5 Application of Thin Layer Chromatography (TLC): Characterization of traditional medicines

5.1 Introduction

In Chapter 4, it was indicated that TLC can be used to identify traditional medicines, however, there is uncertainty if this technique could differentiate between closely related species. In this chapter, the application of the TLC method is expanded.

One of the aims of this chapter is to determine if the TLC method could differentiate between closely related plant medicines. This study was conducted in order to determine the effectiveness of this technique as a tool to identify unknown plant medicines. The Fabaceae family was chosen for this investigation, because of the availability of materials belonging to this plant family. The plants species studied were *Acacia caffra, Acacia karro, Acacia montana and Peltophorum africanum.*

Artemisia species are known to have highest variability amongst the species. *Artemisia afra* species has been used for centuries in traditional medicine for the treatment of fever and malaria (Kohler *et al.* 1997). Darwin's theory of evolution states that species undergo genetic variation with time to adapt to environmental changes (Solomon *et al.,* 1999). Therefore, the same species growing in widely different habitats may drift from the original genetic makeup as a mechanism of adaptation (Hegnauer, 1986). Therefore, same plant species that have been reproducing in different environments may have different chemical profiles. It is, therefore, hypothesized that the variations noted in the previous chapters may result from genetic variation that occurs as a result of environmental influence. *Artemisia afra* is one of the species with high genetic

variation (Balint, 2001). This chapter will also investigate the use TLC to determine the influence of the environment where a plant is grown on the chemical composition of the plant. Furthermore, an investigation of the accuracy of the technique in identifying plant species that were collected from plant is conducted.

Hahn-Deinstrop (2000) and many other workers have made use of TLC and declared this technique to be a good tool for the analysis of drugs substitution and adulteration. Research conducted thus far, has also shown TLC to be a good technique for analysis of botanical products in the investigation of additional substitutions (Ntloedibe, 2002). Little work has been done to develop and validate methods to determine adulteration in traditional medicine markets using TLC. This chapter also investigates the magnitude of adulteration and substitution of traditional medicines in the Pretoria market. Plant medicines chosen for this investigation are *Warburgia salutaris* and *Peltophorum africamum*, because they were traded by every vendor in the market.

5.2 Results and discussion

5.2.1 Application of TLC to differentiate Fabaceae species

The aim of this section was to determine whether the TLC technique can differentiate between closely related medicinal species of the same family. Bark material of species in question was collected from the same geographic area (PNBG). The samples were processed to fine powders as stated in Section 2.2 and then extracted with ethanol, acetone and hexane. The final extracts were separated using three systems: BEA [benzene (80): ethanol (10): ammonium (1)], CEF [chloroform (4): ethyl acetate (3): formic acid (1)] and EMW [ethyl acetate (10): methanol (1.35): water (1)].

The TLC plates analyzed were then sprayed with *p*- anisaldehyde and vanillin sulphuric acid spray reagents, as shown in Figure 5.1 and Figure 5.2, respectively. These chemical profiles indicate that *Acacia karoo* is very similar to *Acacia montana.* Therefore, there are complications that may be encountered when trying to identity plant species of the same family using the TLC method. This implies that when closely related species are sold it is not possible to confirm their identity without botanical evaluation.

5.2.2 Environmental influence on the chemical composition of *Artemisia afra* species

The aim of this section is to investigate the influence of the environment on the chemical profile of *Artemisia afra* species originating from the same seed source but growing in different environments. A farmer from the Delmas district, KwaZulu-Natal approached us to evaluate *Art. Afra* plants grown under widely different cultivation condition for him. This was confirmed by preparing TLC fingerprint of species collected from different geographic areas that originated from different seed sources and have been growing under different environments. These plant samples were collected from the market, Pretoria National Botanical Garden and Agricultural Research Council. The samples and their place of origin are listed in Table 5.1. The leaves were ground up, extracted with ethanol, acetone and hexane, and then developed as outlined in Section 2.3. The developed plates were sprayed with vanillin sulphuric acid, *p*-anisaldehyde and vanillin phosphoric acid spray reagents for detection of extract components.

