

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 polyphenols (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; Bombardelli 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. Bombardelli 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 quercetin (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 $1\ 322 \times g$ 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 H₂O portions. Each step was carried out three times to ensure adequate extraction. All solvents were saturated with distilled H₂O 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 Müller-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 10⁸ 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*.

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

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

(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 $\geq 50\%$ indicate stimulation, $> -50\%$ and $< 50\%$ indicate an indifferent effect and $\leq -50\%$ 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 chloroform-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.

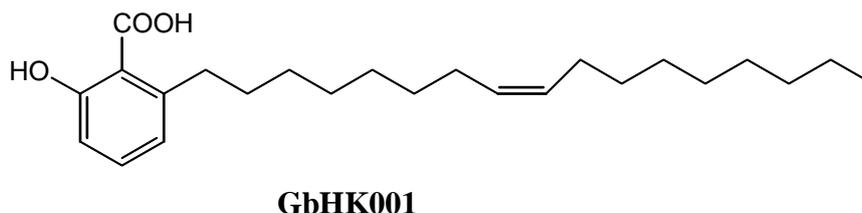


Figure 3. 1. Ginkgolic acid, a 6-alkylsalicylic (anacardic) acid with $C_{17:1}$ alkyl substituent.

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

Isolated compound	van Beek and Winternans (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" will 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 C₆ 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 C₆ 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 C₁₂ and C₁₆. 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 C₁₀ and C₁₂ 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 bonds, 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 derivatives 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).

