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).

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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.

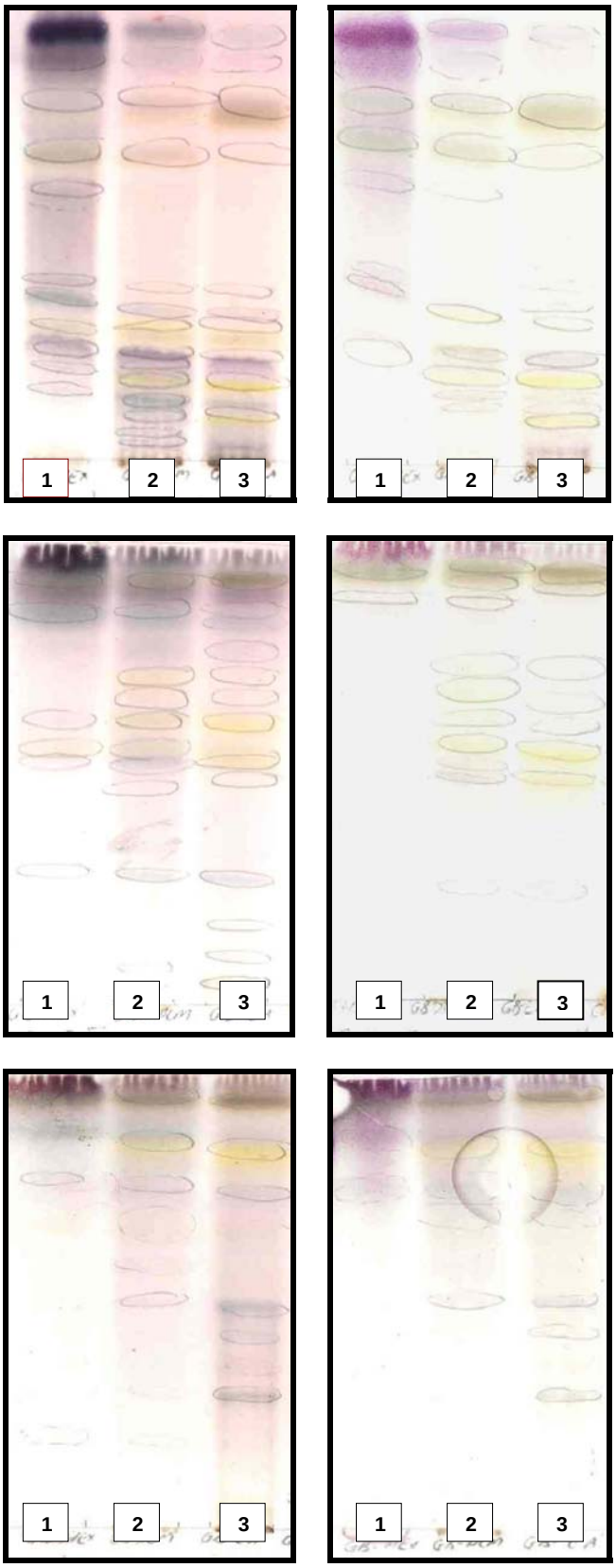


Figure 2. 1. Serially extracted *G. biloba* extracts sprayed with p-anisaldehyde (left) and vanillin (right) with mobile phase BEA (top), CEF (middle) and EMW (bottom)