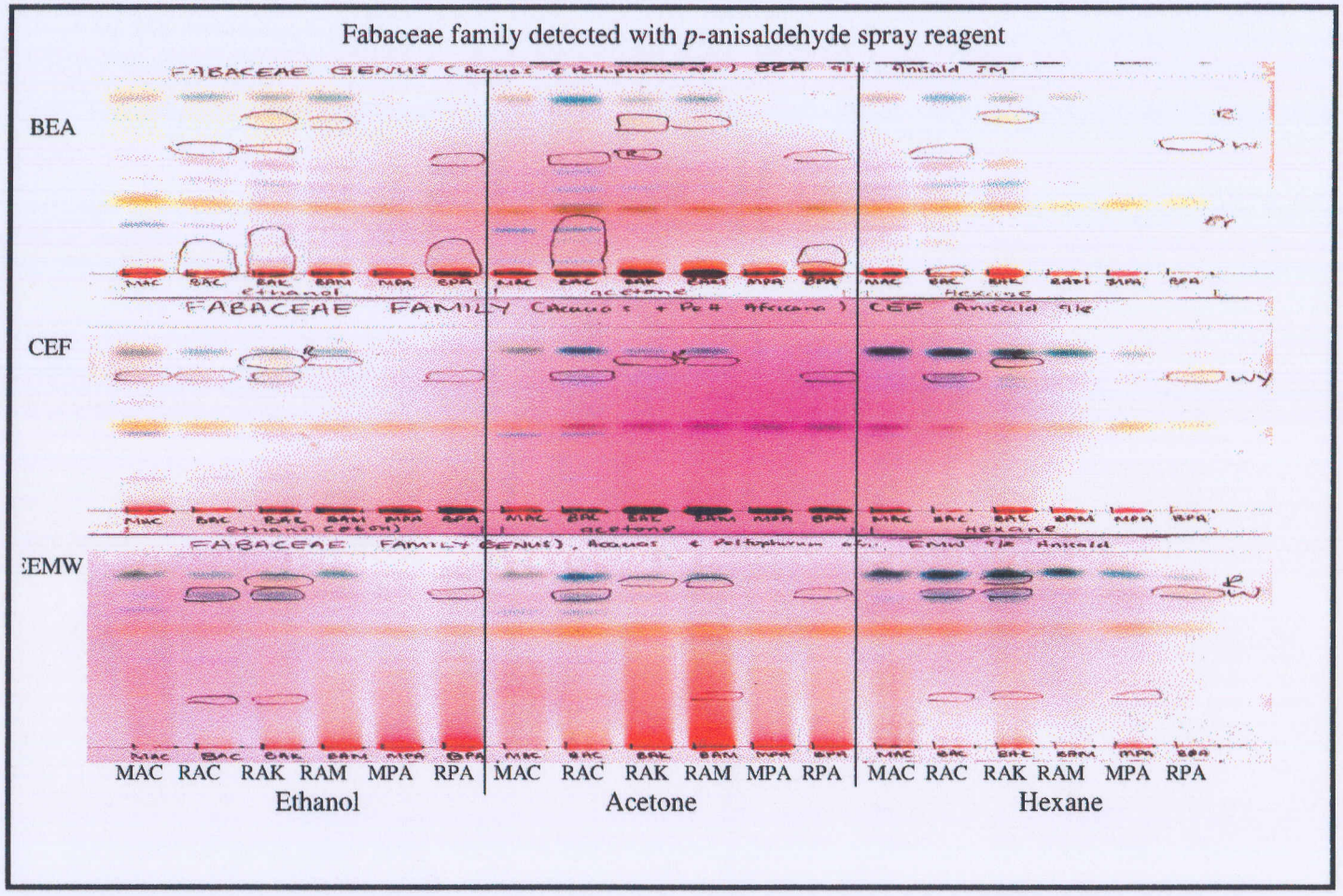

Figure 5.1 TLC profiles of Fabaceae species extracts, analyzed with *p*-anisaldehyde spray reagent. Key: MAC: Market *Acacia caffra*; BAC: PNBG *Acacia caffra,* BAK: PNBG *Acacia karoo,* BAM: PNBG *Acacia montana,* MPA: market *Peltophorum africanum* and BPA: PNBG *Peltophorum africanum*.

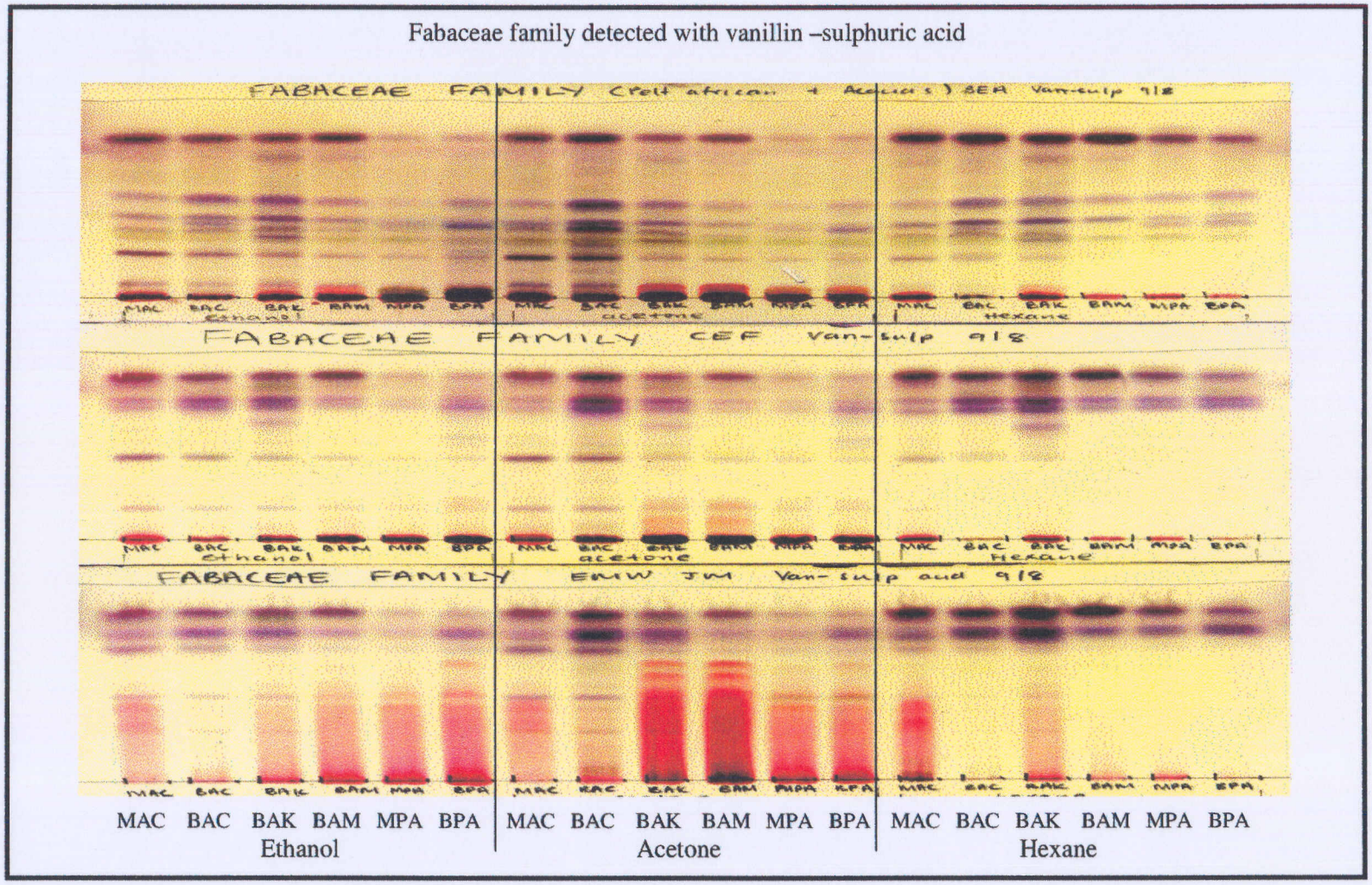

Figure 5.2 TLC profiles of Fabaceae species extracts of ethanol, acetone and hexane sprayed with vanillin sulphuric acid spray reagent. MAC: Market *Acacia caffra*, BAC: PNBG *Acacia caffra,* BAK: PNBG *Acacia karoo,* BAM: PNBG *Acacia montana,* MPA: market *Peltophorum africanum* and BPA: PNBG *Peltophorum africanum.*

