Chapter 4

Solvent-solvent separation

Abstract

The skilful application of fractionation methods exploits the fact that an extract can be separated into groups of compounds sharing similar physico-chemical characteristics. Acetone was chosen as an initial extractant for leaves of *Pteleopsis myrtifolia* because many polar and non-polar compounds are soluble in acetone and the test organisms (bacteria) are not sensitive to 25% acetone in a serial dilution assay. Bioassay-guided fractionation identified that all fractions: n-hexane, carbon tetrachloride, chloroform, 35% water in methanol, n-butanol and water had antibacterial activity against the Gram-positive bacteria, *Staphylococcus aureus* and *Enterococcus faecalis*. The chloroform and 35 % water in methanol extracts were active against the Gram-negative bacteria, *P. aeruginosa* and *E. coli.* The chloroform fraction was antibacterial to all bacteria tested, and had the largest amount of inhibition areas (seven), and thus amount of bacterially active compounds. This fraction was chosen for further purification and to isolate pure compounds from it in future research

4.1 Introduction

4.1.1 Initial extractant for solvent-solvent separation

A variety of extractants was examined for their ability to solubilise antimicrobial compounds from plants. The focus of the study was to provide a more standardised extraction method for a wide variety of researchers working in diverse settings (Eloff, 1998b). Although it is not one of the more frequently used extractants in studies published to date, acetone received the highest overall rating. Acetone had the advantage that it dissolved hydrophilic and lipophylic components from the plants used, was miscible with water, was volatile and had a low toxicity to

the bioassay used. In the table that Eloff (1998b) published, it could be seen from the number of inhibitors that each solvent extracted, that the most active components were not water-soluble. Cowan (1999) referred to the solvents (acetone, methylene dichloride, methanol, ethanol, water) that Eloff (1998b) examined and pointed out that in a review of 48 articles describing the screening of plant extracts for antimicrobial properties in recent years of the *Journal of Natural Products*, the *Journal of Ethnopharmacology*, and the *International Journal of Pharmacognosy*, only one study used acetone as an extractant. That the solvents used most often for initial extractions, like ethanol and methanol, may not demonstrate the greatest sensitivity in yielding antimicrobial chemicals on an initial screening and advised that this disparity should be addressed as the search for antimicrobials intensifies.

4.1.2 Solvent selection to find potentially active phytoconstituents

Physical properties of solvents (availability, detector compatibility, solvent reactivity, boiling point, viscosity, miscibility and safety) to be used for extractions and fractionations need to be carefully considered to make sure the desired compounds are extracted or separated. The total interaction of a solvent molecule with a sample molecule is the result of four interactions: dispersion, dipole, hydrogen bonding and dielectric. The larger these dispersion, dipole, hydrogen bonding and dielectric interactions are in combination, the stronger is the attraction of solvent and solute molecules (Snyder & Kirkland, 1979). Various solvents can be grouped according to their selectivity on a triangular diagram (without considering intermolecular effects) (Snyder & Kirkland, 1979). Solvents are then grouped into clusters of similar selectivity (Figure 4.1).

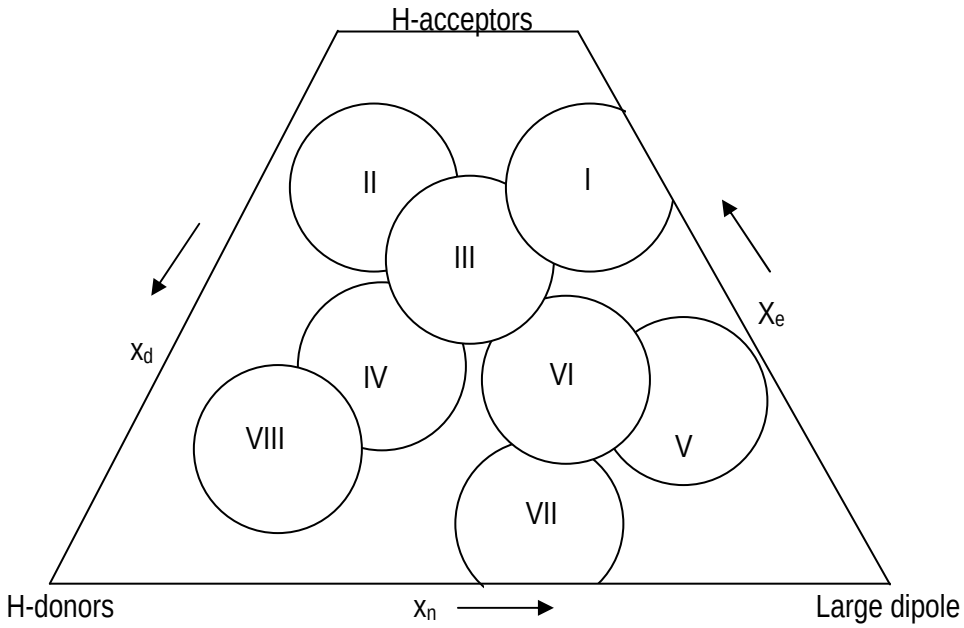


Figure 4.1 Solvents grouped according to selectivity in a selectivity triangle (Snyder & Kirkland, 1979).

