CHAPTER 4

ANTIBACTERIAL AND ANTIOXIDANT ACTIVITY OF ISOLATED COMPOUNDS

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4.1 Introduction

Bacterial diseases continue to present a major threat to human health. Tuberculosis, for instance, rank among the world's leading causes of death. *Streptococcus*, another bacterium, continues to be a frequent cause of life threatening infections during the first two months of life. Food-borne and water-borne bacteria such as *Samonella* and *Campylobacter* are responsible for a recent troubling increase in diarrhoea related diseases. Meanwhile, during the last decade, scientists have discovered many new organisms and new strains of many familiar bacteria, such as *Escherichia coli*. Emerging bacterial diseases present a clear challenge to biomedical researchers (NIAID, 2007; Mandell *et al.,* 2005). Suffering caused by these pathogens is worsened by their development of resistance to antibiotics.

The complexity of this challenge is becoming even clearer as researchers begin to appreciate the many unsuspecting mechanisms that bacteria have for causing trouble to human beings. For example, gene transfer among different strains of bacteria, and even between different species of bacteria, is now understood to be a common means whereby these organisms acquire resistance to antibiotics. Basic research has also discovered that some bacteria may play a major role in certain chronic diseases not formerly associated with bacterial infection. The bacterium *Helicobacter pylori*, for example, have been found to cause stomach ulcers and may contribute to stomach cancer; Guillain Barré syndrome has been associated with prior diarrhoea related diseases caused by *Campylobacter jejuni* (NIAID, 2007; Mandell *et al.,* 2005).

In addition, the frequency of serious nosocomial bacterial and fungal infections is rising due to the use of newer and more powerful antimicrobial agents. As additional new antimicrobial agents are being found, micro-organisms become more resistant to existing chemotherapies. Thus, there is continuous need to develop novel antimicrobial compounds that would be effective against these and other pathogens (Zgoda and Porter, 2001).

4.2 Materials and Methods

4.2.1 Qualitative determination of antibacterial activity

The direct bioautography method described by Begue and Kline (1972) was used. TLC plates were spotted with compounds or fractions and developed in a solvent system: 3-10% methanol in chloroform. The developed plates were allowed to dry overnight under a stream of air to remove residual solvent, which might inhibit bacterial growth. The two cultured bacteria (*Escherichia coli* and *Bacillus cereus*) were then sprayed on the TLC plates (i.e. *E. coli* on one plate and *B. cereus* on another) and incubated at 37℃ in humid conditions. After incubation, plates were sprayed with 2 mg/ml solution of p-iodonitrotetrazolium violet [INT] (Sigma) (Section 2.2.2.2). Clear zones on the chromatograms indicated inhibition of growth after incubation (Fig. 4.1).

4.2.2 Quantitative determination of antibacterial activity

Round bottom sterile 96-well microplates (0.5 ml volume, Fisher Scientific) were used to determine minimal inhibitory concentration (MIC) of the compounds isolated (Eloff, 1998). The stock solution of positive control (streptomycin) and the compounds were dissolved in 5% dimethylsulfoxide (DMSO) (Sigma) so that the concentration of DMSO in the microwells was less than 1% (Langfield *et al*., 2004). The stock solution was kept on ice until used. The concentrations of compounds in the micro-wells ranged from 1.56 to 200.00 µg/ml. The control and compounds were tested in duplicate.

A multipipettor was used to dispense 100 µl of nutrient broth (Merck Chemicals) into all the wells on the microplate. Hundred micro-litres of control were dispensed into each of the first two wells in role A. The same amount of solution containing each of the compounds was introduced into the subsequent two wells on the row. Using a multipipettor set at 100 µl, the control and compounds in the wells were mixed by sucking up and down 6-8 times without splashing. Serial dilutions of the compounds and the control in row A were made by withdrawing 100 µl of the mixture from the wells and transferring these to wells in row B. Similarly, 100 µl of solution in wells on row B were transferred to row C after mixing them for 6-8 times. This procedure

was repeated down the columns to row H. 100 µl of the solutions were withdrawn and discarded from the wells in the last row (H).

