Chapter 5 Optimal extraction method

5.1 Introduction

Tentative sample clean-up (pretreatment) extractants using 20% acetone or ethanol in water mixtures and the possible use of 80% acetone or ethanol in water mixtures as final extractants were identified, while pretreatment with hexane improved on the extractability of compounds present in leaf samples and led to higher antibacterial activity in final extracts. Eighty percent acetone in water and 80% ethanol in water were better extractants than their individual solvents. All this data was utilized to design tentative optimal extraction methods.

Once the pretreatment measures to be employed in tentative optimal extraction methods were identified, the next important questions to be answered that effectively became the objectives of this chapter were:

(1): How many times should pretreatment procedures be performed on leaf sample to ensure best extraction results?

(2): How much activity would be lost in pretreatment procedures?

(3): How much was gained in the final extract?

The pretreatment procedures were designed and performed as outlined in the Table 5.1.

Table 5:1: Table showing the tentative best extraction methods

Extract	Pretreatment	Expected effects	Possible draw backs
Acetone (1a) or Ethanol (1e)	Direct extraction with hexane (hexane – "wash")	• Removes 4% of inactive material that had high R_f values in EMW system. • Increase extractability by breaking cell membranes	• Pretreatment will not remove inactive compounds with low R_f values in EMW system. • Hexane is not miscible with the final extractants; traces of hexane in sample after pretreatment may affect subsequent extractions.
80% acetone (2a) or 80% ethanol in water (2e)	Direct extraction with 20% acetone/ethanol in water (20% - "wash")	• Selectively extract polar compounds with 80% mixture leaving the non-polar inactive material that had high R_f values in EMW system. • 20% mixture will remove the inactive compounds with low R_f values in EMW system. • Presence of water in extractants increases extractability of water soluble compounds.	• Antioxidant activity will decrease because 20% solvent mixtures extracted some antioxidant compounds • If quantities lost are cumulative (4% + 15%), a lot of material will be lost in pretreatment. This increases chances of losing substantial activity
Acetone (3a) or Ethanol (3e)	Serial extraction with hexane first then 20% acetone/ethanol in water	• Hexane "wash" removes inactive material that had high R_f values in EMW system. • 20% mixture will remove inactives with low R_f values in EMW system. • Increased extractability due to effect of hexane and the water mixtures.	• Hexane is not miscible with the final extractant • Loss of antioxidant activity in 20% "wash".
Crude acetone (4a) / Crude ethanol (4e)	Not applicable	• Crude used as control for comparison.	• Inactive compounds not removed

5.2 Pre-treatment procedures

To determine the optimal number of times "washing" should be carried out before final extraction with acetone or ethanol, pretreatment extractions was repeatedly performed on one g samples in 10 ml of extractant until no visible colour change in the extract was observed.

Table 5:2: Amount in mg extracted from one gram of leaf material during 3 serial extraction procedures on leaf sample using hexane, 20% acetone (20% Acn), and 20% ethanol (20% EtOH).

	Hexane	20% Acn	20% EtOH
1st	36	168	189
2nd	10	36	41
3rd	6	16	13
Total	52	220	243

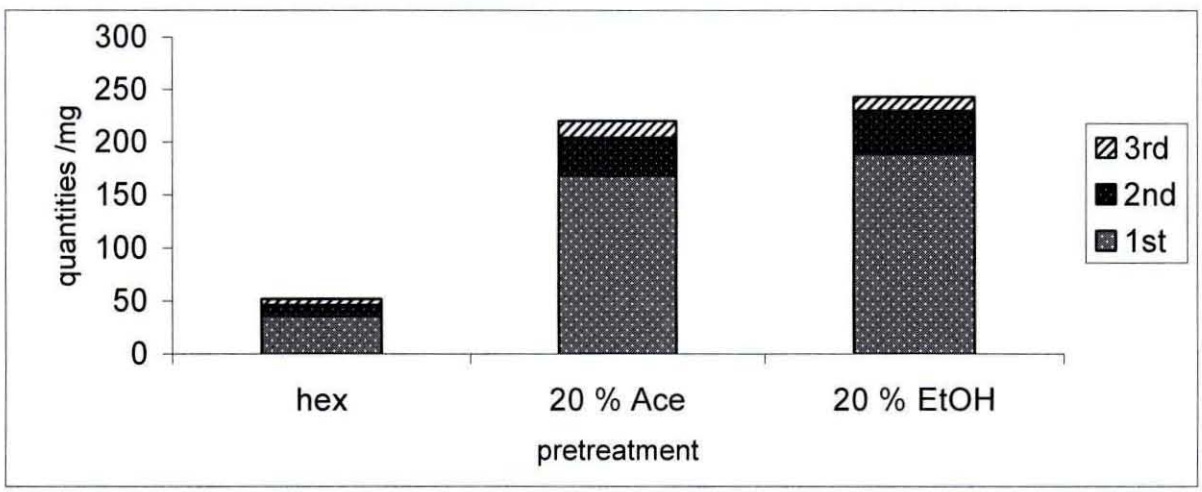

Figure 5:1 Quantities present in the three serial extractions employed in each pretreatment procedure.

Low quantities were extracted by the second and especially the third extractions.