4.1.3 Fractionation

The components of a mixture, such as an extract from a living organism, can be separated into groups of compounds sharing similar physico-chemical characteristics. This process is called fractionation and can be carried out in various ways, each of, which group compounds according to one or more particular features. Solubility, size, shape, electrical charge and several other features may influence grouping (Houghton and Raman, 1998). The skilful application of fractionation methods exploits these differences so that, when two methods are used in sequence, many fractions can be obtained each containing only one or two components. The initial fractionation may be based on solubility differences (e. g. solvent-solvent separation) while the second may utilise molecular size (e. g. Sephadex column chromatography). After a specific plant's activity has been established, the plant extract is fractionised by solvent-solvent separation and activity of different fractions established with bioassay guided fractionation. Utilising this method in initial purification, enables the isolation of

an active fraction and improve the probability to find pure compounds with similar activity above that of working with a crude extract only (Gailliot 1998; Houghton and Raman, 1998).

Acetone was chosen as an initial extractant because many polar and non-polar compounds are soluble in acetone and the test organisms (bacteria) are not sensitive to 25% acetone in a serial dilution assay (Eloff, 1998a). The aim of this investigation was to simplify a leaf extract of *Pteleopsismyrtifolia* by fractionation. Antibacterial activity of the different fractions will be established and quantified. One or more fractions with antibacterial activity will be used in a further investigation to isolate pure compounds from.

4.2 Material and Methods

4.2.1 Plant material

Leaves of *P. myrtifolia* (the same tree that has voucher specimens number 24/2000 in Lowveld NBI herbarium) were collected in the National Botanical Garden of Nelspruit during March 2000 and 2001 and prepared as described in 2.2.1 of Chapter 2.

