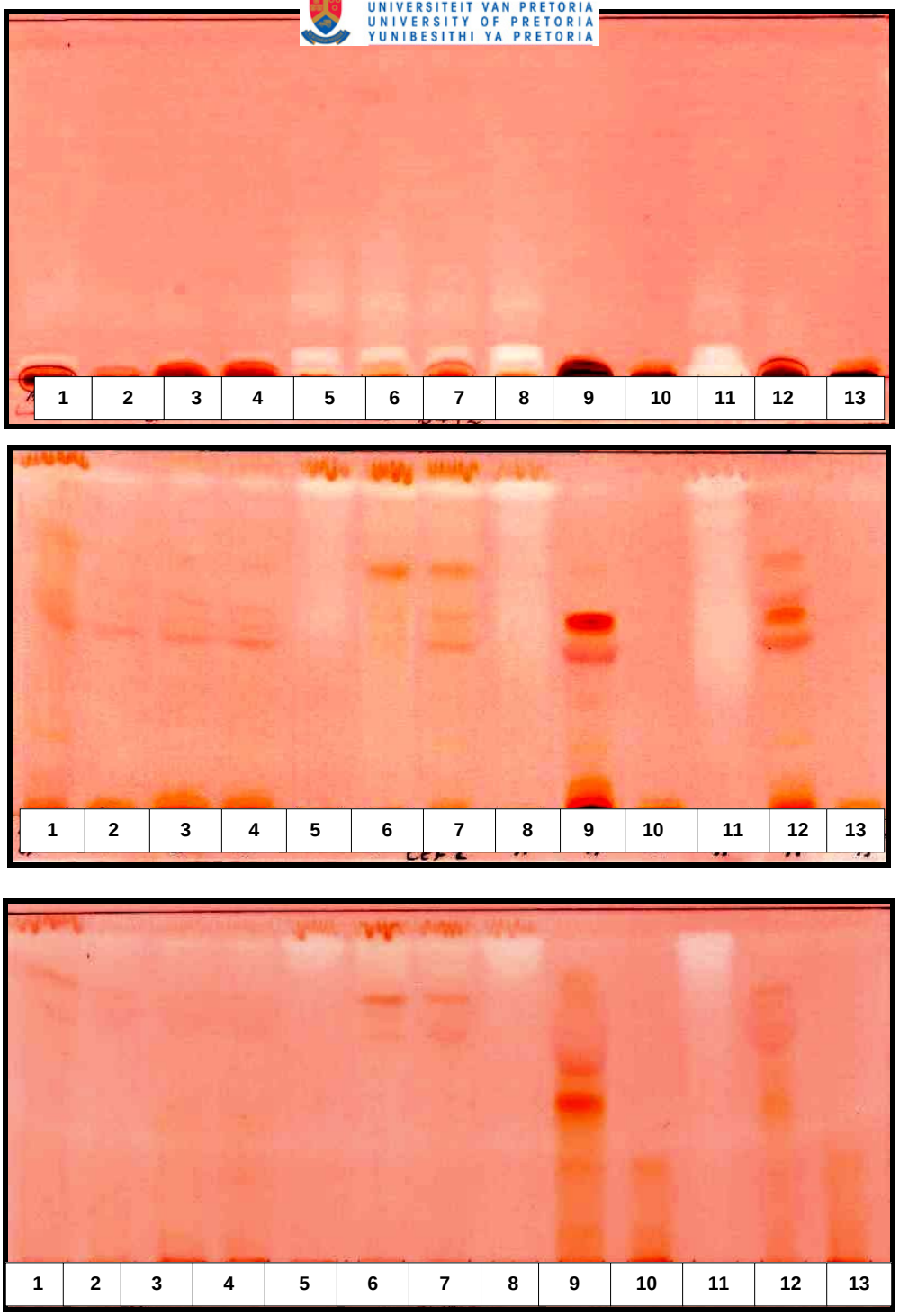


Figure 2.8. *H. perforatum* extracts (see below) sprayed with *S. aureus* with mobile phases BEA (top), CEF (centre) and EMW (bottom)

1) Acetone (100%) extract, 2) Acetone (60%) extract, 3) Ethanol (80%) extract, 4) Methanol (100%) extract, 5) Hexane extract (direct extraction), 6) DCM extract (direct extraction), 7) EA extract (direct extraction), 8) Hexane fraction (solvent solvent-extraction – method 2), 9) EA fraction (solvent-solvent extraction – method 2), 10) Butanol fraction (solvent- solvent extraction – method 2) 11) Hexane fraction (solvent-solvent extraction –method 1), 12) EA fraction (solvent-solvent extraction – method 1), 13) Butanol fraction (solvent-solvent extraction – method 1)

Table 2.4. FIC's of hexane fractions from *G. biloba* and *H. perforatum* to show synergistic (red) or additive (blue) effects of combinations against *E. faecalis* (using Berenbaum's equation in section 2.2.5)

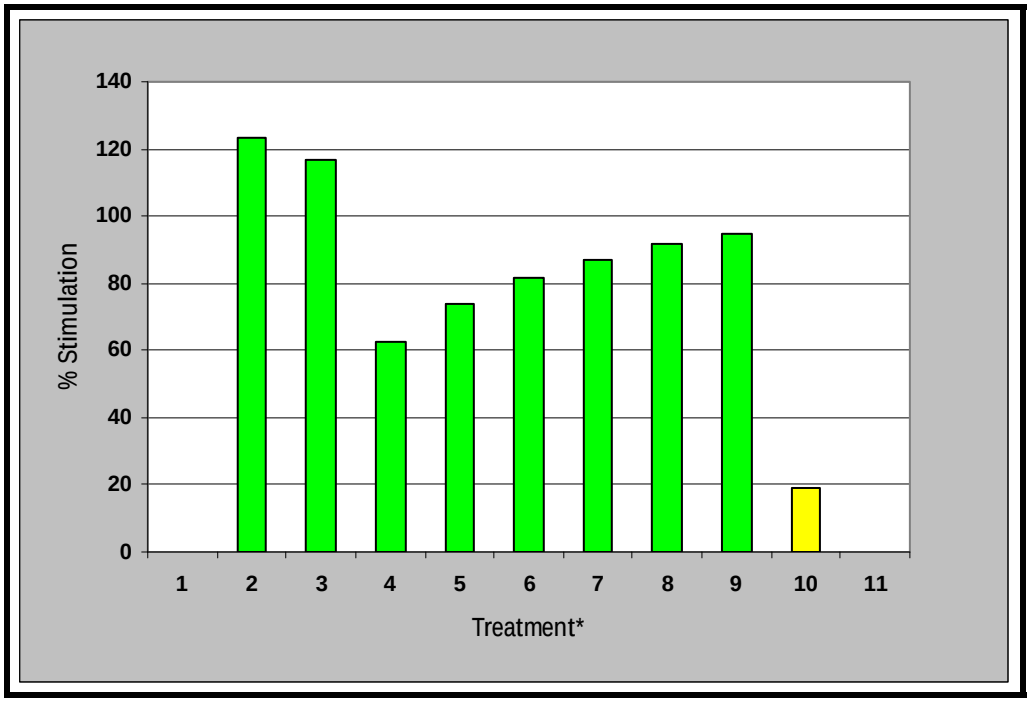
Treatment	% of Test fraction (Combinations)		Expected MIC (mg/ml) (separate values of individual fractions)[1]		Expected MIC (Total)[1]	Actual MIC (mg/ml) (separate values of combined fractions)[2]		Actual MIC (Total)	∑FIC
	a	b	a	b		a	b		
1	100	0	0.208	0	0.208	0.208	0	0.208	1.000
2	90	10	0.187	1	1.187	0.281	0.250	0.531	0.447
3	80	20	0.166	2	2.166	0.166	0.833	0.999	0.461
4	70	30	0.146	3	3.146	0.438	1.500	1.938	0.616
5	60	40	0.125	4	4.125	0.375	2.000	2.375	0.576
6	50	50	0.104	5	5.104	0.313	2.500	2.813	0.551
7	40	60	0.083	6	6.083	0.250	3.000	3.250	0.534
8	30	70	0.062	7	0.062	0.188	3.500	3.688	0.522
9	20	80	0.042	8	8.042	0.125	4.000	4.125	0.513
10	10	90	0.021	9	0.021	0.083	7.497	7.58	0.840
11	0	100	0	10	10	0	10.0	10.0	1.000

a = Hexane fraction of *G. biloba* from solvent-solvent separation technique

b = Hexane fraction of *H. perforatum* from solvent-solvent separation technique

[1]100% values were measured and % fractions were calculated

[2]Separate values were calculated from measured values of combinations (Actual MIC)



*Refer to Table 2.4 for treatment combinations

Figure 2.9. Effect of combining hexane fractions of *G. biloba* and *H. perforatum* on *E. faecalis* (using % Stimulation equation from section 2.2.5.1; green denotes a stimulatory effect and yellow an indifferent effect)

Table 2.5. FIC's of acetone extracts from *G. biloba* and *H. perforatum* to show additive (blue) or indifferent (yellow) effects of combinations against *E. faecalis* (using Berenbaum's equation in section 2.2.5)

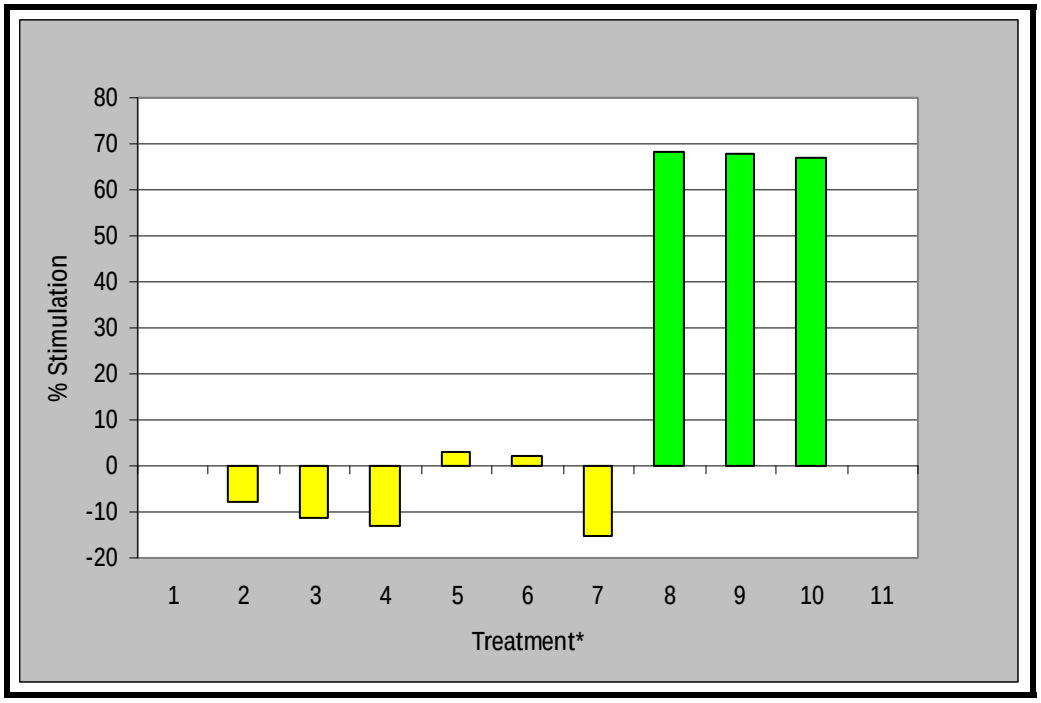
Treatment	% of Test fraction (Combinations)		Expected MIC (mg/ml) (separate values of individual fractions)[1]		Expected MIC (Total)[1]	Actual MIC (mg/ml) (separate values of combined fractions)[2]		Actual MIC (Total)	∑FIC
	a	B	a	b		a	b		
1	100	0	2.50	0.00	2.50	2.50	0.00	2.50	1.00
2	90	10	2.25	1.67	3.92	2.250	2.00	4.250	1.09
3	80	20	2.00	3.33	5.33	2.00	4.00	6.00	1.13
4	70	30	1.75	5.00	6.75	1.75	6.00	7.75	1.15
5	60	40	1.50	6.67	8.18	1.25	6.67	7.92	0.97
6	50	50	1.25	8.33	9.58	1.042	8.33	9.372	0.98
7	40	60	1.00	10.00	11.0	1.00	12.00	13.00	1.18
8	30	70	0.75	11.67	12.42	0.38	7.00	7.38	0.59
9	20	80	0.50	13.33	13.83	0.25	8.00	8.25	0.60
10	10	90	0.25	15.00	15.25	0.13	9.00	9.13	0.60
11	0	100	0.00	16.67	16.67	0.0	16.67	16.67	1.00

a = Acetone extract of *G. biloba*

b = Acetone extract of *H. perforatum*

[1]100% values were measured and % fractions were calculated

[2]Separate values were calculated from measured values of combinations (Actual MIC)



* Refer to Table 2.5 for treatment combinations. Green denotes a stimulatory- and yellow an indifferent effect

Figure 2.10. Effect of combining acetone fractions of *G. biloba* and *H. perforatum* on *E. faecalis* (using % Stimulation equation from section 2.2.5.1; green denotes a stimulatory effect and yellow an indifferent effect)

Table 2. 6. FIC's of hexane fractions from *G. biloba* and *H. perforatum* to show indifferent (yellow) effects of combinations against *C. perfringens* (using Berenbaum's equation in section 2.2.5)

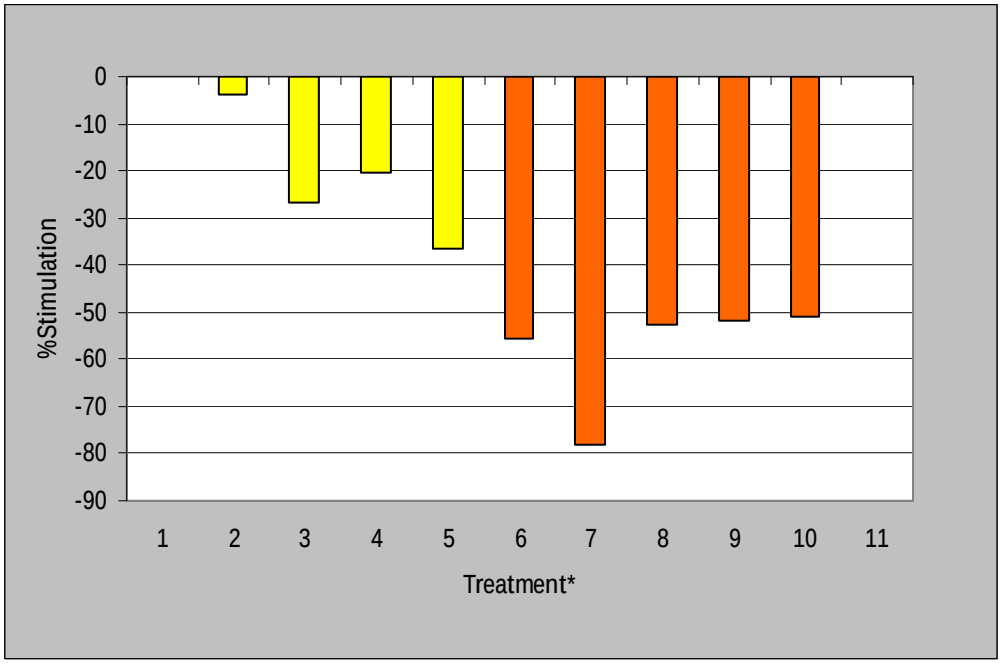
Treatment	% of Test fraction (Combinations)		Expected MIC (µg/ml) (separate values of individual fractions)[1]		Expected MIC (Total)[1]	Actual MIC (µg/ml) (separate values of combined fractions)[2]		Actual MIC (Total)	∑FIC
	a	b	a	b		a	b		
1	100	0	2.23	0.00	2.23	2.23	0.00	2.23	1.00
2	90	10	2.01	1.56	3.57	2.67	1.04	3.71	1.04
3	80	20	1.79	3.13	4.92	3.60	3.12	6.72	1.37
4	70	30	1.56	4.69	6.25	3.15	4.68	7.83	1.25
5	60	40	1.34	6.25	7.59	3.60	8.33	11.93	1.57
6	50	50	1.12	7.81	8.93	4.50	15.63	20.13	2.25
7	40	60	0.89	9.38	10.27	3.60	43.75	20.13	4.61
8	30	70	0.67	10.94	11.61	2.70	21.88	24.58	2.12
9	20	80	0.45	12.50	12.95	1.80	25.00	26.8	2.07
10	10	90	0.22	14.06	14.28	0.90	28.13	29.03	2.03
11	0	100	0.00	15.63	15.63	0.00	15.63	15.63	1.00

a = Hexane fraction of *G. biloba* from solvent-solvent separation technique

b = Hexane fraction of *H. perforatum* from solvent-solvent separation technique

[1]100% values were measured and % fractions were calculated

[2]Separate values were calculated from measured values of combinations (Actual MIC)



* Refer to Table 2.6 for treatment combinations. Yellow denotes an indifferent effect

Figure 2.11. Effect of combining hexane fractions of *G. biloba* and *H. perforatum* on *C. perfringens* (using % Stimulation equation from section 2.2.5.1; yellow denotes an indifferent effect and orange an antagonistic effect)