Table 5.1. List of *Artemisia afra* species originating from different geographic areas and seed sources

Sample code	Environment
A	ARC Well cared for, given water and fertilizer
B	Pretoria National Botanical Garden Natural good soil and climate, naturally conserves environment
D	Wild, cold climate, Mpumalanga
M	Growing in a maize field, good soil, average climate
T	Well cared home garden, Gauteng
V	Wild, warm climate

Figure 5.3 shows the TLC profile of *Artemisia afra* species in Table 5.1 sprayed with anisaldehyde, vanillin sulphuric acid and vanillin phosphoric acid spray reagents. There was a distinct variation amongst the extracts indicating the difference in chemical composition. Variation in biological activity of these species can be expected, since variation in chemical composition was associated with variation in antibacterial activity (Section 3.2). Figure 5.4 presents the total activities of *Artemisia* species in Figure 5.3 against *S. aureus*, *E. faecalis*, *P. aeruginosa* and *E. coli* micro-organisms.

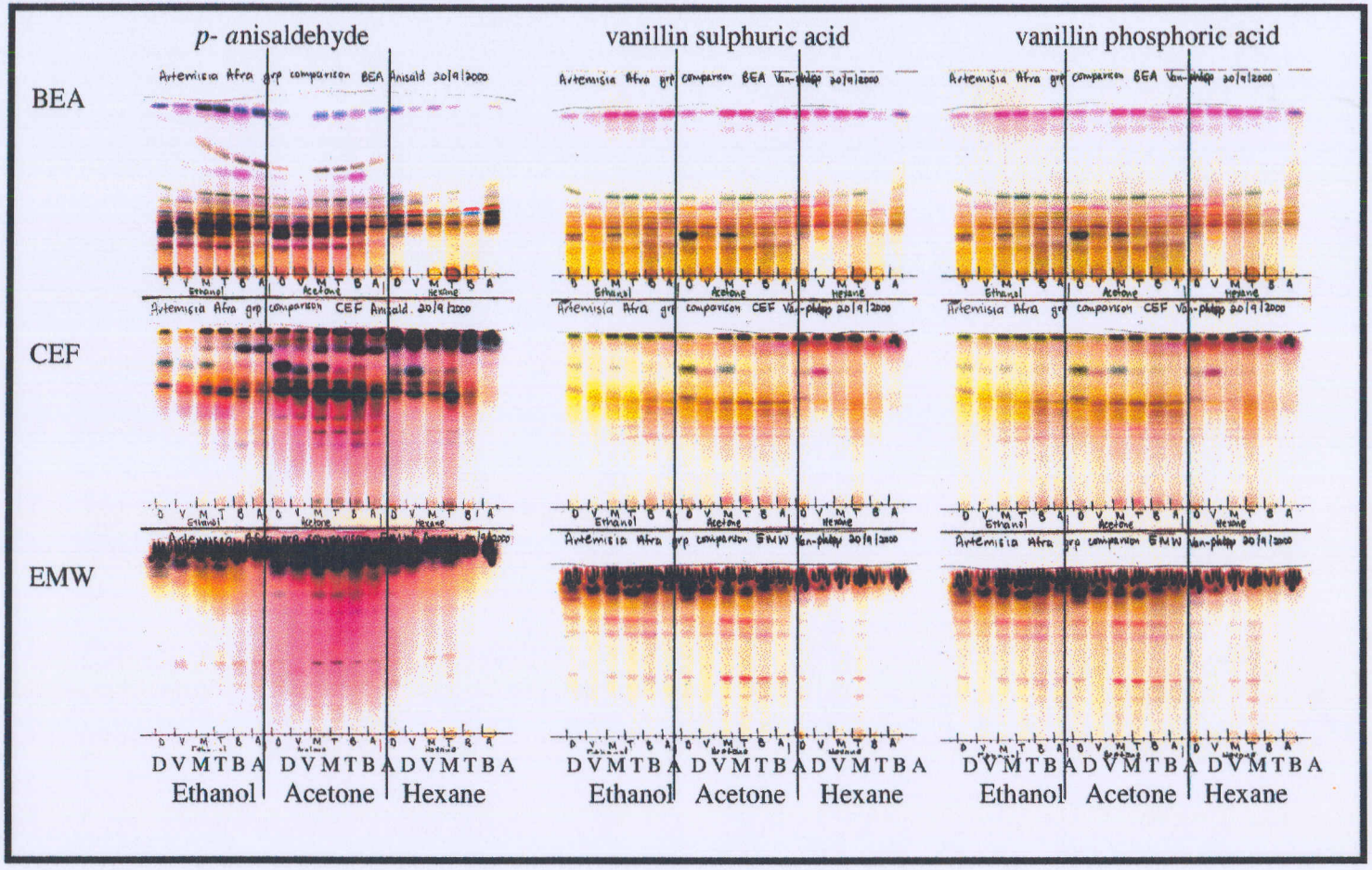

Figure 5.3 TLC profiles of *Artemisia afra* species originating from different geographic area and seed sources, sprayed with: *p*-anisaldehyde, vanillin sulphuric acid and vanillin phosphoric acid spray reagents. Key: D: market sample; V: veldt sample; M: maize land sample; T: private garden sample; B: Pretoria National Botanical Garden sample; A: Agricultural Research Council sample; BEA: benzene, ethanol and ammonium separation system; CEF: chloroform, ethyl acetate and formic acid separation system and EMW: ethyl acetate, methanol and water separation system.