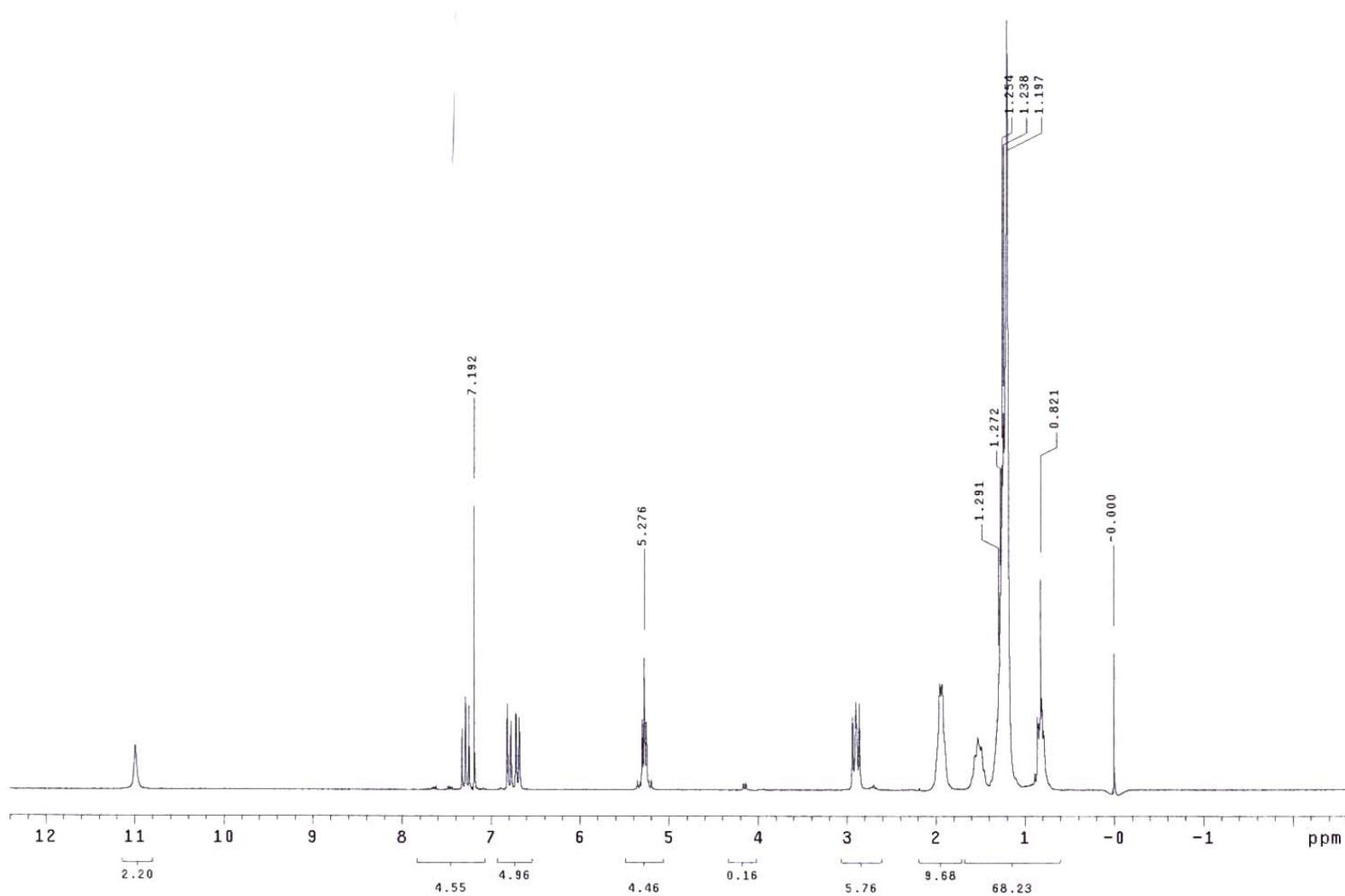


Figure 3. 2. ^1H NMR data for isolated compound GbHK001

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*.

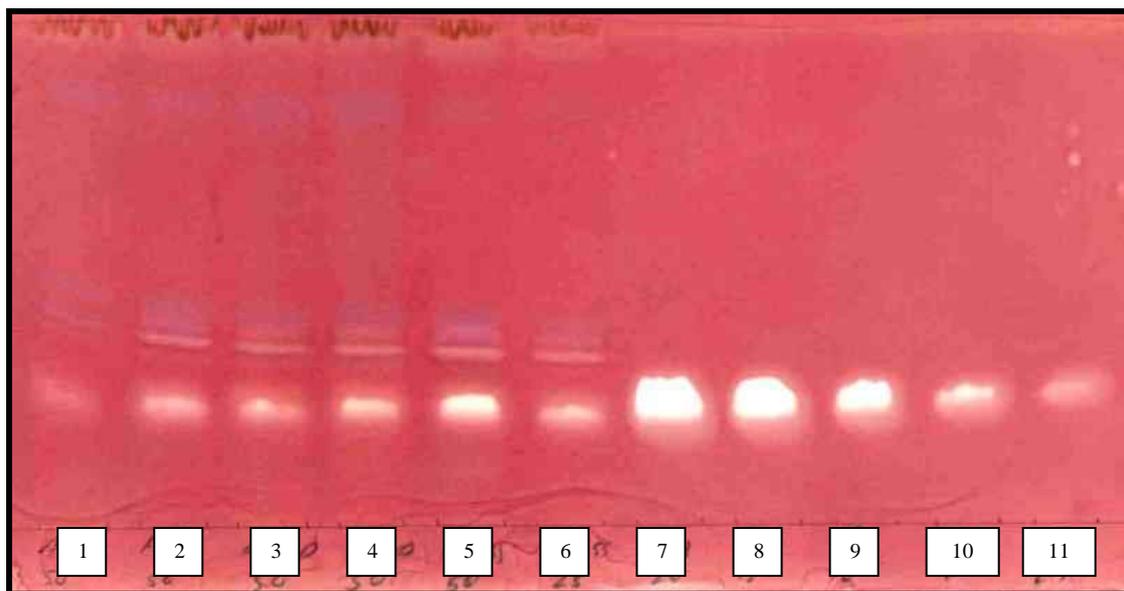


Figure 3. 3. Bioautogram of *G. biloba* extracts (1-6) and different concentrations of the isolated ginkgolic acid (GA) (7-11) sprayed with *S.aureus* with mobile phase DCM:Methanol (19:1)

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 (%)	
	MIC ¹	TA ²	Yield (%)	MIC	TA	Yield (%)	GA in extract/fraction (%)	
<i>E. faecalis</i>								
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
<i>C. perfringens</i>								
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

¹MIC = Minimum inhibitory concentration (mg/ml)

²TA = 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 H₂O 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)