Table 2.7. FIC's of acetone fractions from *G. biloba* and *H. perforatum* to show indifferent (yellow) effects of combinations against *C. perfringens* (using Berenbaum's equation in section 2.2.5)

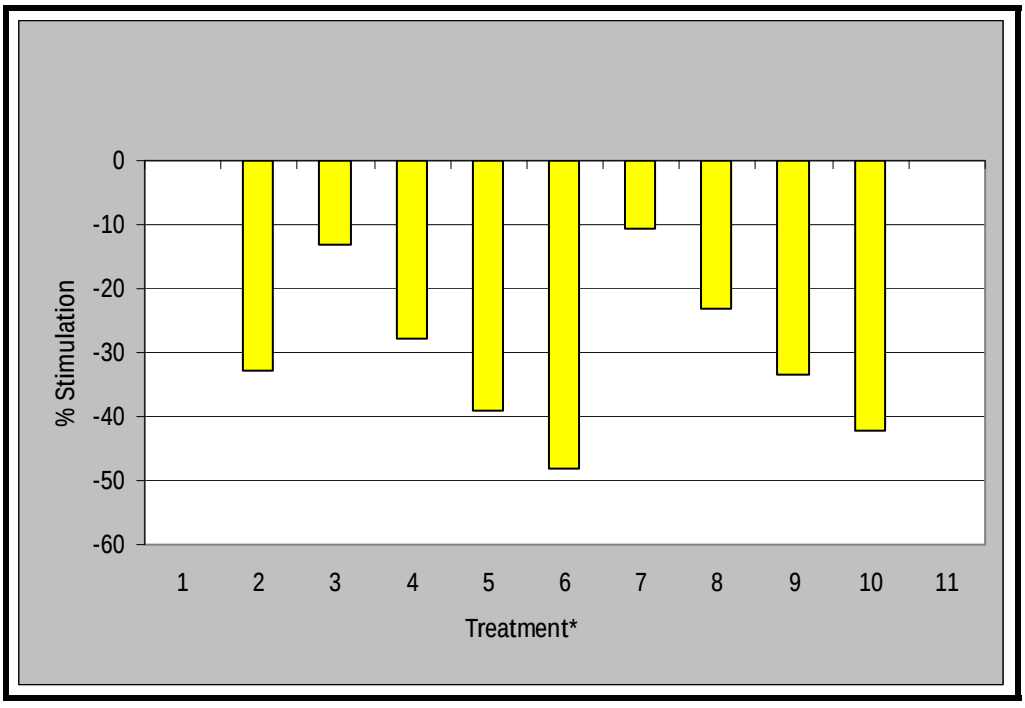
Treatment	% of Test fraction (Combinations)		Expected MIC (mg/ml) (separate values of individual fractions)[1]		Expected MIC (Total)[1]	Actual MIC (mg/ml) (separate values of combined fractions)[2]		Actual MIC (Total)	∑FIC
	a	b	a	b		a	b		
1	100	0	0.14	0.00	0.14	0.14	0.00	0.14	1.00
2	90	10	0.12	0.01	0.13	0.16	0.04	0.20	1.49
3	80	20	0.11	0.02	0.14	0.08	0.06	0.14	1.15
4	70	30	0.10	0.02	0.12	0.07	0.09	0.16	1.38
5	60	40	0.08	0.03	0.11	0.06	0.12	0.18	1.64
6	50	50	0.07	0.04	0.11	0.05	0.16	0.21	1.92
7	40	60	0.06	0.05	0.11	0.02	0.09	0.11	1.12
8	30	70	0.04	0.05	0.09	0.02	0.11	0.13	1.30
9	20	80	0.03	0.06	0.09	0.01	0.12	0.13	1.50
10	10	90	0.01	0.07	0.08	0.01	0.14	0.15	1.73
11	0	100	0.00	0.08	0.08	0.00	0.08	0.08	1.00

a = Acetone extract of *G. biloba*

b = Acetone extract of *H. perforatum*

[1]100% values (rounded off) were measured and % fractions were calculated

[2]Separate values (rounded off) were calculated from measured values of combinations (Actual MIC)



* Refer to Table 2.7 for treatment combinations. Yellow denotes an indifferent effect.

Figure 2.12. Effect of combining acetone fractions of *G. biloba* and *H. perforatum* on *C. perfringens* (using % Stimulation equation from section 2.2.5.1; yellow denotes an indifferent effect)

2.4 Conclusions

The first objective of this study was to evaluate the activity and potentize extracts from *G. biloba* and *H. perforatum* for optimal activity against relevant bacterial pathogens. Extracts of ethyl acetate (EA), hexane, dichloromethane (DCM) and acetone (in order of activity) from the direct extraction procedure of powdered *G. biloba* leaves were active against *E. faecalis, S. aureus* and *C. perfringens*. The EA, hexane and DCM extracts were 2 to 3 times more active than the acetone extract (average total activity 1 728 ml/g dry extract for the 3 pathogens). The DCM, EA, acetone and hexane extracts (in order of activity) from the direct extraction procedure from *H. perforatum* were only active against *C. perfringens* with the first three extracts with a total activity of between 1 026 and 1 333 ml/g dry material and the hexane extract a total activity of 333 ml/g dry material. The spectrum of activity of *G. biloba* corresponds to that of Zn-Bacitracin, which is commonly used as an antibiotic growth promoter in the poultry industry.

When selecting an extractant for the purpose of developing it into an herbal product one should consider its toxicity. According to the Material safety data sheet the LD_{50} (oral rat) values are 28 710, 5 620, 3 000 and 1 600 mg/kg for hexane, EA, acetone and DCM respectively. The low concentration of the residue of the extractant in the dried extract (after evaporation) can thus be considered safe.

The second objective in this study was to combine extracts or fractions of extracts of *G. biloba* and *H. perforatum* to optimise activity against selected bacterial pathogens. A synergistic effect could be observed when combining a ratio of 1:5 of *G. biloba: H. perforatum* (hexane extracts) or 1:15 (acetone extracts) against *E. faecalis* while only an indifferent effect (according to the FIC index) could be observed against *C. perfringens*.

The potential of leaf extracts/fractions of both plant species against Gram-positive bacteria such as *C. perfringens* are of great importance. *C. perfringens* has been associated with necrotic enteritis in poultry, which damage the intestinal mucosa, which result in lower feed intake, mortality and consequently, worsened performance (van Emmerseel *et al.,* 2004). The use of extracts of *G. biloba* and/ or *H. perforatum* may be beneficial as alternatives to the use of growth promoters, which act predominantly against Gram-positive bacteria, in the poultry industry.

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3 Activity of an antimicrobial compound isolated from *Ginkgo biloba*

3.1 Introduction

Ginkgo biloba is among the most sold medicinal plants of this world with estimates of sales in the USA of more than $249 million (de Kosky *et al.,* 2008). Most of the sales are of special extracts from the leaves and are mainly used for the improvement of peripheral and central blood circulation (DeFeudis, 1998). Important constituents present in the leaves are terpene trilactones, i.e., ginkgolides (A, B, C, J) and bilobalide, many flavonol glycosides, biflavones, proanthocyanidins, alkylphenols, simple phenolic acids, 6-hydroxykynurenic acid, 4-O-methylpyridoxine and polyprenols (van Beek, 2002). Egb761 is the first standardised preparation patented by Schwabe (1994) for medicinal use. The content of ginkgolic acids (alkylphenol) in this preparation should not exceed 5ppm because of suspected cytotoxic and allergenic properties (Pan, 2007).

A range of bioactivities such as antiparasitic (Atzori *et al.,* 1993; Bombardellii and Ghione, 1993; Chen *et al.,* 2008), antiviral (de Tommasi *et al.,* 1990), antifungal (Watanabe et al., 1990; Anke and Sterner, 1991) and immunomodulating (Bourguet-Kondracki, 1991) activities have been displayed by compounds from *G. biloba* such as the terpenes (ginkgolides and bilobalides). In addition to these activities, the antibacterial activities of compounds from *G. biloba* leaf extracts have also been investigated. Bombardellii and Ghione (1993) reported 0.01-0.1 µg/ml bilobalide to be active *in vitro* against pathological strains of *Trichomas vaginalis*, *S. aureus*, *E. faecalis*, *E. coli* and *Lactobacillus* spp. Lee and Kim (2002) found kaempferol and quercitin (flavonol glycosides) to be active against *C. perfringens* and/or *E. coli*. In contrast to these findings Mazzanti *et al* (2000) and Lee and Kim (2002) found no antimicrobial activity for bilobalide and ginkgolide A and B as well as for rutin. It should however, be noted that Lee and Kim (2002) used the agar diffusion method which has severe limitations due to the inability of non-polar compounds to diffuse into the aqueous matrix of the agar (Eloff, 1998). Antibacterial activity for the alkylphenols (ginkgolic acid, ginkgols and bilobols) were observed by several investigators: Itokawa *et al*. (1987) observed a weak antimicrobial activity of bilobol and cardanol (alkylphenols) against *S. aureus* and *E. faecalis.* In contrast, Choi *et al* (2009) reported strong activity of hydroxyalkenyl salicylic acids (ginkgolic acid) as low as 2 µg/ml against vancomycin-resistant *Enterococcus* spp. Adawadka and El-Sohly (1981) observed activity of the anacardic acids (ginkgolic acids) against *Mycobacterium smegmatis*. Pan (2007) reported on the activities of ginkgolic acids as low as 25 µg/ml against Gram-positive bacteria including methicillin resistant *S. aureus.*

Due to the fact that antibacterial activity is apparently not confined to a single compound in *G. biloba* extract, the objective of the current investigation was firstly to isolate and identify the major antibacterial compound from *G. biloba* and secondly, to determine whether activity against *E. faecalis* and *C. perfringens* in an extract or fraction

of an extract of *G. biloba* can be attributed to that specific compound or whether synergism or other interactions also play a role in the observed activity.

3.2 Materials and methods

3.2.1 Plant collection

Powders of leaves of *G. biloba* and *H. perforatum* were obtained from Biomox Pharmaceutical (Pty) Ltd – South Africa.

3.2.2 Isolation and identification of active compound

3.2.2.1 Column Chromatography

Two kilograms of finely ground *G. biloba* leaves were extracted with *n*-hexane. The hexane was removed using a rotary evaporator. A bioautogram of this extract developed with BEA (BEA = benzene 90 ml, ethanol 10 ml and ammonium hydroxide 1 ml) and sprayed with *S. aureus* revealed the presence of antibacterial zones.

The main antibacterial compound present in the hexane extract was isolated by bioassay guided fractionation using silica gel 60 Column chromatography with a chloroform-methanol gradient solvent system. Active fraction/s were combined and subjected to Sephadex LH-20 column chromatography eluting with chloroform-methanol (2:1) to isolate the active compound.

3.2.2.2 Structure elucidation

The isolated compound was analyzed using Nuclear Magnetic Resonance (NMR) spectroscopy using the facilities available at the Medical University of South Africa (MEDUNSA).

3.2.3 Plant extracts

Different methods of extraction of *G. biloba* leaves were tested previously (chapter 2). Two extraction methods were selected as they resulted in the best MIC and total activity results against the test pathogens (see microdilution assay). The two extraction methods and extractants used are described briefly:

3.2.4 Extraction of plant material

3.2.4.1 Direct extraction

Dried material was extracted using 100% concentrations of acetone, hexane, dichloromethane (DCM) or ethyl acetate (EA). A ratio of 1:10 dried material: extractant was used in all cases. Mixtures were shaken for 10 min in a Labotec 20.2 shaking machine at high speed. The extracts were centrifuged at 1 322 *x g* for 10 min before decanting into labelled containers. The process was repeated three times on the same material and extractant and the extracts were combined. Extracts were dried at room temperature under a continuous stream of air.

3.2.4.2 Solvent-solvent extraction

Solvent-solvent extraction was carried out in accordance with the method describe by Lee and Kim (2002). Dried material was extracted twice with 60% aqueous acetone at a ratio of 1:10 dried material vs. extractant at room temperature and filtered. The extract was concentrated by using rotary evaporation at 45 °C after which the extract was sequentially partitioned into hexane, EA, butanol and H_2O portions. Each step was carried out three times to ensure adequate extraction. All solvents were saturated with distilled H_2O before use to ensure adequate separation. The solvent portions were concentrated by rotary evaporation at 45°C and further dried at room temperature under a continuous stream of air.

3.2.5 Microdilution assay

In the previous chapter, extracts or fractions of extracts of *G. biloba* were found to be active against *E. faecalis* and *C. perfringens* but not *E. coli, Pseudomonas aeruginosa* and *Salmonella enterica.* Typhimurium with low activity against *S. aureus.* The MIC and total activity (TA) data for *C. perfringens* and *E. faecalis* are presented again in this chapter. The TA is the volume to which 1g of dried plant material (or dried extract in the case of fractions of the extract) can be diluted and still retain activity (Eloff, 2000, 2004). The TA and MIC of the active compound were determined as described in Chapter 2. Zinc-bacitracin was used as a positive control to confirm the sensitivity of the system.

3.2.6 Determination of the concentration of the isolated compound by use of a bioautography method

A bioautography procedure was done according to Begue and Kline (1972) in order to determine the concentration of the active compound in the different extracts/ fractions. Briefly, duplicate TLC plates (10X20cm) were loaded with 50 µg (10 µl of 5 µg/ml) of each of the extracts or fractions. On the other side of the plate, the active compound was loaded with 10 µl each of a series of 20, 15, 10, 5, and 2.5 µg pure compound. The plates were developed in a DCM:Methanol (19:1) mobile system. Chromatograms were dried for 24 h at room temperature to remove the remaining solvent. *S. aureus* were used as the indicator organism to determine the concentration of the active compound in the different extracts/fractions because it has shown to develop clear zones of inhibition with very few complications frequently experienced with bioautography. Cultures of S. *aureus* were grown on Müeller-Hinton (MH) agar and incubated at 37°C overnight. The broth culture was prepared by transferring 2-3 bacterial colonies with a sterile swab from agar into two 250 ml Erlenmeyer flasks each containing 100 ml MH broth. Broth cultures were incubated for 24 h at 37°C. Developed TLC plates were inoculated with a fine spray of the bacterial suspension containing approximately 10^8 cells/ml of actively growing bacteria in a Biosafety Class II cabinet (Labotec, SA). The plates were sprayed until they were just wet and incubated overnight in a chamber at 100% relative humidity in the dark. The plates were subsequently sprayed with a 2 mg/ml solution of INT and incubated for 2-3 h in the same chamber. White areas indicate where reduction of INT to the colored formazan did not take place due to the presence of compound/s that inhibited the growth of the test bacteria. Bioautograms were sealed in clear plastic envelopes and scanned for a permanent record.

3.2.7 Synergy / antagonistic interactions between fractions of the acetone extract of *G. biloba*

The MICs against *E. faecalis* and *C. perfringens* were previously determined (chapter 2). The hexane- and EA fractions from the solvent-solvent separation technique were combined in duplicate and the fractional inhibitory concentration (FIC) index determined (as described in chapter 2) using *E. faecalis* and *C. perfringens*:

$\sum FIC = FIC^{(a)} + FIC^{(b)}$ (Berenbaum, 1978) i.e.

$$\sum FIC = \frac{\text{Actual [MIC (a*) + MIC (b*)]}}{\text{Expected [MIC (a) + MIC (b)]}}$$

(a*) and (b*) = in this study represent the above mentioned hexane- or EA fractions of *G. biloba*.

The FIC index (Schelz *et al*., 2006), is determined as the correlation between the two combined test substances and may be classified as either synergistic (≤0.5), additive (<0.5-1), indifferent (>1 to 4) or antagonistic (≥4).
Determination of % Stimulation:

Percentage Stimulation was determined as described in chapter 2 by combining the hexane- and EA fractions from the solvent-solvent separation technique in duplicate using above mentioned pathogens.

$$\% \text{ Stimulation} = \frac{\text{Expected MIC} - \text{Actual MIC}}{\text{Actual MIC}} \times 100$$

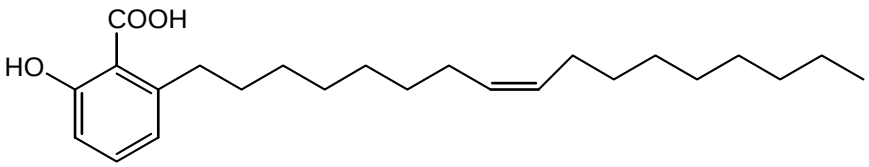
Values ≥ 50% indicate stimulation, > -50% and < 50% indicate an indifferent effect and ≤ -50% denotes antagonism. A 100% stimulation means that the activity is doubled and with a 0% stimulation there was no synergistic or antagonistic activity.

3.3 Results and discussion

3.3.1 Isolation and antibacterial activity of ginkgolic acid from *Ginkgo biloba* leaf extracts

About 36 g of hexane extract was chromatographed over silica gel with a choroform-methanol gradient solvent system resulting in 19 fractions. Fraction 7 contained the most active zone against *S. aureus*. This fraction (4g) was subjected to Sephadex LH-20 chromatography, eluting with chloroform:methanol (2:1), resulting in 25 fractions of 5 ml each. Fractions 11-23 were combined and subjected to Sephadex LH-20, this time eluting with chloroform:methanol (95:5) giving rise to 37 fractions (5 ml). Fractions 30 - 37 (0.4 g) were subsequently chromatographed using a Silica gel column eluting with methanol:DCM (1:9) to yield 270mg of a white, amorphous compound labelled GbHK001.