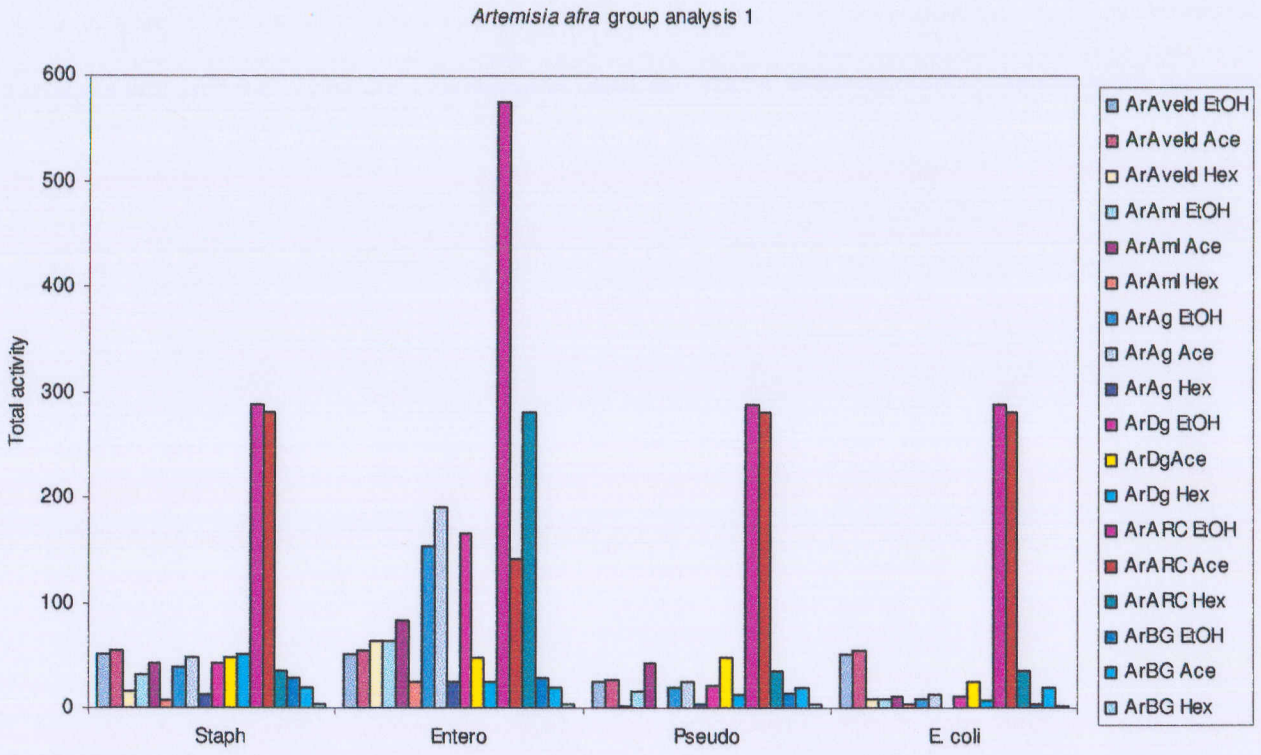

Figure 5.4 Total activities of *Artemisia afra* species tested against *Staphylococcus aureus*, *Pseudonomas aeruginosa, Escherichia coli* and *Enterococcus faecalis* microorganisms. Key: ArAveld: *Artemisia afra* veldt sample; ArAml: *Artemisia afra* maize land sample; ArAg: *Artemisia afra* private garden sample; ArDg: *Artemisia afra* market sample; ArARC: *Artemisia afra* Agricultural Research Council Sample; ArBG: *Artemisia afra* Pretoria National Botanical Garden sample; EtOH: ethanol; Ace: acetone; Hex: hexane.

Variation observed may be due to genetic polymorphism, ecological and geographic differences (Balint, 2001). Figure 5.4 shows the total activity. These results correlate with the variation shown in Figure 5.3. This is because the total activity varies amongst the *Artemisia afra* species examined. A further investigation was done to determine the influence of the environment on the chemical composition of the same species originating from the same seed. The acetone and ethanol extract from ARC show high total activity against all four micro-organisms compared to the other extracts and this is indicated in Figure 5.4. This implies that the environment appears to be an important factor in determining both the chemical composition and biological activity.

Artemisia afra species originating from the same seed source were analyzed using the same experimental approach and then compared. The environments where these species were grown are shown in Table 5.2.

Table 5.2. *Artemisia afra* species originating for the same seed source that were grown in different environments

Sample code	**Environment conditions**
N	Seed source
A	Nutrient rich soil and watered by drop irrigation
D	Soil fertilized with KNO_3
H	Grown under a tree, nutrients and water competition
J	Treated with pyrethrum, an insecticide
M	Grown in a maize field, average climate and soil
L	Grown in a well-cared garden, good soil and climate

The developed TLC plates were sprayed with *p*-anisaldehyde, vanillin sulphuric acid, methanol phosphoric acid, tri-chloroacetic acid (TCA) and toluene spraying reagents. TLC profile of TCA, methanol phosphoric acid and toluene spray reagents were not as good as those sprayed with as *p*-anisaldehyde, vanillin sulphuric acid spray reagents. These are shown in Figure 5.5. Total activities values of these *Artemisia afra* species are shown in Figure 5.6.

The TLC profile of these *Artemisia afra* species originating from the same seed source (N) but exposed to different environmental condition show variation in their chemical composition. Samples A and J are strikingly different form the other samples. N is the least complex showing the least compounds, while A was the most complex. This sample was grown in good soil and treated with drop irrigation regularly. When comparing sample A to N, it can be concluded that well cared plant species yield high quantity chemical components (Jork, *et. al.* 1990). H is open to speculations.

Sample J, treated with pyrethrum shows additional compounds well separated by the BEA system. This species has unique compounds of R_f values of 0.43 and 0.51. The additional compounds show that pyrethrum treatment may result in metabolic changes that result in the accumulation of certain compounds in the plant.

The total activities of the acetone extracts were determined and are represented by the graph in Figure 5.6 The acetone extracts were selected because they contain both the non-polar and polar compounds and showed good resolution. Samples N, A, D, H, J and L show some activity against *E. faecalis* but little activity against *S. aureu, P. aeruginosa* and *E. coli*. On average, *Artemisa afra* has low antibacterial activity.