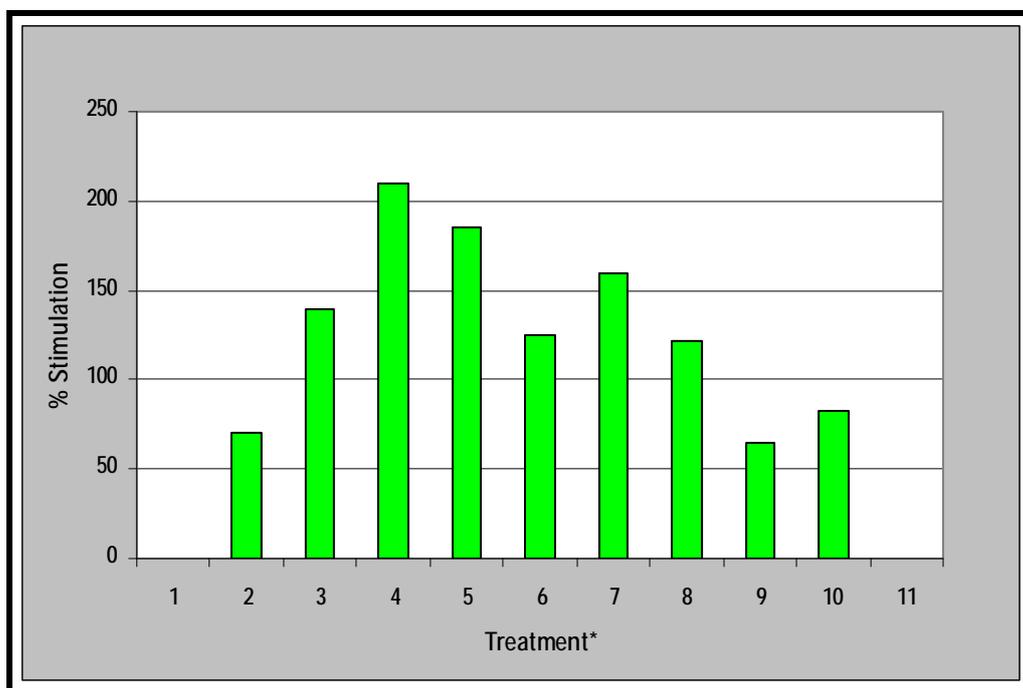
Treatment	% of Test fraction (Combinations)		Expected MIC (mg/ml) (separate values of individual fractions) ¹		Expected MIC (Total) ¹	Actual MIC (mg/ml) (separate values of combined fractions) ²		Actual MIC (Total)	ΣFIC
	a	b	a	b		a	b		
1	100	0	0.08	0.00	0.08	0.08	0.00	0.08	1.00
2	90	10	0.07	0.06	0.13	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

¹100% values (rounded off) were measured and % fractions were calculated

²Separate 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)

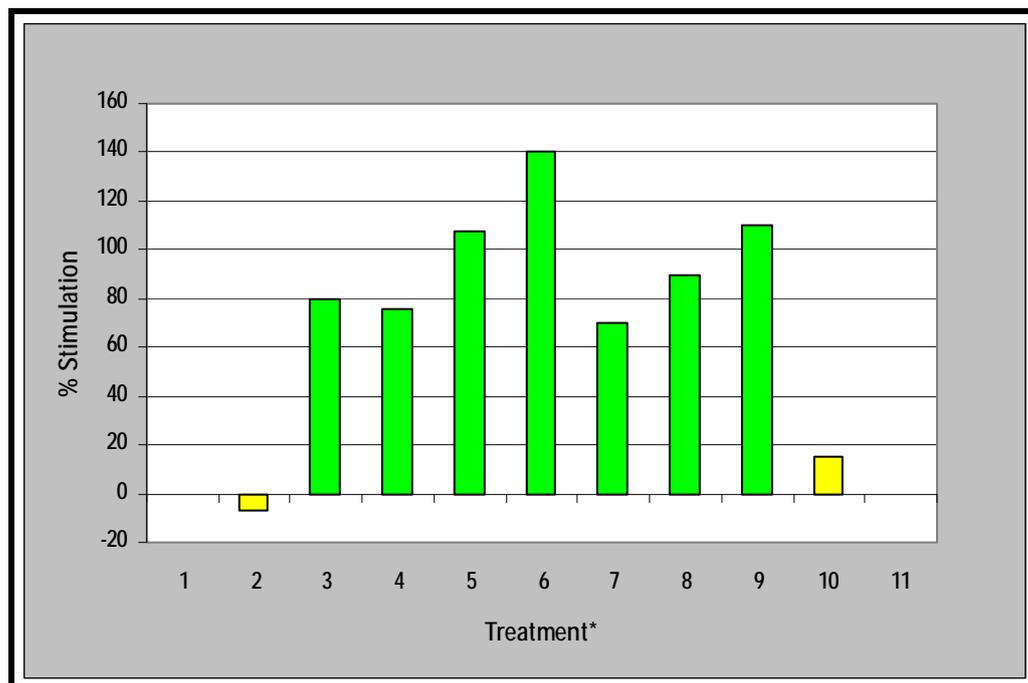
Treatment	% of Test fraction (Combinations)		Expected MIC (µg/ml) (separate values of individual fractions) ¹		Expected MIC's (Total) ¹	Actual MIC (µg/ml) (separate values of combined fractions) ²		Actual MIC (Total)	ΣFIC
	a	b	a	b		a	b		
1	100	0	3.91	0.00	3.91	3.91	0.00	3.91	1.00
2	90	10	3.52	1.95	5.47	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