Compound GbHK001 was identified as ginkgolic acid, a 6-alkylsalicylic acid with $C_{17:1}$ alkyl substituent (Figure 3. 1) by comparison of its spectral data (Table 3.1 and Figure 3.2) with those published in the literature.



GbHK001

Figure 3. 1. Ginkgolic acid, a 6-alkylsalicylic (anacardic) acid with $C_{17:1}$ alkyl substituent.

Table 3. 1. ^{1}H NMR data for isolated compound and ginkgolic acid ($C_{17:1}$).

Isolated compound	van Beek and Wintermans (2001)
5.28	5.33
2.94	2.96
1.97	1.99
1.53	1.59
1.25	1.26
0.82	0.88

Ginkgolic acid (6-alkylsalicylic acid) is an alkylphenol with 3 different classes occurring in *G. biloba* i.e. ginkgolic acids, ginkgols and bilobols. Other synonyms are 2-hydroxy-6-alkylbenzoic acids and anacardic acids (van Beek, 2002). "Ginkgolic acid" wil be used in this report. Ginkgolic acid has also been isolated from various parts of the cashew fruit *Anacardium occidentale* (Anacardiaceae) (Kubo *et al.,* 1995) and recently also from Brazilian propolis (Silva *et al.,* 2008). Activity of ginkgolic acid ($C_{15:1}$, $C_{15:2}$ and $C_{15:3}$) against Gram-positive bacteria including methicillin resistant *S. aureus* (Muroi *et al.,* 2003) and the dental pathogen, *Streptococcus mutans* (Green *et al.,* 2008) with values of 1.56-6.25 µg/ml have been reported. Many studies showed that ginkgolic acids has much stronger activity against Gram-positive than Gram-negative bacteria (Kubo *et al.,* 1993; Yang *et al.,* 2004), except for *Helicobacter pylori*, the causative agent of acute gastritis (Kubo *et al.,* 1999). The structure of the above mentioned compound is similar to that of the compound isolated in the current study except for the length and the number of double bonds of the alkyl side chain.

In this investigation, the isolated ginkgolic acid ($C_{17:1}$) had no activity against the Gram-negative *E. coli*, *S. typhimurium* and *P. aeruginosa*. Activity of 100 µg/ml, 62.6 µg/ml and 1.56 µg/ml was observed for *S. aureus*, *E. faecalis* and *C. perfringens* respectively (Table 3.2). This is comparable to the spectrum of activity of bacitracin (no activity against *E. coli* and *P. aeruginosa* and activity of 0.3, 20 and 40µg/ml against *C. perfringens*, *E. faecalis* and *S. aureus* respectively (results not shown). In general, it is observed that antimicrobial activity of ginkgolic acid is inversely proportional to the length of the C_6 chain and that at a particular critical length it reaches a maximum after which activity greatly diminishes to finally become inactive (Green *et al.,* 2008). Green *et al* (2008) synthesized a series of ginkgolic acids possessing different lengths of the C_6 side chain and found ginkgolic acid ($C_{12:0}$) exhibited the most potent bactericidal activity against *S. mutans*, while ginkgolic acid ($C_{15:0}$) did not show any activity up to 0.8 mg/ml. They noted that although this ginkgolic acid ($C_{15:0}$) was ineffective against *S. mutans*, it nevertheless exhibited potent antibacterial activity against *Propionibacterium acnes* with a MIC of 0.78 µg/ml. Daoud *et al.* (1983) reported that the antimicrobial activity of a series of alkyldimethylbenzylammonium chlorides was a parabolic function of their lipophilicity and maximized with alkyl chain lengths between C_{12} and C_{16}. The penetration of these compounds through cell membranes depends on their lipophilic properties. Substances with low lipid solubility would be unable to cross the lipophilic barriers and remain localized in the first aqueous phase they contact. Conversely, those with high lipid solubility would remain

localized in the lipid regions (Franks and Lieb, 1986). Somewhere between these extremes there would be an optimum point of lipophilicity for transversing the cell barriers. According to Muroi *et al* (2003) this explanation can be applied in the case of the ginkgolic acids. Ginkgolic acid with chain length between C_{10} and C_{12} appears to possess the optimum balance between hydrophilicity and lipophilicity to penetrate cell membranes. In addition to the length, the volume of the lipophilic portions, which is altered by the position, number, and stereochemistry of double bounds, also affects activity (Muroi *et al.,* 2003).

This explanation could probably also apply in the current investigation where the long alkyl chain length of ginkgolic acid ($C_{17:1}$) rendered the ginkgolic acid less effective against *S. aureus* while activity was previously reported to be high (Muroi *et al.,* 2003). It also indicates that the lipophilic character of the bacterial membrane can differ (Kubo *et al.,* 1995) which explains the high activity of this long alkyl chain ginkgolic acid against *E. faecalis* and *C. perfringens*.

The alkylphenols possess contact allergenic, cytotoxic, mutagenic and slight neurotoxic properties (Koch *et al.,* 2000; Baron-Rupert and Luepke, 2001). It should, however, be remarked that there is no solid evidence of a strong allergic reaction when taken orally. For instance, no reports have been filed on the adverse effects of *Ginkgo* homeopathic mother tinctures in spite of the fact that such extracts contain 2.2% ginkgolic acids (van Beek, 2002). The biological activities of the ginkgolic acid derivates have however attracted considerable attention for its molluscicidal activity against *Oncomelania hupensis* (Yang *et al.,* 2008), anti-*Toxoplasma gondii* activity (Chen *et al.,* 2008), antitumor (Kubo *et al.,* 1993), antioxidant (Kubo *et al.,* 2005) and xanthine oxidase inhibitory action (Masuoka and Kubo, 2004).

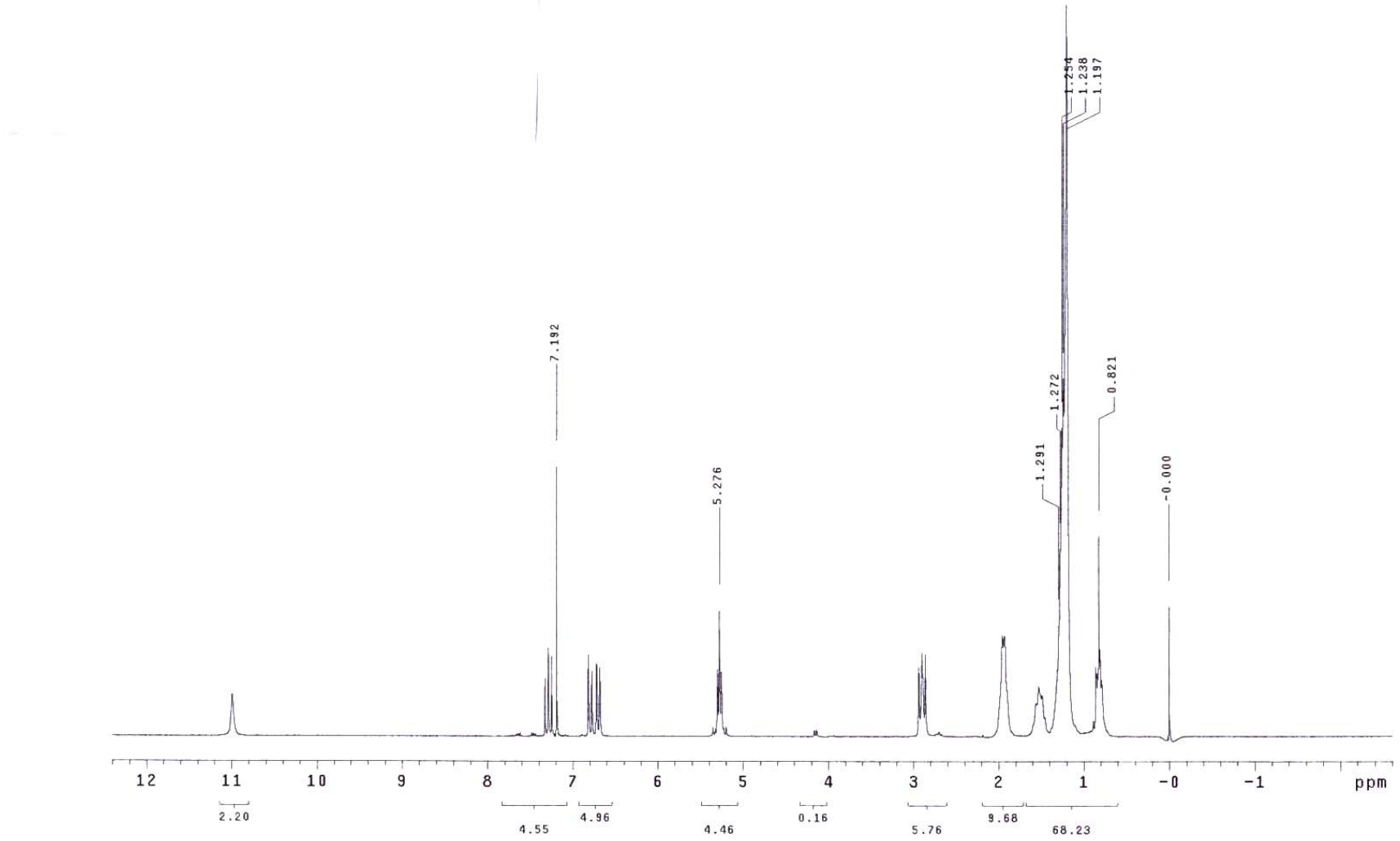


Figure 3. 2· ^{1}H NMR data for isolated compound GbHK001

3.3.2 Determination of the concentration and total activity contribution of ginkgolic acid in the various extracts or fractions of extracts of *G. biloba*

In Table 3.2 the percentage of total activity (TA) ascribed to ginkgolic acid in extracts or fractions of extracts of *G. biloba* against *E. faecalis* and *C. perfringens* are reflected. The percentage of ginkgolic acid in the fractions or extracts was determined by matching the size of the zone of inhibition in the extract or fraction to the corresponding zone of inhibition when loaded with a series of 20, 15, 10, 5, and 2.5µg ginkgolic acid (Figure 3.3).

Ginkgolic acid plays a partial role in the total activity against *E. faecalis* and *C. perfringens*. In the hexane and DCM extracts ginkgolic acid contributes at least 50% to the total activity against both pathogens and in the EA extract 25 and 50% against *E. faecalis* and *C. perfringens* respectively (Table 3.2). The total activity in the acetone extract and the hexane fraction of the acetone extract can be mainly ascribed to ginkgolic acid (100-200 and 50-100% respectively) against *C. perfringens* (see next section for explanation on why activity exceeds 100%). It seems that ginkgolic acid's activity play a smaller role in this acetone extract (12.5-25%) and hexane fraction (20-40%) with regards to the total activity against *E. faecalis*.

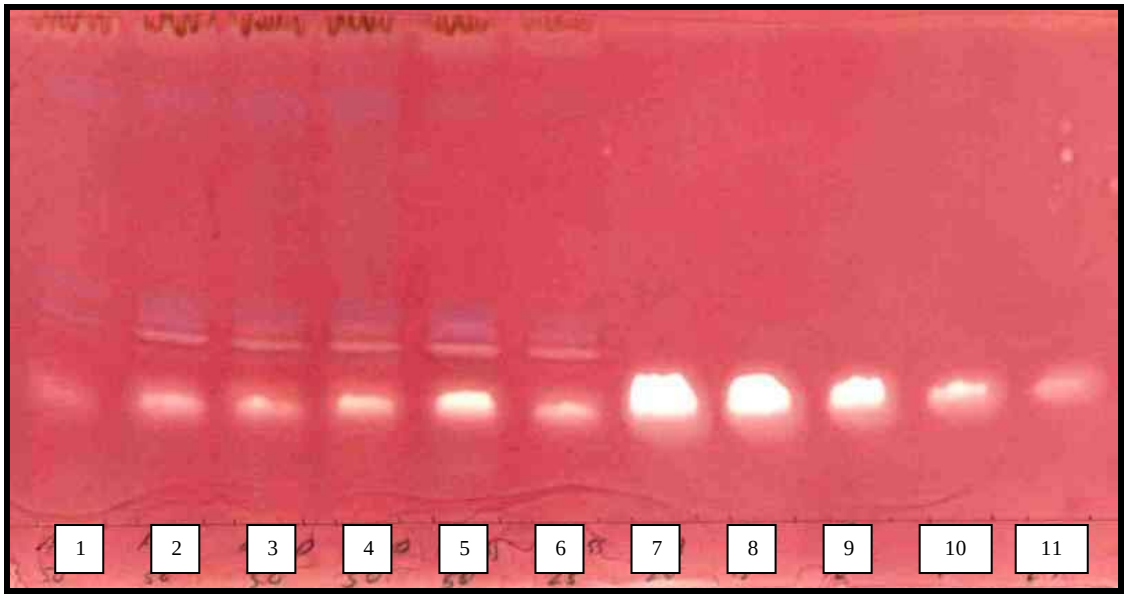


Figure 3. 3. Bioautogram of *G. biloba extracts* (1-6) and different concentrations of the isolated ginkgolic acid (GA) (7-11) sprayed with *S.aureus* with mobile phase DCM:Methanol (19:1)

1) Acetone extract (50µg) 2) Hexane extract (50µg), 3) DCM extract (50µg), 4) EA (50µg), 5) Hexane fraction (50µg), 6) Hexane fraction (25µg), 7) GA (20µg), 8)GA (15 µg), 9)GA (10 µg), 10) GA (5 µg) and 11) GA (2.5 µg).

Table 3.2. Percentage of total activity (TA) ascribed to ginkgolic acid (GA) in extracts or fractions of extracts of *G. biloba* against *E. faecalis* and *C. perfringens*

	Extracts/fractions			Ginkgolic acid				Activity ascribed to GA (%)
E. faecalis	MIC[1]	TA[2]	Yield (%)	MIC	TA	Yield (%)	GA in extract/ fraction (%)	
Hexane	0.3125	321	10.0	0.0625	161	1.0	10.0	50
DCM	0.3125	291	9.1	0.0625	146	0.9	10.0	50
EA	0.156	647	10.1	0.0625	162	1.0	10.0	25
Acetone	0.3125	432	13.5	0.0625	54-108	0.34-0.68	2.5-5.0	12.5-25
Fractions of acetone								
Hexane	0.12675	195	2.5	0.0625	40-79	0.25-0.5	10-20.0	20.5-41
EA	0.3125	289	9.0	0.0625	0	0	0.0	0
Butanol	2.5	24	6.0	0.0625	0	0	0.0	0
C. perfringens								
Hexane	0.0078	12872	10.0	0.00156	6436	1.0	10.0	50
DCM	0.0078	11667	9.1	0.00156	5833	0.9	10.0	50
EA	0.0078	12949	10.1	0.00156	6474	1.0	10.0	50
Acetone	0.03125	4320	13.5	0.00156	2163-4326	0.34-0.68	2.5-5.0	100-200
Fractions of acetone								
Hexane	0.0078	3167	2.5	0.00156	1583-3167	0.25-0.5	10-20.0	50-100
EA	0.039	2313	9.0	0.00156	0	0	0.0	0
Butanol	0.4167	144	6.0	0.00156	0	0	0.0	0

[1]MIC = Minimum inhibitory concentration (mg/ml)
[2]TA = Total activity (ml/g)

3.3.3 Synergy / antagonistic interaction between fractions of the acetone extract of *G. biloba*

The EA fraction does not contain any ginkgolic acid but it clear that at least half of the activity against *E. faecalis* and *C. perfringens* is contained in this fraction. This is not surprising as antimicrobial activity is well reported for other constituents such as the bilobalides and ginkgolide A and B and the flavonol glycosides in *G. biloba* leaves (Bombardelli and Ghione, 1993; Mazzanti *et al.,* 2000; Lee and Kim, 2002). These compounds could have been present at too low concentrations to exhibit a zone of inhibition with the bioautography assay (Chapter 2). In addition to this, a synergistic interaction was observed (Table 3.3 and Figure 3.4; Table 3.4 and Figure 3.5) between these two fractions with regards to activity against *E. faecalis* (FIC index = 0.32 i.e. a 3.1X stimulatory effect) and *C. perfringens* (FIC index = 0.42 i.e. a 2.4X stimulatory effect). It is thus possible that there are synergistic interactions between constituents in the two fractions or that the constituents in the EA fraction enhance the activity of ginkgolic acid. The fact that ginkgolic acid's contribution to the total activity against *C. perfringens* exceeds 100% in the acetone extract indicates that the action of ginkgolic acid was enhanced by other constituents. Green *et al* (2008) observed that antibacterial activity of ginkgolic acid ($C_{15:3}$) against S. *mutans* was enhanced 8-fold (from 6.25 to 0.78µg/ml) when in combination with a sublethal amount (equivalent to ½ MBC) of anethole, a sweet tasting compound from edible *Pimpinella* spp. (Tepe *et al.,* 2006) Synergistic effects were also found to occur in the activity of *Ginkgo* extracts, for example for their anticlastogenic, antioxidant, vasoregulatory, cognition-enhancing, stress alleviating and gene-regulatory effects (Roy *et al.,* 1998; Alaoui-Youssefi *et al.,* 1999; Curtis *et al.,* 1999).

