

Chapter 3

Antibacterial activity of extracts of *Pteleopsis myrtifolia* leaves and fruit and *Quisqualis littorea* leaves.

Abstract

Pteleopsis and *Quisqualis* are two of the less known genera of the Combretaceae plant family that have antibiotic activity. The aim of this research was to investigate several extracts of *Pteleopsis myrtifolia* and *Quisqualis littorea*, thereby facilitating the isolation of compounds from the complex blend of substances in the plant, some of which are antibacterial. Different extractants yielded between 1% and 40.2% of the dry mass and 0.1% to 7 % of the dry mass was acetone soluble. Thin layer chromatography (TLC) indicated that the best separation occurred with an eluent system that separated compounds of medium polarities, like CEF (chloroform: ethyl acetate: formic acid (5:4:1)). The extracts of all plant materials (*P. myrtifolia* leaves and fruit and *Q. littorea* leaves) had antibacterial activity against Gram-positive bacteria, with MIC values between 0.039 and 0.63 mg/ml. With *P. myrtifolia* leaves, the lowest average MIC values were found (in increasing order) for *Enterococcus faecalis* (0.078 mg/ml), *Staphylococcus aureus* (0.195 mg/ml), *Pseudomonas aeruginosa* (0.75 mg/ml), and *Escherichia coli* (>0.97 mg/ml). The Gram-positive bacteria were most sensitive and compared with the MIC values of antibiotics, 0.08 and 0.16 mg/ml for ampicillin and chloramphenicol respectively. With *P. myrtifolia* fruit, the lowest average MIC values were found (in increasing order) for *E. faecalis* (0.13 mg/ml), *S. aureus* (0.13 mg/ml), *P. aeruginosa* (2.4 mg/ml), and *E. coli* (>2.5 mg/ml). With *Q. littorea* leaves the lowest average MIC values were found (in increasing order) for *E. faecalis* (0.09 mg/ml), *P. aeruginosa* (0.31 mg/ml), *E. coli* (0.33 mg/ml) and *S. aureus* (0.58 mg/ml). For *P. myrtifolia* leaves and fruit, the Gram-negative bacteria had higher MIC

values that varied between 5.0 and 1.25 mg/ml. With *Q. littorea* leaves the average MIC value for Gram-negative bacteria was 0.32 mg/ml compared to the average MIC values of 1.86 mg/ml and 2.44 mg/ml for *P. myrtifolia* leaves and fruit respectively. The average antibacterial activity for each bacterium was higher in the leaves than in the fruit. Results obtained in this study make it clear that *P. myrtifolia* leaves and fruit and *Q. littorea* leaves contain several antibacterial compounds. *Q. littorea* had high activity, expressed as total activity, 234.7 ml/g for the Gram-negative bacterium, *E. coli*.

3.1 Introduction

3.1.1 Antibiotic resistance

With the increasing resistance that microbes show against medicine and an increased awareness of toxicities in refined products amongst modern city dwellers, individuals are progressively focusing their attention towards herbal medicine in an effort to find an alternative approach to living healthier. Antibiotic resistance, which resulted from the frequent and unwise use of antibiotics, is a problem in especially hospital environments and can lead to the spread of resistant strains to communities. Resistance is determined by the bacterial genome, which may change rapidly (Berkowitz, 1995). A 'new' antibiotic may have a limited time in which no bacteria has resistance to it and the search for new antibiotics must carry on. The most common bacterial pathogens causing nosocomial infections are *E. coli* (commonest pathogen in adult services), *S. aureus* (commonest pathogen in paediatric and newborn services), *E. faecalis* (antibiotic resistant, some also against Vancomycin) and *P. aeruginosa* (Sacho & Schoub, 1977).

While antibiotic resistance "benefits" the microbes, it presents humans with two big problems: it makes it more difficult to purge infections from the body; and it heightens the risk of acquiring infections in a hospital. The Department of Healthcare Epidemiology and Infection Control at

the University of Pennsylvania Medical Centre reported that antibiotic-resistant “super bugs” cause an estimated 19 000 deaths in the US each year, compared to 372 deaths worldwide that have been attributed to severe acute respiratory syndrome (SARS) since its outbreak in November 2002. According to the centres for disease control and prevention (CDC) statistics:

- Nearly two million patients in the United states get an infection in the hospital each year;
- Of those patients, about 90 000 die each year as a result of their infection (compared to 13 300 patient deaths in the year 1992);
- More than 70% of the bacteria that cause hospital-acquired infections are resistant to at least one of the drugs most commonly used to kill them;
- Persons infected with drug resistant organisms are more likely to have longer hospital stays. In addition, they require treatment with second or third choice drugs that may be less effective, more toxic, and more expensive (<http://www.niaid.nih.gov/factsheets/antimicro.htm>, 2004).

3.1.2 Plant secondary compounds are frequently associated with plant taxons

Plant secondary components are frequently associated with plant taxons. From data provided by Cunningham (1990), Eloff (1998b) calculated that although the Combretaceae is a relatively small family, the scale of use in KwaZulu-Natal is large relative to most other plant families. The Combretaceae thus make up a major group of plants that has potential to offer novel or alternative phytomedicines.

In preliminary investigations of the antibacterial activity of 27 members of the South African Combretaceae family Eloff (1999) found that the MIC's of acetone leaf extracts of *P. myrtifolia* and *Q. littorea* were in the same order as that of other genera in the Combretaceae. In this investigation, one of the highest antibacterial activities found, less than 0.1 mg/ml, was for an acetone leaf extract of *Q. littorea* and *Staphylococcus aureus* (Gram-positive cocci).

In the Combretaceae plant family, traditional healers in Africa have confined themselves almost exclusively to the use of species from the genus *Combretum* and to a lesser extent, *Terminalia*. These species have been used for the treatment of a wide range of disorders, but only about 25% of the African species of *Combretum* have been subjected to scientific study. With the exception of a few species of *Terminalia*, *Annogeissus* and *Guiera*, very little have been reported on the phytochemistry of the remaining genera (Rogers & Verotta, 1997).

3.1.3 Metabolites isolated in Combretaceae

Metabolites isolated in Combretaceae so far include alkaloids, tannins, flavonoids, amino acids; substituted phenanthrenes, triterpenoid acids and their saponins mainly from the cycloartane and oleanane types; unique stilbenes, their glucosides and macrocyclic lactones called combretastatins. Many *Combretum* species exude gums similar to gum Arabic (Rogers & Verotta, 1997). Certain metabolites show cytotoxic, molluscicidal, anti-HIV, antimicrobial and anti-inflammatory activity and several triterpenoid mixtures strongly inhibit seed germination and seedling growth. In nearly all species Carr & Rogers (1987) studied, geographical and seasonal variation had little or no effect on the composition of extracts isolated.

3.1.4 Bioactive properties of *Pteleopsis*

Bioactive properties of *Pteleopsis* spp. are discussed in 1.14 of Chapter 1.

3.1.5 Bioactive properties of *Quisqualis*

Bioactive properties of *Quisqualis* spp. are discussed in 1.15 of Chapter 1.

As mentioned in Chapter 1, previous investigations of species of the Combretaceae at the University of Pretoria, have isolated and determined the structure and biological characteristics of compounds and some extracts had such good activity that commercial applications in the

protection of animal health are currently under way (Kruger, 2004). There is a strong probability that similar activities and applications may be discovered in an investigation of *P. myrtifolia* and

Q. littorea.

The aim of this investigation was to identify extracts with antibacterial activity, from *P. myrtifolia* and *Q. littorea* and to quantify the antibacterial activity of active extracts.

3.2 Material and Methods

3.2.1 Plant material

Plant material was collected and prepared as described in 2.2.1 of Chapter 2.

P. myrtifolia leaves from different environments and or years were compared to establish whether the antibacterial activity in the leaves differ significantly for different environments or different years from the same environment. Groups of leaves compared were from the Lowveld Botanical Garden March 2000, Lowveld Botanical Garden March 2001, Centurion March 2001, Tembe Reserve (KwaZulu-Natal) March 2001 and Durban March 2001.

3.2.2 Extracts

Extracts were prepared as described in 2.2 of Chapter 2.

3.2.3 Thin layer chromatography

Thin layer chromatography (TLC) were carried out as described in 2.3 of Chapter 2.

3.2.4 Determining antibacterial activity

3.2.4.1 Minimum inhibitory concentration

Four test organisms (recommended by the National Committee for Clinical Laboratory

Standards (NCCLS 1990)) were selected on the grounds that they are most often responsible for antibiotic resistance in hospitals (Sacho and Schoub, 1993). Two Gram-positive: *Staphylococcus aureus* American Type Culture Collection [ATCC 29213] and *Enterococcus faecalis* [ATCC 29212] and two Gram-negative: *Pseudomonas aeruginosa* [ATCC 25922] and *Escherichia coli* [ATCC 27853] were used to determine minimum inhibitory concentration (MIC) of the different plant extracts. Many researchers use agar diffusion to assay antibacterial activity. The technique works well with defined inhibitors (Hewitt and Vincent, 1989), but when examining extracts containing unknown components, there are problems leading to false positive and false negative results (Eloff, 1998a). A microplate serial dilution method (as described by Eloff (1998a)) was used. The development of a red formazan indicated bacterial growth (Lund & Lyon, 1975).