5.2.1 Phytochemical analysis

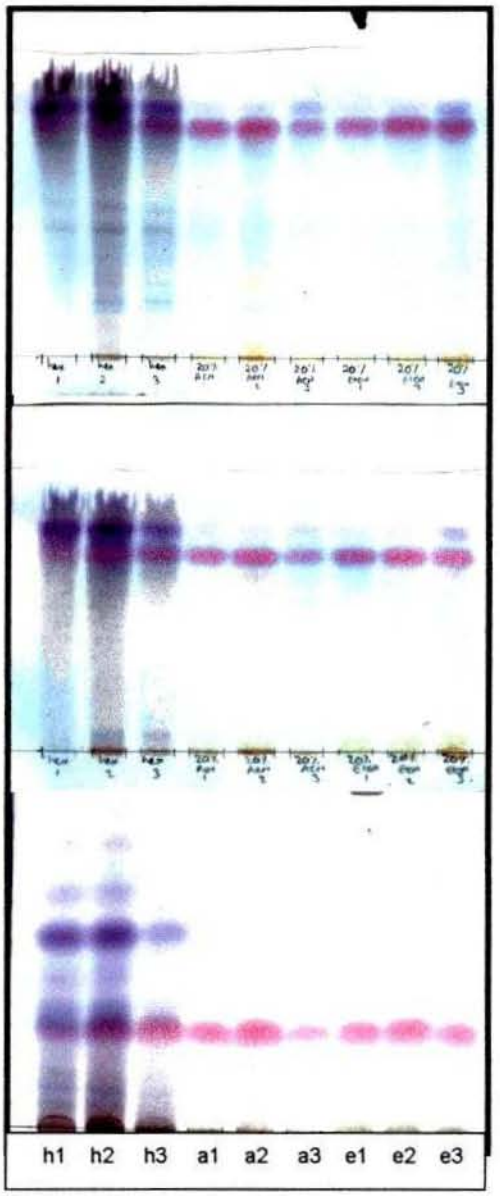

Figure 5:2 TLC profiles of 100 µg of pretreatment extracts developed in EMW (top), CEF (centre) and BEA (bottom) and sprayed with vanillin-sulphuric acid. **Lanes from left to right**: 1st, 2nd and 3rd hexane extracts (h1, h2 and h3 respectively); 1st, 2nd and 3rd 20% acetone in water extracts (a1, a2, a3 respectively) and 1st, 2nd and 3rd 20% ethanol in water extracts (e1, e2, e3 respectively).

The colour of vanillin-active compounds were more intense in the second extractions (h2, a2 and e2) for all pretreatment procedures

5.2.2 DPPH assay

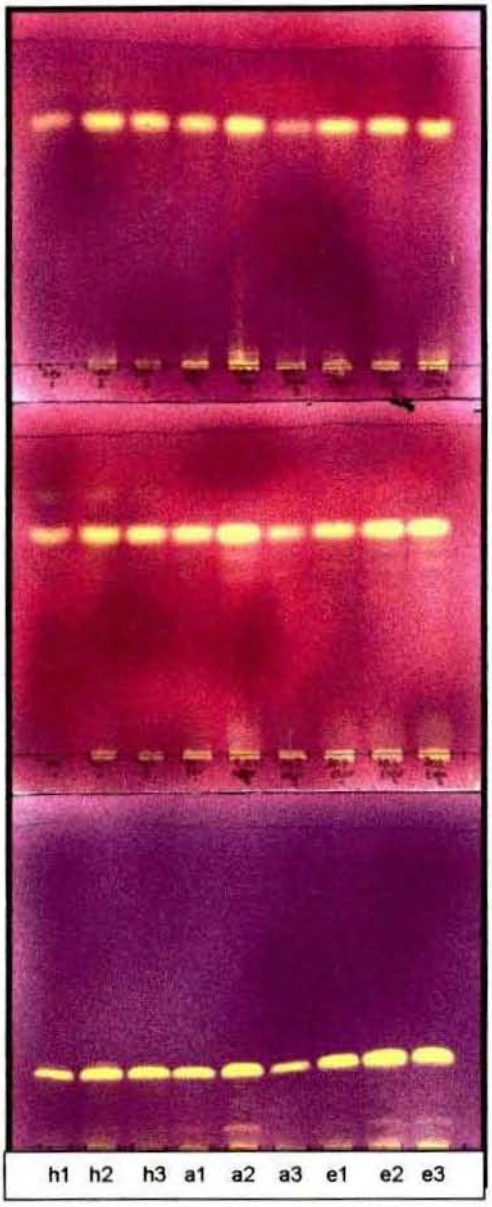

Figure 5:3TLC profiles of 100 µg of pretreatment extracts developed in EMW (top), CEF (centre) and BEA (bottom) and sprayed with 0.2% DPPH in methanol. **Lanes from left to right**: 1st, 2nd and 3rd hexane extracts (h1, h2 and h3 respectively); 1st, 2nd and 3rd 20% acetone in water extracts (a1, a2, a3 respectively) and 1st, 2nd and 3rd 20% ethanol in water extracts (e1, e2, e3 respectively).

All pretreatment extracts contained one of the two major antioxidant compounds, and in all cases, the first extractions (h1, a1, and e1) contained little quantities of this compound relative to the other extracts as evidenced by the smaller size of the inhibition zone.

5.2.3 MIC and total activity lost in pretreatment procedures

To quantify antibacterial activity that would be lost in the serial pretreatment extractions, the MICs of 10 mg/ml extracts after 1st, 2nd, and 3rd extractions in each pretreatment procedure were determined individually using *S. aureus*, *E. coli*, *E. faecalis* and *P. aeruginosa* as test organisms. The MIC method was performed as outlined in 2.5.

Table 5:3: Amount in mg extracted from one g sample in three serial extractions of each pretreatment procedure, MIC values in mg/ml and total activity values (ml) of Hexane "wash", 20% acetone "wash" and 20% ethanol "wash" on *C. woodii* leaves.

	Hexane "wash"			20% Acetone "wash"			20% Ethanol "wash"		
	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd
Quantity	36	46	52	168	204	220	189	230	243
	MIC values (mg/ml)								
E. coli	0.63	0.31	0.31	0.63	0.63	0.63	0.63	0.31	0.31
S. aureus	0.63	0.31	0.31	0.63	0.63	0.63	1.25	1.25	1.25
E. faecalis	0.63	0.31	0.31	0.63	0.31	0.31	0.63	0.31	0.31
P. aeruginosa	0.63	0.63	0.31	0.63	0.63	0.63	0.63	0.63	0.63
Average	0.63	0.39	0.31	0.63	0.55	0.55	0.79	0.63	0.63

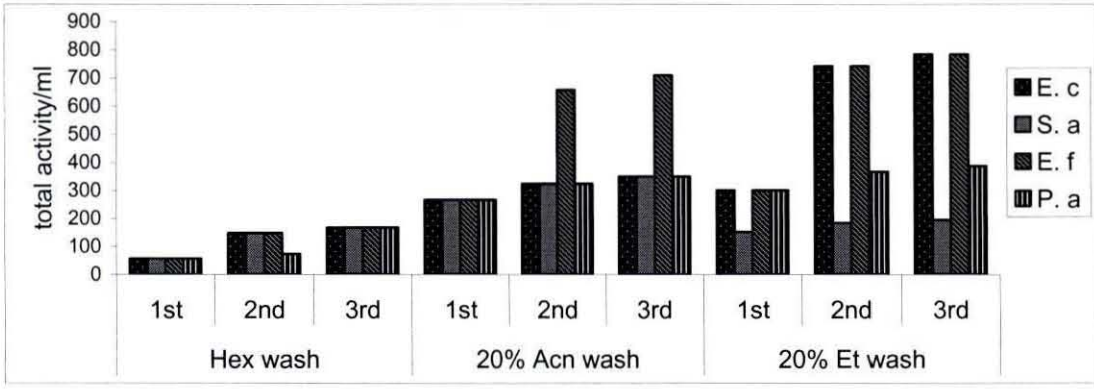

Figure 5:4: Total activity in 1st, 2nd and 3rd serial extracts from the pre-treatment procedures against *S. aureus* (S.a), *E. faecalis* (E.f), *E. coli* (E.c), and *P. aeruginosa* (P.a)

Better MICs were observed in the second and third extractions [Table 5.3] meaning more antibacterial compounds were removed during the second and third extractions.