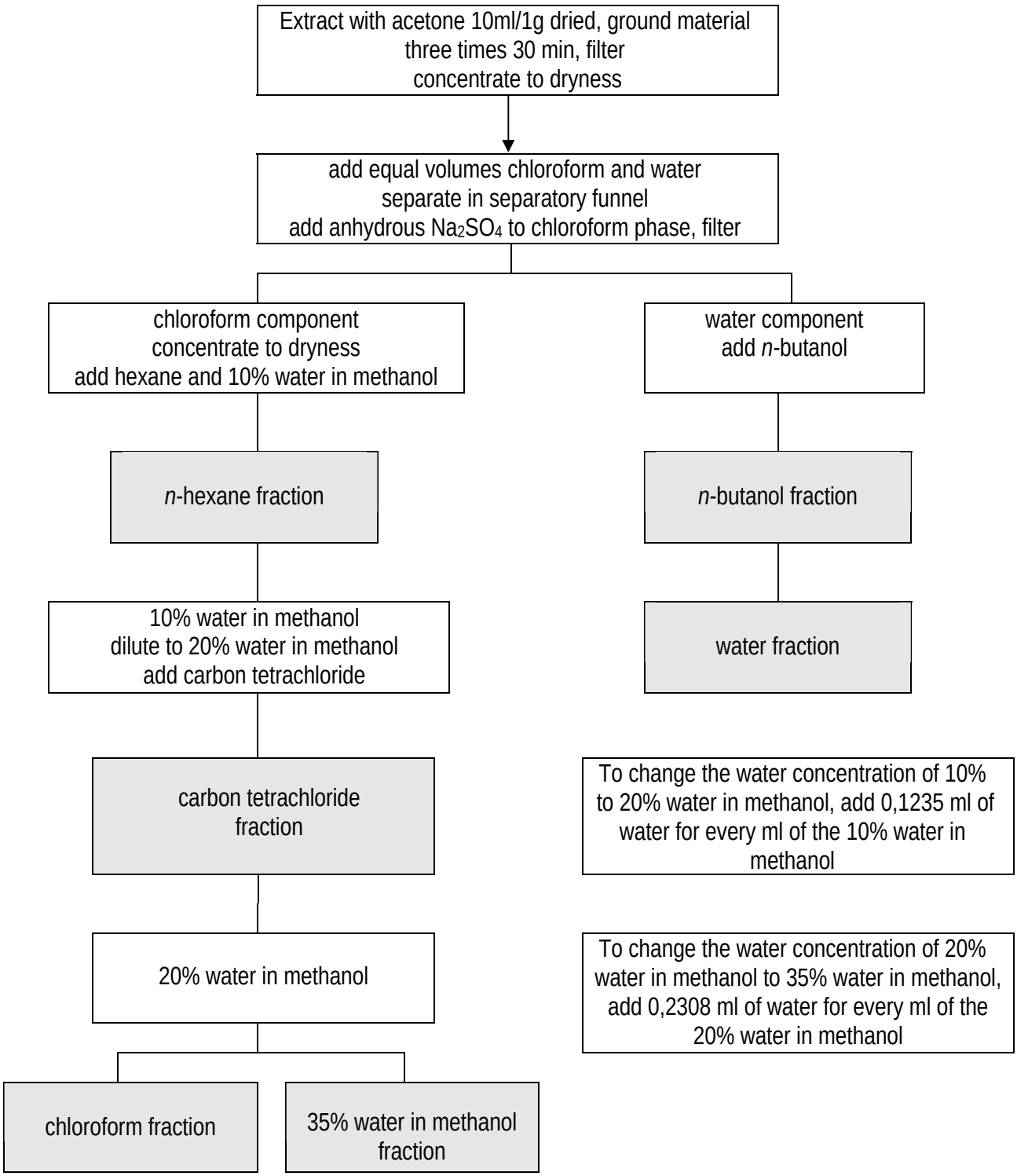
4.2.2 Solvent-solvent separation

The solvent-solvent separation procedure used by the USA National Cancer Institute as described by Suffness and Douros (1979) with a slight variation was used. Finely ground air-dried material (1 kg) of leaves were placed in a 10-liter glass bottle and filled (1:3) w/v with acetone. The bottle with ground leaves and acetone was then carefully shaken on a Labotec model 202 orbital shaker for one hour. Care had to be taken to secure the bottle on the rotary shaker, to avoid momentum and weight to move the bottle because of rotating motion. After an hour, the liquid was removed and filtered through Whatman No 1 quantitative filter paper. Fresh acetone was again added to the ground leaves. The container with leaf material was again shaken for one hour and the supernatant filtered. This was repeated for a third time. The

filtered liquid was dried by vacuum distillation in a Büchi rotary evaporator and the dry weight of the extract determined. The solvent-solvent separation method was followed (diagram below).

A schematic diagram of the method of the solvent-solvent separation procedure is shown in 4.2.2.1.

4.2.2.1 Schematic diagram of method of solvent-solvent separation procedure:



4.2.3 Thin layer chromatography (TLC)

The different fractions, namely: *n*-hexane, carbon tetrachloride, chloroform, 35 % water in methanol, *n*-butanol and water were each separated by thin layer chromatography (TLC) (10 µl of a 10 mg/ml final concentration) on Merck Silica gel F_{254} plates. The same eluent systems (selected to separate high, intermediate and low polarity) as in Chapter 2 were used. Separated compounds were examined under visible and ultraviolet light, marked and sprayed as described in Chapter 2.

To determine antibacterial activity, fractions were used to develop minimum inhibitory concentrations. Duplicate sets of thin layer chromatograms were used to develop bioautograms.

4.2.4 Determining antibacterial activity

4.2.4.1 Minimum inhibitory concentration (MIC)

MIC values were determined as described in 3.2.4.1 of Chapter 3.

4.2.4.2 Bioautography

For bioautography, all the fractions (10 µl of a 10 mg/ml final concentration) were applied on thin layer chromatograms as described in 3.2.4.3 of Chapter 3.

4.3 Results and Discussion

4.3.1 Fractions of group separation

After the third extraction with acetone, most of the green colour was washed out. The large amount of bubbles that originated between two phases of more polar fractions (without movement of the separating funnel) could be an indication of the presence of saponins. Dry

weight of each fraction was (listed in brackets): *n*-hexane (8.7 g), carbon tetrachloride (4.2 g), chloroform (9.8 g), 35% water in methanol (4.1 g), *n*-butanol (11.8 g) and water (5.6 g).

4.3.2 Thin layer chromatography (TLC)

Chromatograms developed by TLC, indicated the complexity of the material (each of the fractions contained several compounds) (Figure 4.2).

4.3.3 Antibacterial activity

4.3.3.1 Minimum inhibitory concentration (MIC) and total activity

MIC values indicated that all fractions had antibacterial activity (Table 4.1). Total activity values were calculated (as described by Eloff (2004) and explained in 3.2.4.2 of Chapter 3) and are listed in Table 4.1.

Table 4.1. Minimum inhibitory concentrations and total activity of *Pteleopsis myrtifolia* leaf fractions from liquid-liquid separation for the bacteria *Staphylococcus aureus* (S), *Enterococcus faecalis* (N), *Pseudomonas aeruginosa* (P) and *Escherichia coli* (E).

Fractions from *P. myrtifolia*	mg/pm from 1 g	MIC (mg/ml)					Tot al activity (ml/g)				
		S	N	P	E	Ave	S	N	P	E	Ave
n-hexane	8.7	0.01	0.002	0.005	0.02	0.009	870	4350	1740	435	1848.8
carbon tetrachloride	4.2	0.02	0.002	0.08	0.02	0.031	210	2100	52.5	210	643.1
chloroform	9.8	0.005	0.004	0.005	0.02	0.009	1960	2450	1960	490	1715.0
35 % water in methanol	4.1	0.01	0.002	0.08	0.002	0.024	410	2050	51.25	2050	1140.3
n-butanol	11.8	0.005	0.002	0.16	0.002	0.042	2360	5900	73.75	5900	3558.4
water	5.6	0.005	0.002	0.08	0.01	0.024	1120	2800	70	560	1137.5
Average	7.4	0.009	0.002	0.034	0.012	0.023	1155	3275	657.9	1607.5	1673.9

P. myrtifolia = *Pteleopsis myrtifolia,* pm = plant material, MIC = minimum inhibitory concentration, Ave = average, S = *Staphylococcus aureus,* N = *Enterococcus faecalis* , P = *Pseudomonas aeruginosa*, E = *Escherichia coli*.