3.2.4.2 Total activity

Minimum inhibitory concentration (MIC) values do not give any indication of the activity present in a plant. A proposal was made that "total activity" should be determined by dividing the quantity extracted (in mg) from 1 g of plant material (redissolved in acetone) by the MIC value in mg/ml (for the specific bacterium). The resultant value in ml/g indicate the degree to which the active compounds in one gram of plant material can be diluted and still inhibit the growth of the tested microorganism (Eloff, 2000). "Antibacterial activity" expressed as "total activity" provides a tool by which different plants can be compared, using the same measuring instruments.

If one compare MIC values of different plants without taking the amount extracted from the plant into account, one can easily make the wrong conclusions (Eloff, 2004). For example: extracts from plants A and B had MIC values of 0.2 mg/ml and 0.1 mg/ml respectively. Looking at MIC values only, one would have concluded that plant B is twice as active as plant A and would be a good source for bioprospecting. However, the calculated antibacterial activity per gram dry

mass for plant A and B, where 200 mg and 20 mg were extracted respectively from 1g, is 200 mg/0.2 mg/ml i.e. 1000 ml/g for plant A and 20 mg/0.1mg/ml i.e. 200 ml/g for plant B. Plant A has a much higher total activity than plant B, and thus much higher activity per gram dry mass. The situation is equivalent to the terms efficacy and potency used in pharmacology. The potency would be the activity in mg/ml of the extract and the efficacy would be the activity of the total plant material in ml/g.

3.2.4.3 Bioautography

For bioautography on the thin layer chromatography (TLC) all the extracts (10 μ l of a 10 mg/ml final concentration) were applied to Merck Silica gel F₂₅₄ plates) and developed with non-polar BEA (benzene: ethanol: ammonia, (90:9:1)), intermediate polar CEF (chloroform: ethyl acetate: formic acid, (5:4:1)) and polar EMW (ethyl acetate: methanol: water, (40:5:4.4)) eluent systems. After TLC separation, chromatograms were dried overnight in a stream of air to remove the last traces of the TLC solvents. The plates were examined under ultraviolet (UV) light and bands of quenching fluorescence (254 nm) or fluorescing (236 nm) were marked with a soft pencil. Subsequently the plates were sprayed with a concentrated solution of actively growing cells of the relevant test organism, - a 24 h old culture in Hinton-Mueller (HM) broth, centrifuged at 3500 r p m for 15 min, the supernatant discarded and the sediment bacteria were resuspended in fresh HM broth. A fine spray was used to spray the bacterial suspension onto the TLC plates. The spraying of the bacteria was done in an extraction cabinet where the front glass panel could slide down. Gloves and a facemask were worn to prevent bacterial infection. The TLC plates were then dried until they appeared translucent and incubated overnight at 37°C and 100% relative humidity. The following day, the TLC plates were sprayed with an aqueous solution of 2.0 mg/ml p-iodonitrotetrazolium violet (INT) solutions and reincubated at 37° C until the development of inhibition zones were complete. Clear zones developed where inhibition was

present and bacterial growth was indicated by the formation of a red formazan (Lund & Lyon, 1975).

3.2.5 Stability of extracts over time

To determine which plant extracts retained activity upon storage, MIC values of freshly made extracts were compared to extracts from material redissolved in acetone and stored at 4° C for 8 months. Total activity of the extracts was compared.

3.2.6 Test for toxicity of tetrahydrofuran

A test for toxic effects of tetrahydrofuran was carried out by drying 15 ml (the amount used for the different extracts) of tetrahydrofuran. CP-grade and AR-quality was used for the experiment. The AR-quality tetrahydrofuran did not dry completely, but formed oil, which would not dry. Three times 5 ml CP-grade of tetrahydrofuran were measured and dried in the same way as the extracts was, quantified and made to a final concentration of 10 mg/ml.

3.3 Results and Discussion

3.3.1 Antibacterial activity

3.3.1.1 Minimum inhibitory concentration

The MIC values for the different extractants and bacteria could be distinguished from the multiwell plates (Figure 3.1, and some controls in Figure 3.2) where bacterial growth was minimally inhibited, values given in Table 3.1.

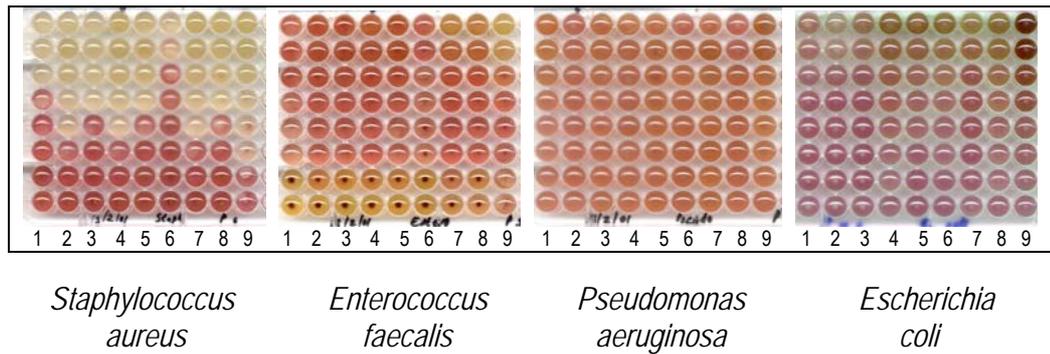


Figure 3.1. 96-Multiwell plates (MWP-96) showing MIC values of *Pteleopsis myrtifolia* fruit for *Staphylococcus aureus* (left), *Enterococcus faecalis* (second from left), *Pseudomonas aeruginosa* (second from right), *Escherichia coli* (right), after 40ul of 0.2 mg/ml p-iodonitrotetrazolium violet solution were added to each well. Lanes from left to right were (1) = *n*-hexane, (2) = *di*-isopropyl ether, (3) = *di*-ethyl ether, (4) = methylene dichloride, (5) = tetrahydrofuran, (6) = ethyl acetate, (7) = acetone, (8) = ethanol, (9) = methanol. All solutions tested were 10 mg/ml, the first row effectively 2.5 mg/ml.

Gentamycin's antibiotic activity (as a positive control) was less effective than a similar quantity of plant extract against all the bacteria tested. All solutions tested were 10 mg/ml, the first row effectively 2.5 mg/ml (Figure 3.2).

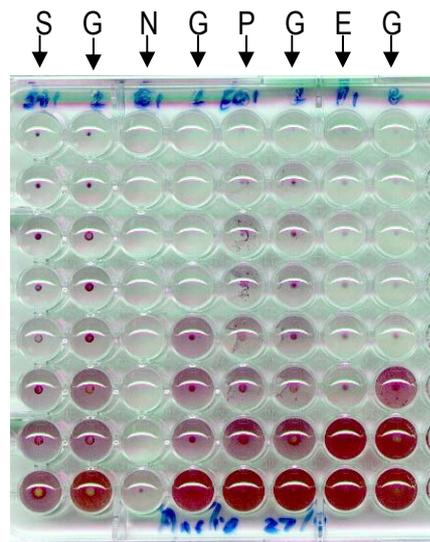


Figure 3.2. Multiwell plate showing minimum inhibitory concentrations of the antibiotic Gentamycin (G), which served as a positive control, (every even column), and a plant extract (every uneven column). The bacteria *Staphylococcus aureus* (S), *Enterococcus faecalis* (N),

Pseudomonas aeruginosa (P) and *Escherichia coli* (E) were used. The amount of colour formation (red formazan) is an indication of the MIC, 2 hours after 40ul of 0.2 mg/ml p-iodonitrotetrazolium violet solution was added to each well. All solutions tested were 10 mg/ml, the first row effectively 2.5 mg/ml.

The extracts of all plant materials (*P. myrtifolia* leaves and fruit and *Q. littorea* leaves) had antibacterial activity for Gram-positive bacteria, with MIC values between 0.039 and 0.63 mg/ml. With *P. myrtifolia* leaves, the lowest average MIC values were found (in increasing order) for *E. faecalis* (0.078 mg/ml), *S. aureus* (0.195 mg/ml), *P. aeruginosa* (0.75 mg/ml), and *E. coli* (>0.97 mg/ml). The Gram-positive bacteria were most sensitive and compared with the MIC values of antibiotics, 0.08 and 0.16 mg/ml for ampicillin and chloramphenicol respectively (Martini, 1998). With *P. myrtifolia* fruit, the lowest average MIC values were found (in increasing order) for *E. faecalis* (0.13 mg/ml), *S. aureus* (0.13 mg/ml), *P. aeruginosa* (2.4 mg/ml), and *E. coli* (>2.5 mg/ml). With *Q. littorea* leaves the lowest average MIC values were found (in increasing order) for *E. faecalis* (0.13 mg/ml), *P. aeruginosa* (0.13 mg/ml), *E. coli* (2.4 mg/ml) and *S. aureus* (>2.5 mg/ml). For *P. myrtifolia* leaves and fruit, the Gram-negative bacteria had higher MIC values that varied between 5 and 1.25 mg/ml. For *Q. littorea* leaves the average MIC value for Gram-negative bacteria was 0.32 mg/ml compared to the average MIC values of 1.86 and 2.44 mg/ml for *P. myrtifolia* leaves and fruit respectively.