¹100% values (rounded off) were measured and % fractions were calculated

²Separate 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

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3.5 References

- Adawadkar, P.D. and El Sohly, M.A. (1981). Isolation, purification and antimicrobial activity of ginkgolic acids from *Ginkgo biloba* fruits. *Fitoterapia* 52: 129-135.
- Alaoui-Youssefi, A., Lamproglou, I., Drieu, K. and Emerit, I. (1999). Anticlastogenic effects of *Ginkgo biloba* extract (Egb 761) and some of its constituents in irradiated rats. *Mutation Research* 445: 99-104.
- Anke, H. and Sterner, O. (1991). Comparison of the antimicrobial and cytotoxic activities of twenty unsaturated sesquiterpene dialdehydes from plants and mushrooms. *Planta Medica* 57: 344-346.
- Atzori, C., Bruno, A., Chichino, G., Bombardelli, E., Scaglia, M. And Ghione, M. (1993). Activity of bilobalide, a sesquiterpene from *Ginkgo biloba*, on *Pneumocystis carinii*. *Antimicrobial Agents and Chemotherapy* 3: 1492-1496.
- Baron-Ruppert, G. and Luepke, N.P. (2001). Evidence for toxic effects of alkylphenols from *Ginkgo biloba* in the hen's egg test (HET). *Phytomedicine* 8: 133-138.
- Begue, W.J., and Kline, R.M. (1972). The use of tetrazolium salts in bioautographic procedures. *Journal of Chromatography* 64: 182-184.
- Berenbaum, M.C. (1978). A method for testing synergy with any number of agents. *The Journal of infectious Diseases* 13: 122-130.
- Bombardelli, E. and Ghione, M. (1993). Use of bilobalide and derivates thereof and pharmaceutical compositions adapted for such use (Patent). European Patent Application EP 556 051 A1.
- Bourguet-Kondracki, M.L., Longeon, A., Morel, E. and Guyot, M. (1991). Sesquiterpene quinones as immunomodulating agents. *International Journal of Immunopharmacology* 13: 393-399.
- Chen, S., Wu, L., Jiang, X., Feng, Y and Cao, J. (2008). Anti-*Toxoplasma gondii* activity of GAS in vitro. *Journal of Ethnopharmacology* 118: 503-507.
- Choi, J.G., Jeong, S.I., Ku, C.S., Sathishkumar, M., Lee, J.J., Mun, S.P. and Kim, S.M. (2009). Antibacterial activity of hydroxyalkenyl salicylic acids from the sarcotesta of *Ginkgo biloba* against vancomycin-resistant *Enterococcus*. *Fitoterapia* 80: 18-20.
- Curtis, P.P., Vere, D. and Fray, P. (1999). Therapeutic value of *Ginkgo biloba* in reducing symptoms of decline in mental function. *Journal of Pharmacy and Pharmacology* 51: 535-541.

Daoud, N.N., Dickinson, N.A. and Gilbert, P. (1983). Antimicrobial activity and physio-chemical properties of some alkyldimethylbenzylammonium chlorides. *Microbios* 37: 73-85.

De Kosky, ST., Jeff, D., Williamson, M.H.S. *et al* (2008). *Ginkgo biloba* for prevention of dementia. A randomized controlled trial. *The Journal of the American Medical Association* 300: 2253-2262.

DeFeudis, F.V. In: *Ginkgo biloba* Extract (Egb761) (1998). From Chemistry to Clinic, Ulstein Medical, Wiesbaden

De Tommasi, N., Pizza, C., Conti, C., Orsi, N. And Stein, M.L. (1990). Structure and *in vitro* antiviral activity of sesquiterpene glycoside from *Calendula arvasensis*. *Journal of Natural Products* 53: 830-835.

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

Eloff, J.N. (2000). A proposal on expressing the antibacterial activity of plant extracts – a small step in applying scientific knowledge to rural primary health care in South Africa. *South African Journal of Science* 96: 116-118.

Eloff, J.N. (2004). Quantification the bioactivity of plant extracts during screening and bioassay guided fractionation. *Phytomedicine* 11: 370-371.

Franks, N.P. and Lieb, W.R. (1986). *Proceedings of the National Academy of Science of the United States of America* 83: 5116.

Green, I.R., Tocoli, F.E., Lee, S.H., Nihei, K and Kubo, I. (2008). Design and evaluation of anacardic acid derivatives as anticavity agents. *European Journal of Medicinal Chemistry* 43: 1315-1320.