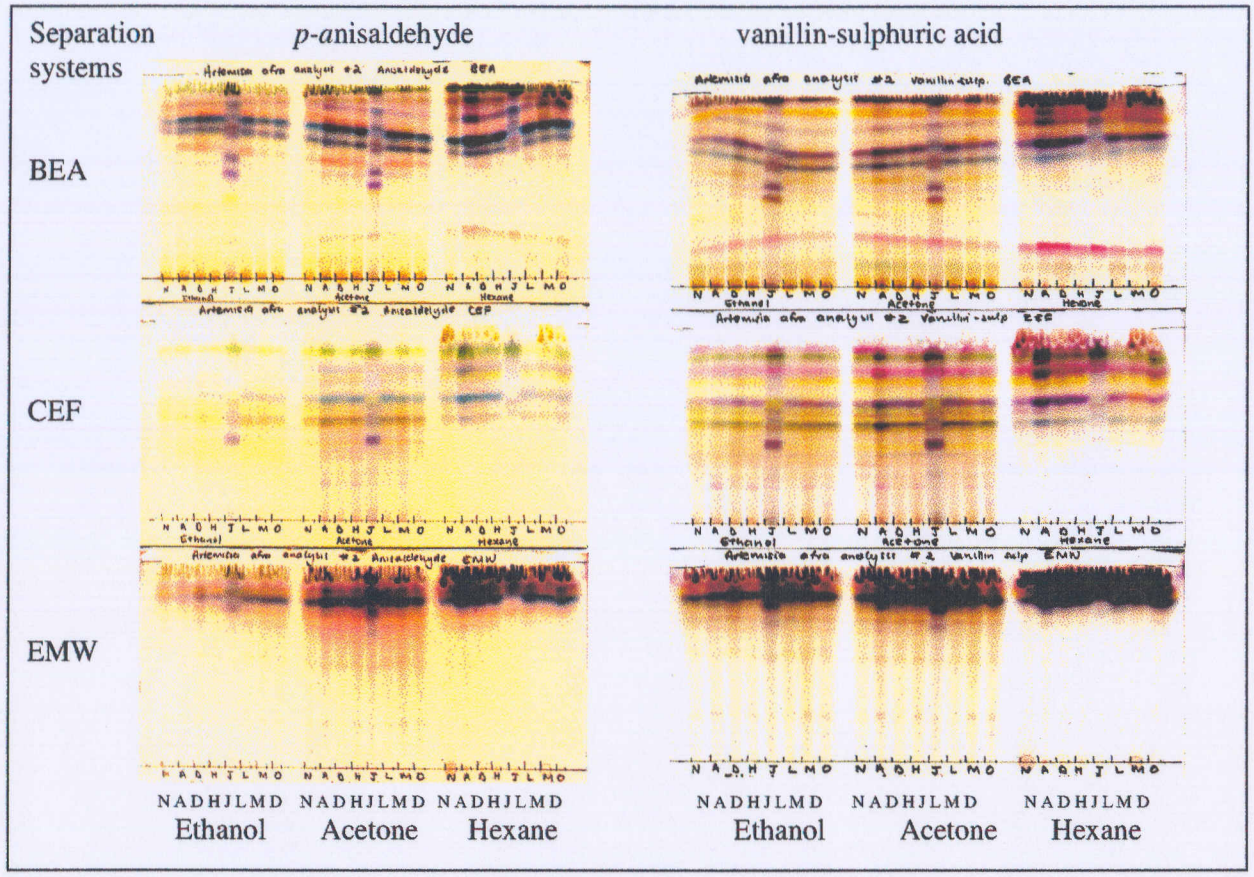

Figure 5.5 TLC Profiles of *Artemisia afra* species originating from the same seed source, sprayed with *p*-anisaldehyde and vanillin sulphuric acid spray reagents. Key: N: seed source sample; A: Agricultural Research Council sample; D: fertilized soil sample; H: shade sample; J: pyrethrum-treated sample; M: maize land sample; L: home garden sample; BEA: benzene, ethanol and ammonium separation system; CEF: chloroform, ethyl acetate and formic acid separation system and EMW: ethyl acetate, methanol and water separation system.