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

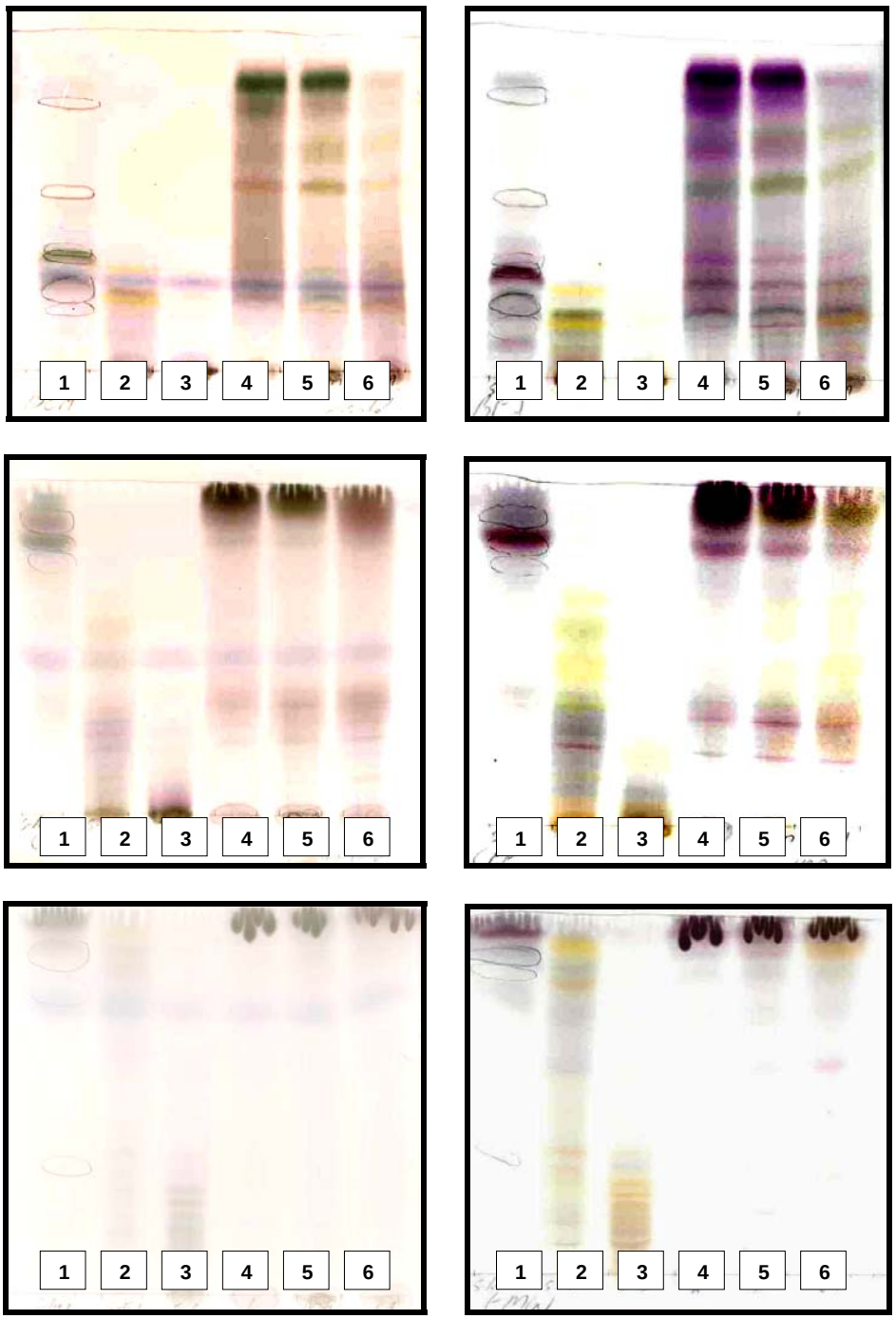


Figure 2.2. *G. biloba* extracts/fractions from the direct- and solvent-solvent extraction procedures sprayed with p-anisaldehyde (left) and vanillin (right) with mobile phase BEA (top), CEF (middle) and EMW (bottom)

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)