A second set of MIC values were calculated which did not differ significantly from the first (results not shown).

3.3.1.2 Total (antibacterial) activity.

To determine which extracts were the most promising as sources of antibacterial compounds, not only the MIC of the extract but also the quantity present in the plant is important. Because the MIC value is inversely related to the quantity of antibacterial compounds present, an

arbitrary measure of the quantity of antibacterial compounds present was calculated by dividing the quantity extracted in milligrams from 1000 mg of leaves by the MIC value in mg/ml (Eloff 1999). The unit of this arbitrary measure is ml/g and Eloff (2004) called it "total activity". This value indicates the volume to which the biological active compounds present in one gram of dried plant material can be diluted and still kill bacteria. Total activity for each type of plant material and all extractants with the four different bacterial strains are listed in Table 3.1 and graphically represented in Figure 3.3.

With *P. myrtifolia* leaves, the highest single and average total activity was found (in decreasing order) for *E. faecalis* (1794.9 and 489.7 ml/g respectively), *S. aureus* (448.7 and 163.4 ml/g respectively), *P. aeruginosa* (223.6 and 74.2 ml/g respectively), and *E. coli* (70.4 and >31.0 ml/g respectively). For the fruit of *P. myrtifolia*, the highest average total activity was found (in

Table 3.1. Milligram plant extracted per gram dry plant material, milligram acetone soluble plant extract per extractant, MIC values and total activity for the different bacterial strains and plant materials.

<i>Pteleopsis myrtifolia</i> leaves												
X-ant	mg x/g	mg a sol/ g	MIC in mg/ ml					Total activity in ml				
			S aur	E fae	P aer	E col	Ave	S aur	E fae	P aer	E col	Ave
Hex	22	16	0.08	0.16	1.25	>4	1.37	205.1	102.6	12.8	>4	81.1
P-e	18	12	0.31	0.16	1.25	1.25	0.74	38.3	76.9	9.6	9.6	33.6
E-e	34	22	0.16	0.08	0.63	2.5	0.84	141.0	282.1	35.2	8.8	116.8
Mdc	64	20	0.16	0.08	0.31	0.63	0.29	128.2	256.4	63.9	32.0	120.1
Thf	88	70	0.16	0.04	0.31	1.25	0.44	448.7	1794.9	223.6	56.0	630.8
E-a	46	28	0.31	0.08	0.31	1.25	0.49	89.5	359.0	89.5	22.4	140.1
Ace	52	38	0.16	0.08	0.31	0.63	0.29	243.6	487.2	121.4	60.8	228.3
Eth	84	44	0.16	0.04	0.31	0.63	0.28	282.1	1128.2	140.6	70.4	405.3
Met	188	14	0.31	0.04	0.31	0.31	0.24	44.7	359.0	44.7	44.7	123.3
H ₂ O	402	2	0.16	0.04	2.5	1.25	0.99	12.8	51.3	0.8	1.6	16.6
Tot	998	266	1.95	0.78	7.50	>9.67	5.98	1634.1	4897.4	742.1	>310.3	1896.0
Ave	99.8	26.6	0.2	0.08	0.8	>0.97	0.60	163.4	489.7	74.2	>31.0	189.6
								Ave g+	326.6	Ave g-	52.6	

<i>Pteleopsis myrtifolia</i> fruit												
X-ant	mg x/g	mg a sol/g	MIC in mg/ ml					Total activity in ml				
			S aur	E fae	P aer	E col	Ave	S aur	E fae	P aer	E col	Ave
Hex	10	10	0.04	0.08	2.5	>2.5	1.28	256.4	128.2	4.0	>4.0	98.2
P-e	14	10	0.08	0.16	2.5	>2.5	1.31	128.2	64.1	4.0	>4.0	50.1
E-e	22	12	0.08	0.16	2.5	1.25	1.00	153.8	76.9	4.8	9.6	61.3
Mdc	114	12	0.08	0.16	2.5	>3.0	1.43	153.8	76.9	4.8	>4.0	59.9
Thf	52	20	0.08	0.04	2.5	>5.0	1.90	256.4	512.8	8.0	>4.0	195.3
E-a	24	14	0.04	0.08	1.25	>2.5	0.97	359.0	179.5	11.2	>5.6	138.8
Ace	30	10	0.16	0.08	2.5	>2.5	1.31	64.1	128.2	4.0	>4.0	50.1
Eth	30	12	0.08	0.16	2.5	>3.0	1.43	153.8	76.9	4.8	>4.0	59.9
Met	116	10	0.63	0.08	2.5	>2.5	1.43	16.0	128.2	4.0	>4.0	38.1
H ₂ O	-	-	-	-	-	-	-	-	-	-	-	-
H ₂ A	184	1.08	0.08	0.313	2.5	0.313	0.80	13.8	3.5	0.4	3.5	5.3
Tot	596	111.1	1.33	1.29	23.75	>25.06	12.86	1555.5	1375.2	50.0	>46.7	756.8
Ave	59.6	11.18	0.13	0.13	2.38	>2.51	1.29	155.6	137.5	5.0	>4.7	75.7
								Ave g+	146.6	Ave g-	4.9	

<i>Quisqualis littorea</i> leaves												
X-ant	mg x/g	mg a sol/g	MIC in mg/ ml					Total activity in ml				
			S aur	E fae	P aer	E col	Ave	S aur	E fae	P aer	E col	Ave
Hex	16	10	0.31	0.04	0.31	0.63	0.323	32.0	255.8	32.0	16.0	84.0
P-e	30	14	0.63	0.04	0.31	1.25	0.557	22.4	358.1	44.8	11.2	109.1
E-e	35	16	0.63	0.08	0.31	0.31	0.332	25.6	204.9	51.2	51.2	83.2
Mdc	152	20	0.16	0.16	0.16	0.04	0.127	128.0	128.0	128.0	511.5	223.9
Thf	56	48	0.31	0.08	0.16	0.08	0.16	153.6	614.6	307.1	614.6	422.5
E-a	30	24	0.31	0.16	0.16	0.16	0.195	76.8	153.6	153.6	153.8	134.5
Ace	40	22	0.31	0.08	0.16	0.08	0.16	70.4	281.7	140.8	281.7	193.7
Eth	50	32	0.31	0.16	0.16	0.08	0.176	102.4	204.7	204.7	409.7	230.4
Met	130	22	0.31	0.08	0.16	0.08	0.16	70.4	281.7	140.8	281.7	193.7
H ₂ O	-	-	-	-	-	-	-	-	-	-	-	-
H ₂ A	260	10	2.50	0.08	1.25	0.63	1.113	4.0	128.0	8.0	16.0	39.0
Tot	799	208	5.78	0.94	3.13	3.32	3.29	685.6	2610.9	1210.9	2347.5	1713.8
Ave	79.9	20.8	0.58	0.09	0.31	0.33	0.33	68.6	261.1	121.1	234.7	171.4
Ave all	79.8	19.5	0.30	0.10	1.15	1.27	0.76	32.0	255.8	32.0	16.0	145.6
								Ave g+	164.9	Ave g-	177.9	

Ave = Average, a sol = acetone soluble, S = *Staphylococcus aureus*, N = *Enterococcus faecalis*, P = *Pseudomonas aeruginosa*, E = *Escherichia coli*, X-ant = extractant, mg x/g = milligram extractant per gram, mg a sol/g = milligram acetone soluble extractant per gram. Hex = *n*-hexane, P-e = *d*-isopropyl ether, E-e = *d*-ethyl ether, Mdc = methylene dichloride, Thf =

tetrahydrofuran, E-a = ethyl acetate, Ace = acetone, Met = methanol, H₂O = water and H₂-A = water: acetone, Ave g⁺ = average for Gram-positive, Ave g⁻ = average for Gram-negative.

decreasing order) for *S. aureus* (155.6 ml/g), *E. faecalis* (137.5 ml/g), *P. aeruginosa* (5.0 ml/g), and *E. coli* (>4.7 ml/g). For both *P. myrtifolia* leaves and fruit, the Gram-positive bacteria were more sensitive and compared well with the values of known antibiotics, 0.08 and 0.16 mg/ml for ampicillin and chloramphenicol. The average antibacterial activity for each bacterium was higher in the leaves than in the fruit.

For *Q. littorea* leaves the highest average total activity were found (in decreasing order) for *E. faecalis* (261.1 ml/g), *E. coli* (234.7 ml/g), *P. aeruginosa* (121.1 ml/g) and *S. aureus* (68.6 ml/g) (Table 3.2 and figure 3.3). Total activity of *Q. littorea* leaves against Gram-negative bacteria is high and it might be worthwhile to cultivate plant material and attempt to isolate pure compounds from a fraction with activity against Gram-negative bacteria.

In a study where Kotzé (2000) investigated antibacterial activity of *Combretum microphyllum* leaves, the lowest MIC values were obtained with extractants ethanol, *di*-isopropyl ether and acetone. Famakin (2002) obtained the lowest average MIC values with extractants ethyl acetate, methylene dichloride, and acetone for *Combretum woodii* leaves. In this study, the lowest average MIC values for *P. myrtifolia* leaves were obtained with extractants methanol, ethanol, and equally acetone and methylene dichloride. Results of the average MIC and total activity values obtained by different researchers while investigating different genera of Combretaceae are listed in Table 3.2.