Since less quantities of material were extracted in the second and third extractions [Table 5.2], and more antibacterial [Table 5.3] and antioxidant [Figure 5.3] activity was being lost in them, it was decided that pretreatment procedures should be performed only once.

Once the optimal number of pretreatment extractions and the antibacterial activity that would be lost in pretreatment was known, the extraction procedures outlined in table 5.1 were adopted as tentative best extraction methods, applying only a single extraction for each pretreatment and allowing the sample to dry before extraction with the final extractant.

Using 10 mg/ml concentrations, phytochemical analysis on the tentative optimal extracts was performed and their antibacterial and antioxidant activity was quantified using MIC method and the TEAC assay as outlined in 2.5 and 2.6 respectively.

5.3 Phytochemical analysis of tentative optimal extracts

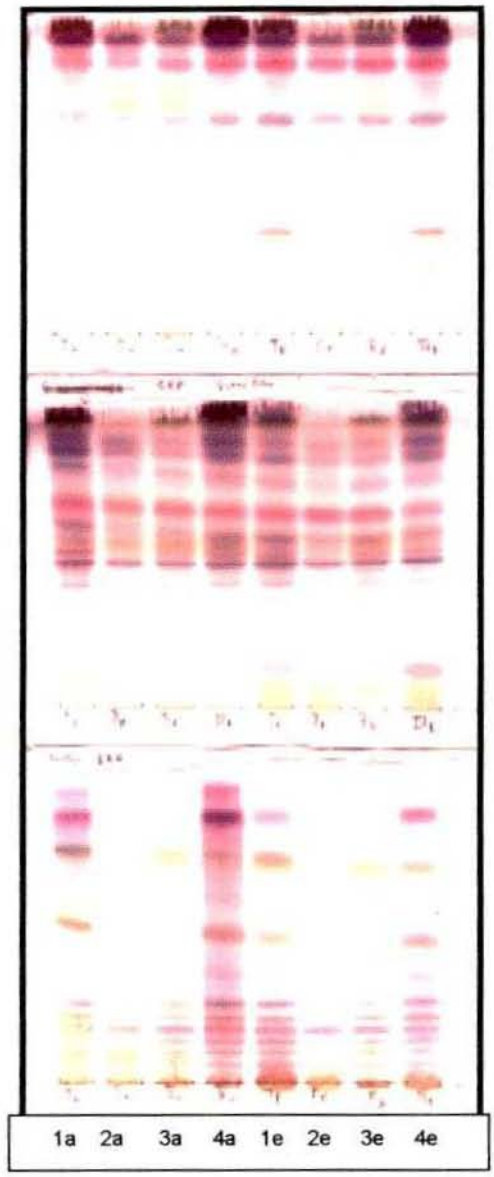

Figure 5:5 TLC profiles of tentative optimal extracts developed in EMW (top), CEF (centre), and BEA (bottom) and sprayed with vanillin-sulphuric acid. **Lanes from left to right:** hexane-pretreated acetone extract (1a), hexane-pretreated ethanol extract (1e), 80% acetone extract pretreated with 20% acetone in water (2a), 80% ethanol extract pretreated with 20% ethanol in water (2e), acetone extract pretreated with hexane first then 20% acetone (3a), ethanol extract pretreated with hexane first then 20% ethanol in water (3e), acetone extract (4a) and ethanol extract (4e).

5.3.1 DPPH assay

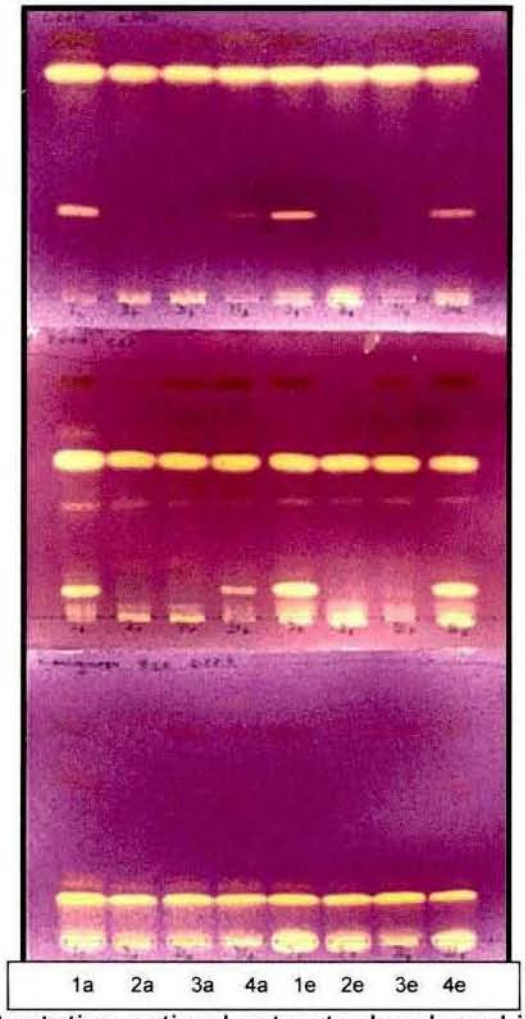

Figure 5:6 TLC profiles of tentative optimal extracts developed in EMW (top), CEF (centre), and BEA (bottom) and sprayed with 0.2% DPPH in methanol. **Lanes form left to right**: hexane-pretreated acetone extract (1a), hexane-pretreated ethanol extract (1e), 80% acetone extract pretreated with 20% acetone in water (2a), 80% ethanol extract pretreated with 20% ethanol in water (2e), acetone extract pretreated with hexane first then 20% acetone (3a), ethanol extract pretreated with hexane first then 20% ethanol in water (3e), acetone extract (4a) and ethanol extract (4e).