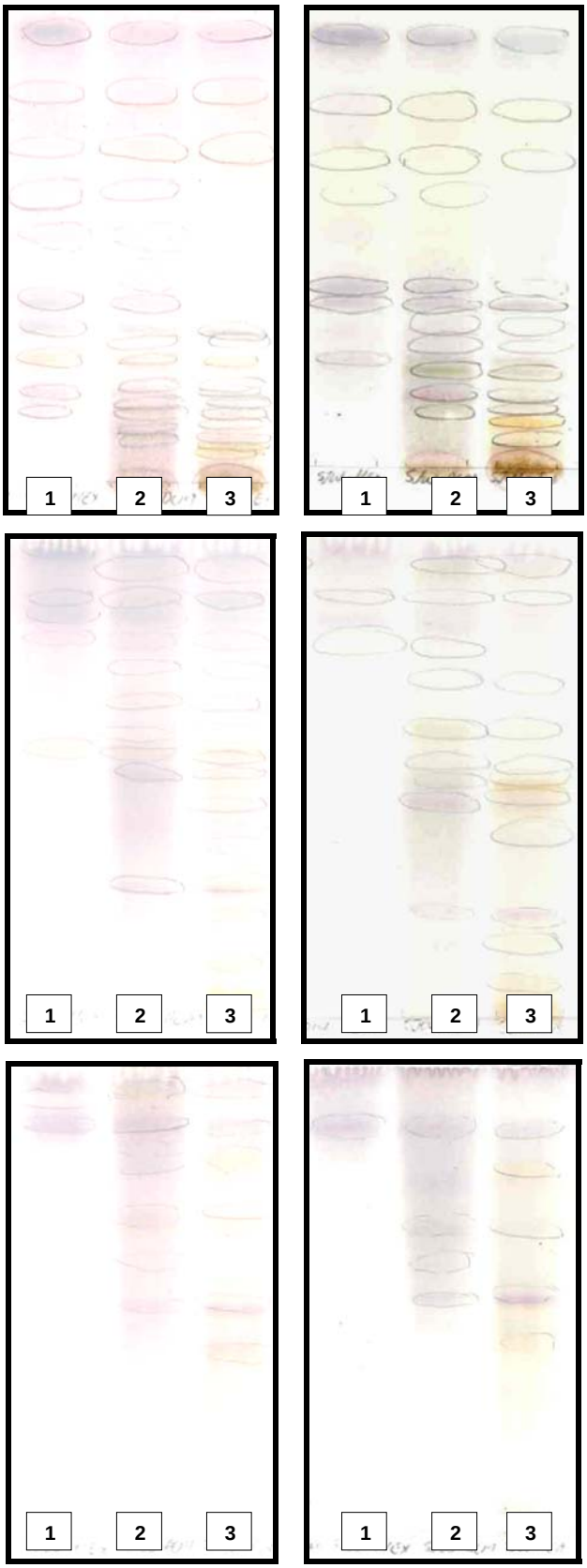


Figure 2.3. Serially extracted *H. perforatum* extracts sprayed with p-anisaldehyde (left) and vanillin (right) with mobile phase BEA (top), CEF (middle) and EMW (bottom)