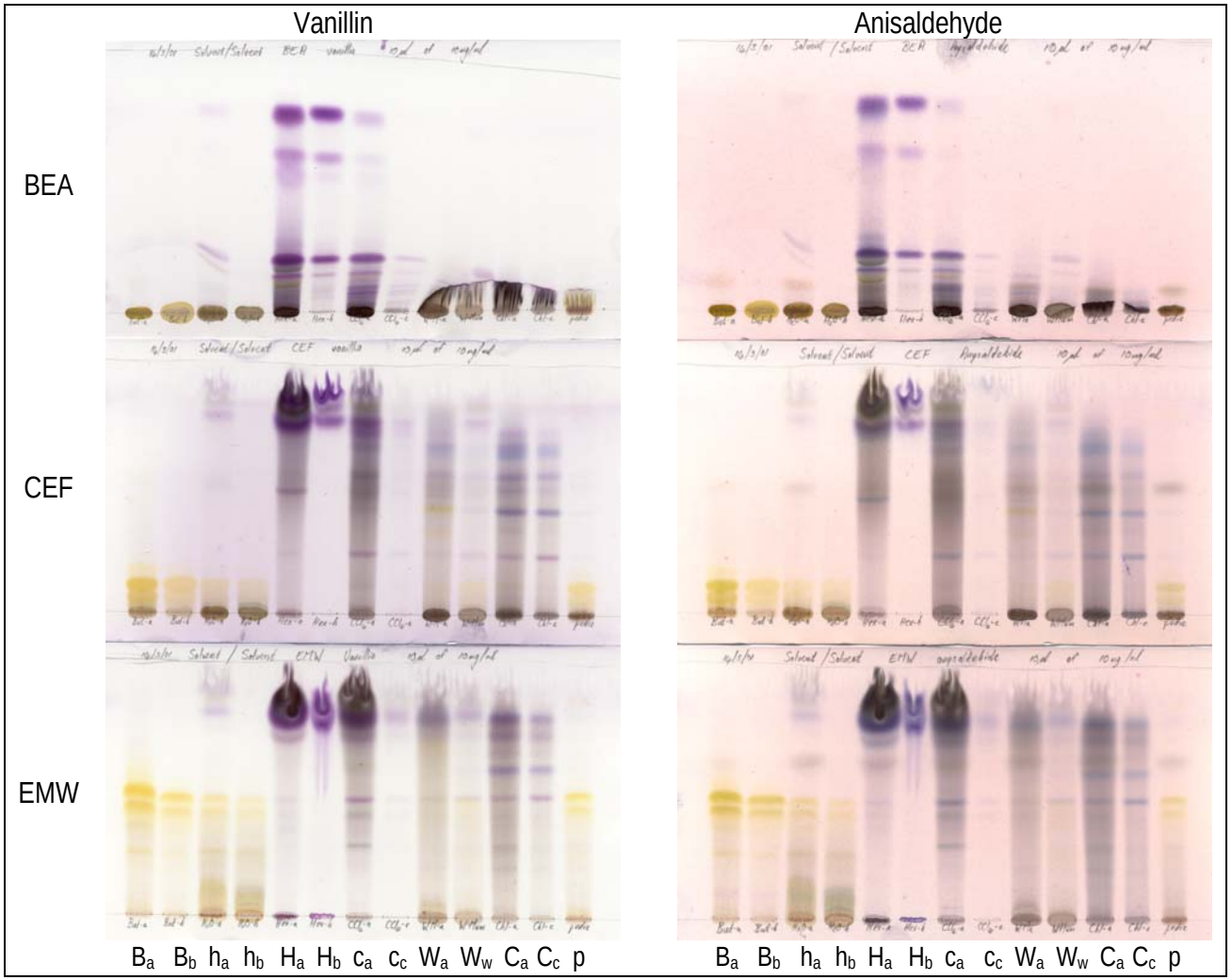


Figure 4.2. Thin layer chromatograms of solvent-solvent fractions from an acetone leaf extract of *Pteleopsis myrtifolia,* developed by the BEA solvent system (top), CEF solvent system (middle) and EMW solvent system (bottom) sprayed with vanillin (left) and anisaldehyde (right). For each thin layer chromatogram, the lanes from left to right were: But-a (B_a), But-b (B_b), H2O-a (h_a), H_2O-h (h_b), Hex-a (H_a), Hex-b (H_b), CCl_4-a (c_a), CCl_4-c (c_c), WM-a (W_a), WM-wm (W_w), Chl-a (C_a), Chl-c (C_c), pell (p). But = butanol, H_2O = water, Hex = *n*-hexane, CCl_4 = carbon tetrachloride, WM = 35% water in methanol, Chl = chloroform, pell = pellicle. Each fraction was redissolved in acetone (indicated by the suffix –a), as well as in the fraction itself (indicated by the first letter the fraction consists of (e.g. Chl-c indicates chloroform redissolved in chloroform). BEA = benzene: ethanol: ammonia (36:5.4:4), CEF = chloroform: ethyl acetate: formic acid (5:4:1), EMW = ethyl acetate: methanol: water (40:5:4.4) .

From the average MIC values of a fraction for all the bacteria (seventh column of Table 4.1), the chloroform fraction had the lowest MIC value, followed by the *n*-hexane fraction. The average MIC values of a bacterium for all the fractions (last row of Table 4.1), *E. faecalis* (N) had the lowest and *P. aeruginosa* (P) the highest MIC values. The average total activity for each fraction (all bacteria together) was calculated and the *n*-butanol, *n*-hexane and chloroform fractions had highest activities. The *n*-hexane and chloroform fractions had the best activity against all the bacteria tested.

4.3.3.2 Bioautography

From the bioautograms developed with different eluent systems and bacteria, the chloroform fraction from the EMW eluent system showed antibacterial activity for all four bacteria tested (Figures 4.3 and 4.4 and Figures 4.5 and 4.6).