One hundred microlitres of cultured bacteria (Section 2.2.2.2) were dispensed into all the wells except the ones in the last two rows (G and H) which were for sterility control and incubated overnight at 37°C. To indicate bacterial growth 40 µl of p-INT solution (0.20 mg/ml) was then added to each well and the plates incubated for an additional ½ hour to 1 hour.

4.2.3 Antimycobacterial testing

The radiometric BACTEC method was used to determine the antimycobacterial activity of the isolated compounds on the H37Rv (reference, drug susceptible) strain of *Mycobacterium tuberculosis* (Lall and Meyer, 2001; Lall *et al.,* 2003). The H37Rv strain was obtained from the American Type Collection (Rockville, MD, USA). The principle of the method is based on the metabolism of the ^{14}C-labelled substrate (palmitic acid) present in the BACTEC 12B broth (7H12 medium) by viable bacteria to produce ^{14}C-labelled carbon dioxide. The amount of $^{14}CO_2$ detected which is reflected by the rate and amount of growth occurring in the sealed vial, is expressed in terms of the growth index (GI) (Mativandlela *et al.*, 2005).

A homogenized culture (0.1 ml) of the strain of *M. tuberculosis* (H37Rv), yielding 1 x 10^4 – 1 x 10^5 colony forming U/ml (CFU per ml), were inoculated in the vials containing the compound, as well as in the control vial. Two compound free vials were used as controls: one vial was inoculated in the same way as the vials containing the compound, and the other was inoculated with a 1:100 dilution of the inoculum (1:100 control), to produce an initial concentration representing 1% of the bacteria population (1 x 10^2 – 1 x 10^3 CFU per ml) found in the vials containing the compound. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of the compound that inhibited more than 99% of the bacterial population.

Inoculated bottles were incubated at 38ºC and each bottle was assayed everyday at about the same time until the cumulative results were interpretable. The difference in the GI values of the last 2 days is designated

as ΔGI. The reading of the vials containing the compound was compared with the control vial, containing a 1:100 dilution of the inoculum. Readings were taken until the control vials containing a 100 times lower dilution of the inoculum, than the vial with the compound, reached a GI of 30 or more. If the ΔGI value of the vial containing the compound was less than the control the population was reported to be susceptible to the compound. All compounds (steenkrotin A, steenkrotin B and the indane) were tested in triplicate (Lall and Meyer, 2001).

4.2.4. Antioxidant testing

The antioxidant activity was assessed by measurement of the scavenging ability of the isolated compounds on the free radical 2, 2'-diphenyl-1-picryldrazyl (DPPH) ($C_{18}H_{12}N_5O_6$). The radical DPPH is reduced to the corresponding colourless hydrazine upon reaction with hydrogen donors (Pandey et al. 2005). Both qualitative and quantitative assays were performed. DPPH was obtained from Fluka Chemie AG, Bucks. Ascorbia acid (Sigma) was used as the control. DPPH (4 mg) was dissolved in MeOH (50 ml) to obtain a concentration of 80 µg/ml.

4.2.4.1 Qualitative assay

The compounds tested were applied on a TLC plate and sprayed with DPPH solution using an atomiser. It was allowed to develop for 30 min. The colour changes (purple on white) were then noted (Kumarasamy *et al.,* 2002) (Fig. 4.2).