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

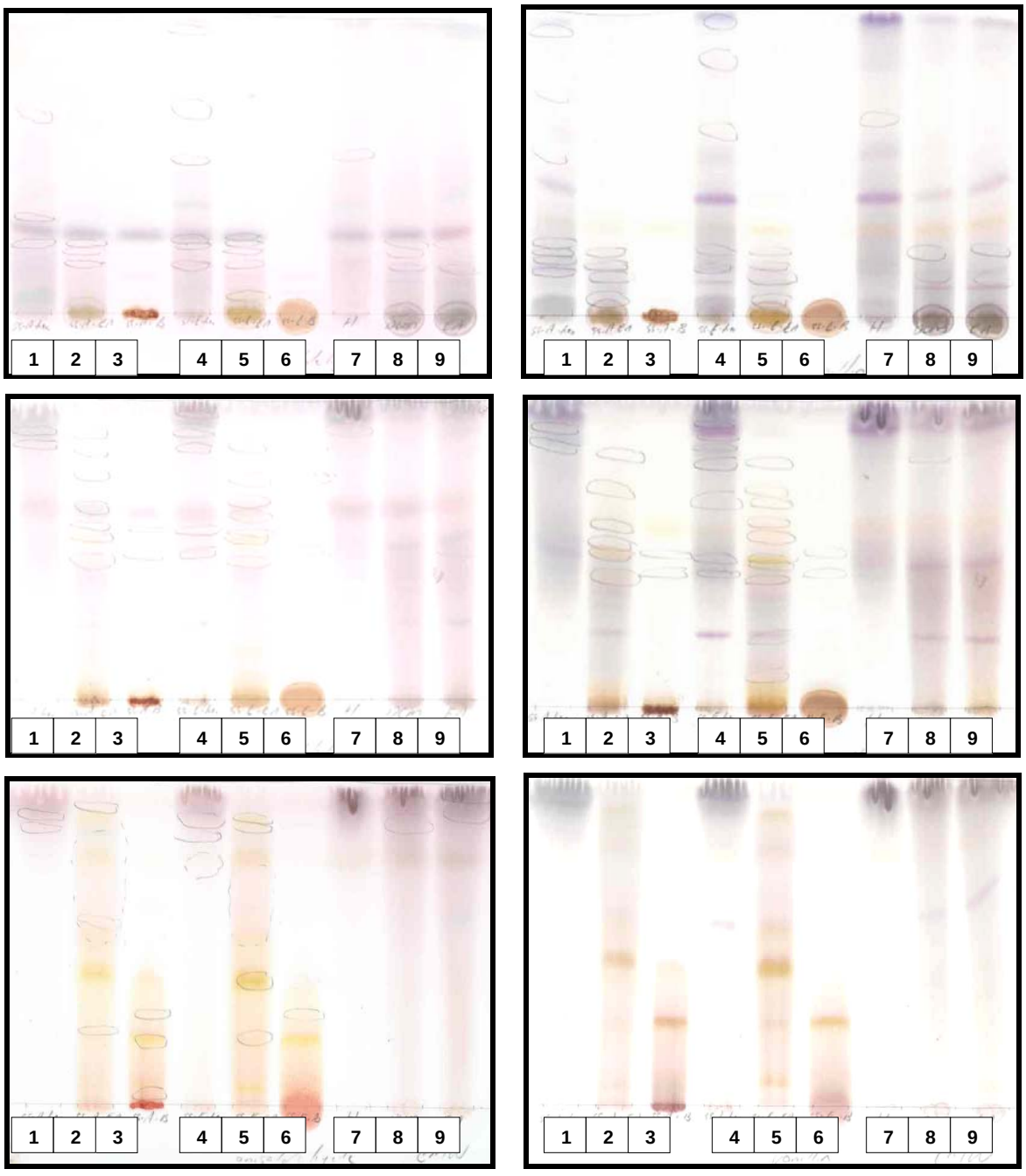


Figure 2.4. *H. perforatum* extracts/fractions from direct- and solvent-solvent separation procedures sprayed with p-anisaldehyde (left) and vanillin (right) with mobile phase BEA (top), CEF (middle) and EMW (bottom)

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)

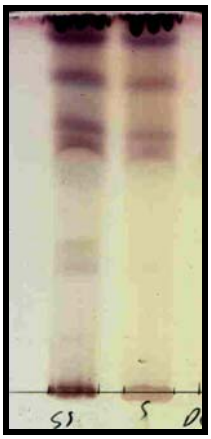


Figure 2.5. Hexane fraction of *G. biloba* from the solvent-solvent extraction procedure (left) and from the direct extraction procedure (right) sprayed with vanillin with mobile phase DCM: methanol (9:1)