Table 3.2. Average MIC and total activity values obtained for each bacterium and extracts of each plant type.

	Average MIC value for each bacterium (mg/ml)				Average total activity for each bacterium (ml/g)			
	S	E	P	E	S	E	P	E
<i>C. microphyllum</i> leaves	0.46	0.29	0.30	0.31	234.6	359.0	296.4	332.0
<i>C. woodii</i> leaves	>0.59	>0.35	>0.80	>0.29	>235.1	>898.2	>246.9	>1155.0
<i>P. myrtifolia</i> leaves	0.2	0.08	0.8	>0.97	163.4	489.7	74.2	>31.0
<i>P. myrtifolia</i> fruit	0.13	0.13	2.38	>2.51	155.6	137.5	5.0	>4.7
<i>Q. littorea</i> leaves	0.58	0.09	0.31	0.33	68.6	261.1	121.1	234.7

S = *Staphylococcus aureus*, N = *Enterococcus faecalis*, P = *Pseudomonas aeruginosa* and E = *Escherichia coli*, *C. microphyllum* = *Combretum microphyllum*, *C. woodii* = *Combretum woodii*, *P. myrtifolia* = *Pteleopsis myrtifolia*, *Q. littorea* = *Quisqualis littorea*.

From Table 3.2, it is clear that different members of Combretaceae have different activities against the different test organisms.

In an investigation where Eloff (1999) investigated antibacterial activity (of acetone leaf extracts) of 27 members of Combretaceae, *P. myrtifolia* and *Q. littorea* included, the MIC values obtained were higher and total activity values lower than the MIC and total activity values obtained in this study (Table 3.3). A possible explanation for variation in values could have been due to the difference in plant material or bacterial cultures from different years.

Table 3.3. MIC and total activity values obtained for extracts of *P. myrtifolia* and *Q. littorea*

Plant and author	MIC (mg/ml)				Total activity (ml/g)	
	S	E	P	E	Ave Gram +	Ave Gram -
<i>P. myrtifolia</i> leaves (Eloff)	0.4	0.4	0.8	1.6	65.8	88.6
<i>P. myrtifolia</i> leaves (Rabie)	0.2	0.08	0.8	>0.97	326.5	>52.6
<i>Q. littorea</i> leaves (Eloff)	<0.7	0.8	1.6	0.8	14.8	19.0
<i>Q. littorea</i> leaves (Rabie)	0.58	0.09	0.31	0.33	164.8	177.9

Ave Gram + = average Gram-positive, Ave Gram - = average Gram-negative, S = *Staphylococcus aureus*, N = *Enterococcus faecalis*, P = *Pseudomonas aeruginosa*, E = *Escherichia*

coli.

Graphs drawn of the values in Table 3.1 give a 2-dimensional representation of the total activity for all plant material (Figure 3.3).

Results with *Pteleopsis* plant material indicated that on average the extractants tetrahydrofuran, ethanol and acetone gave the highest total activity for leaves and the extractants tetrahydrofuran, ethyl acetate and *n*-hexane gave the highest total activity for fruit. For *Q. littorea* leaves on average the extractants tetrahydrofuran, ethanol and methylene dichloride gave the highest total activity for leaves (Table 3.2). For all plant material types water or (1: 1) (water: acetone) gave the lowest total activity. Water probably did not extract acetone soluble material because it could not pass the lipids in the membranes. A mixture of water and acetone however, could break the plant membranes and extract some soluble cell contents.

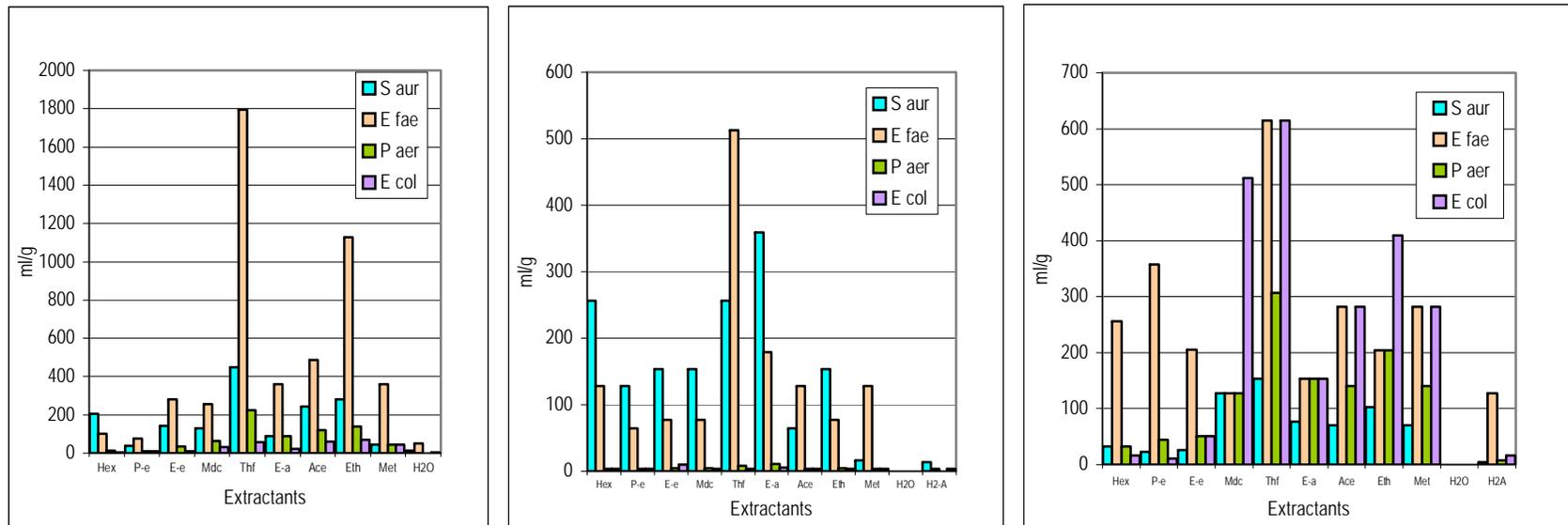


Figure 3.3. Total activity for *Pteleopsis myrtifolia* leaves (left), fruit (middle) and *Quisqualis littorea* leaves (right) for the bacteria *Staphylococcus aureus* (*S aur*), *Enterococcus faecalis* (*E fae*), *Pseudomonas aeruginosa* (*P aer*) and *Escherichia coli* (*E col*) in m/g, with extractants *n*-hexane (Hex), *di*-isopropyl ether (P-e), *di*-ethyl ether (E-e), methylene dichloride (Mdc), tetrahydrofuran (Thf), ethyl acetate (E-a), acetone (Ace), ethanol (Eth), methanol (Met) and water (H₂O) or water: acetone (1:1) (H₂-A).

The average antibacterial activity (expressed as total activity) for each bacterium was higher in the leaves than in the fruit (although TLC separated more compounds in the fruit). The lowest average antibacterial activity was found for *P. aeruginosa* (Figure 3.4(a)).

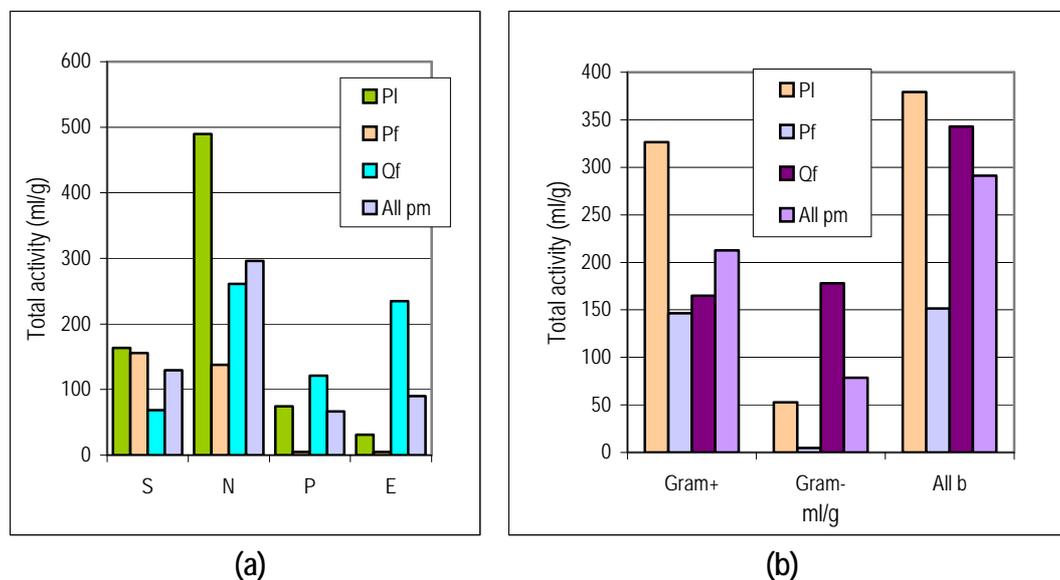


Figure 3.4. Average of total activity for each bacterium type and type of plant material ((a) left), and average total activity for Gram-positive (Gram+), Gram-negative (Gram-) and all bacteria (All b) for each plant material type as well as for all plant material (All pm) ((b) right). S = *Staphylococcus aureus*, N = *Enterococcus faecalis*, P = *Pseudomonas aeruginosa*, E = *Escherichia coli*, PI = *Pteleopsis myrtifolia* leaves, Pf = *Pteleopsis myrtifolia* fruit, QI = *Quisqualis littorea* leaves.