It was observed that when the acetone extract, with TA values of 432 ml/g dry material against *E. faecalis* and 4 320 ml/g dry material against *C. perfringens* were fractionated, the resulting fractions had a combined total activity of 508 and 5 624 ml/g extract (Table 3.2). Considering that synergism was observed between the hexane and EA fractions and the fact that the total activity in the combined fractions exceeds that of the whole acetone extract (both pathogens) one can extrapolate that antagonistic interactions are also at play. These could be from the highly polar compounds in the H_20 fraction, which were not investigated in this study.

Table 3.3. Raw data and FIC's of combined hexane- and EA fractions from *G. biloba* to show synergistic (red) or additive (blue) effects against *E. faecalis* (using Berenbaum's equation in section 2.2.5)

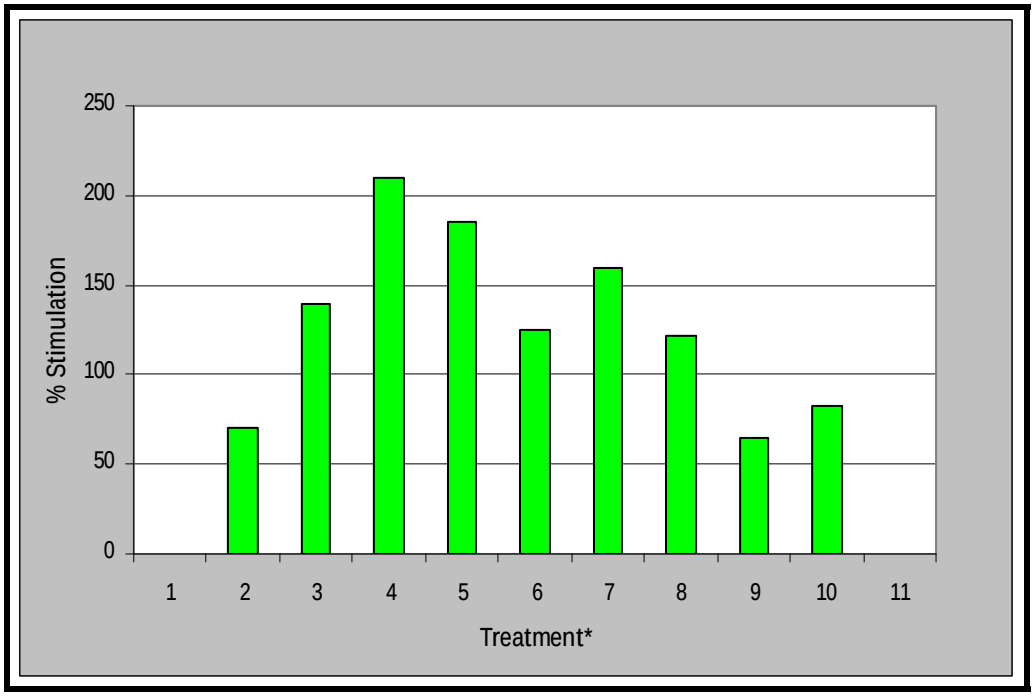
Treatment	% of Test fraction (Combinations)		Expected MIC (mg/ml) (separate values of individual fractions)[1]		Expected MIC (Total)[1]	Actual MIC (mg/ml) (separate values of combined fractions)[2]		Actual MIC (Total)	∑FIC
	a	b	a	b		a	b		
1	100	0	0.08	0.00	0.08	0.08	0.00	0.08	1.00
2	90	10	0.07	0.06	0.13	0.07	0.01	0.08	0.59
3	80	20	0.06	0.13	0.19	0.06	0.02	0.08	0.42
4	70	30	0.05	0.19	0.24	0.05	0.02	0.07	0.32
5	60	40	0.05	0.25	0.30	0.06	0.04	0.10	0.35
6	50	50	0.04	0.31	0.35	0.08	0.08	0.16	0.44
7	40	60	0.03	0.38	0.41	0.06	0.09	0.15	0.38
8	30	70	0.02	0.44	0.46	0.06	0.15	0.21	0.45
9	20	80	0.02	0.50	0.52	0.06	0.25	0.31	0.61
10	10	90	0.01	0.56	0.57	0.03	0.28	0.31	0.55
11	0	100	0.00	0.63	0.63	0.00	0.63	0.63	1.00

a = Hexane fraction of *G. biloba* from solvent-solvent separation technique

b = EA fraction from *G. biloba* from solvent-solvent separation technique

[1]100% values (rounded off) were measured and % fractions were calculated

[2]Separate values(rounded off) were calculated from measured values of combinations (Actual MIC)



* Refer to Table 3.3 for treatment combinations.

Figure 3.4. Effect of combining hexane and EA fractions of *G. biloba* on *E. faecalis* . (using % Stimulation equation from section 2.2.5.1; green denotes a stimulatory effect)

Table 3.4. Raw data and FIC's of combined hexane- and EA fractions from *G. biloba* to show synergistic (red) or additive (blue) effects against *C. perfringens* (using Berenbaum's equation in section 2.2.5)

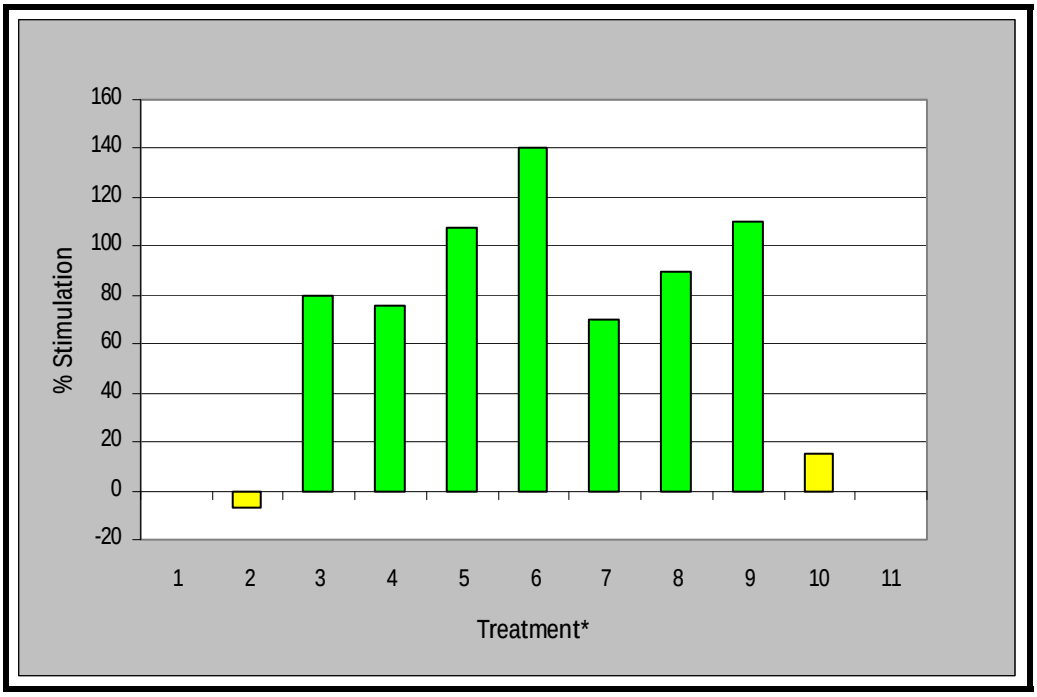
Treatment	% of Test fraction (Combinations)		Expected MIC (µg/ml) (separate values of individual fractions)[1]		Expected MIC's (Total)[1]	Actual MIC (µg/ml) (separate values of combined fractions)[2]		Actual MIC (Total)	∑FIC
	a	b	a	b		a	b		
1	100	0	3.91	0.00	3.91	3.91	0.00	3.91	1.00
2	90	10	3.52	1.95	5.47	5.27	0.59	5.86	1.07
3	80	20	3.13	3.91	7.04	3.13	0.78	3.91	0.56
4	70	30	2.73	5.86	8.59	3.42	1.46	4.88	0.57
5	60	40	2.34	7.81	10.5	2.93	1.95	4.88	0.48
6	50	50	1.95	9.77	11.72	2.44	2.44	4.88	0.42
7	40	60	1.56	11.72	13.28	3.13	4.69	7.82	0.59
8	30	70	1.17	13.67	14.84	2.34	5.47	7.81	0.53
9	20	80	0.78	15.63	16.41	1.56	6.25	7.81	0.48
10	10	90	0.39	17.58	17.93	1.56	14.06	15.62	0.87
11	0	100	0.00	19.53	19.53	0.00	19.53	19.53	1.00

a = Hexane fraction of *G. biloba* from solvent-solvent separation technique

b = EA fraction from *G. biloba* from solvent-solvent separation technique

[1]100% values (rounded off) were measured and % fractions were calculated

[2]Separate values(rounded off) were calculated from measured values of combinations (Actual MIC)



* Refer to Table 3.4 for treatment combinations

Figure 3.5. Effect of combining hexane and EA fractions of *G. biloba* on *C. perfringens* (using % Stimulation equation from section 2.2.5.1; green denotes a stimulatory effect and yellow an indifferent effect)

3.4 Conclusions

The objective of the current investigation was firstly to isolate and identify the major antibacterial compound from *G. biloba* and secondly, to determine whether activity against *E. faecalis* and *C. perfringens* in an extract or fraction of and extract of *G. biloba* can be attributed to that specific compound or whether synergism or other interactions also play a role in the observed activity.

The isolated long-chain ginkgolic acid ($C_{17:1}$) plays a partial role in the observed activity against *E. faecalis* and *C. perfringens*. Synergistic interactions were observed between constituents in the hexane and EA fraction, with the last mentioned fraction not containing any ginkgolic acid. These results support the use of the whole extract as opposed to isolated compounds as antimicrobial agents against pathogenic organisms. The use of isolated active compounds prove not only expensive and time-consuming, but also exclude positive interactions between constituents in whole extracts which could considerably enhance activity.

The method followed in this study i.e. isolation of active compounds, determination of ml/g TA of isolated compound vs. that of the whole extract followed by detection of interactions between fractions of the whole extract (determination of the FIC-index), are useful parameters in providing answers to determine the role of the isolated compound in the specific extract.

3.4 Acknowledgements

Dr Ladislaus Mdee assisted with the isolation and characterization of ginkgolic acid.

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4 Pharmacodynamic aspects of hexane extract and ginkgolic acid from *G. biloba* using *E. faecalis*

4.1 Introduction

Antimicrobial drugs have a widespread use in human and veterinary medicine, animal husbandry, aquaculture, agriculture and food technology (Barbosa and Levy, 2000). The total number of antimicrobials used worldwide is estimated in 100 000 to 200 000 tonnes per year (Kümmerer, 2003) and it therefore exerts a strong selective pressure for the emergence of resistance in both pathogenic and commensal pathogens.

Since the 1950's, antibiotic growth promoters (AGPs) have been used as growth promoters in broiler and pig production, allowing adequate productivity of animals raised under high density growth conditions (Jones and Ricke, 2003). Despite the observed improvement in animal weight gains, the use of AGPs has been criticized due to its possible role in the occurrence of antibiotic resistance in human pathogens. *Enterococcus* spp. are of specific concern due to their widespread occurrence i.e. enterococci are typical lactic acid bacteria and are part of the autochthonous (resident) intestinal flora of humans and production animals and consequently also part of the microflora of fermented food and feed. The transfer of glycopeptide resistant enterococci (GRE) from food to the human intestine is well documented rendering glycopeptides ineffective as the last choice of treating nosocomial infections of the immunocompromised host with enterococci (Klein, 2003).

Directive 183/2003 of the European Parliament, issued on September, 22nd, 2003, determined the ban of the use of all antimicrobials as growth promoters in the European Union as of January 1st, 2006. The new context caused an increase in the search for alternative growth promoters (Barreto *et al.,* 2008).

Plant derived products as alternative to AGPs are currently under investigation because plants constitute a rich source of bioactive chemicals and many of them are largely free from harmful effects (Rios and Recio, 2005). These compounds are produced by the plants for defence against external factors, such as physiological stress, environmental factors, and protection against predators and pathogens. Extracts of *Ginkgo biloba* are being investigated for its potential to replace AGPs in broiler production with ginkgolic acid (alkylphenol) being an important contributor to the antibacterial activity observed in the extract (Chapters 3 and 5).

There is little or no information regarding the development of resistance to plant extracts or the cause of increases in resistance to antibiotics. Hübner (2003) has shown that *Staphylococcus aureus* is able to acquire resistance against hyperforin, the major antibacterial compound from *Hypericum perforatum*. This resistance has, however, not led to cross resistance against clinically used antibiotics. This is in contrast to Ward *et al.*

(2002) who illustrated that garlic products increased the MIC of the norfloxacin marker above baseline against *S. aureus* and also with the ampicillin marker against *Escherichia coli.*

If a promising plant extract also leads to the development of resistance when used prophylactically as an AGP it would only present a short-term solution. Plant extracts have the potential advantage over antibiotics with respect to development of resistance firstly because many plant extracts contain several antibacterial compounds - up to as many as 14 (Martini and Eloff, 1998) and secondly because of synergism between compounds (with or without antimicrobial activity) enhancing activity against pathogens (Hemaiswarya *et al.,* 2008) It is thus possible that the potential of development of resistance against plant extracts would be much lower if the different antimicrobial compounds had different targets in the pathogen.

The objective of the study was to study resistance development to a hexane extract and ginkgolic acid from *G. biloba* against *E. faecalis.* A secondary objective was to study the time-kill dynamics of this hexane extract over 24 h against *E. faecalis* in order to evaluate pharmacodynamic interactions.

4.2 Materials and methods

4.2.1 Ginkgolic acid

Ginkgolic acid ($C_{17:1}$) was isolated (chapter 3) and identified and was prepared for subsequent studies by mixing pure compound with a 1:1 acetone and water mixture.

4.2.2 Preparation of hexane extract from *G. biloba*

Powders of leaves of *G. biloba* and *H. perforatum* were obtained from Biomox Pharmaceutical (Pty) Ltd – South Africa. The hexane fraction was prepared by using a solvent-solvent extraction method described by Lee and Kim (2002) (Chapter 2). Briefly, dried material was extracted twice with 60% aqueous acetone at a ratio of 1:10 dried material vs. extractant at room temperature and filtered. The extract was concentrated by using rotary evaporation at 45 °C after which the extract was sequentially partitioned into hexane, EA, butanol and H_2O portions. Each step was carried out three times to ensure adequate extraction. All solvents were saturated with distilled H_2O before use to ensure optimal separation. The hexane fraction was selected and concentrated by rotary evaporation at 45°C and further dried at room temperature under a continuous stream of air for further experiments. The hexane fraction was prepared for subsequent studies by mixing dried extract (10 mg/ml) with a 1:1 acetone and water mixture.

4.2.3 Preparation of *E. faecalis* inoculum

Enterococcus faecalis (ATCC 9212) was sub-cultured from the original strain, stored at -70°C at the Bacteriology Laboratory in the Faculty of Veterinary Science. The culture was transferred to Blood Tryptose agar (BTA) (obtained from Onderstepoort Biological Products) and incubated at 37°C in an incubator.

Three to 5 colonies of the test bacteria from an 18 – 24 h agar plate culture were inoculated into 2ml sterile distilled water with 0.02% Tween 80 (BDH). After thorough mixing, 10-100 µl was transferred to 100 ml Müeller-Hinton (MH) broth to give a final concentration of approximately 5 x 10^5 CFU/ml.

4.2.4 *In vitro* emergence of resistance studies

Selection of resistant *E. faecalis* against the test substances was performed in a series of test tubes. In the primary passage experiment, hexane extract of *G. biloba*, ginkgolic acid or gentamicin (1 ml of 5 mg/ml, 0.5 mg/ml or 25 µg/ml acetone /water mixture respectively) was serially diluted to a ratio of 1:1 with water in a series of 8 (A – H) duplicate test tubes: In the first test tube, 1 ml of the test substance (2 repetitions per extract) was added to 1 ml water and mixed, after which 1 ml was taken from A to B and also mixed. The process was repeated up to test tube H and the final 1 ml discarded. One ml of the bacterial suspension (10^3 - 10^5 cell/ml) was added to each test tube, sealed and incubated at 37ºC at 100% relative humidity overnight. A quantity of 0.1 ml from each test tube was plated out onto BTA agar to detect growth. The *E. faecalis* colonies on the plate representing the test tube containing the highest concentration of test substance were selected (Figure 4. 1) and identity was confirmed by streaking out on BTA, McConkey agar and inoculation into aesculin broth (Figure 4. 2). The selected *E. faecalis* strains were used to perform the secondary passage experiment for all test substances. For each passage, an extract-free culture (growth control) and an uninoculated medium sterility control were included. The process was repeated for the number of times necessary to observe growth in a test substance at a concentration ≥4 times the original MIC (Lister, 2006) or for up to 9 passages. Results were confirmed by using the microplate dilution assay of Eloff (1998) (in accordance with the recommendations by the Clinical Laboratory Standards Institute (CLSI, 2008) for determining the MIC's of the test substances against the selected *E. faecalis* strains obtained from the series of passage experiments. Cultures obtained after each passage were stored at -70ºC.