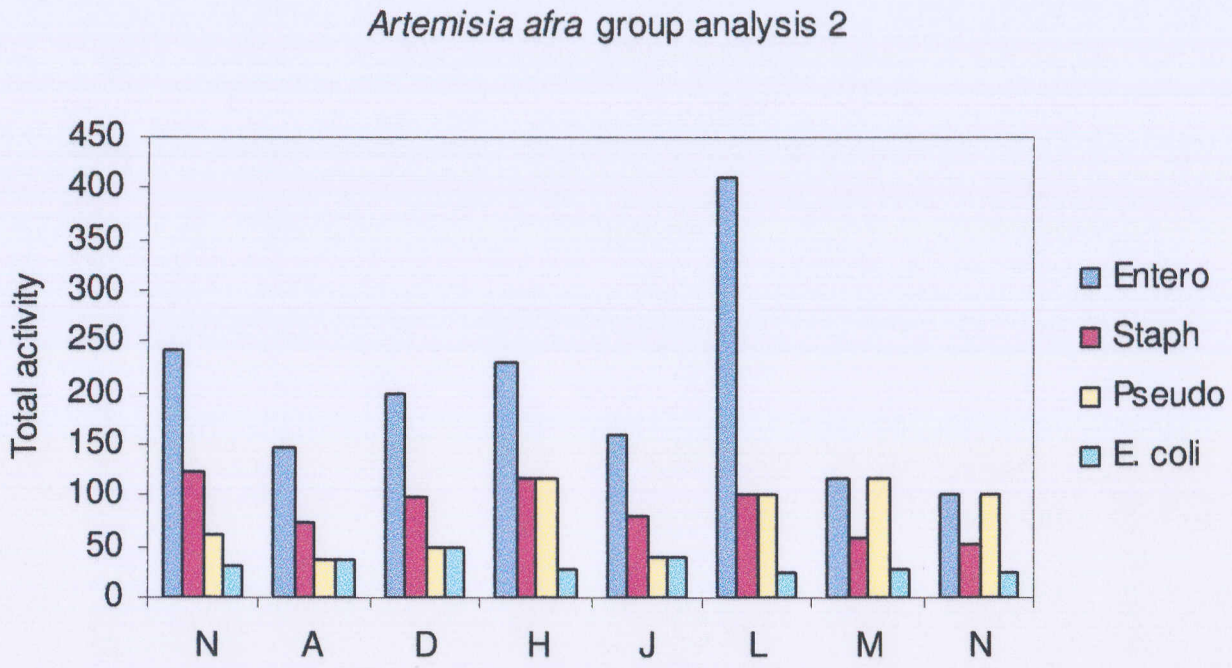

Figure 5.6 Total activities of acetone extracts of the *Artemisia afra* species tested against *Staphylococcus aureus*, *Pseudonomas aeruginosa, Escherichia coli* and *Enterococcus faecalis* microorganisms. Key: N: seed source sample; A: Agricultural Research Council sample; D: fertilized soil sample; H: shade sample; J: pyrethrum-treated sample; M: maize land sample; L: home garden sample; BEA: benzene, ethanol and ammonium separation system; CEF: chloroform, ethyl acetate and formic acid separation system and EMW: ethyl acetate, methanol and water separation system.

Sample L, which was grown in a well-cared garden showed the highest activity against *E. faecalis. P. aeruginosa* and *E. coli.*

5.2.3 Determination of adulteration in traditional medicine markets

The aim of this section was to determine the magnitude of adulteration in traditional medicines traded in the market. The results of this investigation reflect the true extent of adulteration in Pretoria markets, which would have health implications.

A series of investigations were conducted to answer this question. Amongst plant species selected for identification in Section 3.1, *Warburgia salutaris* (Molaka) and *Peltophorum Africanum* (Mosetlha) were selected for this investigation since almost every trader in the market sold these species. Plant materials of these species were bought from at least four different traders situated at different outlets in Pretoria traditional medicine markets. It was assumed that the collectors are different. The weights of the materials received were determined (Table 5.3) and pictures of the bark samples were taken before the materials were processed and these are shown in Figure 5.7 and Figure 5.8.

Table 5.3. A list of *Warburgia salutaris* and *Peltophorum africanum* species bought from different traders for the same price (R5.00)

Trader's code	Plant species	Weight (gram)
Vendor A	*Warburgia salutaris* *Peltophorum africanum*	28.76 27.00
Vendor B	*Peltophorum africanum*	66.33
Vendor C	*Warburgia salutaris* *Peltophorum africanum*	41.2 111.29
Vendor D	*Warburgia salutaris* *Peltophorum africanum*	17.01 24.52
Vendor E	*Warburgia salutaris* *Peltophorum africanum*	81.18 48.17

The weight in Table 5.3 represents a wide variation in pricing. Vendor C gave more quantity of *P. africanum* than the other traders and Vendor E gave the most quantity of *Warburgia salutaris*. There is no pattern that can be deduced from these values. It is well known that traditional traders and healers don't often use measurements, as indicated by Figure 5.7 (*Warburgia salutaris*) and Figure 5.8 (*Peltophorum africanum*). The weight of the materials may not play a major role in identifying adulteration because it could reflect the water content of the plant.

Thus a fresh bark cannot be compared to a dry bark. In this study, all bark material received were dry, therefore, the weight of the materials could be compared in this case. The materials received from Vendor D have the lowest weight for both species. The significant variation in mass could be an indication of possible adulteration. Pricing variation may be a result of market force e.g. location, rental income of clientele and buying price.

Figure 5.7 Photographs of the bark of *Warburgia salutaris* bought from vendor A, C, D and E.

Figure 5.8 Photographs of the bark *Peltophorum africanum* (Mosetlha) bought from vendors A, B, C, D and E.

5.2.3.1 Determination of adulteration in *Peltophorum africanum* (Mosetlha) species

The bark material from five different traders was extracted with three solvents: ethanol, acetone and hexane. TLC plated loaded with the extracts were developed as before and then sprayed with *p*-anisaldehyde and vanillin sulphuric acid in Figures 5.9 and Figure 5.10, respectively. This figures show similarities in the extracts of all five samples of Vendors A to E. There is slight variation in the intensity of the bands detected, even though all samples have similar compounds. This appears to show variable concentration. The total activity results were also variable, confirming the results obtained in Chapter 4.