Only extracts labelled 1a and 1e contained the same number of antioxidant active compounds as the crude acetone (4a) and crude ethanol (4e) extracts, while the other extracts contained fewer zones of inhibition. This meant that antioxidant activity was being lost in extraction procedures that resulted in extracts labelled 2 and 3 for both acetone (a) and ethanol (e).

5.3.2 Bioautography

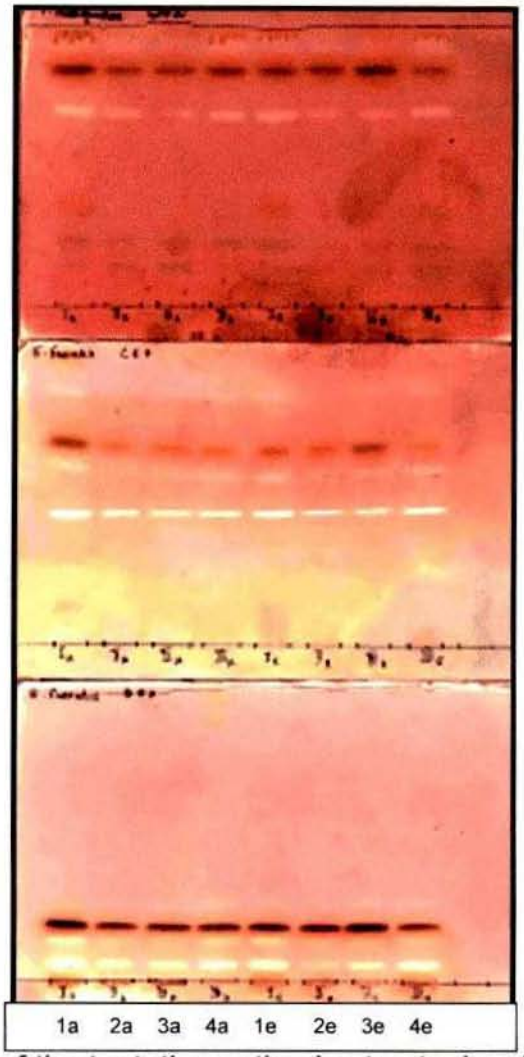

Figure 5:7: Bioautography of the tentative optimal extracts developed in EMW (top), CEF (centre), and BEA (bottom) solvent systems and sprayed with *S. aureus*. **Lanes from left to right:** hexane-pretreated acetone extract (1a), hexane-pretreated ethanol extract (1e), 80% acetone extract pretreated with 20% acetone in water (2a), 80% ethanol extract pretreated with 20% ethanol in water (2e), acetone extract pretreated with hexane first then 20% acetone (3a), ethanol extract pretreated with hexane first then 20% ethanol in water (3e), acetone extract (4a) and ethanol extract (4e).

Extracts 1a and 1e had the same number of antibacterial compounds (3 zones of inhibition) as did acetone (4a) and ethanol (4e) extracts on plates developed in CEF solvent system while extracts 2 and 3 had fewer (2) zones of antibacterial compounds by bioautography.

5.4 MIC and total activity values of the tentative best extraction method

Qualitative analysis on bioautography had shown extracts 2 and 3 to possess lower numbers of antibacterial compounds compared to the crude acetone and ethanol extracts while hexane pretreated extracts had the same numbers of antibacterial compounds [Figure 5.7], however, bioautography results were not sufficient for one to identify the extract that contained the most antibacterial potency. The MIC method was employed to quantitatively discern the antibacterial activity of the different extracts. Ten mg/ml concentrations of tentative best extracts were tested against *S.aureus*, *E. coli*, *E. faecalis* and *P. aeruginosa* as described in 2.5.

5.4.1 Results

Table 5:4: Amount in mg extracted from one gram of leaf sample, the MIC values in mg/ml and total activity values in ml of the hexane-pretreated acetone extract (1a), hexane-pretreated ethanol extract (1e), 80% acetone extract pretreated with 20% acetone in water (2a), 80% ethanol extract pretreated with 20% ethanol in water (2e), acetone extract pretreated with hexane first then 20% acetone (3a), ethanol extract pretreated with hexane first then 20% ethanol in water (3e), acetone extract (4a) and ethanol extract (4e).

	1a	2a	3a	4a	1e	2e	3e	4e
Quantity	84	119	128	82	87	139	110	92
MIC values in mg/ml								
E. coli	0.08	0.16	0.16	0.16	0.08	0.16	0.31	0.16
S. aureus	0.04	0.16	0.16	0.08	0.08	0.16	0.31	0.16
E. faecalis	0.04	0.08	0.16	0.04	0.04	0.16	0.16	0.08
P. aeruginosa	0.16	0.16	0.31	0.31	0.16	0.16	0.63	0.31
Average MIC	0.08	0.14	0.20	0.15	0.09	0.16	0.35	0.18
Total activity values in ml								
	1a	**2a**	**3a**	**4a**	**1e**	**2e**	**3e**	**4e**
E. coli	1050	744	800	513	1088	869	355	575
S. aureus	2100	744	800	1025	1088	869	355	575
E. faecalis	2100	1488	800	2050	2175	869	688	1150
P. aeruginosa	525	744	413	265	544	869	175	297
Average	1444	930	703	963	1223	869	393	694

Table 5:5: Changes in average MIC values of the tentative optimal extracts compared to their crude extracts.

	Activity lost in pre-treatment (ave MIC)	Activity of untreated extract (ave MIC)	Activity of developed extract (ave MIC)	% Improvements in ave MIC.
1a	0.63	0.15	0.08	87.5
2a	0.63	0.15	0.14	7.14
3a	0.63	0.15	0.20	-25
1e	0.63	0.18	0.09	100
2e	0.79	0.18	0.16	12.5
3e	0.71	0.18	0.35	-48.6

5.4.2 Discussion and conclusions

Improvement in average MIC values were realized in hexane-pretreatment acetone and ethanol extracts (1a and 1e, respectively) and 80% acetone or ethanol in water extracts developed by pretreatment with 20% "wash" (2a and 2e, respectively) while extracts labelled 3 had lower antibacterial activity compared to their crude extracts [Table 5.5].

The most significant improvement in antibacterial activity was realized in extracts labelled 1 (87.5% and 100% for the hexane-pretreated acetone (1a) and ethanol (1e) extracts respectively), though the most antibacterial activity was lost in their pretreatment procedure (average MIC value of 0.63 mg/ml) relative to the other extraction procedures (0.71 and 0.79 mg/ml). The improvement in average MIC for extracts labelled 2 were 7.14% and 12.5% for the acetone and ethanol extracts respectively [Table 5.5]. Antibacterial activity was lost in pretreatment procedures employed in development of extracts labelled 3 as illustrated by the poor average MIC values compared to the crude extracts.