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

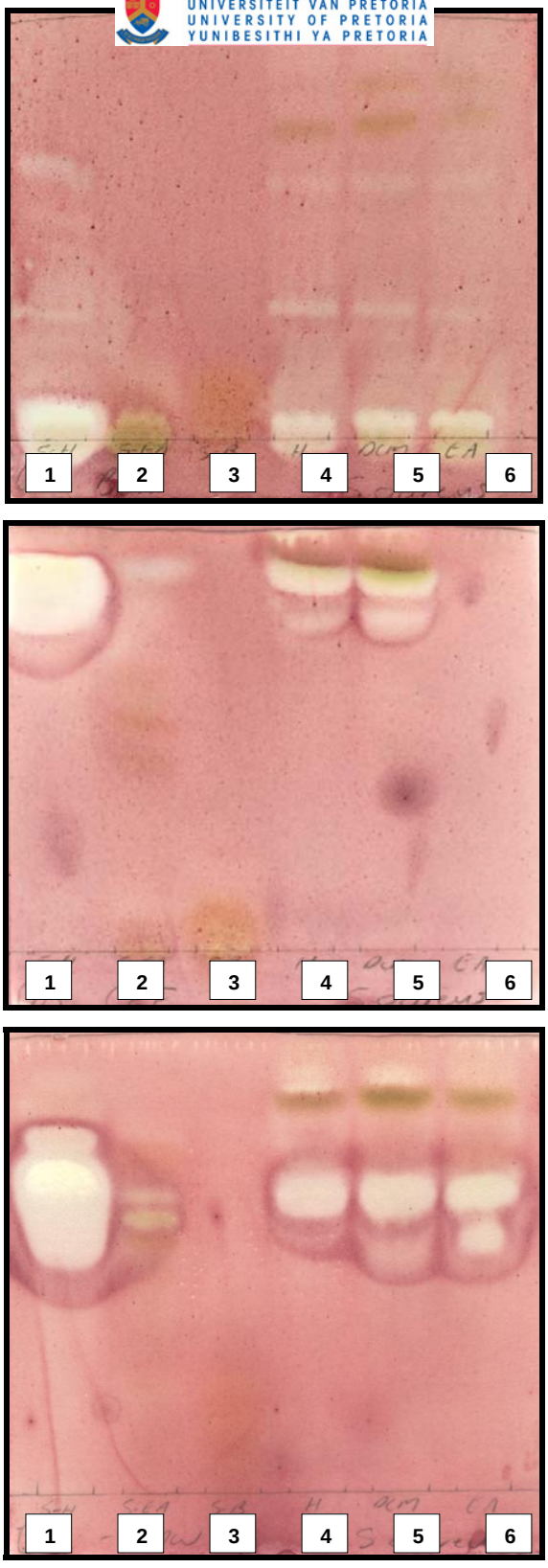


Figure 2.7. *G. biloba extracts* sprayed with *S. aureus* with mobile phases BEA (top), CEF (centre) and EMW (bottom)

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)