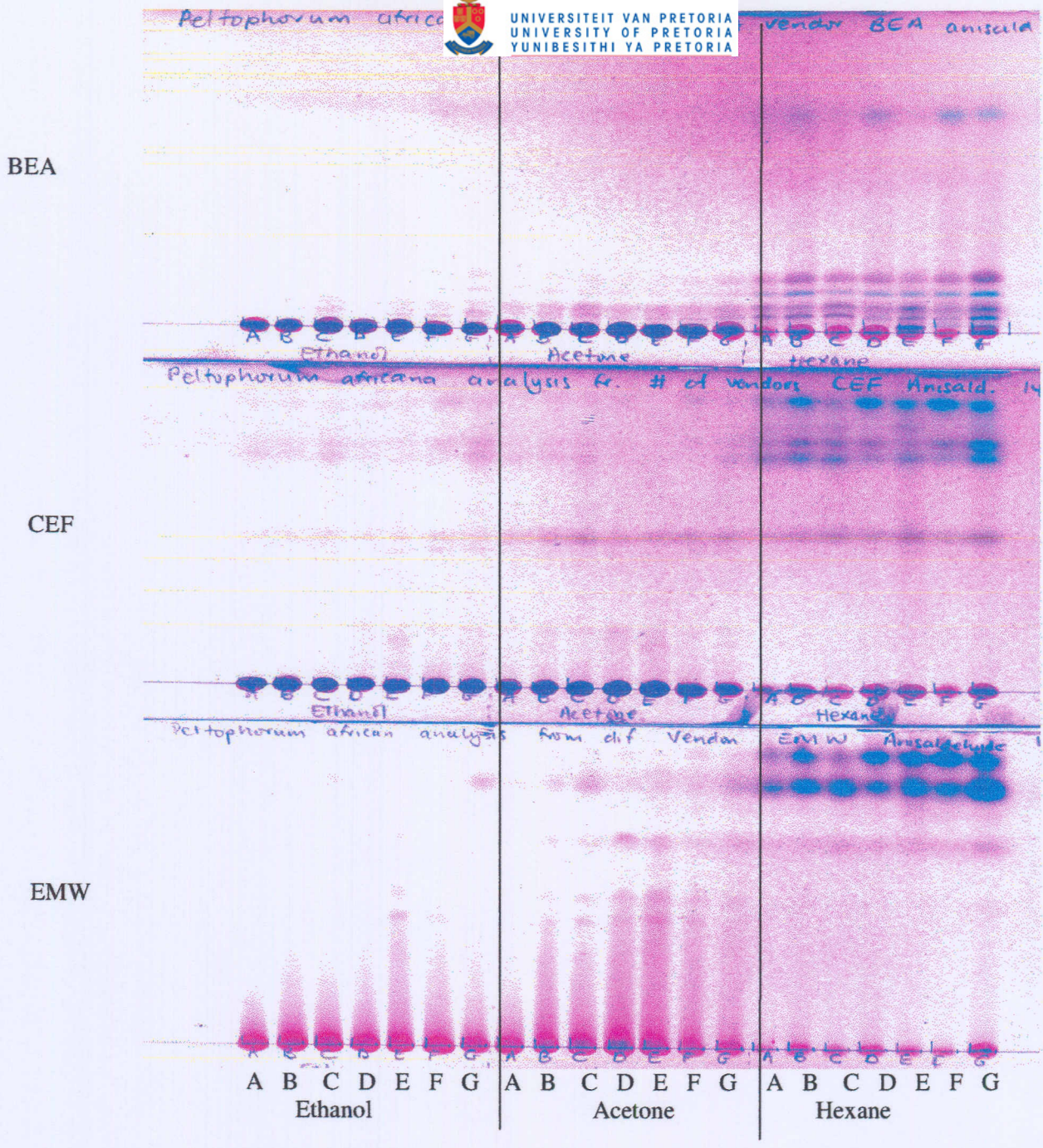

Figure 5.9 TLC profiles of *Peltophorum africanum* bark materials obtained from different vendors (A, B, C, D, E, F) and G extracted with ethanol, acetone and hexane and detected with *p*-anisaldehyde. Key: BEA: benzene, ethanol and ammonium separation system; CEF: chloroform, ethyl acetate and formic acid separation system and EMW: ethyl acetate, methanol and water separation system.

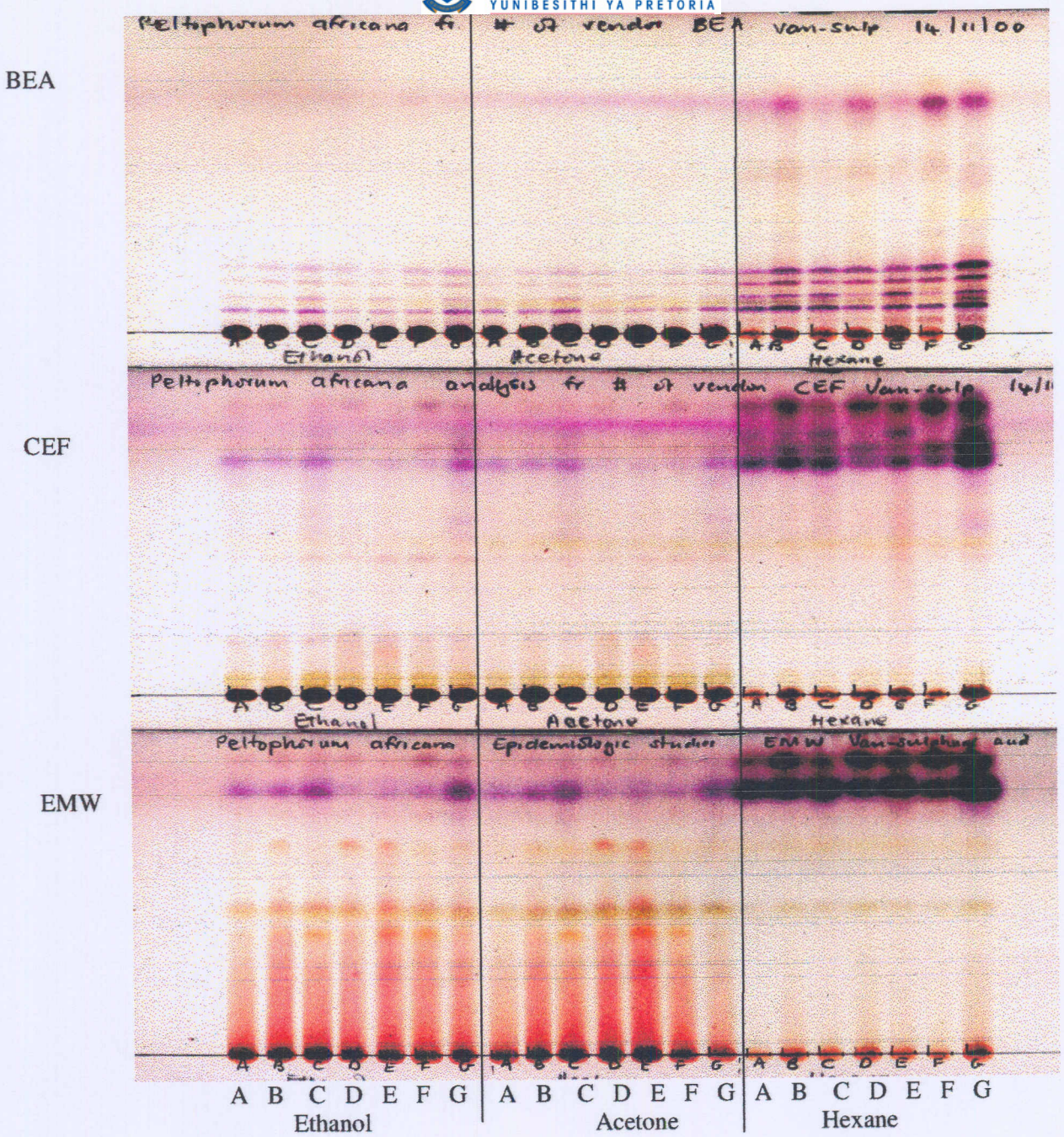

Figure 5.10 TLC profiles of *Peltophorum africanum* bark materials obtained from different vendors (A, B, C, D, E, F and G) extracted with ethanol, acetone and hexane and detected with vanillin sulphuric. Key: BEA: benzene, ethanol and ammonium separation system; CEF: chloroform, ethyl acetate and formic acid separation system and EMW: ethyl acetate, methanol and water separation system.