Based on these results, extraction procedures labelled 1 were identified as possibly the best procedures. Since the extraction procedures of extracts labelled 2 resulted in improvements in average MICs and the principle aim was to develop an extract with high antibacterial and antioxidant activity, this extraction procedure would be considered if the antioxidant activity of extracts prepared by procedure 2 far outweighs that of extracts prepared by procedure 1.

5.5 Quantification of the antioxidant activity of tentative best extraction method

5.5.1 Introduction

Generally the DPPH assays on TLC of the developed extracts were similar with at least two major antioxidant compounds visible in all extracts. Differences in antioxidant activity, if any, would therefore be discerned by methods that quantify antioxidant activity.

The most widely used methods for measuring antioxidant activity are those that involve the generation of a radical species and measurement of a range of end points at a fixed time or over a range (Re *et al.*, 1999). Two types of approaches may be followed, namely the inhibition assays where the extent of the scavenging by hydrogen or electron donation of a preformed free radical is the marker of antioxidant activity as well as assays involving the presence of antioxidant system during the generation of the radical (Re *et al.,* 1999). One difficulty in assessing antioxidant activity is to select which method to be used. Different methods seem to give different results and methods have their advantages and disadvantages.

This study made use of the Trolox equivalent antioxidant capacity (TEAC) assay. This assay is frequently used to rank antioxidants and for the construction of structure activity relationships. It has the major advantage that it is applicable to both aqueous and lipophilic systems (Re *et al.,* 1999). TEAC is a decolourization assay that measures antioxidant activity in relation to Trolox, a water-soluble vitamin E analogue (Re *et al.*, 1999).

5.5.2 Method

The TEAC assay was performed as outlined in 2.6.2.

5.5.3 TEAC results

A Trolox standard line was prepared by plotting percentage inhibition of the $ABTS^+$ radical against concentration of Trolox. The curve had a gradient of 169.98 and a percentage fit of 98.5%.

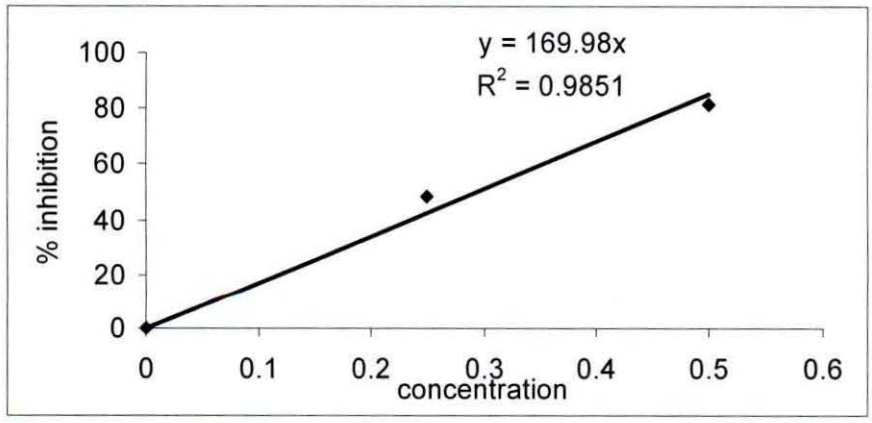

Figure 5:8 Standard curve of% inhibition of $ABTS^+$ against concentration of Trolox after 6 minutes of reaction time.

Data from the tentative optimal extracts was analyzed in a similar manner and the gradient obtained for the different extracts was divided by the gradient of Trolox resulting in a Trolox equivalent value.

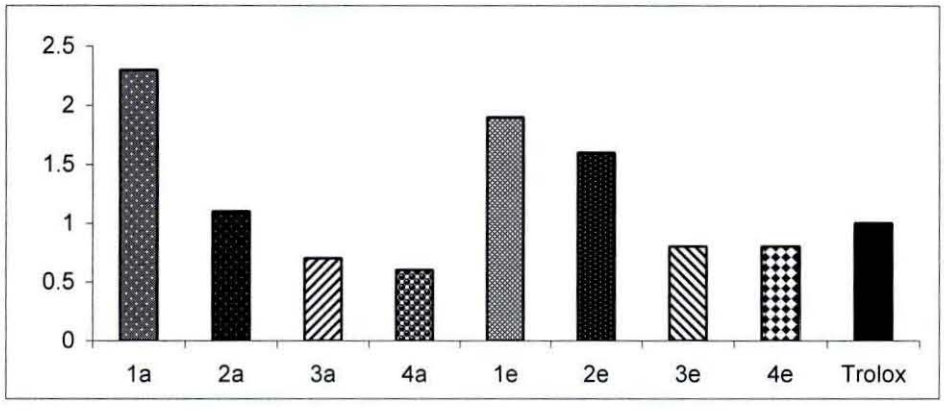

Figure 5:9 Comparison of TEAC values of the hexane-pretreated acetone extract (1a), hexane-pretreated ethanol extract (1e), 80% acetone extract pretreated with 20% acetone in water (2a), 80% ethanol extract pretreated with 20% ethanol in water (2e), acetone extract pretreated with hexane first then 20% acetone (3a), ethanol extract pretreated with hexane first then 20% ethanol in water (3e), acetone extract (4a) and ethanol extract (4e).

Table 5:6: Magnitude of change in TEAC values of the tentative optimal extracts compared to their respective crude extracts.

Extract	TEAC value	Un-pretreated (crude) extracts	% Increase In TEAC
1a	2.3	0.6	283.3
2a	1.1	0.6	83.3
3a	0.7	0.6	16.7
1e	1.9	0.8	137.5
2e	1.6	0.8	100
3e	0.8	0.8	0

Except for extract labelled 3e, the tentative optimal extracts had higher antioxidant activity compared to their respective crude extracts. Extract labelled 1a had the highest antioxidant activity with a TEAC value of 2.3, and a percentage increase in TEAC value of 283% compared to the crude acetone extract, while extract labelled 1e was second best with a TEAC value of 1.9 and percentage improvement of 137.5% from the crude ethanol extract.