4.2.5 Time – kill methodology

Death kinetics of the hexane extract of *G. biloba* were performed on *E. faecalis* (ATCC 9212). The culture was transferred to MH agar and incubated at 37°C for 24h in an incubator. The stock culture was prepared by inoculating 3 to 5 colonies of the test bacteria into 2 ml sterile distilled water with 0.02% Tween 80. Hexane

fraction was incorporated into duplicate 100ml MH broth in Erlenmeyer flasks at concentrations of 0, 0.039 mg/ml, 0.078 mg/ml and 0.156 mg/ml and *E. faecalis* was inoculated into the broth at a final inoculum of $6.9X10^5$ cells/ml. The flasks containing the broth cultures were incubated at 37 ºC in an incubator. At pre-determined time intervals ranging from 0 min – 24 h (0, 30, 90, 120, 240 min, 8, 16 and 24 h) aliquots of 1ml were transferred to 9 ml sterile Ringers solution and serially diluted. From each dilution 0.1 ml was plated onto MH-agar. The plates were incubated at 37°C for 24h in an incubator and colony-forming units (CFU/ml) counted. Death kinetics was expressed in $\log_{10}$ reduction time-kill plots.

4.3 Results and discussion

According to Lister (2006), emergence of resistance can be defined as outgrowth of subpopulations of bacteria exhibiting significantly decreased sensitivity to the antibacterial agent of interest. This must be evaluated by plating samples on/in agar/broth containing the antibacterial agent at a concentration ≥4-fold above the MIC. No decreased sensitivity to the hexane fraction (Table 4. 1, Figure 4. 3) or ginkgolic acid from *G. biloba* against *E. faecalis* could be observed after 9 and 10 passages respectively. The positive control showed that the system used to select for resistant bacteria was capable of selecting for mutant strains: the MIC value for gentamicin increased from 0.78 to 6.25 µg/ml, an 8 fold increase after 7 passages compared to the parent strain (Figure 4. 3). Because no decreased sensitivity was observed against the test extacts, no cross-resistance studies could be performed against other antibiotics. Apart from the fact that the results indicate a low potential for developing resistance against *E. faecalis* if *G. biloba* extracts are to be used as substitute for AGPs in poultry, it also indicated a low potential for resistance developing in patients using *G. biloba* extracts as a phytopharmaceutical clinically for the improvement of blood circulation and treatment of dementia.

Table 4. 1. Susceptibility of *E. faecalis* against hexane extract and ginkgolic acid from *G. biloba* and gentamicin

Test substance	MIC* (Parent strain)	No of passages	MIC* (end)	Increase (*fold)
Hexane extract of *G. biloba*	0.056	10	0.056	1
Ginkgolic acid	0.03125	9	0.03125	1
Gentamicin	0.78	6	6.25	8

*MIC measured in mg/ml for the hexane extract and ginkgolic acid, and in µg/ml for gentamicin

Figure 4. 1. Selection of resistant strain (C) from plate representing the highest concentration of test substance, with A–F in order from the highest to the lowest concentration

Figure 4. 2. *E. faecalis* streaked out on McConkey agar (left) and BTA (middle) and inoculated into Aesculin-broth (right) to confirm identity

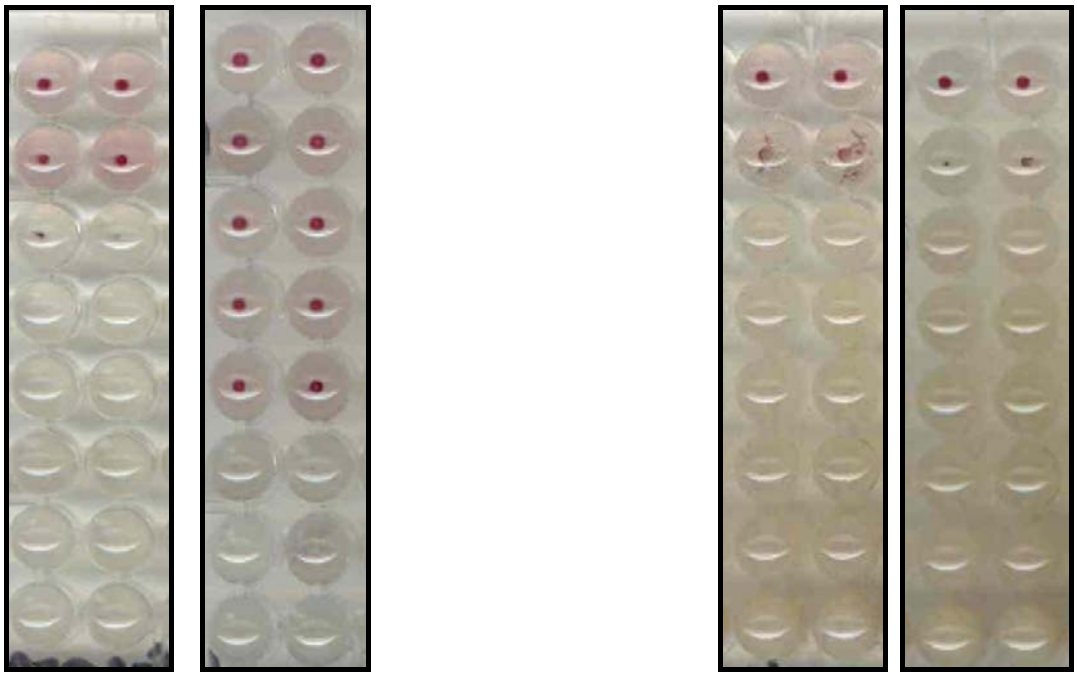

Figure 4. 3. Increase in *E. faecalis* resistance to Gentamicin developed within 7 passages (P) (left) compared to no resistance development to hexane extract of GB (right)

Time-kill dynamics revealed that the hexane extract had a bactericidal effect and was able to kill 99.9% (log three reduction is considered the minimum for significant bactericidal activity (Lister, 2006)) of the starting inoculum in 7 and <3 h at a concentration of 0.078 and 0.156 mg/ml respectively (Figure 4. 4). The time required to decrease viable counts below the level of detection was <5 h at a concentration of 0.156 mg/ml and 24 h at a concentration of 0.078 mg/ml (Figure 4.5). At a concentration of 0.039 mg/ml only a 10 fold decrease in bacterial numbers was observed after 24 h. Bacterial growth of up to 10^8 cells/ml was observed in the control. No regrowth was observed after 24 h (up to 48 h - results not shown) at concentrations of 0.078 and 0.156 mg/ml. This indicates firstly that the hexane extract retained its activity up to 48 h and secondly that no outgrowth of mutant subpopulation(s) occurred (as supported by the resistance studies).

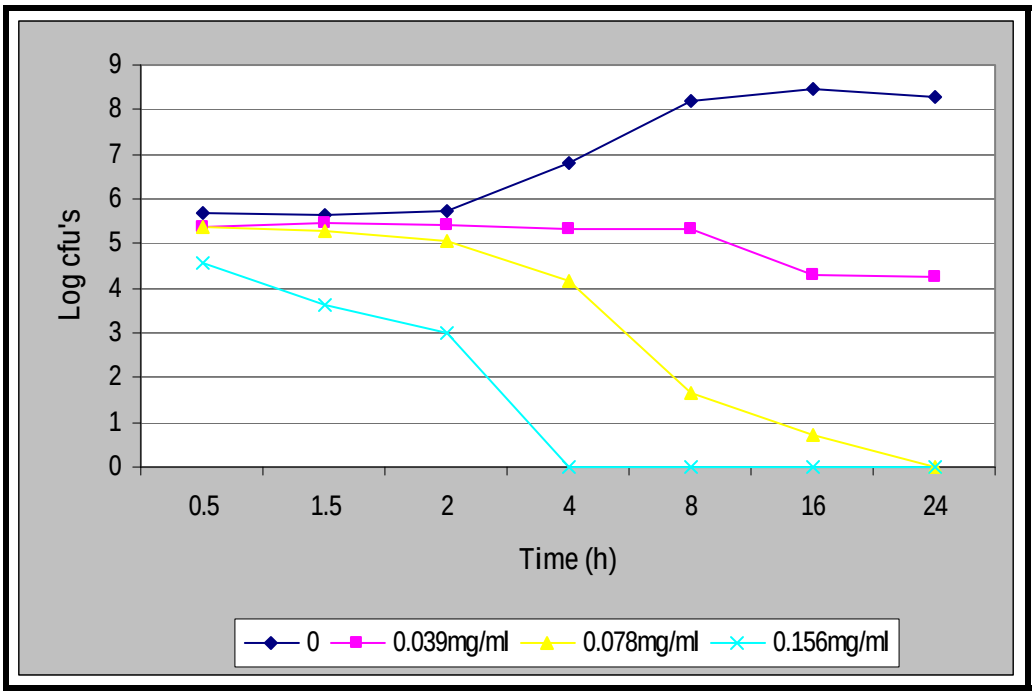

Figure 4. 4. Death kinetics of *E. faecalis* with exposure to different concentrations of hexane extract of *G. biloba* as seen over a 24 h period

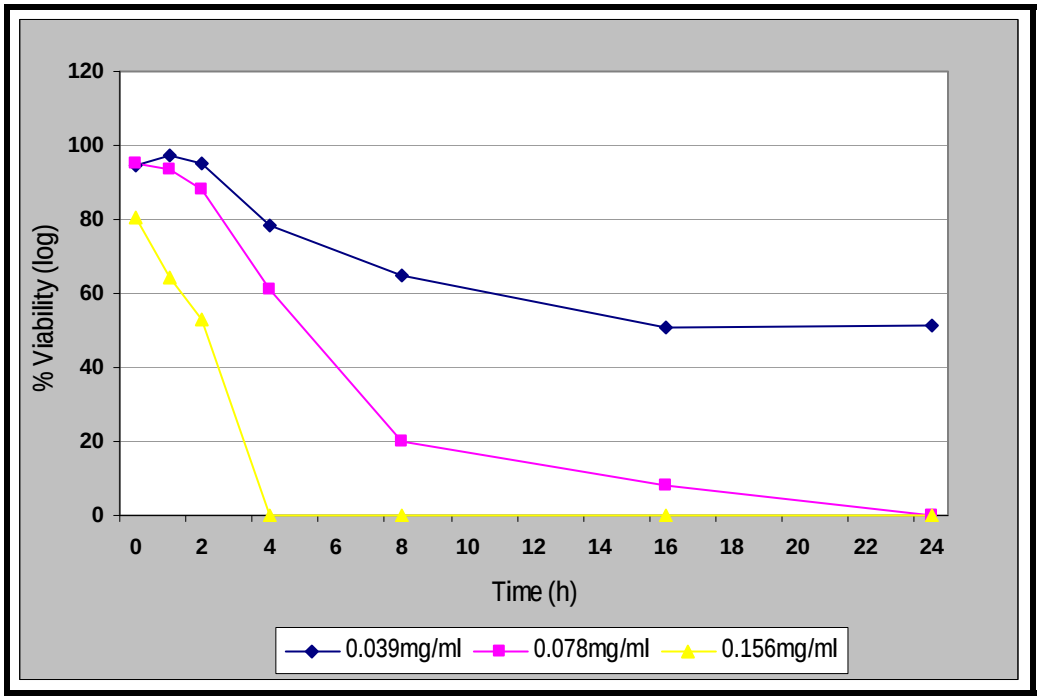

Figure 4. 5. Percentage viability ((Log cfu in treatment/log cfu in control) X100) of *E. faecalis* exposed to different concentrations of hexane extract of *G. biloba* over 24 h

4.4 Conclusion

The objective of the study was to study resistance development to a hexane extract and ginkgolic acid from *G. biloba* against *E. faecalis.* A secondary objective was to study the time-kill dynamics of this hexane extract over 24 h against *E. faecalis* in order to evaluate pharmacodynamic interactions.

No decreased susceptibility of the hexane fraction or ginkgolic acid from *G. biloba* was observed. With a history of over 200 million years, *G. biloba* is the sole survivor of the Ginkgoaceae family and is considered the oldest plant as a "living fossil" to survive on earth (Kleenex and Knipschild, 1992). Ginkgolic acid, one of the known antimicrobial compounds in *G. biloba*, has been treated as a hazardous compound with suspected cytotoxic and allergenic properties and thus the content of ginkgolic acids should not exceed 5ppm in the standardized preparation. Many studies have however unveiled that ginkgolic acids have wide bioactivities, which may endow *Ginkgo* some remarkable properties and genetic tenacity surviving over millions of years, e.g. resistance to pests (Pan, 2007).

The measured pharmacodynamic endpoints (extent of bacterial killing, emergence of resistance) are important parameters to consider early in the development of a new antimicrobial agent. The bactericidal nature of the hexane extract and ginkgolic acid from *G. biloba* as well the absence of decreased susceptibility in the resistance studies against *E. faecalis* indicate that this extract has potential to be exploited as an alternative to AGPs in the poultry industry.

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5 *Ginkgo biloba* and *Hypericum perforatum* extracts used as growth promoters in broilers

5.1 Introduction

Antibiotic growth promoters (AGPs) are substances that are added to feed in sub-therapeutic levels in intensive animal production to improve weight gain and conversion of feed into body mass (Dibner and Richards, 2005). Since January 2006, legislation has been in place in Europe to prohibit the use of antibiotics as growth promoters due to concerns that the use of antibiotics in feedstuffs contribute to the spread of antibiotic-resistance genes by promoting the selection of antibiotic-resistance bacteria in animals (Anonymous, 2003; Dibner and Richards, 2005; Castanon, 2007). As a result, economically important diseases, such as necrotic enteritis of chickens have become more prevalent in these countries.

Necrotic enteritis caused by the virulent net B producing strains of *Clostridium perfringens* is clinically characterized by inappetence, depression and increased mortality (Helmboldt and Bryant, 1971; Long, 1973). The chronic damage to the intestinal mucosa leads to decreased digestion and absorption reduced weight gain and increased feed-conversion ratios (Elwinger *et al.,* 1992; Kaldhusdal *et al.,* 2001). The disease is therefore associated with important economic losses in the poultry industry (Dahiya *et al.,* 2006).

The number of publications on the efficacy of possible replacements of AGPs to control the occurrence of NE has been steadily growing. The mode of action of these supplements can be divided into four basic groups with distinct strategies: 1) improvement of nutrient utilization by the host, 2) stimulation/modulation of the immune system, 3) stimulation of introduction of beneficial bacteria and 4) direct reduction of pathogens (Kocher and Choct, 2008). The antimicrobial effect of plants/plant extracts is well known (Rios and Recio, 2005) and their potential as an alternative to AGPs due to the direct effect on pathogenic bacteria has been investigated (Mitsch *et al.,* 2004). A beneficial effect on production traits has also been illustrated. Examples for chickens or quail include an increase in average daily weight gain (ADG) of 7% with oregano (Florou-Paneri *et al.,* 2006), 6% with thyme (Denli *et al.,* 2004) and between 1 and 8% with coriander (Güler *et al.,* 2005). Chikoto (2006) illustrated a significant increase of 4.8% in mean live body mass of broilers with *G. biloba* extract (included into feed at 14 ppm) in combination with grapeseed extract. A non-significant increase of 3% was obtained when *H. perforatum* extract was added to that mixture. These results were achieved in spite of the fact that grape seed extract on its own had a deleterious effect on growth promotion (Chikoto, 2006).

The current investigation is a continuation of the work of Chikoto (2006). Chikoto (2006) established that the acetone extracts of *G. biloba* and *H. perforatum* could be tolerated by broilers at a rate of 2 and 10 mg/kg respectively. Furthermore, the extracts were found to be stable in feed and water and antimicrobial activity was

found to be unaffected under varying pH conditions. It was thus decided to continue with the acetone extracts due to the absence of adverse *in vitro* and *in vivo* effects. It was also decided to increase the concentration of *H. perforatum* from the 14ppm used in Chikoto's (2006) experiment to 28ppm as this dose was found to be safe in *in vivo* tolerance studies. Grape seed was excluded due to the deleterious effects previously observed (Chikoto, 2006). The objective of the study was to determine the effect of a combination of extracts of *G. biloba* (14ppm) and *H. perforatum* (28ppm) on the performance of broiler chickens over a 35 day period. The effect on *C. perfringens* in the intestine was also investigated.