4.2.4.2 Quantitative assay

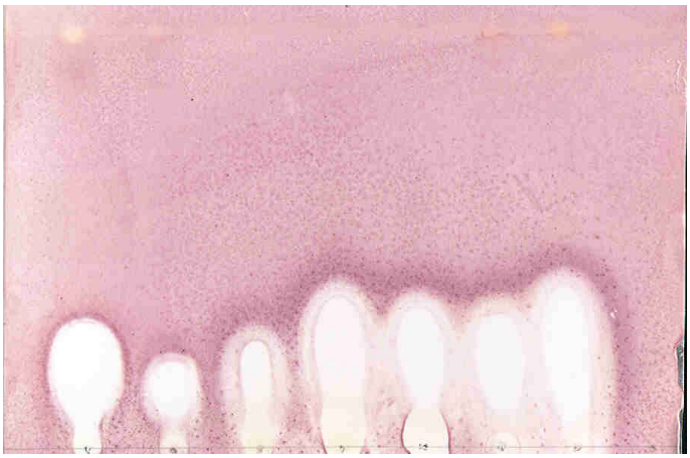
The ethanol extract and compounds were dissolved in MeOH to obtain a concentration 3 mg/ml and 1 mg/ml respectively. Serial dilution of the extract, compounds and control were made on a 96-welled plate. The extract was diluted to obtain final concentrations of 1 000.0, 500.0, 250.0, 125.0, 62.5, 31.3, 15.6 and 7.8 µg/ml. The compounds (except quercetin), ethanol extract and control (ascorbic acid) (Table 4.2) were diluted to obtain final concentrations of 500.0, 250.0, 125.0, 62.5, 31.3, 15.6, 7.8 and 3.9 µg/ml. The quercetin was tested at a final concentration of 0.05 µg/ml. The diluted

solutions (100 µl) were mixed with DPPH (100 µl) and allowed to stand for half an hour to allow for any reaction to occur. A negative control (blank) was also included in the assay. The wells for the negative control contain solvent, MeOH (100 µl) and DPPH (100 µl) (Kumarasamy *et al.,* 2002) (Fig.4.3).

The program (KC Junior) was used to read the plate on an ELISA plate reader at 550 nm. The experiment was done in triplicate and the absorption was noted for each concentration. The percentage decrease of the absorbance was calculated by the formula: $I = [(A_B-A_A)/A_B] \times 100$, where I = % inhibition, A_B = absorbance of the blank sample (t = 0), A_A = absorbance of the test sample at the end of the reaction (t = 30 min). The IC_{50} was extrapolated from the standard curve of the percentage inhibition against concentration (Paixão *et al.,* 2007).

4.3 Results and Discussion

All compounds tested with bioautography on TLC plates (Fig. 4.1) showed interesting activity against Gram-negative bacteria (*E. coli*) and Gram-positive bacteria (*B. cereus*). Therefore, the MIC of the compounds on these pathogens was determined with a serial dilution microplate assay (Table 4.1).



1 2 3 4 5 6 7

Figure 4.1: Bioautogram of the indane in lanes 1-7. A TLC plate was developed and sprayed with *B. cereus*, incubated overnight then sprayed with INT. Growth inhibition is indicated by the lighter zones on the lanes.

Table 4.1: MIC of compounds against *B. cereus* and *E. coli*.

Compound	**MIC (µg/ml)** ***E. coli***	**MIC (µg/ml)** ***B. cereus***
Eriodictyol	100.0	100.0
Quercetin	50.0	50.0
Steenkrotin A	50.0	50.0
Steenkrotin B	50.0	50.0
The indane	100.0	50.0
Streptomycin	10.0	10.0

The MIC of the compounds on both pathogens was promising against Gram negative and positive bacteria. The observed activity of quercetin corresponds to the findings of Bylka *et al.* (2004). The possibility of synergistic activity of the compounds need further investigation, this is because the bioautography of the fractions indicated more intense inhibition of the bacteria than that obtained for pure compounds (Fig. 2.2b and 2.3).