5.5.4 Discussion and conclusions

The TEAC assay is widely applied to assess the total amount of radicals that can be scavenged by an antioxidant, i.e. the antioxidant capacity, however TEAC values reported in the literature are variable. It appears that the TEAC value largely depends on the assay conditions. The most important reason for this variation is that the reaction of an antioxidant with $ABTS^{+}$ usually does not reach completion within the time span applied (van den Berg *et al.*, 1999). Furthermore, the TEAC assay measures the antioxidant capacity of the parent compound plus that of the reaction products. These reaction products may have a considerable contribution to the TEAC value (Arts *et al.*, 2004).

The major limitation of this procedure is that the concentration range of the antioxidant that can be used is relatively small. A too high antioxidant concentration would need too much $ABTS^{+}$, giving a too high absorbance for a reliable fit of the curve. A too low concentration of the antioxidant would result in a decrease in $ABTS^{+}$ absorbance too low to be measured accurately (van den Berg *et al.*, 1999).

Despite its drawbacks, the TEAC assay is a useful tool for tracking down unknown antioxidants in complex mixtures. For this application, the TEAC assay has been used with success (van Overveld *et al.*, 2000).

Though the majority of enriched extracts showed an increase in antioxidant activity as evidenced by their improved TEAC values compared to their respective crude extracts, only extracts labelled 1a, 2a, 1e and 2e had higher TEAC values than Trolox. Extract 1a had a TEAC value 2.3 times greater than that of the Vitamin E analogue. The lower TEAC values in the extracts developed by pretreatment procedures involving the use of 20% acetone or ethanol in water mixtures could be explained by the fact that DPPH assay on TLC showed 20% acetone or ethanol in water mixtures to retain one of the two major antioxidant compounds in large quantities as evidenced by the large zone of inhibition. This meant that one of the two major antioxidant compounds was lost in these pretreatment procedures hence the lower TEAC values.

Based on MIC values and the results obtained from the TEAC assay, the hexane-pretreated acetone extract (extract labelled 1a) was selected as the optimal extract.

Chapter 6 *In vitro* antibacterial and toxicity tests on the optimal extract

6.1 Introduction

The optimal extract is intended for commercial application as a feed additive in the broiler industry thus, the next important steps were to test its *in vitro* activity against common poultry pathogens and to ascertain the toxicity profile of the enriched extract.

Some enteric bacteria in poultry are zoonotic and may cause diseases in humans e.g. *Campylobacter spp.* cause abdominal pain and diarrhoea in man and *Salmonella spp.* cause typhoid (Aarestrup, 1999). A newsletter article from the meat industry insights dated October 23 1997 stated that *Salmonella enteritidis, Campylobacter jejuni* and *Clostridium perfringens* have been found in 79% of fresh and frozen chickens bought in butcheries and supermarkets in the United States of America. The continued supplementation of antibiotics in feed may lead to development of resistant strains of these bacteria that can be transmitted to humans via the food chain (Aarestrup, 1999). Perpetual use of existing drugs also results in the development of multi drug-resistant strains of bacteria.

The best way to circumvent the proliferation of resistance would be to develop novel antimicrobial agents, which inhibit these new bacterial strains (Coleman, 2004). The effectiveness of the optimal extract in this regard was assessed against two strains each of *Campylobacter jejuni, Clostridium perfringens, Salmonella enteritidis, E. coli* and multi drug-resistant *E. coli* isolated from chickens.

To establish whether the optimal extract had the potential to be toxic in poultry, basic toxicity screening test were performed using the brine shrimp assay and the MTT assay on cell culture. These assays were chosen because they are relatively cheap and easy to conduct.

6.2 Antibacterial activity of the best extract against poultry enteric bacteria

6.2.1 Introduction

Campylobacter jejuni is one of the most common bacterial causes of diarrhoeal illness in humans. It grows best at the body temperature of a bird, and seems to be well adapted to birds, which carry it without becoming ill. The bacterium is fragile; it cannot tolerate drying and can be killed by oxygen. It grows only if there is less than the atmospheric concentration of oxygen present. Many chicken flocks are infected with the organism but show no signs of illness. *Campylobacter* can be easily spread from bird to bird through a common water source or through contact with infected faeces. When an infected bird is slaughtered, *Campylobacter* can be transferred from the intestines to the meat (Frediani-Wolf and Stephan, 2003).

Clostridium perfringens is an anaerobic, spore-forming rod. It is widely distributed in the environment and frequently occurs in the intestines of humans and many domestic and feral animals. Spores of the organism persist in soil, sediments, and areas subject to human or animal faecal pollution (Johansson *et al.*, 2004).

Escherichia coli is a Gram-negative bacterium. There are hundreds of strains of this bacterium. Although most of them are harmless and live in the intestines of healthy humans and animals, some strains produce toxins and can cause severe illness. Infection often leads to bloody diarrhoea, and occasionally to kidney failure. Most illness has been associated with eating undercooked, contaminated meat. Infection can also occur after drinking raw milk and after swimming in or drinking sewage-contaminated water (Schroeder *et al.*, 2004).

Most types of *Salmonella* live in the intestinal tracts of animals and birds and are transmitted to humans by contaminated foods of animal origin. *Salmonella enteritidis* infects the ovaries of healthy appearing hens and contaminates the eggs before the shells are formed, it can therefore be inside normal-appearing eggs, and if the eggs are eaten raw or undercooked, the bacterium can cause illness (Wierup, 2000).

6.2.2 Method

The MIC values were determined as described in section 2.5. Antibacterial activity of the optimal extract was tested against two strains each of *Campylobacter jejuni, Clostridium perfringens, Salmonella enteritidis, E. coli* and multi-drug resistant *E. coli* isolated from chickens obtained from the Microbiology Laboratory, Faculty of Veterinary Science, University of Pretoria. *E. coli* ATCC 28922 was used as a control organism and was tested against gentamicin. The organisms were all grown under aerobic conditions, therefore gentamycin, which requires oxygen for activity, was a valid choice as positive control.

6.2.3 MIC results and discussion

Table 6:1: MIC values in mg/ml, total activity in ml and the mean and standard deviation (SD) of the MIC values.