5.2 Materials and methods

5.2.1 Experimental design

The trial ran in the form of a 2 X 5 factorial design (Table 5.1). Treatments consisted of the following:

- Treatment 1: Broiler ration without growth promoter/ antibiotic (Not challenged with *C. perfringens* i.e. the negative control)
- Treatment 2 Broiler ration with growth promoter/ antibiotic Zn-bacitracin (75ppm) (challenged with *C. perfringens* i.e. the positive control)
- Treatment 3: Broiler ration without growth promoter/ antibiotic (*C. perfringens* challenged negative control)
- Treatment 4: Treatment 3 supplemented with *G. biloba* (14ppm)
- Treatment 5: Treatment 3 supplemented with a combination of extracts of *G. biloba* (14ppm) and *H. perforatum* (28ppm)

Table 5.1. Summary of the experimental design

C. perfringens challenge (days)	Lesion /microbial scoring (days)	Diet	Weight monitoring (days)	Duration of trial (days)
15,23,24, 27	14,21,29	Maize-soya	13, 20, 27, 35	35

5.2.2 Experimental animals and housing

The total of 700 (600 + 100 replacement birds) sexed (female) Ross 788 one day old, non-vaccinated chicks were obtained from a commercial hatchery from a single parent flock and randomly allocated to the 20 pens (initially 35 chickens/pen, 4 pens/treatment) (Figure 5.6). All birds were individually tagged on day 13 prior to oral

administration of *C. perfringens*. Birds were weighed on a per pen basis on day 1 and weekly on individual basis (from 14 days) until 35 days of age after which the birds were sent to a slaughterhouse.

The protocol for the project was approved by the University of Pretoria's Research committee and Animal Use and Care Committee (Protocol nr. V038-08) before commencement of the experiment. The experiment was conducted at the Poultry Researdh unit, Poultry Reference Centre of the Faculty of Veterinary Science, Onderstepoort. The part of the facility used consisted of 2 broiler houses, one with 4 pens (treatment 1) and the other with 16 pens (treatment 2 – 5) (Figure 5.1). Treatment 1 was kept separately to minimize contamination with *C. perfringens.* All birds were housed in pens bedded with wood shavings (Figure 5.6). The maximum stocking density of birds was 23 kg/m^2. Stocking densities were above those recommended by the South African National Standard: The use of animals for scientific purposes (10 386), but well below the minimum standards of 33 kg/m^2 (or 39 kg/m^2 with good ventilation and a temperature control system) agreed on by the European Commission (European Commission, 2007). With these stocking densities we were able to imitate commercial conditions where AGPs are normally required, without unnecessarily sacrificing the welfare of the chickens.

Environmental control was facilitated by means of electrical heaters (Figure 5.3) and extractor fans. On day one the house temperature control was set to 32°C, but decreased after 3 days to 28°C. The temperature was then kept constant for the remainder of the growing period. Records were kept in terms of daily min-max temperatures and daily procedures.

Pens were checked twice daily and morbidity or mortality noted. Sick birds were removed and euthanized. All mortalities were submitted for necropsy.

5.2.3 Feed and feeding

On day one, chicks' beaks were dipped in water from the fountain drinker to ensure that the swallow reflex was intact (Figure 5.2). Each pen was equipped with a feeding tray and chicken font drinker for the first 7 days (Figure 5.4) after which tube feeders (Figure 5.1) and nipple drinkers were supplied. All feed consisted of two phases i.e. a starter and a grower ration and were supplied *ad libitum*. Birds were fed on a commercial maize-soya diet from day 1 until three weeks (starter) after which a grower mix was supplied (Table 5.2). The feed contained no (other than mentioned) commercial antimicrobial growth promoters or coccidiostats.

Ginkgo biloba extract was incorporated into a calcium carbonate premix at a rate of 14 mg per kg (14 ppm) and *H. perforatum* extract at a rate of 28 ppm (in combination with the *G. biloba* extract) of both the starter and the finisher feed.

All feed was weighed daily and the weight of left over feed subtracted from the total feed administered in order to determine feed intake per pen. Feed intake together with average mass gain was used to determine the feed conversion ratio (FCR) and the European Performance Efficiency Factor index (EPEF). The data were corrected for mortality.

$$\text{FCR} = \frac{\text{Feed consumed per bird}}{\text{Weight gain per bird}}$$

$$\text{EPEF} = \frac{\text{Mean live mass on specific day of trial x \% survivors x 100}}{\text{FCR x number of days}}$$

Table 5.2. Composition of broiler grower feed

Broilers grower ingredients	Percentage
Yellow maize	64.0
Soya	24.5
Fish meal	5.0
Fat	2.5
Limestone	1.2
Salt	0.25
Dicalcium phosphate	1.4
Lysine HCL	0.03
Methionine	0.17
Threonine	0.05
Vitamin and Minerals	0.6
Sodium bicarbonate	0.25

5.2.4 Challenge with *C. perfringens*

5.2.4.1 Preparation of inoculum

Clostridium perfringens type A culture was obtained from Dr J. Picard from Department of Veterinary Tropical Diseases at the Faculty of Veterinary Science, University of Pretoria. This strain had been originally isolated from a bird that died whilst suffering from necrotic enteritis and had been used for the experimental induction of necrotic enteritis on several occasions including the experiments of Chikoto (2006). Frozen isolates were thawed, streaked out on Horse Blood Columbia agar (BCA) (Selecta Media) and incubated at 37°C overnight in an anaerobic chamber. Cultures were checked for purity and inoculated into Erlenmeyer flasks containing sterilised BHI - broth (brain-heart-infusion broth) (Oxoid) augmented with 0.04% cysteine and incubated aerobically for 24 h at 37°C. Cultures were resuspended at a rate of 1 x 10^8 cells/ml each (optical density (OD) of 1 measure on the spectrophotometer at 540 nm) in new sterilised BHI-broth augmented with 0.04% cysteine and distributed in 16 bottles containing 50 ml cultured broth each. Four clearly marked bottles containing sterile broth were kept separately for the non challenged controls. One bottle per pen was used. Cultures were kept at room temperature at the broiler facility and used within 6 h of preparation.

5.2.4.2 Inoculation of chickens

Feed was withdrawn from all the birds (including the non challenged treatment) for 24 h prior to oral administration of *C. perfringens* broth culture to minimize the occurrence of regurgitation (adapted from Collier *et al*., 2003). On days 15, 23, 24 and 27, all chickens received 1ml of either broth containing 1 x 10^8 cells/ml *C. perfringens* (challenged group) or uninoculated sterile BHI broth (non challenged control) by oral gavage using a sterile syringe (Figure 5.7). Feeding was resumed as soon as administration of *C. perfringens* was completed.

5.2.5 Collection of samples

5.2.5.1 Necrotic enteritis lesion scoring

On days 14, 21 and 29 three birds per pen (all treatments) were randomly selected and euthanized by CO_2. The abdomen was opened and the gastro-intestinal tract exposed and examined for lesions and scored. Lesion scoring was performed as follows (Chalmers *et al.,* 2007):

0 = Small intestine grossly normal

1 = Small intestine wall grossly thinner than normal and break or tear easily under mild tension but with no evidence of mucosal necrosis or other abnormalities

2 = One or more focal round or oval areas of acute full thickness mucosal necroses of the small intestine. These foci can vary in diameter from approximately 1 – 5 mm. The surface of these lesions is raised above the surrounding tissue and consists of grey or white necrotic debris. Alternatively, when superficial necrotic material is removed, the lesions are slightly depressed with grey or white colour.

3 = Irregularly shaped confluent areas of full thickness mucosal necrosis of the small intestine >5mm in diameter but affecting <25% of the small intestine surface area. The surface of these lesions appears to be raised above the surrounding tissue and consist of orange/brown necrotic debris. When portions of the superficial necrotic material are removed, the lesions can be slightly depressed from the surrounding tissue.

4 = Large confluent areas of full thickness mucosal necrosis of the small intestine affecting 25% or more of the small intestinal surface area and involving the entire internal circumference of the affected small bowel. The surface of these lesions is generally raised above the surrounding tissue and consists of orange/brown necrotic debris.

5.2.5.2 Microbial scoring

5.2.5.2.1 *C. perfringens* culture

A 5 cm portion of the duodenum (Figure 5.8) was opened aseptically and an area 0.5 by 2cm (by using a new plastic template for each sample) was abraded with a sterile bacteriological swab and placed in 1ml sterile BHI broth augmented with 0.4g/l cysteine. The sample was cultured anaerobically on BCA augmented with 0.1g/l neomycin by streaking into 4 quadrants, sterilizing the loop between quadrants. Plates were incubated at 37°C for 24 h.

Colonies on blood agar plates were identified as *C. perfringens* if they exhibited a double-zone β-haemolysis (Chalmers, 2007). The number of colonies meeting these criteria were counted within each quadrant, and the density scored as 4+, 1 or more colonies in the fourth quadrant; 3+, 0 colonies in the fourth quadrant, 1-10 in the third quadrant; 2+, 0 colonies in the fourth and third quadrants,1-10 colonies in the second quadrant; 1+, 0 colonies in the second, third and fourth quadrant, 1-10 colonies in the first quadrant; 0, no colonies in any quadrant (Chalmers, 2007).

5.2.5.2.2 Identification and quantification of *C. perfringens*

A volume of 0.01 ml from the above mentioned BHI broth was spread on a designated area (using a circular template with a diameter of 16 mm) on a microscopic slide, air dried and stained using Gram's stain. The number of Gram-positive rods (magnification 1000X) was counted in 10 microscopic fields and graded as 0, no Gram-positive rods; 1, 1-10 gram positive rods (Figure 5.11); 2, 11-20 Gram-positive rods; 3, 21-30 Gram-positive rods; 4, >30 Gram-positive rods (Figure 5.12) (Chalmers, 2007).

5.2.6 Statistical analysis

Statistical analysis of the data was performed using the SPSS 17 programme. For all parameters (FCR, live body mass, % survival and EPEF) significant differences between groups were detected using a one-way analysis of variance (ANOVA). Post-hoc significance was determined using Bonferroni. Significance was set at P = 0.05. For the microbial scoring system significant differences between the groups were determined using the non-parametric Kruskal-Wallis (non-challenged and challenged control, *G. biloba*-, *G. biloba* + *H. perforatum*- and Zn-bacitracin treatments). Post-hoc comparison to the non-challenged control group was determined using a Mann-Witney (P = 0.05). In tables and figures, values differing at a P = 0.05 level are indicated with different superscripts.

Figure 5.1. Broiler facility where challenged treatments were housed

Figure 5.2. Day old chicks' beaks are being dipped in water to ensure swallow reflex is intact

Figure 5.3. Heater hanging from the roof

Figure 5.4. Day old chicks in and around the feeding tray and water fountain

Figure 5. 5. Nine day old chicks around tube feeder

Figure 5.6. Cages (each cage represent one treatment replicate)

Figure 5.7. Oral gavage of 15 day old chicken with *C. perfringens*

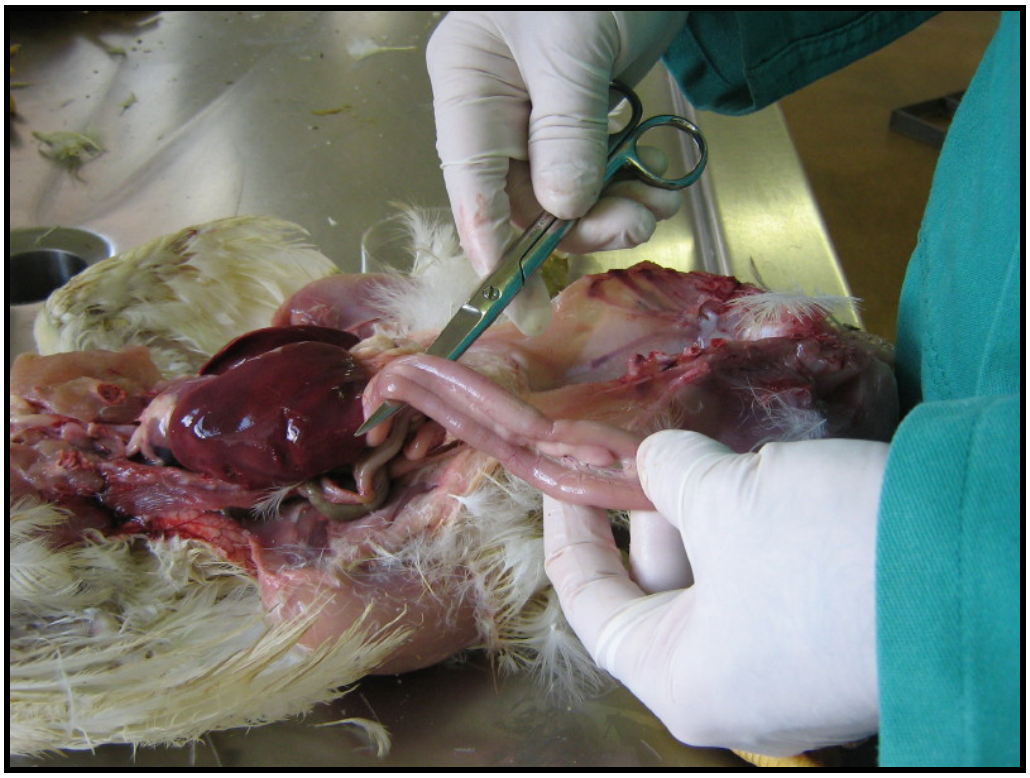

Figure 5.8. Section of intestine of chick where a sample were taken for smears and culturing of *C. perfringens*

5.3 Results and discussion

5.3.1 Mortality

Each pen initially contained 35 broilers and a total of 9 broilers per pen were sacrificed for the purpose of lesion scoring on days 14, 21 and 29. Mortality of 1.3% (results not shown) did occur before the *C. perfringens* challenge on day 13 but necropsy analysis revealed that this was unrelated to treatment. No significant differences were found between challenged and non-challenged treatments on the different days (Table 5.3) although a trend towards higher survival rates in the Zn-bacitracin group could be observed. Similarly, this trend was also observed with respect to cumulative daily mortality after challenge with *C. perfringens* i.e. treatment with Zn-bacitracin resulted in lower mortality (similar to non-challenged negative control) compared to other treatments (Figure 5.9). Survival rate (on day 35) that ranged between 92.1 and 94.8% for the non-challenged and challenged broilers was within the normal ranges and provides evidence of the safety of the feed additives administered. No lesions were observed in any of the broilers that died during the experiment ruling out necrotic enteritis due to *C. perfringens* as a cause of death.

Table 5.3. Percentage survivors of broiler chickens fed different combinations of feed additives

Treatments	% Survivors		
	Day 20	Day 27	Day 35
Neg control (non challenged)	100[a]	98.2[a]	94.8[a]
Neg control (challenged)	98.4[a]	95.5[a]	92.1[a]
Zn-bacitracin*	99.2[a]	97.3[a]	97.1[a]
*G. biloba**	99.2[a]	94.8[a]	92.3[a]
G. biloba + *H. perforatum**	97.6[a]	93.9[a]	93.2[a]
P-value	0.234	0.502	0.660

*Challenged

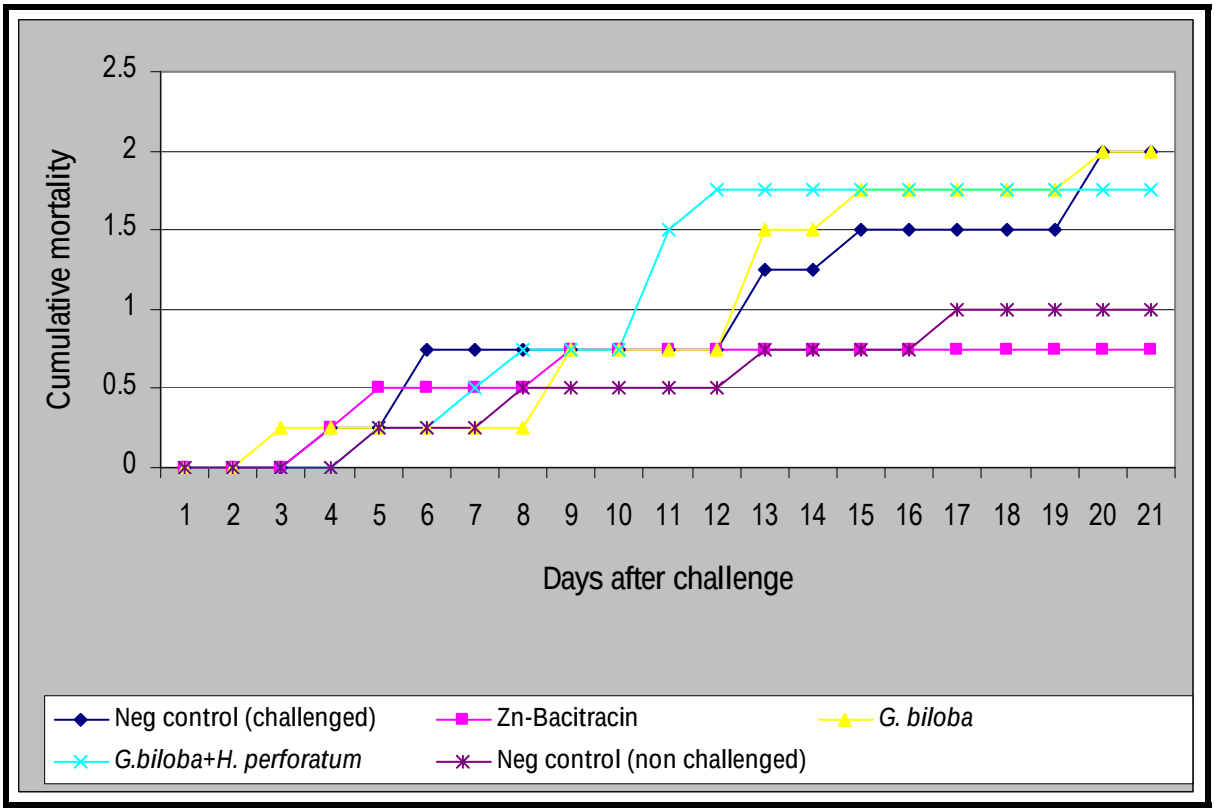

Figure 5.9. Cumulative mortality associated with different treatments after challenge with *C. perfringens*

5.3.2 Lesion scores

No lesions (lesion score zero) were observed in the intestines (jejunum, duodenum, ileum or colorectum) of broilers examined on the different days, indicating that the disease was not successfully induced. Necrotic

enteritis of broilers is characterized by gross lesions in the jejunum (Parish, 1961a; Kaldhusdal and Hofshagen, 1992), sometimes extending to the duodenum (Helmboldt and Bryant, 1971; Kaldhusdal and Hofshagen, 1992) or the ileum (Parish, 1961a; Broussard *et al.,* 1986). Rarely, lesions also occur in the colorectum or the caecal tonsils or necks (Long *et al.,* 1973). Typical focal lesions are sharply demarcated from the surrounding mucosa (Parish, 1961a; Kaldhusdal and Hofshagen, 1992). In severe cases, lesions are longitudinally expanded along the intestines, and the epithelium is eroded and detached (Williams, 2005).