Itokawa, H., Totsuka, N., Nakahara, K., Takeya, K., Lepoittevin, J.P. and Asakawa, Y. (1987). Antitumor principles from *Ginkgo biloba* L. *Chemical and Pharmaceutical Bulletin* 35: 3016-3020.

Koch, E., Jaggy, H. and Chatterjee, S.S. (2000). Evidence for toxic effects of alkylphenols from *Ginkgo biloba* L. leaf extracts using the popliteal lymph node assay in the mouse. *International Journal of Immunopharmacology* 22: 229-236.

Kubo, I., Muroi, H. and Kubo, A. (1995). Structural functions of antimicrobial long-chain alcohols and phenols. *Bioorganic and Medicinal Chemistry* 3: 873-880.

Kubo, I., Masuoka, N., Joung Ha, T. and Tsujimoto, K. (2005). Antioxidant activity of anacardic acids. *Food Chemistry* 99: 555-562.

- Kubo, I., Ochi, M., Veira, P.C. and Komatsu, S. (1993). Antitumour agents from the cashew (*Anacardium occidentale*) apple juice. *Journal of Agricultural and food Chemistry* 41: 1012-1015.
- Kubo, J., Lee, J.R., and Kubo, I. (1999). Anti-*Helicobacter pylori* agents from the cashew apple. *Journal of Agricultural and Food Chemistry* 47: 533-537.
- Lee, H. and Kim, M. (2002). Selective responses of three *Ginkgo biloba* leaf-derived constituents on human intestinal bacteria. *Journal of Agricultural and Food Chemistry* 50: 1840-1844
- Masuoka, N. and Kubo, I. (2004). Characterization and xanthine oxidase inhibition by anacardic acids. *Biochemical and Biophysical Acta* 1688: 245-249.
- Mazzanti, G., Mascellino, M.T., Battinelli, L., Coluccia, D., Manganaro, M. and Saso, L. (2000). Antimicrobial investigation of semi purified fractions of *Ginkgo biloba* leaves. *Journal of Ethnopharmacology* 71: 82-88.
- Muroi, H and Kubo, I. (1993). Bactericidal activity of anacardic acids against *Streptococcus mutans* and their potentiation. *Journal of Agricultural Food Chemistry* 41: 1780-1783.
- Muroi, H., Nihei, K., Tsujimoto, K. And Kubo, I. (2003). Synergistic effects of anacardic acids and methicillin against methicillin resistant *Staphylococcus aureus*. *Bioorganic and Medicinal Chemistry* 12: 583-587.
- Pan, J. (2007). Antibacterial actions of ginkgolic acids and related mechanisms. In: *New biocides development*. Chapter 19 pp 389-410.
- Roy, S., Kobuchi, H., Sen, C.K., Gohil, K., DroyLefaix, M.T. and Packer, L. 1998. *Ginkgo biloba* extract (Egb 761): antioxidant properties and regulation of gene expression. *Ginkgo biloba* extract Egb 761 lessons from cell biology 7: 1-17.
- Schelz, Z., Molnar, J. and Hohmann, J. (2006). Antimicrobial and antiplasmid activities of essential oils. *Fitoterapia* 77: 279-285.
- Schwabe, K.P. (1994). Extract from *Ginkgo biloba* leaves, its method of preparation and pharmaceuticals containing the extract. European Patent EP 0 431 535 B1.
- Silva, M.S.S., DeLima, S.G., Oliveira, E.H., Lopez, J.A.D., Chaves, M.H., Reis, F.A.M. and Citó, A.M.G.L. (2008). Anacardic acid derivatives from Brazilian propolis and their antibacterial activity. *Ecletica Quimica* 33: 53-58.
- Tepe, B., Akpulat, H.A., Sokmen, M., Daferera, D., Yumrutas, O., Aydin, E., Polissiou, M. and Sokmen, A. (2006). Screening of the antioxidative and antimicrobial properties of the essential oils of *Pimpinella anisetum* and *Pimpinella flabellifolia* from Turkey. *Food Chemistry* 97: 719-724

van Beek, T.A. (2002). Chemical analysis of *Ginkgo biloba* leaves and extracts. *Journal of Chromatography* 967: 21-55.

van Beek, T.A. and Wintermans, M.S. (2001). Preparative isolation and dual column high-performance liquid chromatography of ginkgolic acids from *Ginkgo biloba*. *Journal of Chromatography* 930: 109-117.

Watanabe, N., Yamagishi, M., Mizutani, T., Kondon, H., Omura, J., Hanada, K. and Kushida, K. (1990). CAF 603: a new antifungal carotene sesquiterpene. Isolation and structure elucidation. *Journal of Natural Products* 53: 1176-1181.