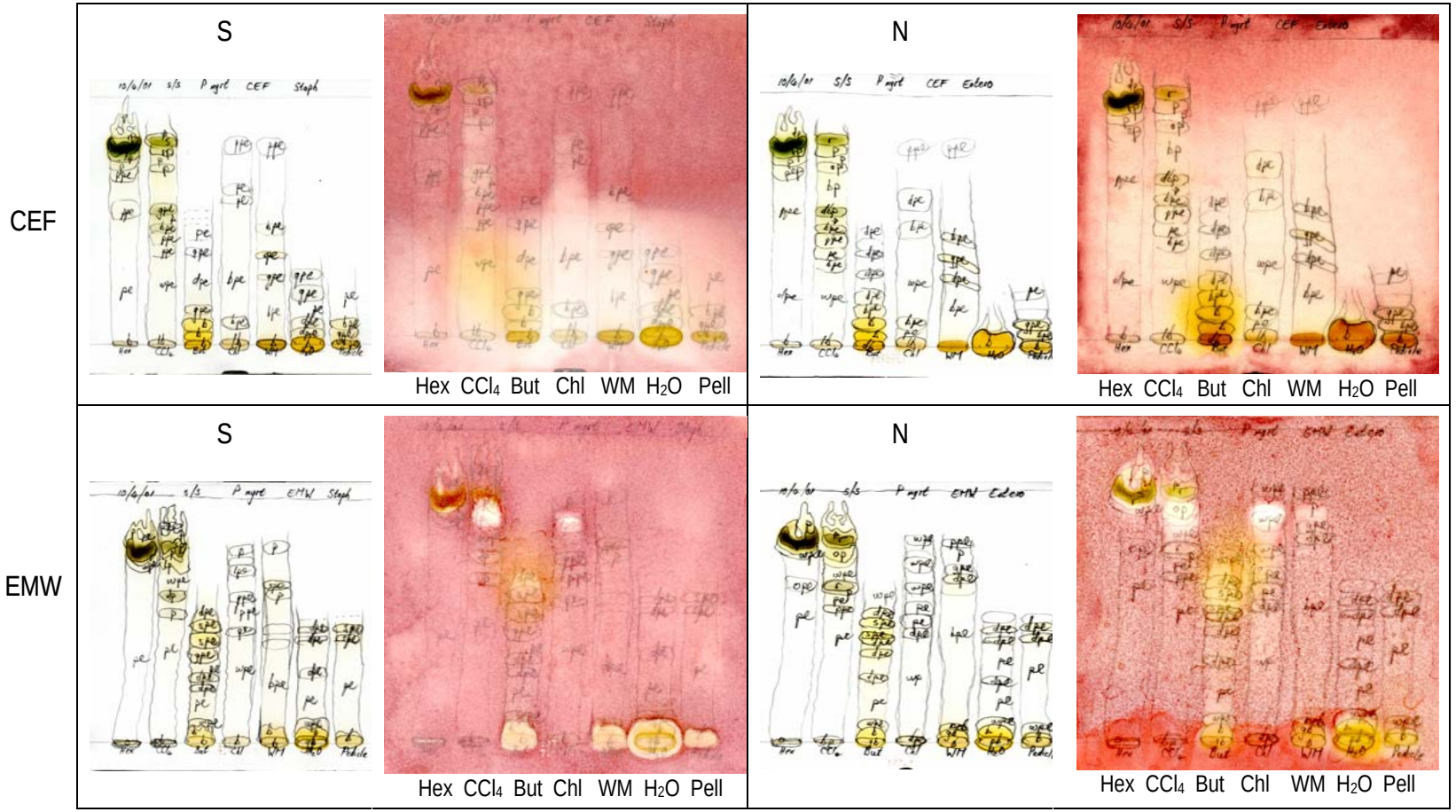


Figure 4.3. Bioautographs of fractions from *Pteleopsis myrtifolia* leaves and the bacteria *Staphylococcus aureus* (S) (left) and *Enterococcus faecalis* (N) (right). In each set, the top chromatogram was developed with the CEF eluent system and the bottom one by an EMW eluent system, and the one to the left, without bacteria. Fractions applied from lanes left to right were: Hex = *n*-hexane, CCl_4 = carbon tetrachloride, But = *n*-butanol, Chl = chloroform, WM = 35% water in methanol, H_2O = water, Pell = pellicle or foamy layer between phases of polar fractions. CEF = chloroform: ethyl acetate: formic acid (5:4:1), EMW = ethyl acetate: methanol: water (40:5:4.4).