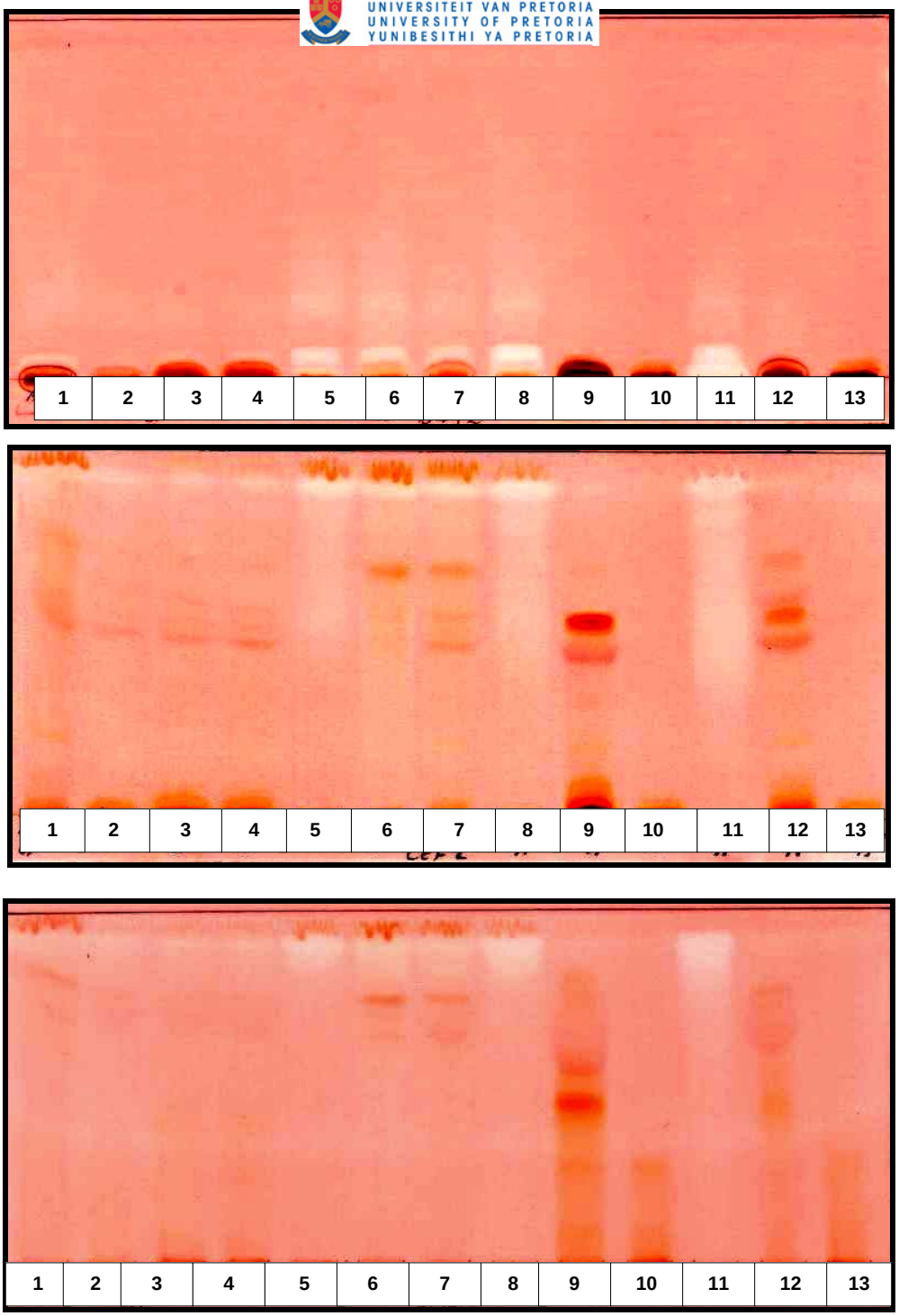


Figure 2.8. *H. perforatum* extracts (see below) sprayed with *S. aureus* with mobile phases BEA (top), CEF (centre) and EMW (bottom)

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)

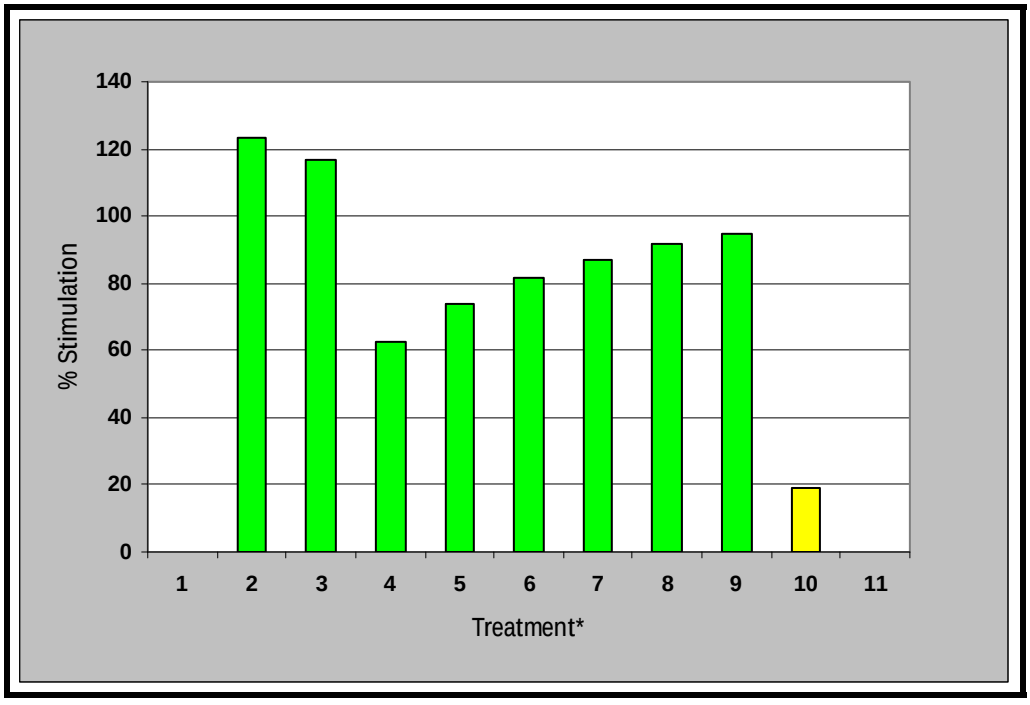
Treatment	% of Test fraction (Combinations)		Expected MIC (mg/ml) (separate values of individual fractions)[1]		Expected MIC (Total)[1]	Actual MIC (mg/ml) (separate values of combined fractions)[2]		Actual MIC (Total)	∑FIC
	a	b	a	b		a	b		
1	100	0	0.208	0	0.208	0.208	0	0.208	1.000
2	90	10	0.187	1	1.187	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)