On average *E. faecalis* was more sensitive to leaf material and *S. aureus* was more sensitive to fruit material of *P. myrtifolia*. The Gram-negative bacteria *P. aeruginosa* and *E. coli* were more sensitive to *Q. littorea* leaf material (Figure 3.4(a)). *P. myrtifolia* leaves and fruit support the observation of Vlietinck *et al.* (1995) - that plant extracts are frequently more active against Gram-positive bacteria. This was not true for *Q. littorea* leaves. The average total activity of *Q. littorea* leaves against Gram-negative bacteria was higher than for Gram-positive bacteria. The average total activity for Gram-positive bacteria was highest for *P. myrtifolia* leaf material and

the average total activity for Gram-negative bacteria was highest for *Q. littorea* leaf material (Figure 3.4 (b)).

The aim in an investigation of this type, is extracting the largest quantity of antibacterial activity, therefore not only the MIC, but also the (acetone soluble) quantity extracted should be considered. A very complex extract will not yield a lot of pure compound, therefore the main objective is to find a highly active extract with a low concentration of other compounds - found by looking for extracts with a low MIC and a large quantity of extract that redissolves in acetone. According to Table 3.2, the best extractants for this purpose are tetrahydrofuran, ethyl acetate, acetone and ethanol (631, 140, 228, 405 ml/g) for *P. myrtifolia* leaves, *n*-hexane, *di*-ethyl ether, tetrahydrofuran and ethyl acetate (98, 61, 195, 139 ml/g) for *P. myrtifolia* fruit and methylene dichloride tetrahydrofuran, ethanol and jointly acetone and methanol (224, 423, 230, 194 ml/g) for *Q. littorea* leaves.

To determine if the selectivity group of the extractants (Snyder and Kirkland, 1979) has a major effect, the results were grouped according to selectivity group (Table 3.4). A solvent's selectivity is the result of the interaction of three forces x_d , x_e and x_n , shown in more detail in Figure 4.1 of Chapter 4. When these latter values for each solvent are plotted in a triangular diagram (in Figure 4.1 of Chapter 4), it is found that various solvents are grouped into clusters of similar selectivity. All the solvents in the same cluster belong to the same 'selectivity group'. For example, group I will include all the H-acceptors such as amines and ethers, group II includes donor acceptors such as the alcohols, and group VIII consists of pure donor solvents such as chloroform. A graph was drawn with the solvents arranged according to selectivity group (Figure 3.5).

Table 3.4. The extractants listed within their selectivity groups of Snyder and Kirkland (1979), solvent strength, average amounts of plant material extracted per gram dry weight (third column) and acetone soluble (fourth column).

Extractant	Sel grp	Solvent strength	Ave all pm x/g	Ave all pm AS
Hex	-	0	16.0	12.0
P-e	I	2.4	20.7	12.0
E-e	I	2.8	30.3	16.7
Eth	II	4.3	54.7	29.3
Met	II	5.1	144.7	15.3
Thf	III	4.0	65.3	46.0
Mdc	V	3.1	110.0	17.3
E-a	Vla	4.4	33.3	22.0
Ace	Vla	5.1	40.7	23.3
H ₂ O	VIII	10.2	134.0	0.7
50H_A	-		148.0	3.7

Sel grp = selectivity group, Ave = average, pm = plant material, x/g = extracted per gram, AS = acetone soluble. Hex = *n*-hexane, P-e = *d*-isopropyl ether, E-e = *d*-ethyl ether, Mdc = methylene dichloride, Thf = tetrahydrofuran, E-a = ethyl acetate, Ace = acetone, Met = methanol, H₂O = water and H₂-A = water: acetone.

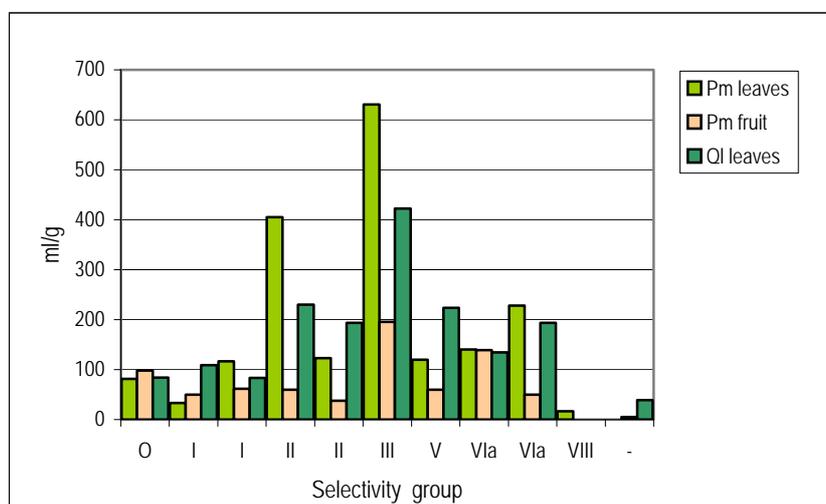


Figure 3.5. Graph of total activity for extractants in selectivity groups for each plant material type. (Pm = *Pteleopsis myrtifolia*, Ql = *Quisqualis littorea*).

Extractants in selectivity groups II, III, V and Vla had good activity. These extractants had the required solvents strengths to extract active compounds from the plant materials used. These selectivity groups correspond to extractants with intermediate polarity.

MIC values of *Pteleopsis* leaves from different environments were determined and did not differ significantly (results not shown).

3.3.1.3 Bioautography

Bioautograms were made of the different extractants and bacteria to confirm MIC values, as well as to see if the same compounds (R_f values) were responsible for antibacterial activity for the different bacteria (Figures 3.6, 3.7 and 3.8). In each of Figures 3.6-3.8, the top row is the plant material of *P. myrtifolia* leaves, the middle row is *P. myrtifolia* fruit and bottom row is *Q. littoria* leaves. In Figures 3.6-3.8, the white areas indicate growth inhibition of bacteria and thus antibacterial activity of plant substance on the specific area (R_f) of the TLC.

Areas of growth inhibition were not the same for all bacteria. In figure 3.6 the R_f (of the areas of growth inhibition (1-4)) value in brackets behind each bacterium's name, for the hexane extract of *P. myrtifolia* leaves, differed for the bacteria *S. aureus* ((1) no R_f), *E. faecalis* ((2a) 0.04 and (2b) 0.28), *P. aeruginosa* ((3) 0.28), and *E. coli* ((4) 0.58). This indicated that (except for some similarities) different compounds were responsible for antibacterial activity for the different bacteria. In cases where the R_f values are the same, it would indicate one compound having a broad antibacterial spectrum. It could also be seen that the *n*-hexane extract of leaf material (*Pteleopsis* and *Quisqualis*) showed no or slight inhibition of growth of *S. aureus* and the water extract of *Pteleopsis* leaves and fruit showed no or slight inhibition of growth of *P. aeruginosa*.

For all plant material types, the CEF eluent system indicated areas of bacterial growth inhibition the clearest. In some cases (e.g. *E. coli* with *P. myrtifolia*) there is a good correlation between low MIC and clear areas of inhibition. In other cases (e.g. *E. coli* with *Q. littorea*) however, the clear area of inhibition on the bioautogram, is not as large as one would expect from a low MIC value. This can be explained if the active compounds are volatile, then they may have