Yang, X., Chen, S., Xia, L. and Chen, J. (2008). Molluscicidal activity against *Oncomelania hupensis* of *Ginkgo biloba*. *Fitoterapia* 79: 250-254.

Yang, X., Zhu, W., Chen, J., Ain, Z. and Xie, J. (2004). Study on anti-bacterial activity of ginkgolic acids and their monomers. *Zhong Yao Cai* 27:661-663. Abstract Medline.

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 H₂O portions. Each step was carried out three times to ensure adequate extraction. All solvents were saturated with distilled H₂O 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 5×10^5 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 37°C 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 -70°C.

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 6.9×10^5 cells/ml. The flasks containing the broth cultures were incubated at 37 °C 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 $\mu\text{g/ml}$, an 8 fold increase after 7 passages compared to the parent strain (Figure 4. 3). Because no decreased sensitivity was observed against the test extracts, 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 <i>G. biloba</i>	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 $\mu\text{g/ml}$ for gentamicin

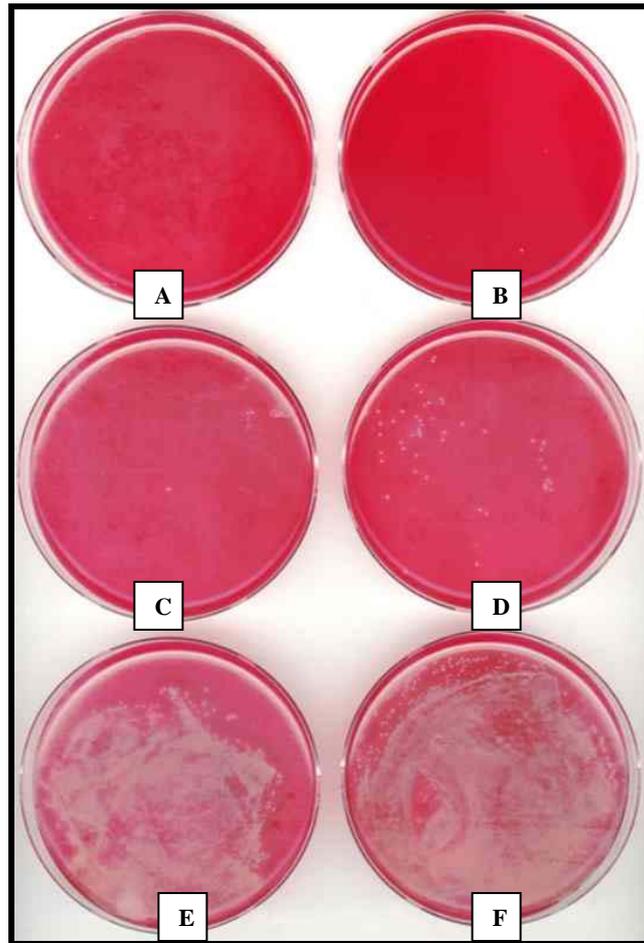


Figure 4. 1. Selection of resistant strain (C) from plate representing the highest concentration of test substance, with A–F in order from the highest to the lowest concentration