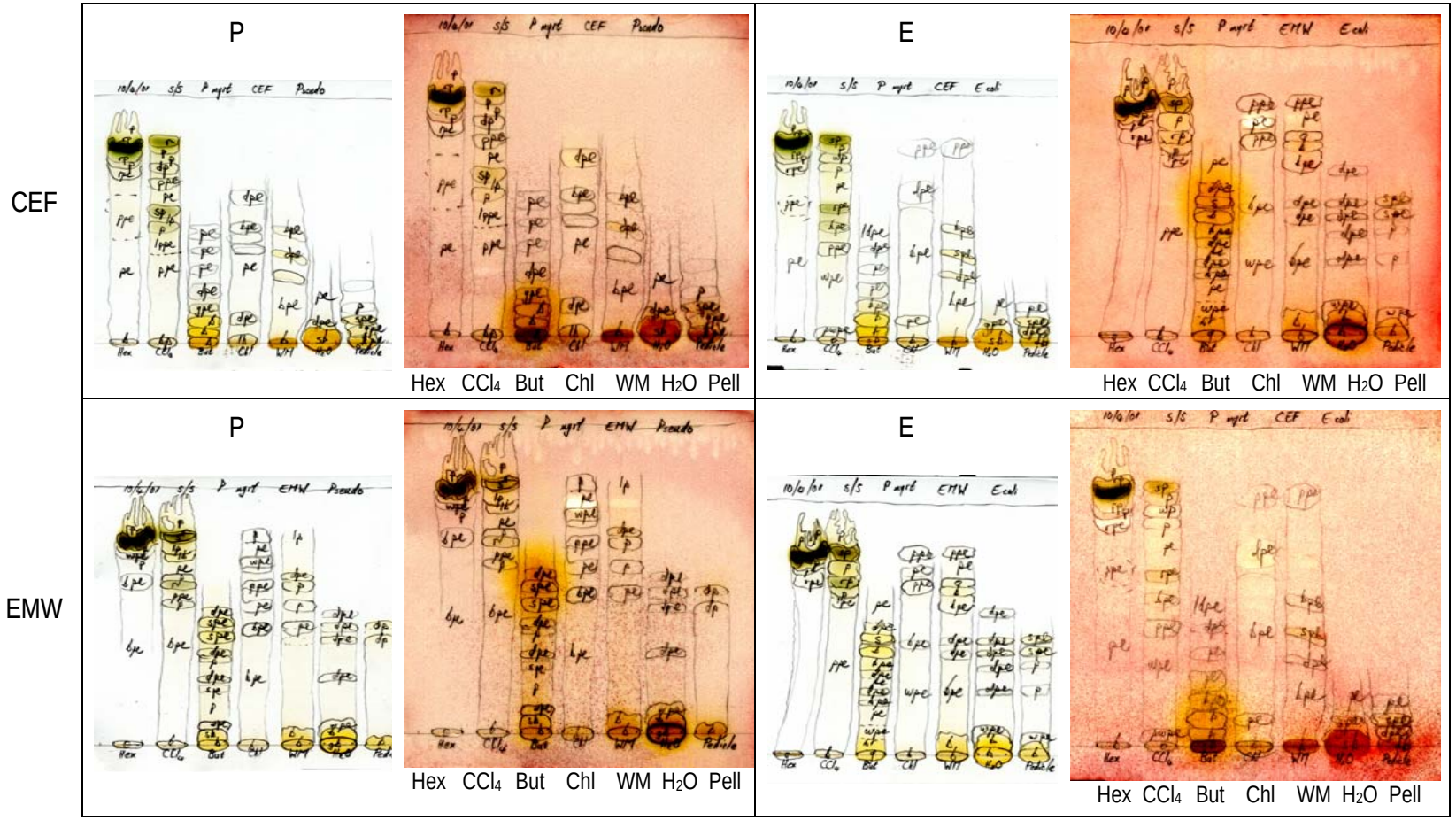


Figure 4.4. Bioautographs of fractions from *Pteleopsis myrtifolia* leaves and the bacteria *Pseudomonas aeruginosa* (P) (left) and *Escherichia coli* (E) (right). In each set, the top chromatogram was developed with the CEF eluent system and the bottom one by an EMW eluent system, and the one to the left, without bacteria. Fractions applied from lanes left to right were: Hex = *n*-hexane, CCl_4 = carbon tetrachloride, But = *n*-butanol, Chl = chloroform, WM = 35 % water in methanol, H_2O = water, Pell = pellicle or foamy layer between phases of polar fractions. CEF = chloroform: ethyl acetate: formic acid (5:4:1), EMW = ethyl acetate: methanol: water (40:5:4.4).