	MIC values (mg/ml)			Mean	S.D	Total
	1	2	3			activity (ml)
Campylobacter jejuni 26/7	0.08	0.04	0.08	0.07	0.02	1260
Campylobacter jejuni B603/96	0.16	0.16	0.31	0.21	0.07	400
Clostridium perfringens B805/96	0.04	0.04	0.08	0.05	0.02	1575
Clostridium perfringens Bs2	0.08	0.08	0.08	0.08	0.00	1050
E. coli ATCC 28922	0.08	0.08	0.08	0.08	0.00	1050
E. coli P1016/04	0.16	0.16	0.16	0.16	0.00	525
E.coli P1008/04	0.31	0.16	0.16	0.21	0.07	400
E. coli P1493 (multi resistant)	0.63	0.63	0.63	0.63	0.00	133
E. coli P1497 (multi resistant))	0.63	0.63	1.25	0.84	0.29	100
Salmonella enteritidis 215	1.25	1.25	1.25	1.25	0.00	67
Salmonella enteritidis 57/2001	1.25	1.25	1.25	1.25	0.00	67

The MIC can give an indication as to the concentration of antimicrobial needed at the site of infection to inhibit the growth of the pathogen. MIC values of the optimal extract against poultry pathogens were understandably higher than values expressed for single antibiotics as outlined by the National Committee for Clinical Laboratory Standards (NCCLS, 1994). Values for enrofloxacin for example, varied between 0.03 µg/ml and 4 µg/ml, oxytetracycline from 0.5 µg/ml to 64 µg/ml and for gentamicin between 0.25 and 32 µg/ml against the same ATCC strains of *E. coli, S. aureus* we used and other common pathogens.

Campylobacter jejuni and *Clostridium perfringens* were relatively sensitive with MICs varying from 40 µg/ml to 80 µg/ml. Though less sensitive than *Campylobacter jejuni* and *Clostridium perfringens*; *E. coli* and *Salmonella enteritidis* also showed some sensitivity with MIC values of 1250 µg/ml for both strains against to the optimal extract.

6.3 *In vitro* toxicity tests of the optimal extract

In vitro assays are important and useful tools in toxicity assessment of various compounds and extracts not only because they significantly reduce evaluation time, but also because they are usually less expensive, more quantitative and more reproducible than *in vivo* studies. They are also useful and necessary for screening purposes to define dose and time-dependent cytotoxicity, considered primarily as the potential of a compound to induce cell death, in different cell types (Eisenbrand *et al.*, 2002). On the other hand, the possibility of obtaining a false positive result is of particular concern when using bioassays. False positives may also be due to compounds that are apparently non-toxic to higher animals, but toxic to lower biological organisms, tissue or cells (Pangrahi, 1993). For the reversed argument false negatives may also be obtained. This study made use of the brine shrimp assay and the MTT assay on monkey kidney cells to assess *in vitro* cytotoxicity of the optimal extract.

6.3.1 The brine shrimp assay

The brine shrimp assay is an indicator of cytotoxicity and may be a predictor of effects on cancer cells. Fang *et al.*, (1991) found a positive correlation between brine shrimp toxicity and 9KB (human nasopharyngeal carcinoma) cytotoxicity. Although the brine shrimp assay is rather inadequate regarding the elucidation of the mechanism of action, it is a useful tool in assessing the toxicity of extracts.

6.3.1.1 Method

The brine shrimp assay was performed as outlined in 2.7.1.

6.3.1.2 Results

Brine shrimp assay results were analysed by plotting percentage mortality of brine shrimps against the different concentrations of the extract. The curve had a percentage fit of 93.39% and the equation of the curve was, $y = 27.452 \text{ Ln } (x) + 54.045$.

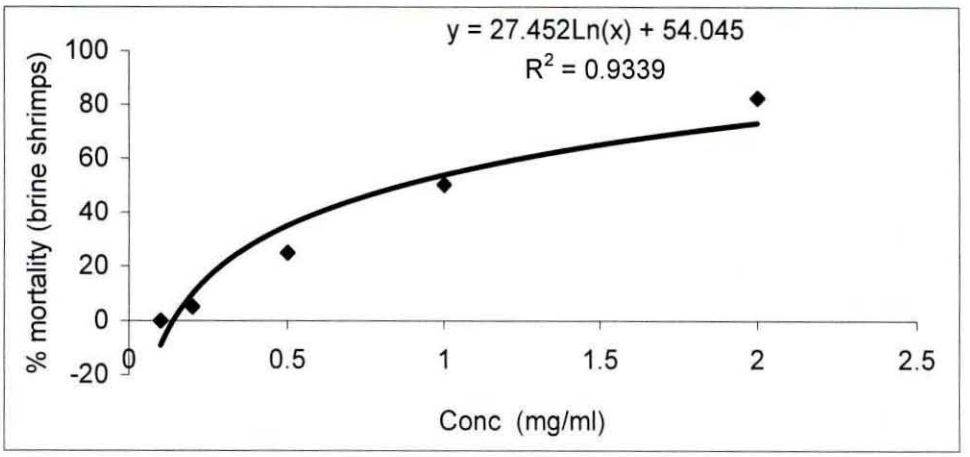

Figure 6:1 Brine shrimp assay curve.

The LC_{50} value was calculated by substituting 50% for y into the equation of the curve. The optimal extract was relatively non-toxic with an LC_{50} value of 863 µg/ml compared to 7 µg /ml for the Podophyllotoxin standard.

6.3.2 The MTT cytotoxicity assay

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, first described by Mosmann (1983), is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form dark blue formazan crystals which are largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. Solubilisation of the cells by the addition of a detergent results in the liberation of the crystals that are also solubilized. The number of surviving cells is directly proportional to the level of the formazan product created. The colour can then be quantified using a simple colorimetric assay. The results were read on a multiwell scanning spectrophotometer (Mosmann, 1983).

6.3.2.1 Method

The MTT assay was performed as outlined in section 2.7.

6.3.2.2 Results

The curve for Berberine chloride standard had a 91.66% fit and gave an equation of y = -0.3234x + 0.6284. The LC_{50} was calculated by substituting for y half the value of absorbance at 540 nm for the control (0.459)

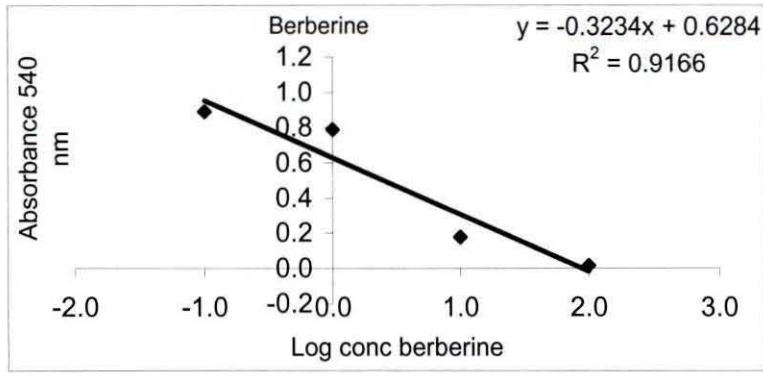

Figure 6:2 MTT cytotoxicity assay curve for Berberine chloride.