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

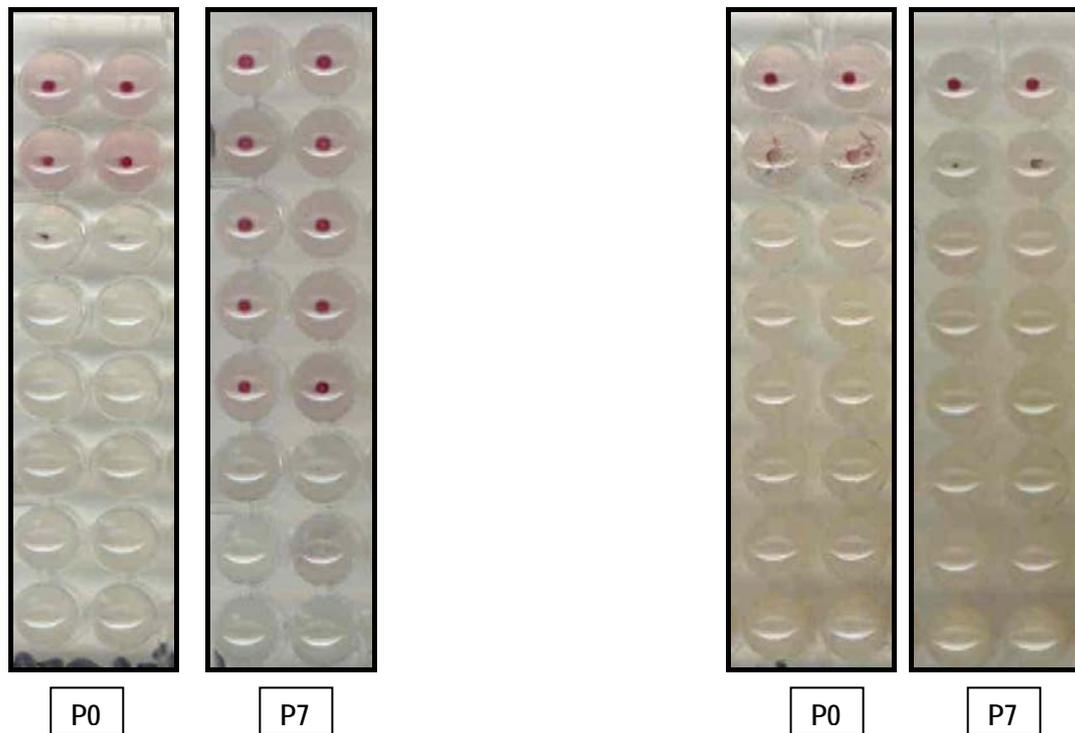


Figure 4. 3. Increase in *E. faecalis* resistance to Gentamicin developed within 7 passages (P) (left) compared to no resistance development to hexane extract of GB (right)

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).

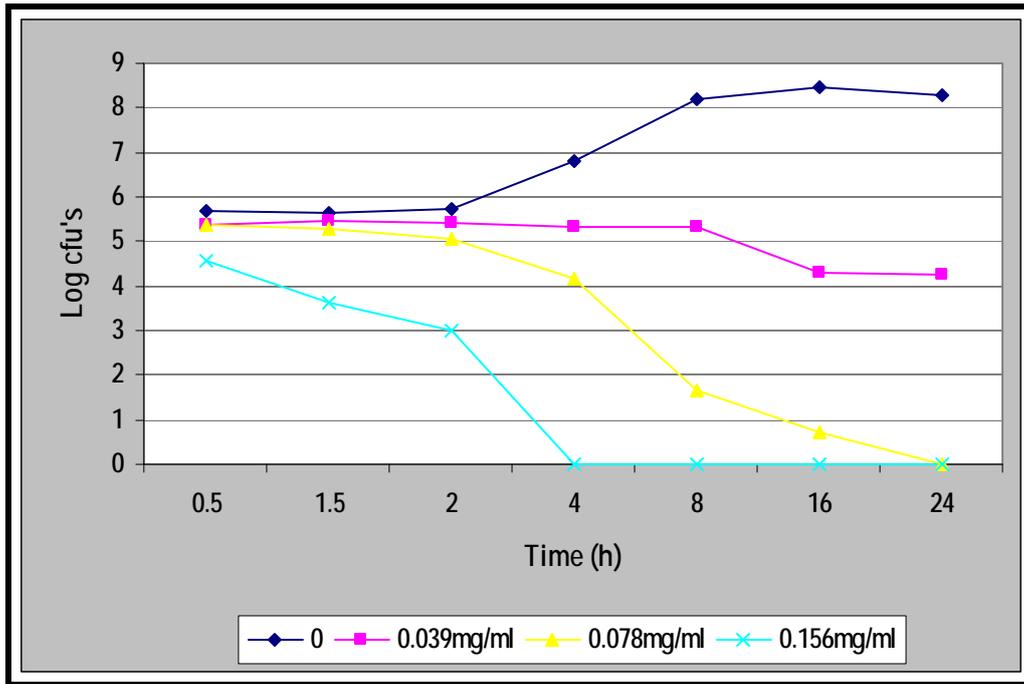


Figure 4. 4. Death kinetics of *E. faecalis* with exposure to different concentrations of hexane extract of *G. biloba* as seen over a 24 h period