BEA eluent system

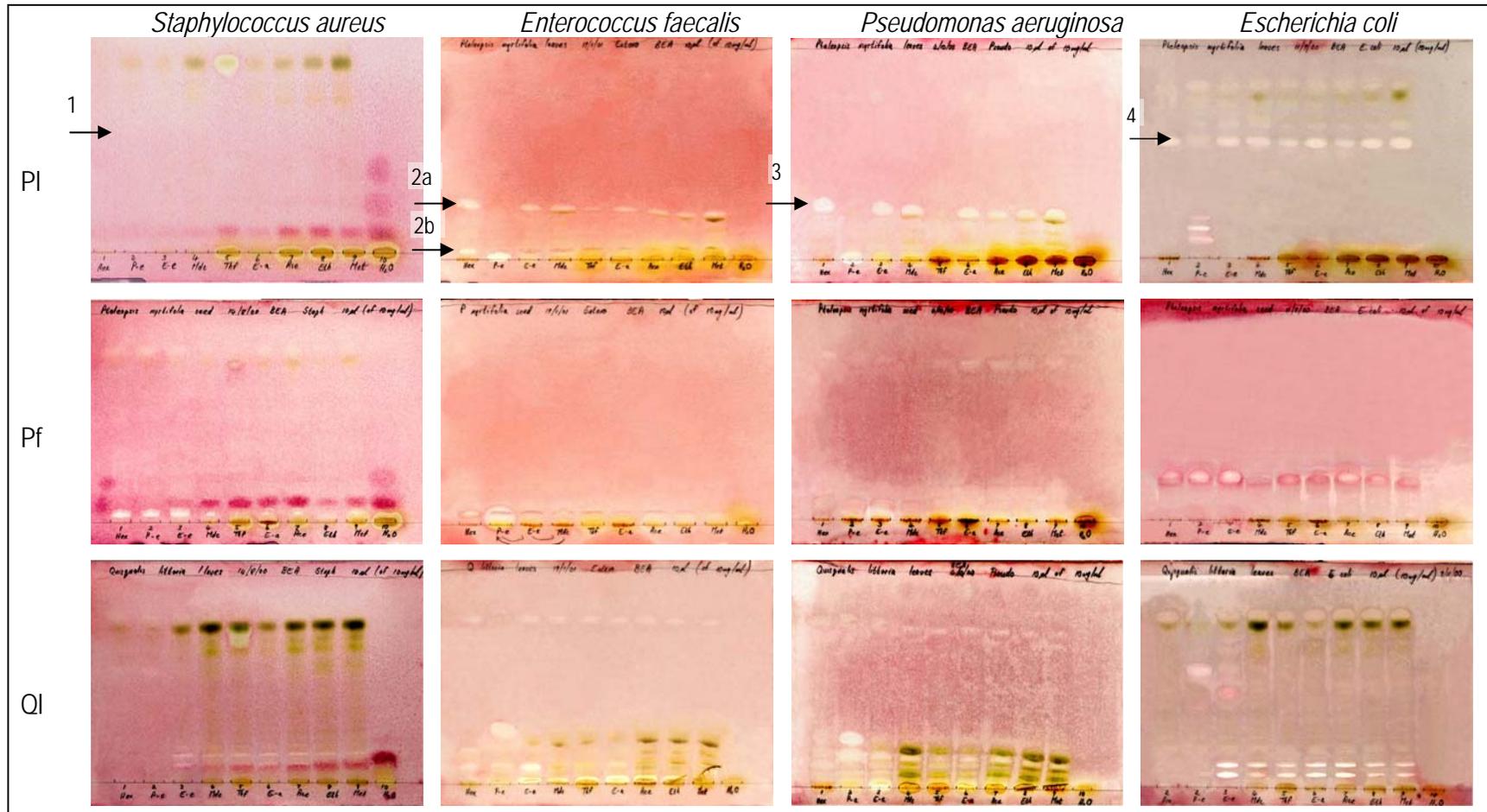


Figure 3.6. Bioautograms of PI (*Pteleopsis myrtifolia* leaves), Pf (*Pteleopsis myrtifolia* fruit) and QI (*Quisqualis littorea* leaves) developed with the BEA eluent system, sprayed with bacteria (columns) and 2.0 mg/ml p-iodonitrotetrazolium violet solution. For each bioautogram, lanes from left to right are: Hex = *n*-hexane, P-e = *d*-isopropyl ether, E-e = *d*-ethyl ether, Mdc = methylene dichloride, Thf = tetrahydrofuran, E-a = ethyl acetate, Ace = acetone, Met = methanol, H₂O = water or H₂-A = water: acetone. (BEA = benzene: ethanol: ammonia (36:5.4:4)).

CEF eluent system

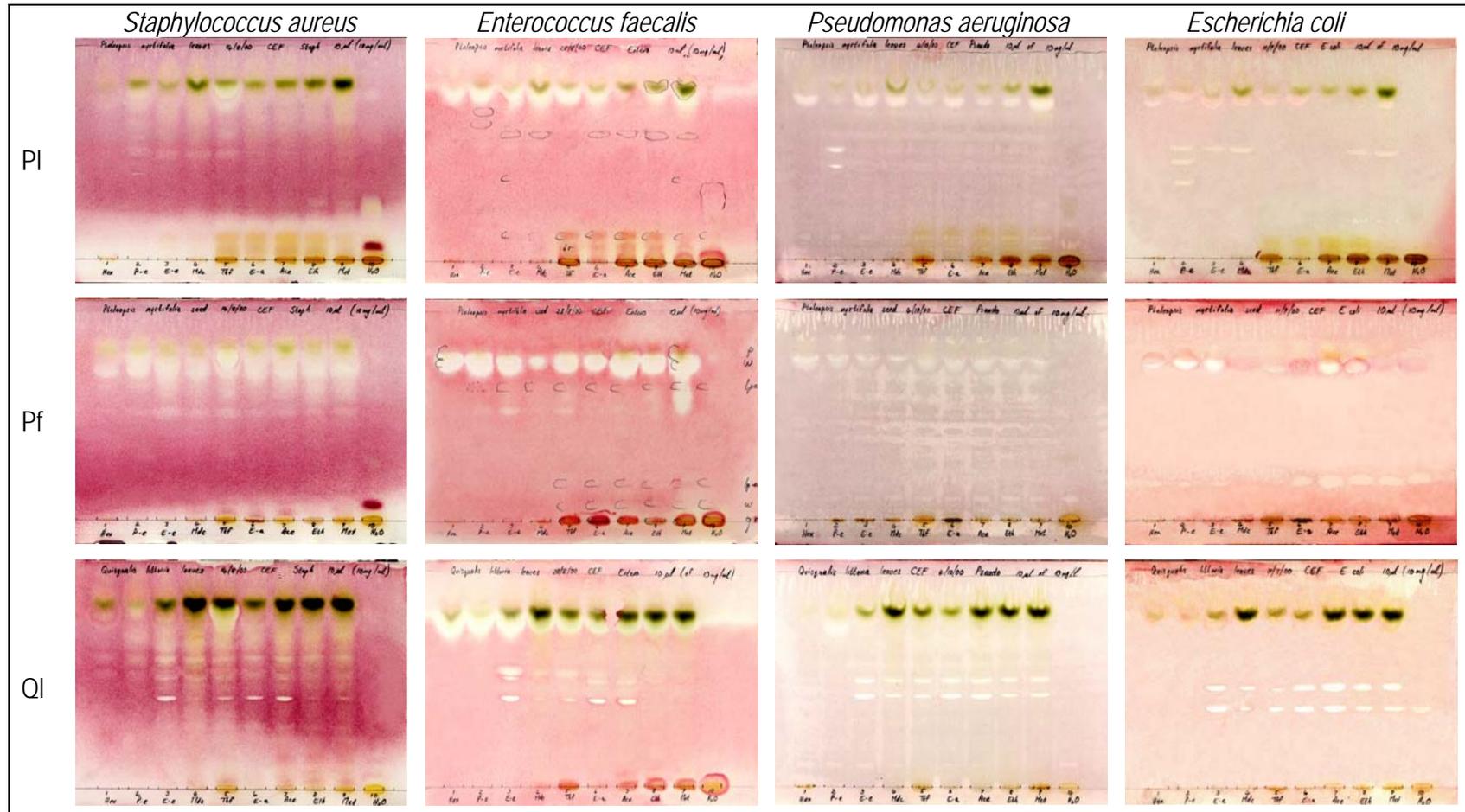


Figure 3.7. Bioautograms of PI (*Pteleopsis myrtifolia* leaves), Pf (*Pteleopsis myrtifolia* fruit) and QI (*Quisqualis littorea* leaves) developed with the CEF eluent system, sprayed with bacteria (columns) and 2.0 mg/ml p-iodonitrotetrazolium violet solution. For each bioautogram, lanes from left to right are: Hex = *n*-hexane, P-e = *d*-isopropyl ether, E-e = *d*-ethyl ether, Mdc = methylene dichloride, Thf = tetrahydrofuran, E-a = ethyl acetate, Ace = acetone, Met = methanol, H₂O = water or H₂-A = water: acetone. (CEF = chloroform: ethyl acetate: formic acid (5:4:1)).