The LC $_{50}$ value for Berberine chloride was therefore 3.3 µg/ml (published results give an LC_{50} value of 10 µg/ml).

Cytotoxicity of the best extract was analysed at 540 nm for 1 mg/ml, 0.1, 0.01 and 0.001 mg/ml concentrations.

Table 6:2: Results showing absorbance values at 540 nm for the various optimal extract concentrations

Conc.	Log conc.	Ave abs 540nm	SD
1	0.000	0.290	0.029
0.1	-1.000	0.485	0.040
0.01	-2.000	0.946	0.042
0.001	-3.000	0.926	0.052

The results were analysed by plotting the logarithm of different concentrations of the extract versus absorbance values at 540nm. The curve had a percentage fit of 87.67% and the equation of the curve was, y = -2368 (x) + 0.3062.

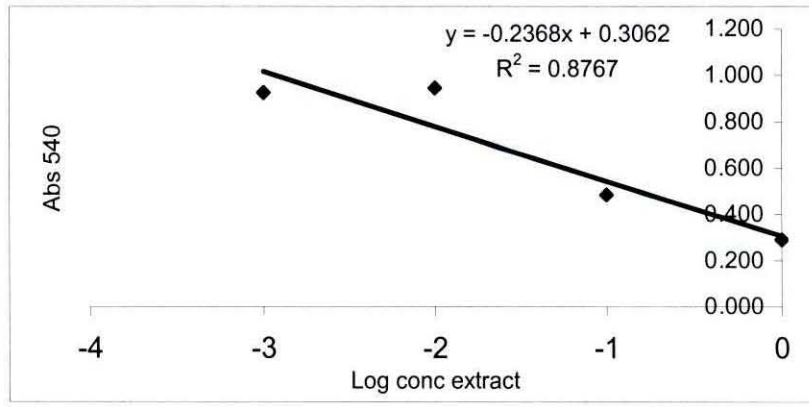

Figure 6:3 MTT cytotoxicity assay curve for the best extract.

LC_{50} was calculated by substituting for y by half the value of absorbance at 540 nm for the control (0.459). The LC_{50} was a relatively non-toxic value of 226 µg/ml compared to 3.3 µg/ml of the Berberine chloride standard.

6.4 Discussion and conclusions

The optimal extract had cytotoxic effects in the brine shrimp assay with an LC_{50} of 863 µg /ml. The MTT cytotoxicity assay on monkey kidney cells gave an LC_{50} value of 226 µg /ml. The LC_{50} values from these two assays are close and comparable.

According to the prevailing view of the MTT assay, respiratory chain activity is the sole MTT reducing activity and thus the marker for viable cells. However, in certain cell types the situation seems to be more complex than that. In a study with a cell line derived from rat brain tumours it was reported that the mitochondria are not the exclusive site of MTT reduction (Liu *et al.*, 1997). It was observed that various sub-cellular fractions could reduce MTT when supplied with NADH or NADPH and the intracellular MTT formazan granules did not accumulate in mitochondria, endoplasmic reticulum, or Golgi apparatus, but partially co-localize with endosomes or lysosomes. Furthermore, based on inhibition experiments it was concluded that the investigated cellular MTT-reductase is an N-ethylmaleimide sensitive flavin oxidase. Although these studies made the exclusive role of mitochondria in MTT reduction questionable, they did not question the validity and usefulness of the MTT assay because even if the MTT assay measures endocytosis, it would be based on a fundamental feature of living cells (Liu *et al.*, 1997).

A crucial point in discussing the relevance of LC_{50} values obtained in the brine shrimp assay is the question of whether the mortality data can be tied to a more specific activity. A general correlation of brine shrimp toxicity with special types of bioactivity seems invalid. However, in various cases it was possible. Fang *et al.*, (1991) investigated the usefulness of the brine shrimp assay as an antitumour pre-screen for plant extracts and was able to determine a positive correlation between brine shrimp lethality and cytotoxicity towards 9KB cells (cell line derived from the human carcinoma of the nasopharynx used as an assay for antineoplastic agents), while Solis *et al.*, (1993) found the brine shrimp assay to be predictive of KB-cell cytotoxicity except for compounds requiring metabolic activation, since the brine shrimp lack the necessary cytochrome P-450 enzyme.

Several cytotoxic stilbenes have been isolated from *C. caffrum* a plant closely related to *C. woodii* and these compounds have different degrees of *in vitro* cytotoxicity against various tumor cell lines and are considered potential lead compounds for the development of new chemotherapeutic agents. A *cis* – stilbene, combretastatin B5 has also been isolated from *C. woodii* by Famakin (2002) but its cytotoxicity was not evaluated, however, the wide application of combretastatins in cancer therapy is owing to their antineoplastic activity. The toxic effect of the optimal extract on monkey kidney cells and brine shrimp may be due to the antineoplastic or antimitotic activity of combretastatin B5.

Relative safety margin indicates of the number of times the effective concentration is lower than the LC_{50} concentration of the optimal extract and is calculated using the LC_{50} and MIC values.

Table 6:3 Relative safety margin (using LC_{50} value from the brine shrimp assay) of the optimal extract.

	Average MIC (mg/ml)	LC_{50} / MIC
Campylobacter jejuni	0.14	6.2
Clostridium perfringens	0.07	12.3
E. coli (ATCC 28922)	0.08	10.8
E. coli	0.19	4.5
Multi-resistant *E. coli*	0.79	1.1
Salmonella enteritidis	1.25	0.7

Although the MIC values of the optimal extract are above the MIC values of currently used antibiotics, the optimal extract was relatively non-toxic, the latter result means that the relative safety margin (LC_{50}/MIC) of the optimal extract was large, this allows for large quantities of the optimal extract to be incorporated in feed without causing toxic reactions in the broilers The effectiveness of the extract as a feed additive would further be aided by its high antioxidant activity.

Plant extracts contain thousands of compounds; an activity 10-1000 times lower on mass basis than currently used antibiotics is still exciting. Because a pure compound with inactive components removed may have excellent biological activities. In the next section an attempt was made to isolate the main antioxidant compound that seems to occur at a very high concentration.