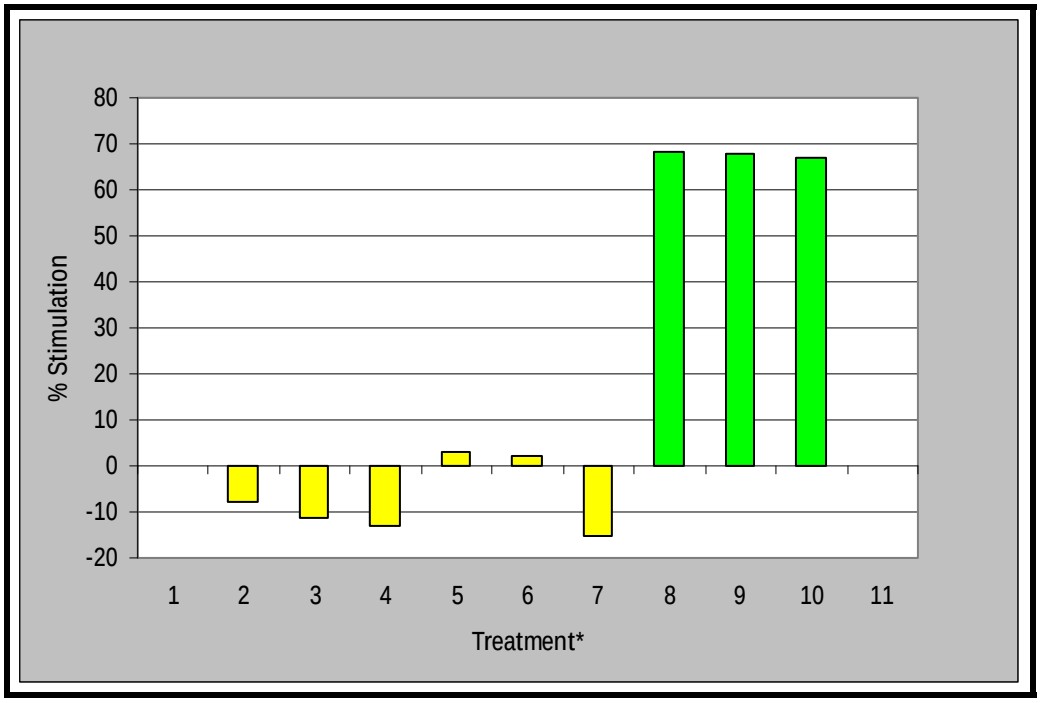
Treatment	% of Test fraction (Combinations)		Expected MIC (mg/ml) (separate values of individual fractions)[1]		Expected MIC (Total)[1]	Actual MIC (mg/ml) (separate values of combined fractions)[2]		Actual MIC (Total)	∑FIC
	a	B	a	b		a	b		
1	100	0	2.50	0.00	2.50	2.50	0.00	2.50	1.00
2	90	10	2.25	1.67	3.92	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)