Several researchers have reported difficulties in inducing necrotic enteritis under experimental conditions (Gholamiandehkordi *et al.,* 2007; Kocher and Choct, 2008). The model in this study was based on that of Chikoto (2006) who observed a significant increase in broilers with lesions in the challenged groups compared to those in the non-challenged controls using an identical *C. perfringens* isolate. Other researchers used different infection protocols, most of which use predisposing factors to induce disease. These predisposing factors include coccidial infection, immunosuppression and manipulation of the diet with regards to type of cereal and protein content (Kaldhusdal and Hofshagen, 1992; Kaldhusdal and Skjerve, 1996). Intestinal damage caused by coccidial infection can cause leakage of plasma proteins and thus provide growth factors for the proliferation of *C. perfringens* (van Immerseel *et al.,* 2004). Diets based on wheat, rye, oats or barley increase digestra viscosity, which increases the intestinal passage time (van der Klis and van Voorst, 1993) which in turn may allow anaerobic bacteria to proliferate and produce enterotoxins (Parish, 1961a; Vahjen *et al.,* 1998). Immunosuppression by exposure to infectious bursal disease, chicken infectious anaemia virus and Marek's disease as well as non-specific stress, may predispose birds to necrotic enteritis (Schuring & van Gills, 2001). The presence of one or more predisposing factors such as a natural coccidia infection or other non-specific stress factors could be the reason why Chikoto's (2006) model worked at the different location where the experiment was performed. This is supported by the fact that up to 80% of broilers had lesions even before the *C. perfringens* challenge (Chikoto, 2006).

5.3.3 Microbial scores

Although no lesions were observed, *C. perfringens* were more frequently isolated from the intestines of broilers of the challenged treatments compared to the non challenged control as indicated by the microbial scores on the cultured plates (Table 5.4 and Figure 5.10). The presence of *C. perfringens* in the intestine of the non-challenged chickens can be explained by the fact that this organism occurs naturally in a healthy chicken's intestine (Barnes *et al.,* 1972). Spores of *C. perfringens* are ubiquitous in the environment (van Immerseel *et al.,* 2008) and can also be present in feed especially when fish-meal is included in the diet (Wijewanta and Seneviratna, 1971). It is exremely difficult to ensure that poultry feed is spore-free, because spores are resistant to heat and can tolerate 100°C for 2 h (Parish, 1961b).

Necrotic enteritis results from the high frequency of adhesion by *C. perfringens* to the damaged mucosa (Baba *et al.,* 1992) facilitating bacterial proliferation and toxin production. The pathogenicity of the *C. perfringens* strain is however also an important factor in disease development. Chalmers *et al* (2007) found considerable variances in the ability of *C. perfringens* stains from field outbreaks to reproduce the disease in an experimental model (no coccidial challenge were used). Only one out of five field isolates was able to cause 33.7% necrotic enteritis-associate mortalities and produced an average lesion score of 1.83 while the other isolates resulted in mortality of 0-3.8% and lesion scores of between 0.33 and 1.17 in the sacrificed birds. Treatment with the virulent isolate also resulted in higher culture and smear scores (3.6) in mortalities compared to the scores from mortalities from treatment with avirulent strains (average 0-1.3) (Chalmers *et al.,* 2007). It is thus possible that the *C. perfringens* strain used in my study did not display sufficient virulence to induce disease. Chikoto (2006) did not perform any quantitative or qualitative studies on *C. perfringens* in the intestines of broilers challenged with the same *C. perfringens* strain used in my study. It is therefore not possible to comment on the association between *C. perfringens* colonization and severity of the disease (lesions in 79% of the challenged birds vs. 67% in the non-challenge birds 9 days after *C. perfringens* challenge) (Chikoto, 2006). Chalmers (2007) found that a high degree of genetic relatedness or the presence of virulent genes does not necessarily imply analogous virulence in the challenge model. It is thus not possible to predict the virulence level of the strain based on genetic relatedness to known virulent strains.

There was a general trend (not on a statistical significant level) towards a reduction in microbial scores in the feed supplemented with *G. biloba* and *H. perforatum*. Extracts of *G. biloba* and *H. perforatum* have an *in vitro* antimicrobial effect on *C. perfringens* (Chapter 2-unpublished data; Ahn *et al.,* 1994; Lee and Kim, 2002) and could therefore have a effect on the reduction of *C. perfringens* in the intestine of broiler chickens. Direct effects of an AGP on the microflora have the potential to decrease competition for nutrients and to reduce microbial metabolites that depress growth (Visek, 1978; Anderson *et al.,* 1999). Mitsch *et al.* (2004) were able to illustrate a reduction in the *C. perfringens* concentrations in the intestines and faeces of broilers in field trials using essential oil blends containing a combination of thymol, eugenol, curcumin and peperin or a combination of thymol, carvacrol, eugenol, curcumin and piperin.

Table 5.4. Mean microbial scores of *C. perfringens* on plates (cultures) and slides (smears) from the intestines of challenged broilers fed different feed additives

Treatments	Day 14*		Day 21		Day 29	
	Plate	Slide	Plate	Slide	Plate	Slide
Neg control (non-challenged)	0	0.06 [a]	0.08 [a]	0.13 [a]	0.08[b]	0.48 [a]
Neg control (challenged)	0	0.12 [a]	0 [a]	0.09 [a]	0.58[a]	0.95 [a]
Zn-bacitracin	0	0.04 [a]	0 [a]	0.06 [a]	0.75[a]	0.93 [a]
G. biloba	0	0.03 [a]	0.08 [a]	0.08 [a]	0.75[a]	1.03 [a]
G. biloba + *H. perforatum*	0	0.09 [a]	0 [a]	0.17 [a]	0.42[ab]	0.65 [a]
P-value[1]	-	0.860	0.549	0.922	0.034	0.447

*Before challenge; [1]F-probability value; (Scoring system: Slides: 0, no Gram-positive rods; 1, 1-10 Gram positive rods; 2, 11-20 Gram positive rods; 3, 21-30 Gram positive rods; 4, >30 Gram positive rods : Plates: 4+, 1 or more colonies in the fourth quadrant; 3+, 0 colonies in the fourth quadrant, 1-10 in the third quadrant; 2+, 0 colonies in the fourth and third quadrants,1-10 colonies in the second quadrant; 1+, 0 colonies in the second, third and fourth quadrant, 1-10 colonies in the first quadrant; 0, no colonies in any quadrant (Chalmers, 2007).

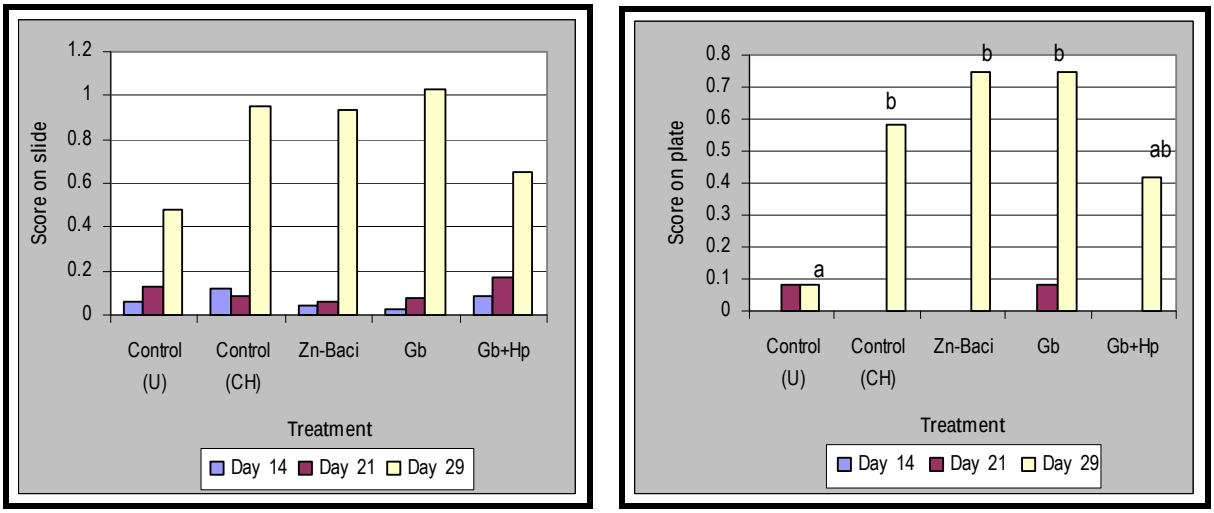

Figure 5.10. Smear (left) and culture (right) scores of C. perfringens indicating colonization of intestines of broiler fed different feed additives. U = non challenged control; CH = challenged control; Zn-Baci= Zn-bacitracin; Gb = *G. biloba*; Gb+Hp = *G.biloba* + *H. perforatum*. n = 12 for each treatment group

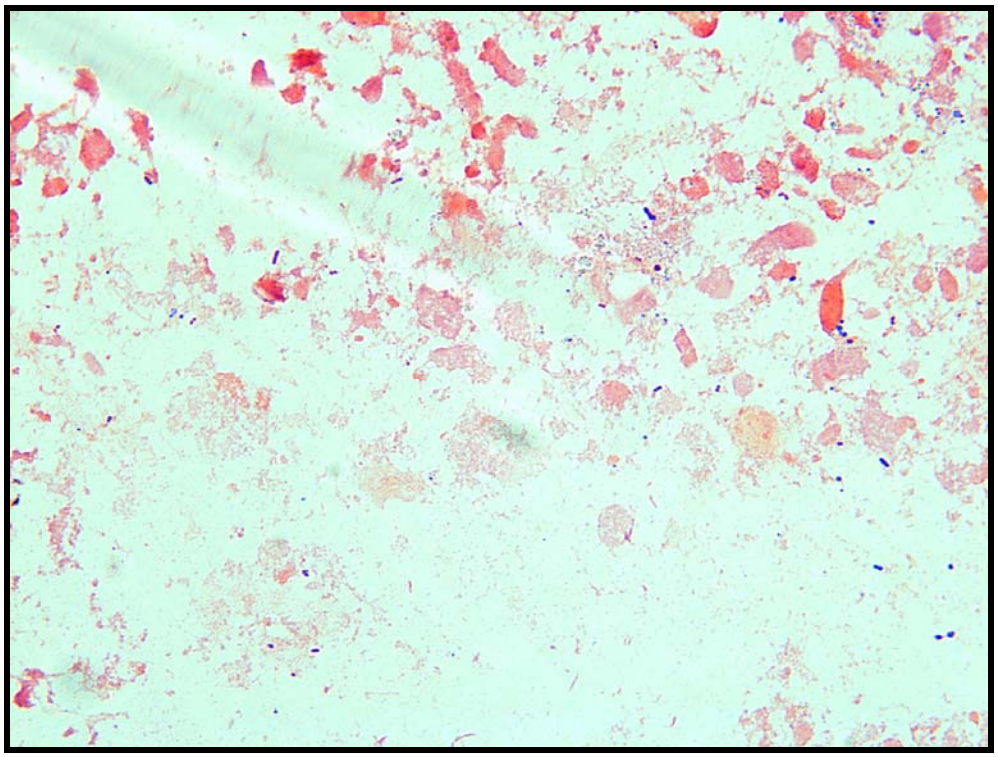

Figure 5.11. Slide showing a low concentration of *C. perfringens* (score = 1)

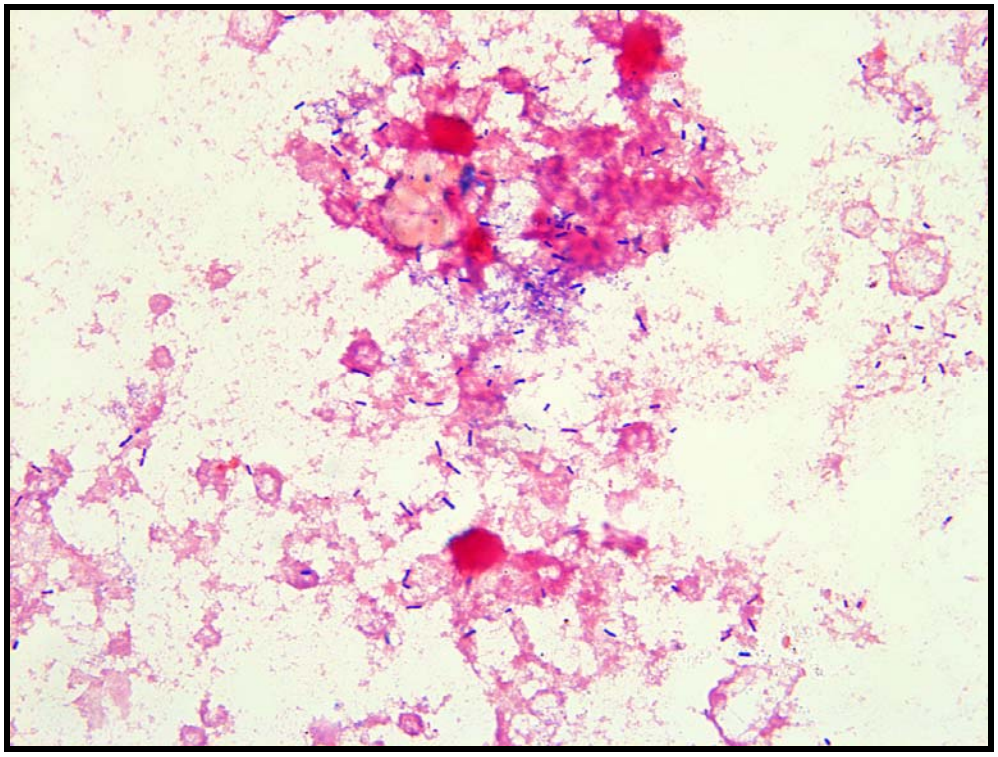

Figure 5.12. Slide showing high concentrations of *C. perfringens* (score = 4)

5.3.4 Live body mass

No differences were observed in body mass of *C. perfringens* challenged broiler chickens fed different feed additives or Zn-Bacitracin (Table 5.5). Oral antibiotics do not have growth-promoting effects in healthy animals (Coates *et al.,* 1955, 1963) This probably indicates that disease was not succesfully induced in this experiment. The non-challenged birds were kept in a different growth room under apparently similar conditions to inhibit cross infection. There was however a slower growth rate up to day 27 which is not easy to explain. The average live body masses of the non-challenged group were 263 g (SEM 3.03), 593 g (6.67), 915 g (8.97) and 1479 g (15.81) on day 13, 20, 27 and 35 respectively. These values were not included in the statistical analysis.

Chikoto (2006) previously illustrated a growth stimulatory effect of 4.9% (on a statistically significant basis) in broilers with experimentally induced necrotic enterits and fed with grapeseed extract in combination with *G. biloba* extract. Grapeseed extract in combination with *G. biloba* and *H. perforatum* extract also resulted in a 2.9% (not statististically significant) increase in body mass compared to the untreated control. In contrast with these findings, no differences in live body mass were observed in the same experiment after a 42 day period in broilers fed different combinations of these extracts (as well as with the Zn-bacitracin control) not challenged with *C. perfringens*. It can however be said that inclusion of any of the feed additives in this experiment did not have any negative effect on production. Çabuk *et al* (2006) were also unable to demonstrate any growth stimulatory response in healthy broilers treated with essential oil extract from a combination of oregano, laurel leaf, sage leaf, myrtle leaf, fennel seed and citrus peel. Similarly, Madrid *et al*. (2003) found that the oral administration of a blend of plant extracts containing oregano, cinnamon and pepper oil did not have any effext on body weight of the broilers. In contrast, positive effects of dietary essential oils on body weight were observed by Alcicek *et al* (2003) and Denli *et al* (2004). Hernandez *et a*l (2004) also found that the addition of two plant extracts to a broiler diet significantly improved body weight at 35 days of age. Moreover, Jamroz *et al.* (2003) found that the inclusion of 150 or 300mg/kg of a plant extract containing capsaicin, carvacrol and cinnamicaldehyde in a diet improved body weight by 5.4 and 8.1% respectively.