In literature, derivatives of the indane and diterpenes have been reported to be active against *M. tuberculosis* (Turan-Zitouni *et al.,* 2008; Berrue *et al.,* 2007; Dettrakul *et al.,* 2003). However, the IC_{50} for the three compounds: steenkrotin A, the indane (2,6-dimethyl-1-oxo-4-indanecarboxylic acid) and steenkrotin B tested for antimycobacterial activity was greater than 10 µg/ml (highest concentration in well) while that of the control RMP was 0.12 µg/ml. Since the compounds tested did not show activity at highest concentration, they are classified as inactive against *M. tuberculosis*. Although the activity of RMP used as positive control against *M. tuberculosis* (H37Rv) was much higher than those for the novel compounds tested, it would be interesting to study the synergistic effect, if any, when these compounds are tested in combination with established antitubercular drugs.

The crude extract and all the isolated compounds tested showed DPPH radical scavenging activity (Fig. 4.2, 4.3 and 4.4).

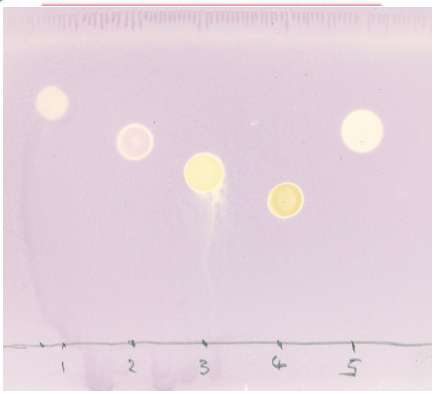
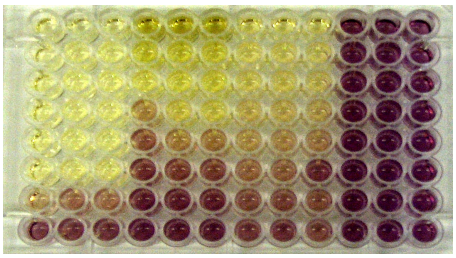


Figure 4:2 Qualitative antioxidant assay. All compounds tested showed antioxidant activity. The indane in lane 1, steenkrotin A in lane 2, tamarixetin in lane 3, quercetin in lane 4 and steenkrotin B in lane 5.



1 2 3 4 5 6 7 9 10 11 12 13

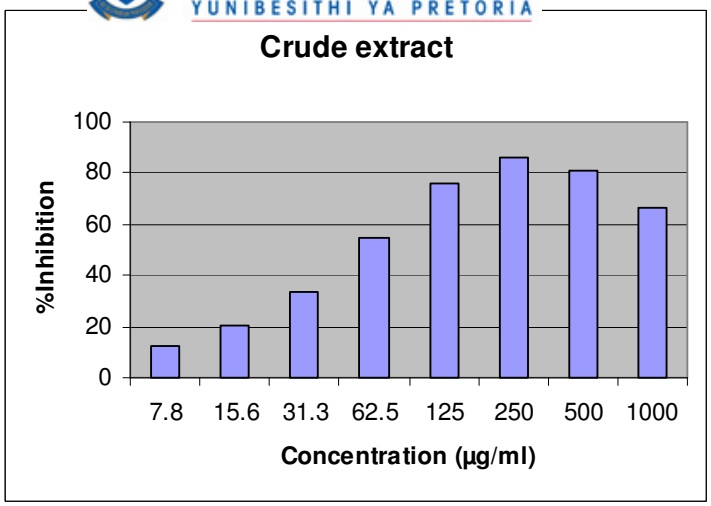
Figure 4.3 Quantitative antioxidant assay. Lane 1-3: ascorbic acid, lane 4-6: crude extract, lane 7-10: steenkrotin B and lane 11-12: negative control (blank).