EMW eluent system

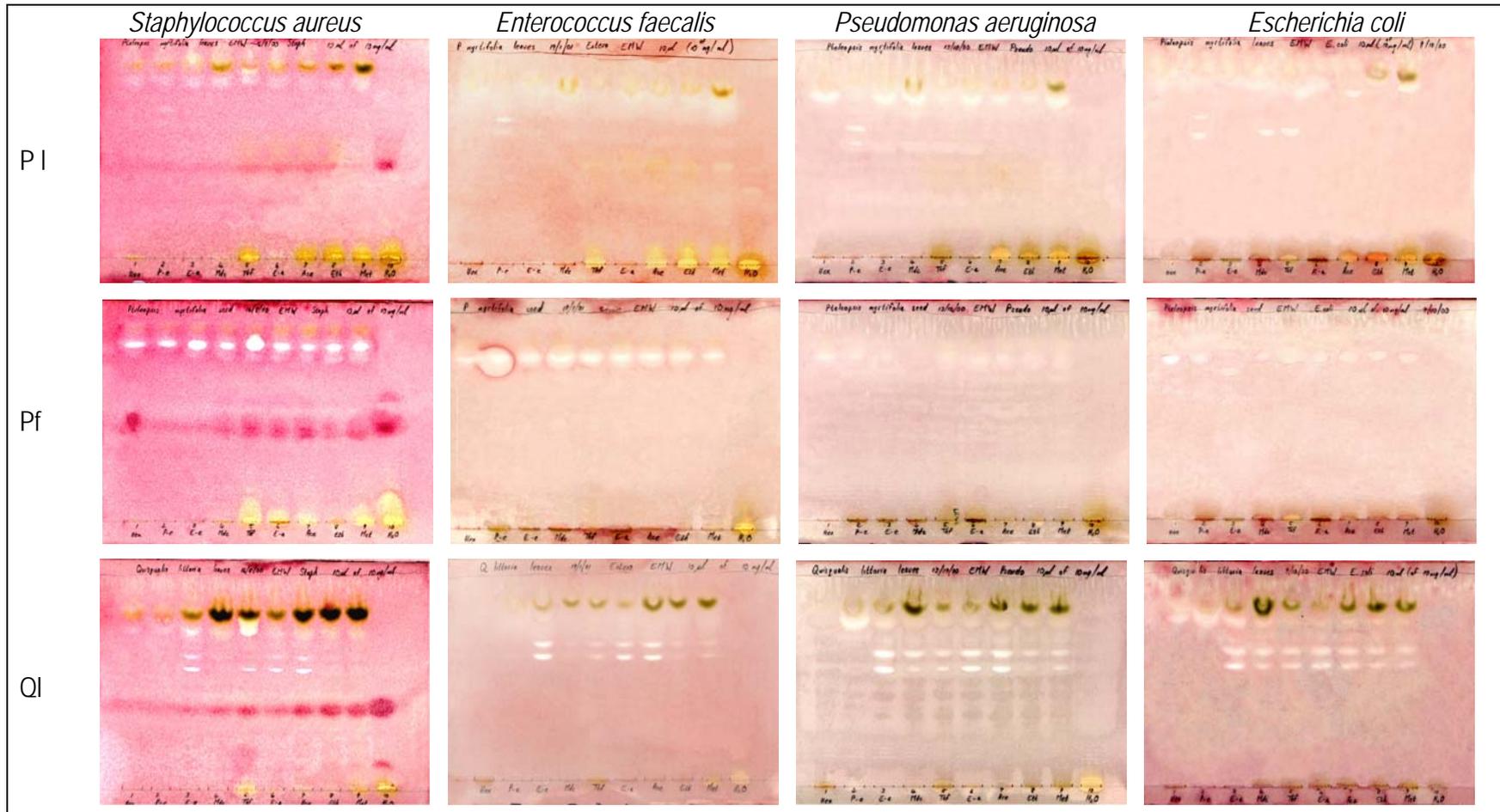


Figure 3.8. Bioautograms of PI (*Pteleopsis myrtifolia* leaves), Pf (*Pteleopsis myrtifolia* fruit) and QI (*Quisqualis littorea* leaves) developed with the EMW eluent system, sprayed with bacteria (columns) and 2.0 mg/ml p-iodonitrotetrazolium violet solution. For each bioautogram, lanes from left to right are: Hex = *n*-hexane, P-e = *di*-isopropyl ether, E-e = *di*-ethyl ether, Mdc = methylene dichloride, Thf = tetrahydrofuran, E-a = ethyl acetate, Ace = acetone, Met = methanol, H₂O = water or H₂-A = water: acetone. (EMW = ethyl acetate: methanol: water (40:5:4.4)).

evaporated from the overnight drying of the chromatograms before treatment with bacteria.

The bacterium *S. aureus* also indicated areas (on all three of the eluent systems, clearer with BEA and EMW (Figure 3.6 and 3.8)) of growth promotion (areas that stained darker red than the rest of the plate) and all plant materials. For *S. aureus* the MIC values obtained may be an average of the growth inhibitory and growth promoting areas.

Above-mentioned areas of bacterial growth inhibition (clear, but show white, as the TLC plate is white) may not be the only ones. Brown areas formed after application (on the baseline of the plates) of the more polar extracts like tetrahydrofuran, ethyl acetate, acetone, ethanol, methanol and water or 50% water and 50% acetone. In Figure 3.7 it can be seen that they were not in all cases (like with *E. faecalis*) clearly covered by a red formazan colour after sprayed with INT, and they probably contribute to the extracts' antibacterial activity.

Areas of antibacterial inhibition were tabulated according to R_f values. While tabulated, it was almost impossible to make a reasonable interpretation about the R_f values. The R_f values were therefore plotted in graphs to find visual presentations of antibacterial activity. Figures 3.9 and 3.10 are visual presentations of some R_f values found for the CEF and EMW respectively. For the CEF system the R_f values of the methylene dichloride, acetone and ethyl acetate extracts are represented in Figure 3.9, and for the EMW system the *d*-isopropyl ether, acetone and methanol extracts are represented in Figure 3.10. Similar R_f values (within one eluent system), indicate which extracts isolated the same compound, for example, the methylene dichloride, acetone and ethyl acetate extractants all isolated the compounds '1' and '2' in Figure 3.9, and compounds '3' and '4' in Figure 3.10. Compounds with similar R_f values that inhibited more than one bacterium, indicate compounds with broad antibacterial spectra, for example, "a" and "e" from *Pteleopsis* leaves, "b" and "c" from *Pteleopsis* fruit, and "d" from *Quisqualis* leaves (Figures 3.9 and 3.10). In Figure 3.9, only methylene dichloride isolated a

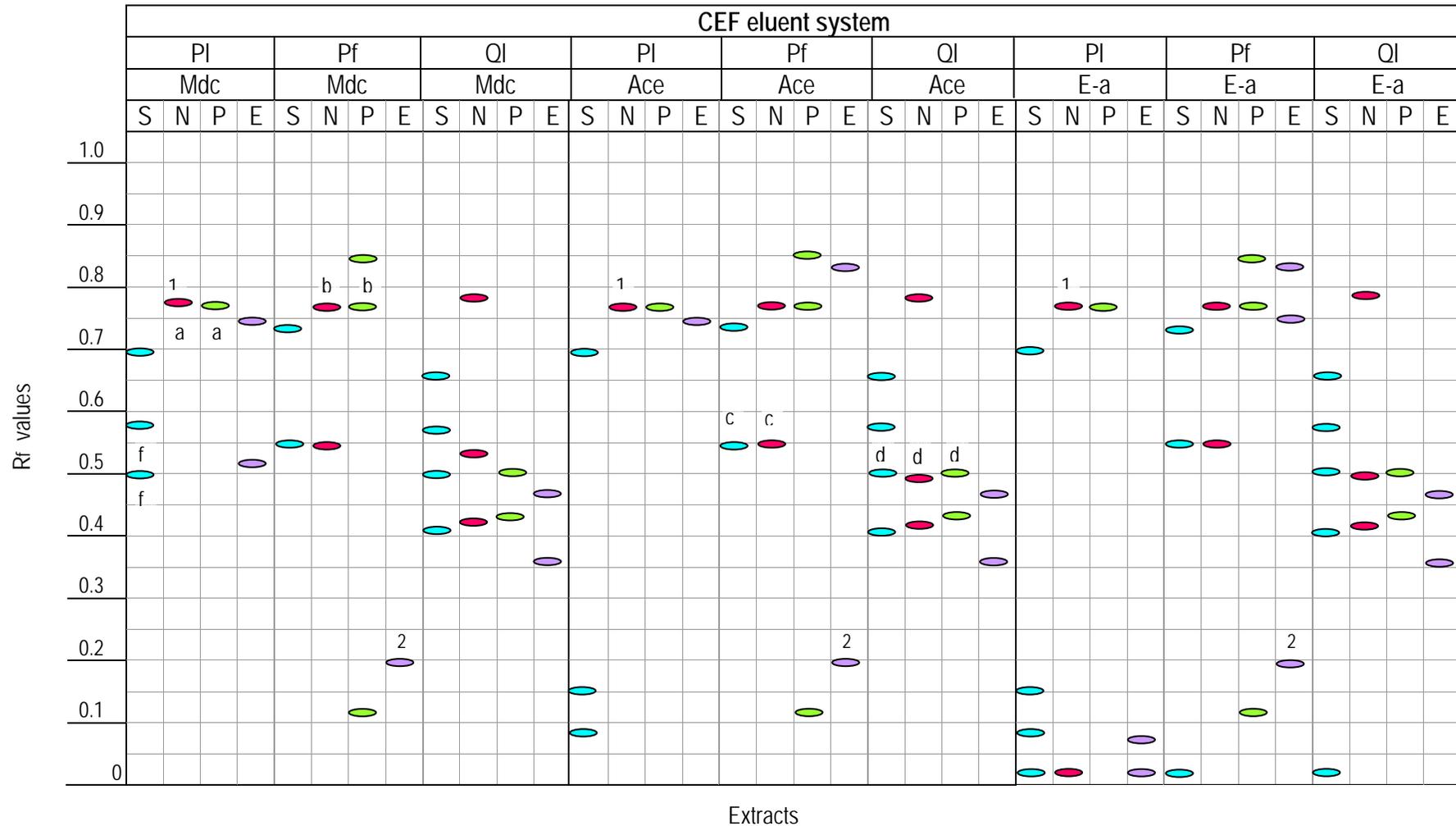


Figure 3.9. Visual presentation of R_f values of the methylene dichloride, acetone and ethyl acetate extracts on bioautograms developed with the CEF eluent system. PI = *Pteleopsis* leaves, Pf = *Pteleopsis* fruit, QI = *Quisqualis* leaves, S = *Staphylococcus aureus* (●), N = *Enterococcus faecalis* (●), P = *Pseudomonas aeruginosa* (●), E = *Escherichia coli* (●), Mdc = methylene dichloride, Ace = acetone, E-a = ethyl acetate, CEF = chloroform: ethyl acetate: formic acid (5:4:1).