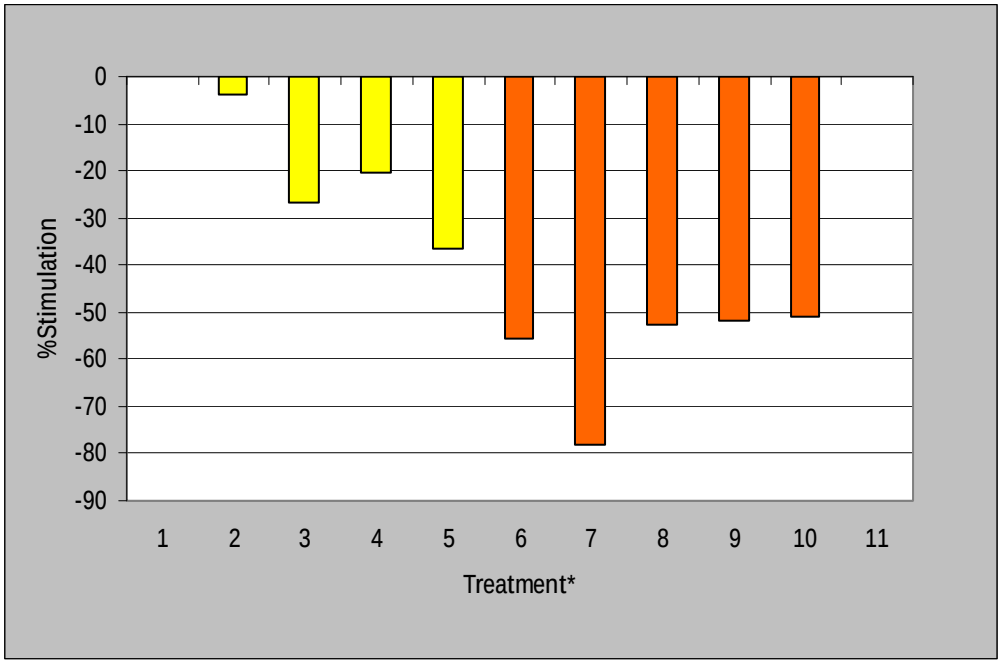
Treatment	% of Test fraction (Combinations)		Expected MIC (µg/ml) (separate values of individual fractions)[1]		Expected MIC (Total)[1]	Actual MIC (µg/ml) (separate values of combined fractions)[2]		Actual MIC (Total)	∑FIC
	a	b	a	b		a	b		
1	100	0	2.23	0.00	2.23	2.23	0.00	2.23	1.00
2	90	10	2.01	1.56	3.57	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)

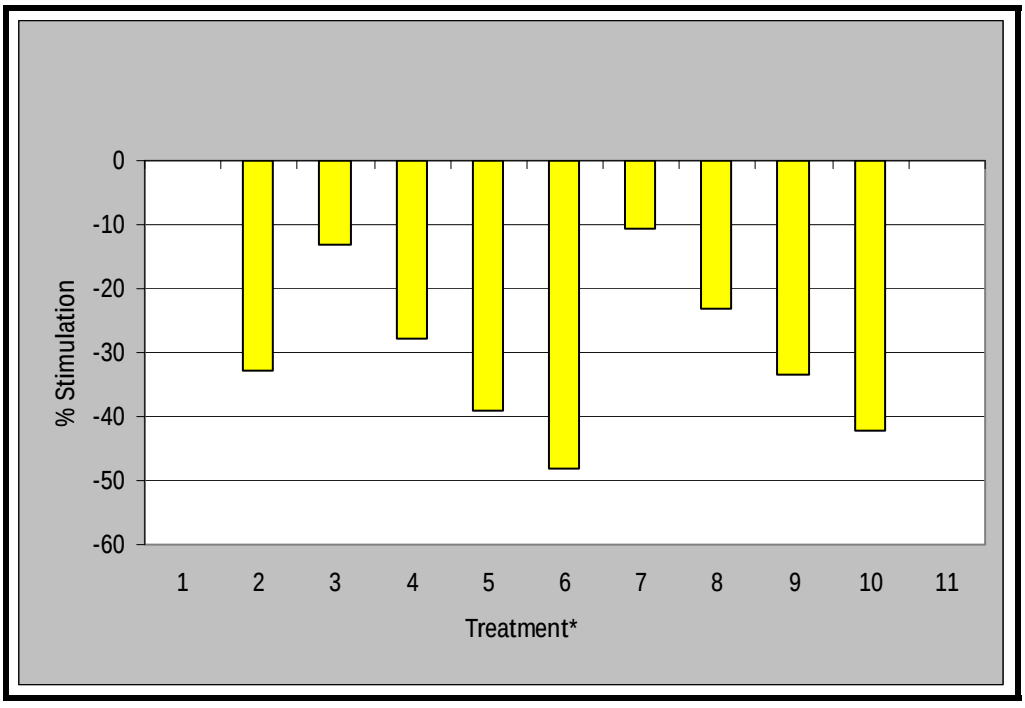
Treatment	% of Test fraction (Combinations)		Expected MIC (mg/ml) (separate values of individual fractions)[1]		Expected MIC (Total)[1]	Actual MIC (mg/ml) (separate values of combined fractions)[2]		Actual MIC (Total)	∑FIC
	a	b	a	b		a	b		
1	100	0	0.14	0.00	0.14	0.14	0.00	0.14	1.00
2	90	10	0.12	0.01	0.13	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.

2.5 References

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