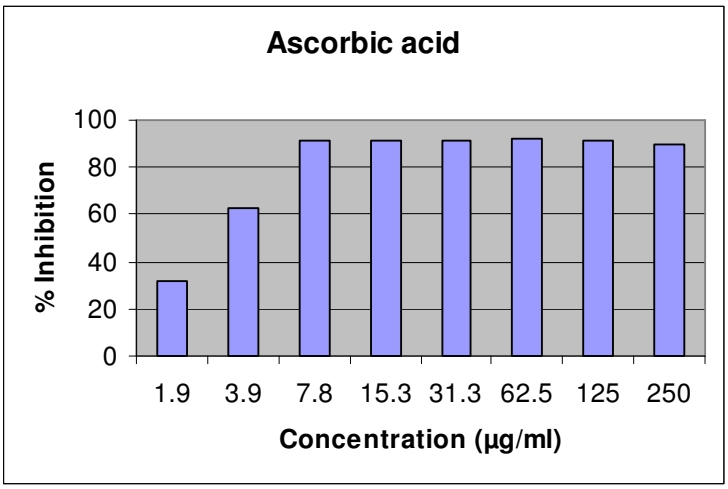
Quercetin was the most active of all the substances tested (Table 4.2). This can be attributed to the presence of the phenolic group in its molecule (Kumarasamy *et al.*, 2002). From the IC_{50} values, it can be seen that the

Table 4.2 Quantitative antioxidant activities of the ethanol crude extract and the isolated compounds.

Substance	**IC_{50} (µg/ml)**	**Ascorbic equivalents**
Ethanol crude extract	76.01	0.04
Indane	> 280.00	> 0.01
Quercetin	0.05	66.00
Steenkrotin A	> 280.00	> 0.01
Steenkrotin B	274.00	0.01
Ascorbic acid	3.30	1.00

increasing order of activity was quercetin > ascorbic acid > crude extract > steenkrotin B > steenkrotin A and indane. It was established that the DPPH scavenging activity of quercetin was 66 times more than that of ascorbic acid, 1 535 times more than that of the crude extract, 5 500 times more than steenkrotin B and over 5 500 times more active than that of steenkrotin A and indane. The moderate antioxidant activity of the ethanol extract was probably due to its rich phenolic compounds content (Narasimhan *et al*. 2005).





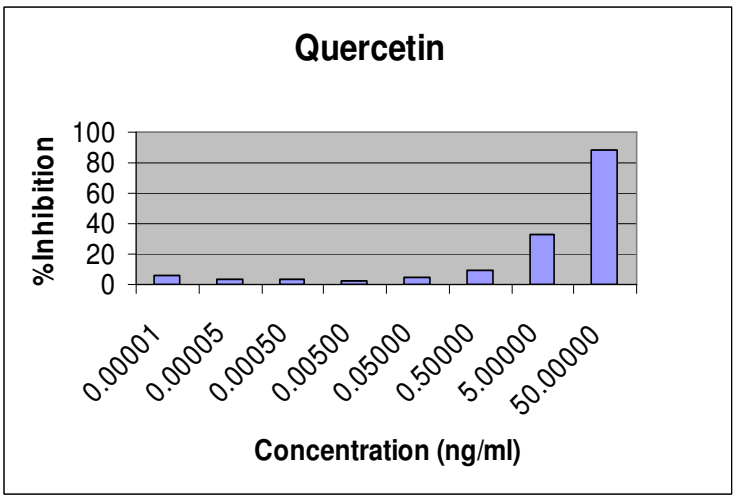


Figure 4.4 Antioxidant activities of the crude extract and compounds.

4.4 References

Berrue, F., Thomas, O.P., Laville, R., Prado, S., Golebiowski, J., Fernandez, R. and Amade, P. 2007. The marine sponge *Plakortis zyggompha*: a source of original bioactive polyketides. Tetrahedron 63(10):2328-2334.

Begue, M.J. and Kline, R.M., 1972. The use of tetrazolium salts bioautographic procedures. J. Chrom. 64:182-184.

Dettrakul, S., Kittakoop, P., Isaka, M., Nopichai, S., Suyarnsestakorn, C., Tanticharoen, M. and Thebtaranonth, Y. 2003. Antimycobacterial pimarane diterpenes from the Fungus *Diaporthe* sp. Bioorg. Med. Chem. Lett. 13(7):1253-1255.