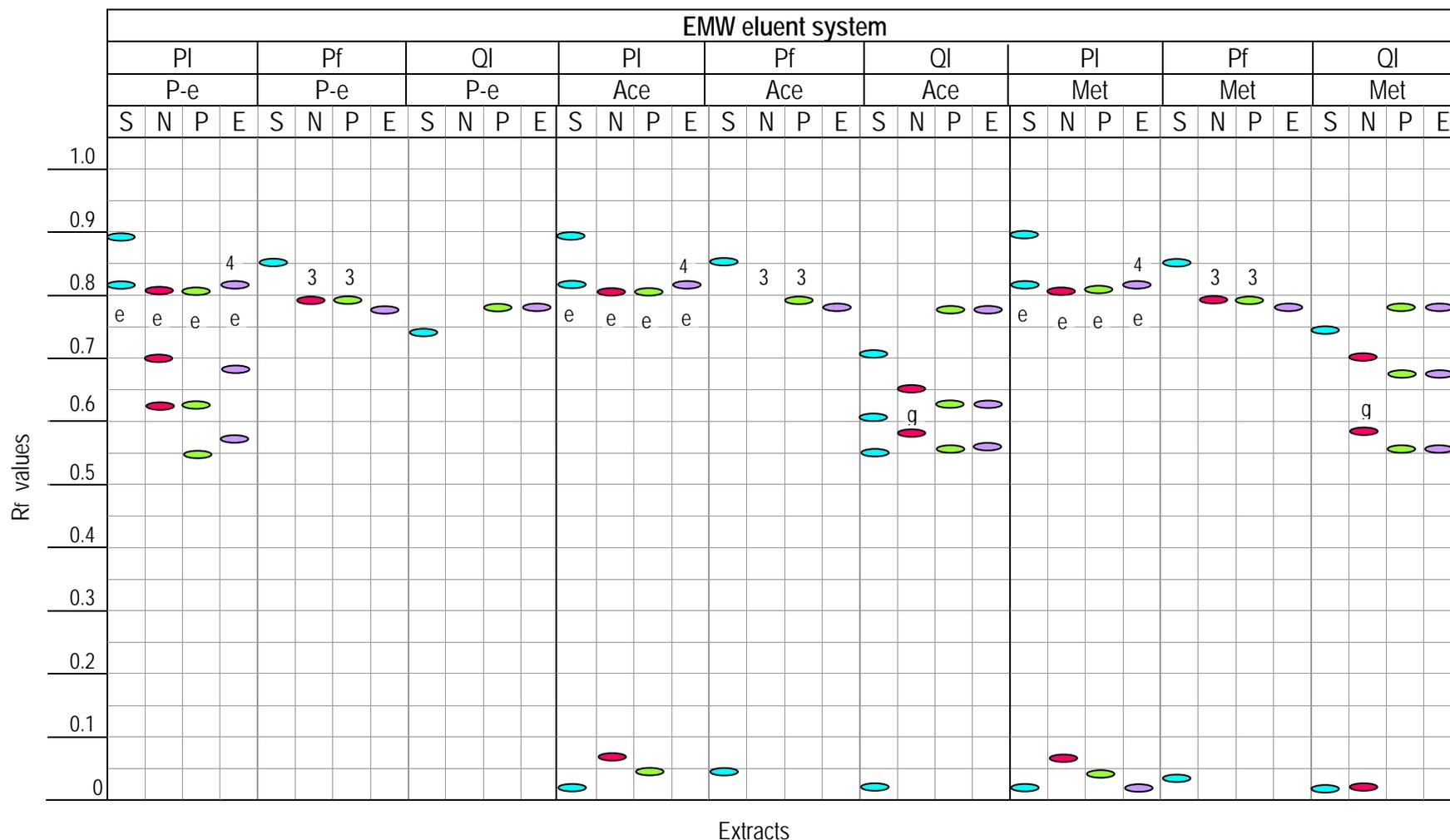


Figure 3.10. Visual presentation of R_f values of the *di*-isopropyl ether, acetone and methanol extracts on bioautograms developed with the EMW eluent system. PI = *Pteleopsis* leaves, Pf = *Pteleopsis* fruit, QI = *Quisqualis* leaves, S = *Staphylococcus aureus* (●), N = *Enterococcus faecalis* (●), P = *Pseudomonas aeruginosa* (●), E = *Escherichia coli* (●), P-e = *di*-isopropyl ether, Ace = acetone, Met = methanol, EMW = ethyl acetate: methanol: water (40:5:4.4).

compound indicated by " f ", from *Pteleopsis* leaves to which *S. aureus* was sensitive, and acetone and ethyl acetate did not. Similarly, only the acetone and methanol extracts isolated a compound indicated by " g ", from *Quisqualis* leaves to which *E. faecalis* was sensitive, and the *d*-isopropyl ether did (Figure 3.10).

3.3.2 Stability of extracts over time

MIC values of *P. myrtifolia* leaf extracts that were determined when freshly prepared, were compared to leaf extracts extracted and redissolved in acetone 8 months previously. The amount extracted was taken into account and total activity was calculated. For the extractants investigated: tetrahydrofuran, ethyl acetate, ethanol and methanol, the total activity for *S. aureus* was reduced within 8 months. The total activity for *E. faecalis*, *P. aeruginosa* and *E. coli* increased for tetrahydrofuran, ethyl acetate, acetone and ethanol after an 8-month period (Figure 3.11).

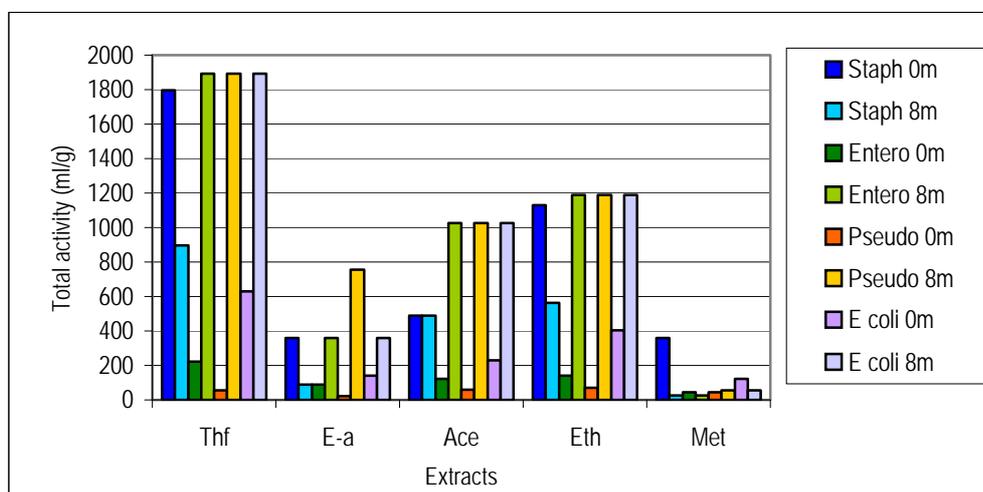


Figure 3.11. Total activity of *Pteleopsis myrtifolia* leaves against *Staphylococcus aureus* (Staph), *Enterococcus faecalis* (Entero), *Pseudomonas aeruginosa* (Pseudo) and *Escherichia coli* (E coli) and the extractants tetrahydrofuran (Thf), ethyl acetate (E-a), acetone (Ace), ethanol (Eth), and methanol (Met) at 0 and 8 months.

The loss of activity of certain extracts after storing in the cold could have been due to chemical modification of active compounds or to their precipitation over time. Since the extracts were kept in screw-capped containers and the volume was checked and evaporation losses corrected before testing their antibacterial activity, the surprising increased antibacterial activity could be explained if some inhibitory compounds were volatile or unstable over time, and this would explain the increased activity. The mechanism of the enhancement of potency and subsequent stability should be investigated (Eloff, 1999).

3.3.3 A test for toxicity of tetrahydrofuran

The CP grade of tetrahydrofuran (which was used for the assays) gave only 100 µl of a 10 mg/ml final concentration and when tested, MIC values were not increased, compared to a control where only water was added to the wells with bacteria (*S. aureus*, *E. faecalis*, *P. aeruginosa* and *E. coli*). A bigger volume than 15 ml did increase the MIC values and there was therefore decided not to use this extractant for a large extract. If one would use tetrahydrofuran for large extracts, activity might be mistakenly ascribed to its measurable toxicity in larger volumes.

3.4 Conclusions

Results obtained in this study clearly show that *P. myrtifolia* leaves and fruit and *Q. littorea* leaves contain several antibacterial compounds. The majority of plants do not have activity against Gram-negative bacteria (Vlietinck *et al.*, 1995), but *Q. littorea* leaf extracts have good activity against the Gram-negative bacterium, *E. coli*.

Establishing measurable toxicity to the test organisms when more than 15 ml tetrahydrofuran extractant is used, further indicate (except the reasons discussed in Chapter 2) that acetone is the extractant of choice for antibacterial assays and will be used in future investigations

(Chapter 4).

Further research to isolate pure compounds from antibacterial active fractions, as well as establishing toxicities of extracts of this plant, may offer medicinal uses for the indigenous population (at little cost) or offer a structure (of a pure compound) for Pharmaceutical development. If extracts would be used on a large scale for medicinal purposes, it is of utmost importance that cultivation and conservation of this plant accompany the use for medicinal purposes.

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