Table 5.5. Mean live body mass (g) on days 13, 20, 27 and 35 (each chicken analysed as an experimental unit) of *C. perfringens* challenged broiler chickens fed different feed additives

Treatments	Mean live body mass (g)							
	Day 13*	n	Day 20	n	Day 27	n	Day 35	n
Neg control (challenged)	289.7 (2.94)[a]	136	616.4 (6.27)[a]	122	910.0 (8.59)[a]	106	1490.4 (13.14[a])	91
Zn-bacitracin	291.9 (3.36)[a]	138	624.3 (6.59)[a]	125	911.4 (8.88)[a]	111	1502.3 (13.56)[a]	98
G. biloba	292.7 (2.80)[a]	141	627.1 (5.42)[a]	128	916.0 (7.60)[a]	111	1497.0 (11.75)[a]	97
G. biloba + H. perforatum	295.2 (2.72)[a]	141	630.0 (6.18)[a]	126	921.9 (8.41)[a]	109	1494.5 (15.07)[a]	96
P-value	0.633		0.438		0.748		0.939	

*Before challenge

5.3.5 Feed conversion ratios

There were no statistical difference in FCRs in the different challenged treatments except on day 13 (before challenge) where treatment with Zn-bacitracin resulted in better FCRs (Table 5.6). These differences can not be attibuted to differences in live body mass (Table 5.5) and are thus due to differences in rate of consumption i.e. higher rates of consumption (but less effective conversion into body mass) in the untreated, *G. biloba* and combination of *G. biloba* and *H. perforatum* treatments only up to day 13. FCRs were lower for the non-challenged group which were kept in a separate facility room. This could be ascribed to food wastage because a ring was not placed on the tube feeder. These values were not included in the statistical analysis.

Chikoto (2006) found no differences in FCRs of non-challenged broilers after treatment with combinations of grapeseed, *G. biloba*- or *H. perforatum* extract as well as the Zn-bacitracin control. Treatment with a combination of grapeseed- and *G. biloba* extract resulted in a significantly better FCR (4.3% improvement relative to the untreated control) in broiler chickens with experimentally induced necrotic enteritis. This improvement was achieved in spite of the fact that grapeseed extract on its own had a negative effect on FCR (Chikoto, 2006).

The current findings on FCR are in agreement with those of Lee *et al.* (2003), Botseglou *et al.* (2004) and Hernandez *et al.* (2004) who reported that addition of plant extracts or essential oils to the diet had no beneficial effect on feed intake or FCR. In contrast, Lee *et al.* (2003), who studied carvacrol from oregano, Madrid *et al.* (2003) who studied the effect of plant extracts and Alcicek *et al.* (2004) who used 48 mg/kg of an essential oil mixture in the diet of the broiler, found an improvemenent in FCR when these were included in the diet of broilers.

Table 5.6. Feed conversion ratios (FCR) of broilers challenged with *C. perfringens* and fed different feed additives

Treatments	FCR			
	Day 13*	Day 20	Day 27	Day 35
Neg control	1.75 (0.018)^{ac}	1.68 (0.021)^{a}	1.85 (0.028)^{a}	1.68 (0.028)^{a}
Zn-bacitracin	1.52 (0.029)^{bc}	1.66 (0.022)^{a}	1.86 (0.011)^{a}	1.69 (0.005)^{a}
G. biloba	1.94 (0.019)^{ab}	1.64 (0.013)^{a}	1.84 (0.026)^{a}	1.62 (0.054)^{a}
G. biloba + *H. perforatum*	1.88 (0.043)^{a}	1.60 (0.039)^{a}	1.80 (0.036)^{a}	1.66 (0.018)^{a}
P-value	0.00	0.283	0.427	0.383

*Before challenge

5.3.6 European Performance Efficiency Factor index

Means of the EPEF index were not significantly different between any of the treatment of the non-challenged broilers although the trend observed on day 35 was an improvement compared to the negative control of 2.1% with *G. biloba* + *H. perforatum* , 5.0% with *G. biloba* and 5.5% with Zn-Bacitracin (Table 5.7). Chikoto (2006) also observed a trend in the C*. perfringens* challenged group that treatment with Zn-bacitracin and treatments containing *G. biloba* and/or *H. perforatum* resulted in higher EPEF values than in the negative control. His values of 234 for the negative control vs. 248.4 and 244 for the treatments containing *G. biloba* + grapeseed and a combination of *G. biloba* + *H.perforatum* + grapeseed respectively on day 42 are comparable to what was found in this study. It should be noted that the grapeseed treatment on its own in Chikoto (2006)'s experiment resulted in values lower than the untreated control (challenged and non-challenged groups).

Table 5.7. European Performance efficiency index (EPEF) of broilers challenged with *C. perfringens* and fed different feed additives

Treatments	EPEF		
	Day 20	Day 27	Day 35
Neg control	181.2^{a}	174.6^{a}	234.0^{a}
Zn-bacitracin	187.2^{a}	176.3^{a}	246.9^{a}
G. biloba	190.2^{a}	174.7^{a}	245.6^{a}
G. biloba + *H. perforatum*	192.2^{a}	178.7^{a}	239.6^{a}
P-value	0.416	0.942	0.839

5.4 Conclusions

The objective of the study was firstly to determine the effect of a combination of extracts of *G. biloba* and *H. perforatum* on the performance of broiler chickens over a 35 day period and secondly to investigate the effect on *C. perfringens* in the intestine of broilers.

No significant differences were found with relation to any of the production parameters studied although a trend towards more favourable EPEF values were observed for treatments containing *G. biloba* (5% improvement) or a combination of *G. biloba* and *H. perforatum* (2.1% improvement) compared to the untreated control. Similarly, Zn-bacitracin resulted in a 5.5% improvement compared to the untreated control. This trend was also previously observed by Chikoto (2006) with treatments containing the same extracts.

C. perfringens was more frequently isolated from the intestines of broilers of the challenged treatments compared to the non challenged control in spite of the fact that necrotic enteritis was not succesfully induced in this experiment. There was a general trend (not on a statistical significant level) towards a reduction in microbial scores in the feed supplemented with *G. biloba* and *H. perforatum* which can probably be ascribed to the direct antimicrobial effect. The rate of colonization was too low to cause infection and have an impact on production probably due to the absence of predisposing factors and the possibility that the challenge strain was insufficiently virulent. It is necessary for an effective disease model to be developed in order for the efficacy of any new treatment method to be properly evaluated. Such a model will require a much higher incidence of disease and reproducibility than was achieved in this project.

5.5 Acknowledgements

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5.6 References

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6 General conclusions

The commercial rearing of broilers has to date depended on the inclusion of in-feed antibiotic growth promoters (AGP's). This practice is, however, disappearing due to the threat of development of antibiotic resistant microbes with public concern forcing the poultry industry to consider alternatives. Strategies to control necrotic enteritis in the absence of AGPs have focused on dietary and management practices and alternative feed additives. Among the candidate replacements for antibiotics are the inclusion of plant extracts into the diet. No single non-antibiotic measure has been identified yet, even though the *in vitro* antimicrobial effect of plant extracts against intestinal pathogens has been demonstrated (Kamel, 2000). Our study group joined this search for a commercially viable antibacterial phytogenic product from *Ginkgo biloba* and *Hypericum perforatum* with a low potential to develop resistance, as an alternative to AGPs in poultry production.

The objectives of the study were to:

- Evaluate the activity and potentize extracts from *G. biloba* and *H. perforatum* for optimal activity against selected bacterial pathogens (Chapter 2).
- Combine extracts or fractions of extracts of *G. biloba* and *H. perforatum* for optimising activity against selected bacterial pathogens (Chapter 2).
- Isolate and identify the major antibacterial compound in *G. biloba* (Chapter 3).
- Evaluate certain pharmacodynamic aspects of *G. biloba* extracts i.e. evaluate the potential of *E. faecalis* to develop *in vitro* resistance against a fraction/compound of *G. biloba* extracts and study the time-kill kinetics of this fraction using above mentioned pathogen over a 24 h period (Chapter 4).
- Determine the effect of extracts of *G. biloba* and/or *H. perforatum* on growth promotion and *Clostridium perfringens* in the intestines of broiler chickens challenged with *C. perfringens* (Chapter 5).

In order to prove the combination of *G. biloba* and *H. perforatum's* potential as an alternative to AGPs in poultry, the quantitative and qualitative aspects surrounding the *in vitro* antimicrobial effect needed to be investigated. Extracts of ethyl acetate (EA), hexane, dichloromethane (DCM) and acetone (in order of activity) from the direct extraction procedure of powdered *G. biloba* leaves were active against *E. faecalis, S. aureus* and *C. perfringens*. The EA, hexane and DCM extracts were 2 to 3 times more active than the acetone extract (average total activity 1728 ml/g dry extract for the 3 pathogens). The DCM, EA, acetone and hexane extracts (in order of activity) from the direct extraction procedure from *H. perforatum* were only active against *C. perfringens* with the first three extracts having a total activity of between 1 026 and 1 333 ml/g dry material and the hexane extract a total activity of 333 ml/g dry material. The spectrum of activity of *G. biloba* corresponds to that of Zn-Bacitracin which is commonly used an antibiotic growth promoter in the poultry industry.

Combination of the hexane fractions of *G. biloba* and *H. perforatum* from the solvent-solvent extraction procedure had a synergistic effect (activity enhanced 2.2 times when a 1:15 combination of *G. biloba: H. perforatum* were used) against *E. faecalis* but a neutral (neither synergistic nor antagonistic) effect against *C. perfringens.* An indifferent to additive effect was observed when combining the acetone extracts from the two plant species against *C. perfringens* or *E. faecalis*. Biological activity may thus be enhanced through the use of a combination of phytochemicals with different metabolic effects.

At least one compound was consistently present in above-mentioned active extracts of *G. biloba* as observed by using the bioautography technique. This compound was isolated and identified as ginkgolic acid, a 6-alkylsalicylic acid with $C_{17:1}$ alkyl substituent belonging to the alkylphenol group of compounds. Ginkgolic acid plays a role in the activity of these extracts against *E. faecalis* (12.5-50%) and *C. perfringens* (50-200%). The enhanced activity of ginkgolic acid against *C. perfringens* can be ascribed to proven synergistic interactions (2.4 times) between the hexane- and EA fractions of the acetone extract, with only the hexane extract containing ginkgolic acid. Combination of these two fractions resulted in a MIC 3.1 times lower against *E. faecalis* compared to the sum of the MIC of the individual fractions. The potential use of isolated active compounds as antimicrobial products proved not only expensive and time-consuming, but also exclude positive interactions between constituents in whole extracts which could considerably enhance activity. According to Yuan and Lin (2000), the National Cancer Institute of the USA and the USD Department of Agriculture screened 35 000 samples from different tissues from plant species and only three new drugs were discovered. Nevertheless, the ancient Oriental pharmacopoeias contain thousands of therapeutic formulations, indicating that the biological activity of these preparations might result from synergy of active compounds rather than a single chemical entity.

In chapter 4 the potential of *E. faecalis* to develop resistance against the hexane fraction (containing the highest concentration of ginkgolic acid) as well as ginkgolic acid from *G. biloba* was evaluated. No decreased susceptibility of the hexane fraction or ginkgolic acid was observed even after 10 and 9 passages respectively while an 8-fold increase was observed with the gentamycin control within 7 passages. This is not surprising as *G. biloba* has survived over 200 million years probably due to its ability to resist pests and diseases. Previous studies have indeed shown the presence of antibacterial compounds in *G. biloba* (chapter 2 – unpublished data; Mazzanti *et al.,* 2000; Pan, 2007) as well as synergistic interactions between constituents which enhancesantimicrobial activity (chapter 4 – unpublished data).

In addition to the emergence of resistance studies performed in chapter 4, another pharmacodynamic end point i.e. the extent of bacterial killing, was also investigated, as this is an important parameter to consider early in the development of a new antimicrobial agent. The bactericidal nature of the hexane extract from *G. biloba* as well the absence of decreased susceptibility in the resistance studies against *E. faecalis* indicated that this extract has potential to be exploited as a alternative to AGPs in the poultry industry.

Chapter 5 was a continuation of the work of Chikoto (2006). The objective was to determine the effect of a combination of *G. biloba* and *H. perforatum* on the performance of broiler chickens over a 35-day period. The effect on *C. perfringens* in the intestine was also investigated. In spite of the fact that the hexane, DCM and EA extracts from *G. biloba* yielded the best results in terms of antimicrobial activity against *E. faecalis* and *C. perfringens* (chapter 2), the acetone extract was used in this experiment. The reasons were three-fold: firstly Chikoto (2006) established that the acetone extracts of *G. biloba* and *H. perforatum* can be tolerated by broilers at a rate of 2 and 10mg/kg respectively and secondly the extracts were found to be stable in feed and water. Antimicrobial activity was also found to be stable under varying pH conditions. No significant differences were found after treatment with *G. biloba* and/or *H. perforatum* with relation to live body weight, feed conversion ratio (FCR), mortality or European Performance efficacy factor (EPEF) although a trend towards more favourable EPEF values were observed for treatments containing *G. biloba* (5% improvement) or a combination of *G. biloba* and *H. perforatum* (2.1% improvement) compared to the untreated control. Similarly, Zn-Bacitracin resulted in a 5.5% improvement compared to the untreated control. This trend was also previously observed by Chikoto (2006) with treatments containing the same extracts.

C. perfringens was more frequently isolated from the intestines of broilers of the challenged treatments compared to the non challenged control in spite of the fact that necrotic enteritis was not succesfully induced in this experiment. There was a general trend (not on a statistically significant level) towards a reduction in microbial scores in the feed supplemented with *G. biloba* and *H. perforatum,* which can probably be ascribed to the direct antimicrobial effect. The rate of colonization was too low to cause infection and have an impact on production probably due to the abence of predisposing factors. It is necessary for an effective disease model to be developed in order for the efficacy of any new treatment method to be properly evaluated. Such a model will require a much higher incidence of disease and reproducibility than was achieved in this project.

The use of a product containing ginkgolic acid as active ingredient raises some questions regarding safety as ginkgolic acids and related alkylphenols have been recognized as hazardous compounds with suspected cytotoxic and allergenic properties (Pan, 2007). The acetone extract from *G. biloba* was included into the chicken feed at a rate of 14 ppm. Considering that the chickens in this trial consumed up to 170 g of feed/kg live weight of chicken / day the maximum dose of the acetone extract from *G. biloba* extract was 2.4 mg/kg/day. The active ginkgolic acid constitutes up to 5% of the acetone extract (chapter 3), thus relating to 0.12 mg/kg/day (0.12 ppm). For reasons of drug safety a maximum concentration of ≤5 ppm of ginkgolic acids is recommended by the Monograph of the Commission E of the former German Federal Health Agency (Baron-Ruppert and Luepke, 2001). Therefore the effect of residues on consumers of chicken products would probably be negligible. During production of the standard *Ginkgo* extract EGb761®, alkylphenols are largely eliminated as water insoluble compounds (decanter sludge) from the primary acetone extract. Baron-Ruppert and Luepke (2001) evaluated different fractions from the decanter sludge for their embryotoxic effects in the hen's egg test (HET). A fraction enriched for ginkgolic acids (16%) and biflavones (6.7%) was found to induce death of 50% of the chick embryos

(LD_{50}) at a dose of 1.8 mg/egg (≈33 ppm). A similar strong lethal effect (LD_{50}:3.5 mg/egg; 64 ppm) was observed for a fraction which contained 58% ginkgolic acids but less than 0.02% biflavones. Some scientists believe that the safety hazard of ginkgolic acids was overemphasized by the Schwabe company as part of a marketing exercise (personal communication: Prof. J.N. Eloff). Hausen (1998) for example found that though the guinea pigs in his study were sensitized successfully with pure ginkgolic acids, the animals could not be sensitized with the leaf extract. Leaf extracts of *G. biloba* taken orally or given by infusion to treat diffuse cerebral disturbances can be considered safe, even when they contain up to 1000 ppm of the sensitizing ginkgolic acids (Hausen, 1998). The absence of adverse effects when alkylphenols are taken orally are supported by the fact that no reports have been filed on adverse effects of *Ginkgo* mother tinctures in spite of the fact that such extracts contain 2.2% (22000 ppm) of ginkgolic acids and have been on the market for several years (van Beek, 2002).

The biological activities of the ginkgolic acid derivates have attracted considerable attention for their molluscicidal activity against *Oncomelania hupensis* (Yang *et al.,* 2008), anti-*Toxoplasma gondii* activity (Chen *et al.,* 2008) antitumor (Kubo *et al.,* 1993) antioxidant (Kubo *et al.,* 2005) and xanthine oxidase inhibitory action (Masuoka and Kubo, 2004). Ginkgolic acids or extracts containing high concentrations thereof can thus be exploited for it's benefits in many different fields such as the agricultural and pharmaceutical industry.

The combination of extracts of *G. biloba* and *H. perforatum* holds promise as a potential growth promoter in poultry production. Better results may be achieved if potentized extracts are used and compared with Zn-Bacitracin and a negative control under industrial growth conditions where the birds are stressed and natural infections would take place.

6.1 References

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