Eloff, J.N., 1998. A sensitive and quick micro-plate method to determine the minimal inhibitory concentration of plant extract for bacteria. Planta Med. 64 (8):711-713.

Greenwood, D., Slack, R.C.B. and Peutherer, J.F., 1997. A guide to microbial infections: Pathogenesis, immunity, laboratory diagnosis and control. Medical Microbiology, 15th edn. Ch. 3. p. 25-35. Churchill Livingstone.

Kumarasamy, Y., Fergusson, M.E., Nahar, L. and Satyajit, S.D. 2002. Bioactivity of Moschamindole from *Centaurea moschata*. Pharm. Biol. 40(4):307-310.

Lall, N. and Meyer, J.J.M. 2001. Inhibition of drug-sensitive and drug-resistant strains of *Mycobacterium tuberculosis* by diospyrin, isolated from *Euclea natalensis,* J. Ethnopharmacol. 78:213-219.

Lall, N., Das Sarma, M., Hazra, B. and Meyer, J.J.M. 2003. Antimycobacterial activity of diospyrin derivatives and a structural analogue of diospyrin against *Mycobacterium tuberculosis* in vitro. J. Antimicrob. Chemother. 51:435-438.

Langfield, R.D., Scarano, F.J., Heitzman, M.E., Kondo, M., Hammond, G.B. and Neto, C.C. 2004. Use of a modified microplate bioassay method to investigate antibacterial activity in the Peruvian medicinal plant *Peperomia galioides*. J. Ethnopharmacol. 94:279-281.

Luczaj, W. and Skrzydlewska, E. 2005. Antioxidant properties of black tea. Prev. Med. 40:910-918.

Mandell, G.L., Bennett, J.E. and Dolin, R. 2005. Principles and practice of infectious diseases. 6th edn. Ostroff, S.M., McDade, J.E., LeDuc, J.W. and Hughes, J.M. In: Emerging and re-emerging infectious disease threats. Ch. 13. pp.173-192. Philadelphia, Pennsylvania: Churchill Livingstone.

Mativandlela, S.P.N., Lall, N. and Meyer, J.J.M. 2005. Antibacterial, antifungal and antitubercular activity of the roots of *Pelargonium reniforme* (CURT) and *Pelargonium sidoides* (DC) (Geraniaceae) root extracts. S. Afr. J. Bot. 72(3):349-352.

Middlebrook, G., Reggiards, Z. and Tigertt, W.D. 1977. Automable radiometric detection of growth of *Mycobacterium tuberculosis* in selective media. Am. Rev. Respir. Dis. 115:1067-1069.

Narasimhan, S., Govindarajan, R., Vijayakumar, M. and Mehrotra, S. 2005. Free radical scavenging potential of *Chlorophytum tuberosum* Baker. J. Ethnoharmacol. 104: 423-425.

National Institute of Allergy and Infectious Diseases (NIAID). Microbes in sickness and in health. Viewed 20 October 2007, http//www.niaid.nih.gov/.

Paixão, N., Perestrelo, R., Marques, J.C. and Cāmara, J.S. 2007. Relationship between antioxidant capacity and total phenolic content of red, rosé and white wines. Article in press: www.sciencedirect.com.

Pandey, M. M., Govindarajan, R., Rawat, A.K. and Pushpangadan, P. 2005. Free radical scanvenging potential of *Saussarea costus*. Acta Pharm. 55:297-304.

Turan-Zitouni, G., Ozdemir, A., Kaplanikli, Z.A., Benkli, K., Chevallet, P. and Akalin, G. 2008. Synthesis and antituberculosis activity of new thiazolylhydrazone derivatives. Eur. J. Med. Chem. 43(5):981-985.

Zgoda, J.R. and Porter, J.R. 2001. A convenient microdilution method for screening natural products against bacteria and fungi. Pharm. Biol. 39(3):221-225.