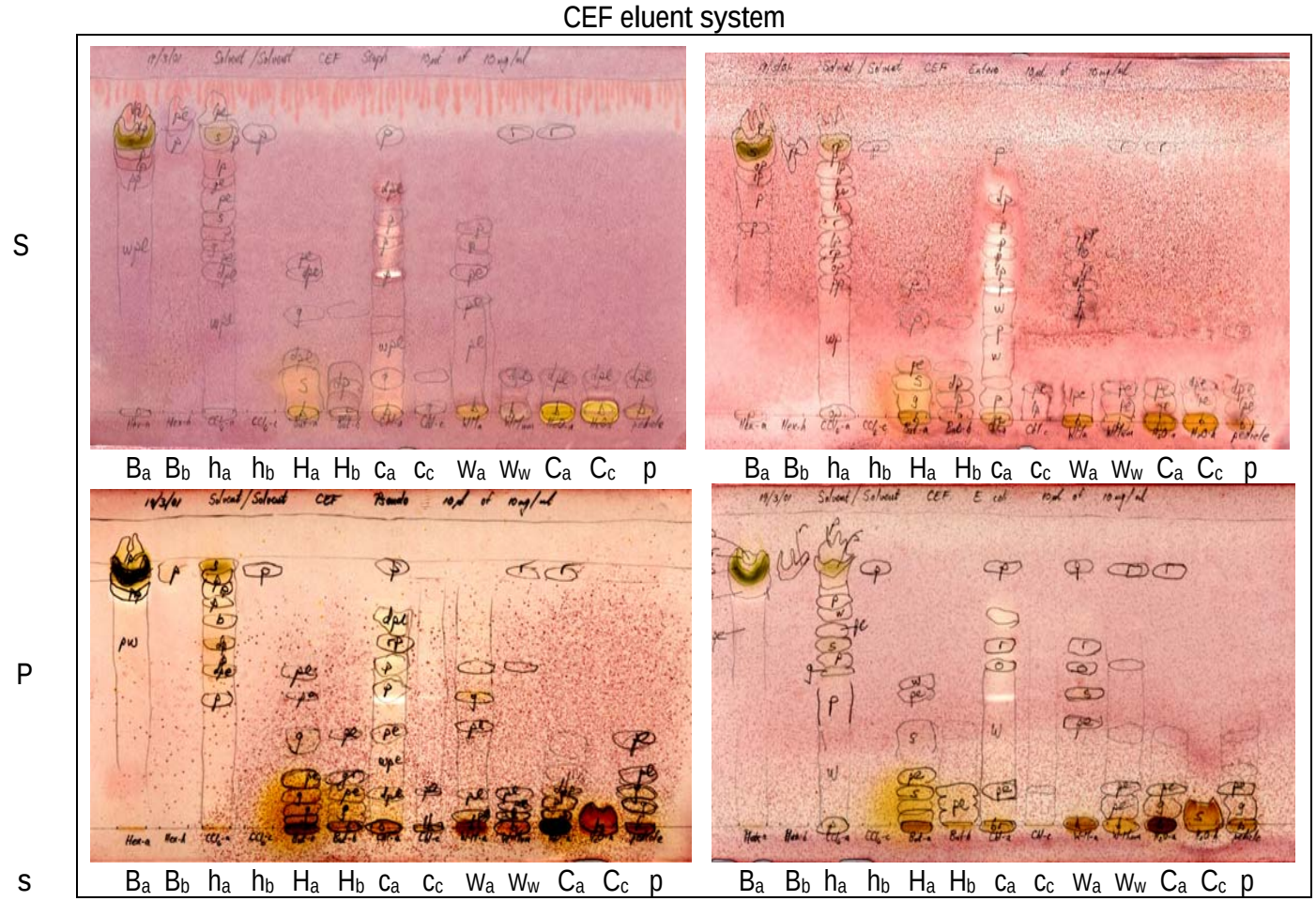
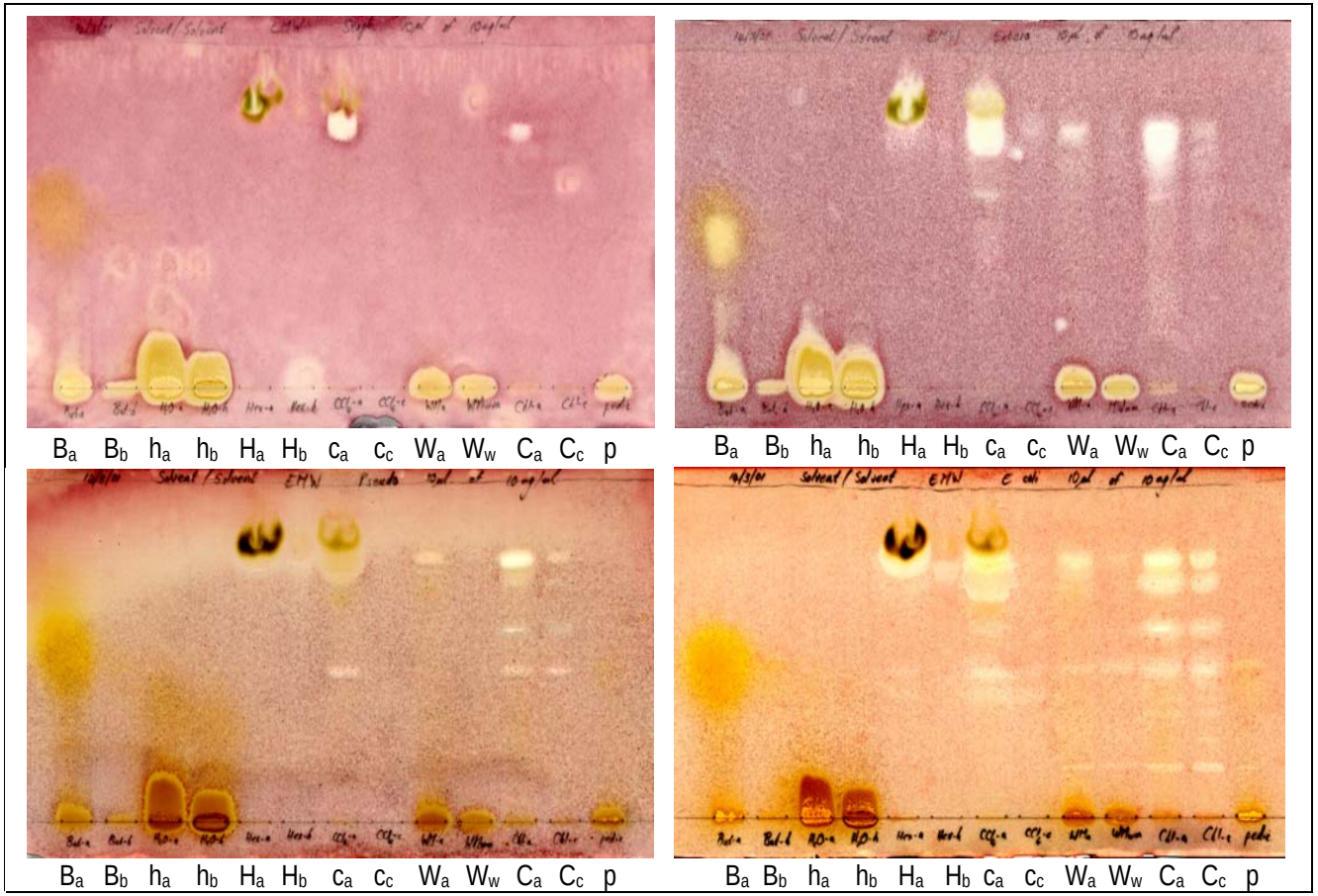