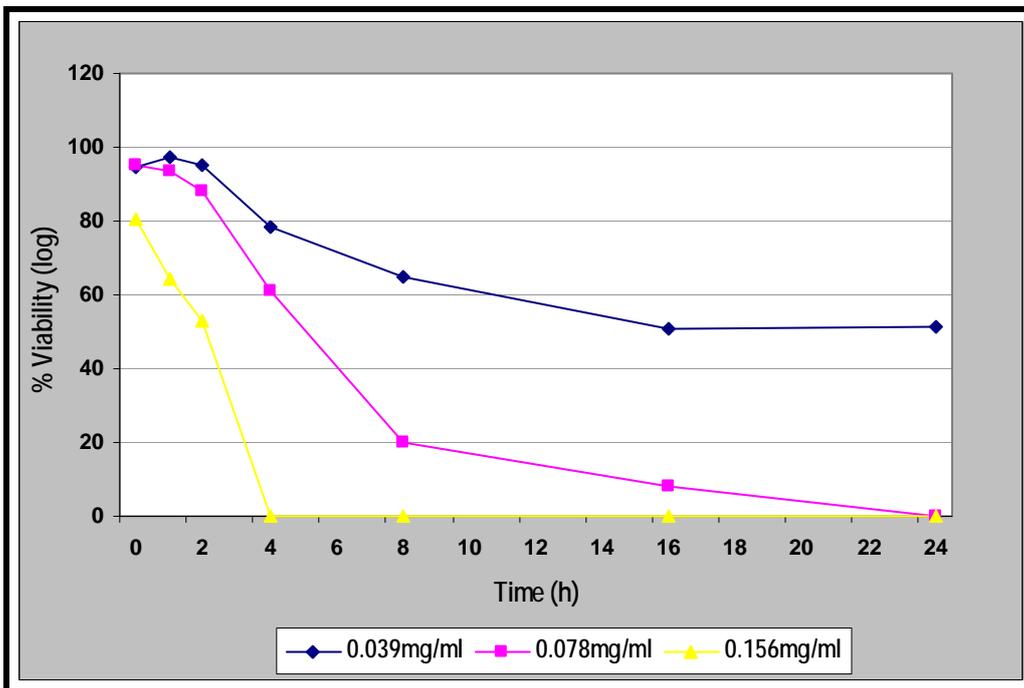


Figure 4. 5. Percentage viability ((Log cfu in treatment/log cfu in control) X100) of *E. faecalis* exposed to different concentrations of hexane extract of *G. biloba* over 24 h

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.

4.5 References

- Barbosa, T.M. and Levy, S.B. (2000). The impact of antibiotic use on resistance development and persistence. *Drug Resistance Update* 3: 303-311.
- Barreto, M.S.R., Menten, J.F.M., Racanicci, A.M.C., Pereira, P.W.Z. and Rizzo, P.V. (2008). Plant extracts used as growth promoters in broilers. *Brazilian Journal of Poultry Science* 10: 109-115.
- The Clinical and Laboratory Standards Institute (CLSI) (2008). Performance Standards for Antimicrobial Tests For Bacteria Isolated from animals. Approved Standard – Third Edition. CLSI document M31-A3 (ISBN 1-56238-659-X), Pennsylvania 19087-1898 USA.
- Eloff, J.N. (1998). A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. *Planta Medica* 64: 711-713
- Hemaiswarya, S., Kruthiventi, A.K. and Doble, M. (2008). Synergism between natural products and antibiotics against infectious diseases. *Phytomedicine* 15: 639-652.
- Hübner, A.T. (2003). Treatment with *Hypericum perforatum* L. does not trigger decreased resistance in *Staphylococcus aureus* against antibiotics and Hyperforin. *Phytomedicine* 10: 206-208.
- Jones, F.T. and Ricke, S.C. (2003). Observations on the history of the development of antimicrobials and their use in poultry feeds. *Poultry Science* 82: 613–617.
- Kleenex, J. and Knipschild, P. (1992). *Ginkgo biloba*. *The Lancet* 340: 1136-1139.
- Klein, G. (2003). Taxonomy, ecology and antibiotic resistance of enterococci from food and the gastro-intestinal tract. *International Journal of Food Microbiology* 88: 123-131.
- Kümmerer, K. (2003). Significance of antibiotics in the environment. *Journal of Antimicrobial Chemotherapy* 52: 5-7.
- Lee, H. and Kim, M. (2002). Selective responses of three *Ginkgo biloba* leaf-derived constituents on human intestinal bacteria. *Journal of Agricultural and Food Chemistry* 50: 1840-1844
- Lister, P.D. (2006). The role of pharmacodynamic research in the assessment and the development of new antibacterial drugs. *Biochemical Pharmacology* 71: 1057-1065.

Martini, N. and Eloff, J.N. (1998). The preliminary isolation of several antibacterial compounds from *Combretum erythrophyllum* (Combretaceae). *Journal of Ethnopharmacology* 62: 255-263.

Pan, J. (2007). Antibacterial actions of ginkgolic acids and related mechanisms. In: *New biocides development*. Chapter 19 pp 389-410.

Rios, J.L. and Recio, M.C. (2005). Medicinal plants and antimicrobial activity. *Journal of Ethnopharmacology* 100: 80-84.

Ward, P.M.L., Fasitsas, S. and Katz, S.E. (2002). Inhibition, resistance development and increased antibiotic and antimicrobial resistance caused by nutraceuticals. *Journal of Food Protection* 65: 528-533.