Figure 4.5. Bioautograms of *Pteleopsis myrtifolia* leaf fractions developed with the CEF eluent system and sprayed with bacteria, top left: *Staphylococcus aureus* (S), top right: *Enterococcus faecalis* (N), bottom left: *Pseudomonas aeruginosa* (P) and bottom right: *Escherichia coli* (E). For each thin layer chromatogram, the lanes from left to right were: But-a (B_a), But-b (B_b), H2O-a (h_a), H_2O-h (h_b), Hex-a (H_a), Hex-b (H_b), CCl_4-a (c_a), CCl_4-c (c_c), WM-a (W_a), WM-wm (W_w), Chl-a (C_a), Chl-c (C_c), pell (p). But = butanol, H_2O = water, Hex = *n*-hexane, CCl_4 = carbon tetrachloride, WM = 35% water in methanol, Chl = chloroform, pell = pellicle. Each fraction was redissolved in acetone (indicated by the suffix –a), as well as in the fraction solvent itself (indicated by the first letter the fraction consists of (e.g. Chl-c indicate chloroform redissolved in chloroform). (CEF = chloroform: ethyl acetate: formic acid (5:4:1)).

EMW Eluent system

S

N



P

E

Figure 4.6. Bioautograms of *Pteleopsis myrtifolia* leaf fractions developed with the EMW eluent system and sprayed with bacteria, top left: *Staphylococcus aureus* (S), top right: *Enterococcus faecalis* (N), bottom left: *Pseudomonas aeruginosa* (P) and bottom right: *Escherichia coli* (E). For each thin layer chromatogram, the lanes from left to right were: But-a (B_a), But-b (B_b), H2O-a (h_a), H_2O-h (h_b), Hex-a (H_a), Hex-b (H_b), CCl_4-a (c_a), CCl_4-c (c_c), WM-a (W_a), WM-wm (W_w), Chl-a (C_a), Chl-c (C_c), pell (p). But = butanol, H_2O = water, Hex = *n*-hexane, CCl_4 = carbon tetrachloride, WM = 35% water in methanol, Chl = chloroform, pell = pellicle. Each fraction was redissolved in acetone (indicated by the suffix –a), as well as in the fraction itself (indicated by the first letter the fraction consists of (e.g. Chl-c indicate chloroform redissolved in chloroform). (EMW = ethyl acetate: methanol: water (40:5:4.4)).

The fact that the inhibition areas were better developed with the first set of bioautograms could be explained if the growth of the bacterial cultures differed for the two experiments. It is interesting to note that the fractions that were redissolved in acetone had more colour bands compared to fractions redissolved in the original extractant (for example the *n*-hexane, water and carbon tetrachloride fractions redissolved in acetone had 4, 5 and 5 colour bands respectively, compared to the *n*-hexane, water and carbon tetrachloride fractions redissolved in chloroform, which had 3, 1 and 3 colour bands respectively (The EMW eluent's chromatograms spayed with vanillin in Figure 4.2)). This confirmed the fact that acetone is miscible with polar and non-polar compounds and a larger variety of compounds, which led to more and clearer inhibition areas (e.g. the chloroform fraction of Figure 4.6 redissolved in acetone had clear areas of inhibition for *E. faecalis* and *P. aeruginosa* compared to the chloroform fraction of Figure 4.6 redissolved in chloroform where the inhibition areas for *E. faecalis* and *P. aeruginosa* were not clear at all.

All fractions had antibacterial activity against the Gram-positive bacteria, *S. aureus* and *E. faecalis* (bottom row of Figure 4.3 and top row of Figure 4.6). The chloroform and 35 % water in methanol extracts were active against the Gram-negative bacteria, *P. aeruginosa* and *E. coli* (bottom row of Figure 4.4 and bottom row of Figure 4.5). The largest amount of inhibition areas (seven) was observed with the chloroform fraction and *E. faecalis* and *E coli*. The chloroform fraction was antibacterial to all bacteria tested, and had the largest amount of inhibition areas, and thus amount of bacterially active compounds.

4.4 Conclusions

Bioassay-guided fractionation identified that all fractions: n-hexane, carbon tetrachloride, chloroform, 35% water in methanol, n-butanol and water had antibacterial activity against at least two of the bacteria tested. The chloroform fraction was antibacterial to all bacteria tested,

and had the largest amount of inhibition areas (seven), and thus amount of bacterially active compounds. This fraction was chosen for further purification and to isolate pure compounds from it in future research (Chapter 7 and 8).

4.5 Literature references

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