5.2.3.2 Determination of adulteration in *Warburgia salutaris* (Molaka) species

Bark material of *Warburgia salutaris* was bought from four vendors and the photographs of these materials are shown in Figure 5.7. The samples received from Vendor C and E were separated into two groups since the bark material did not look similar on visual examination. The samples were coded C_1, C_2, E_{21} and E_2 respectively. Reference sample obtained from the Pretoria National Botanical Garden was also loaded as control.

The bark was ground to fine powder extracted with ethanol, acetone and hexane and then separated using the three systems (BEA, CEF and EMW) outlined in Section 2.3. The developed TLC plates were sprayed with *p*-anisaldehyde and vanillin sulphuric acid and are shown in Figures 5.11 and 5.12, respectively.

There was no major difference between the samples analyzed, except the samples received from Vendor D. This sample lacks most of the compounds visualized in the other samples, although the hexane extracts were similar in all cases. The plate sprayed with *p*-anisaldehyde show this similarity better. It can, therefore, be concluded from this that Vendor D is not selling *W. salutaris.*

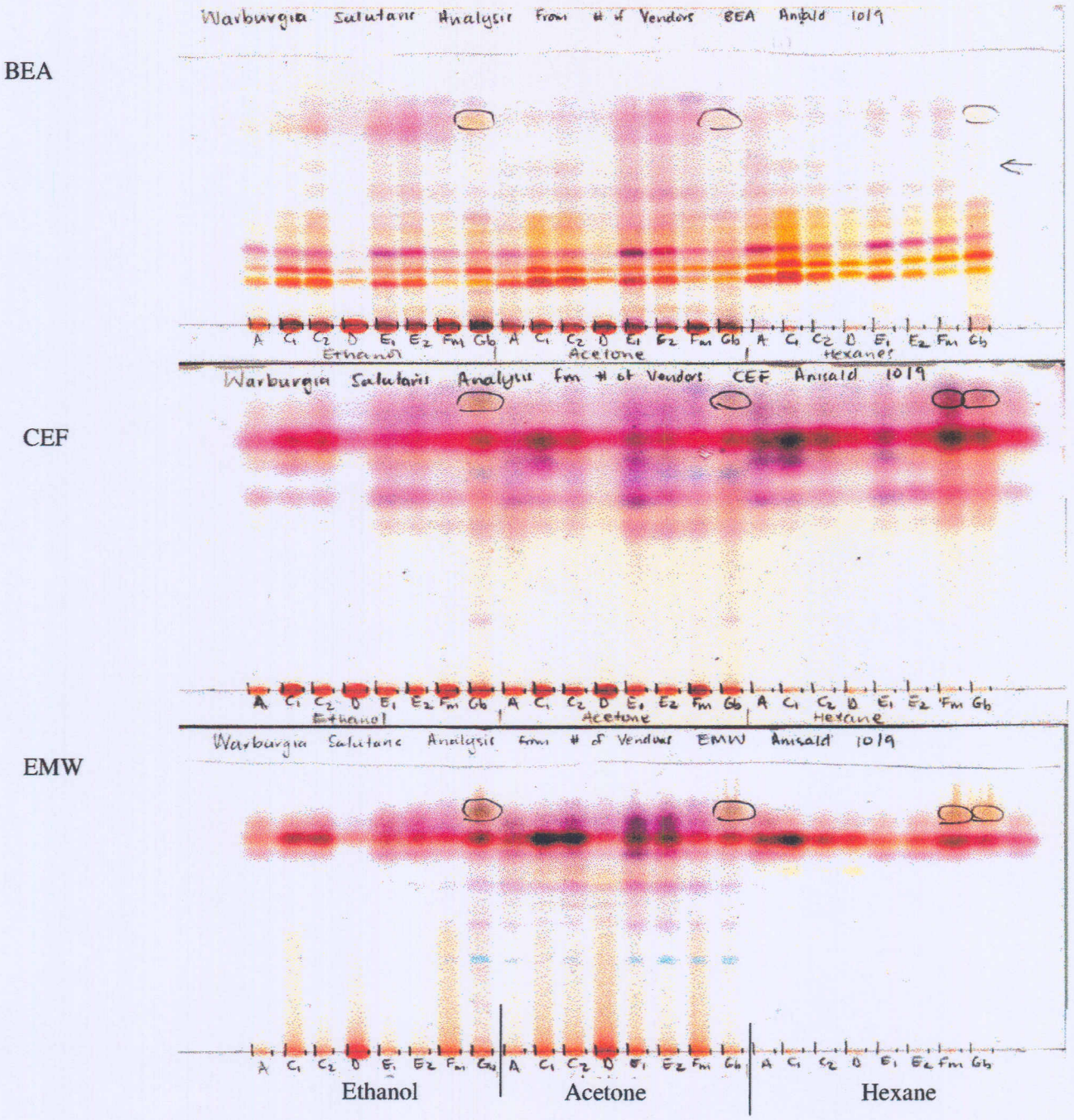

Figure 5.11 TLC profile of *Warburgia salutaris* species from different vendors (A, C, D, E and G) extracted with ethanol, acetone and hexane. The extracts' chemical components on the TLC plate were sprayed with *p*-anisaldehyde. Key: BEA: benzene, ethanol and ammonium separation system; CEF: chloroform, ethyl acetate and formic acid separation system and EMW: ethyl acetate, methanol and water separation system.

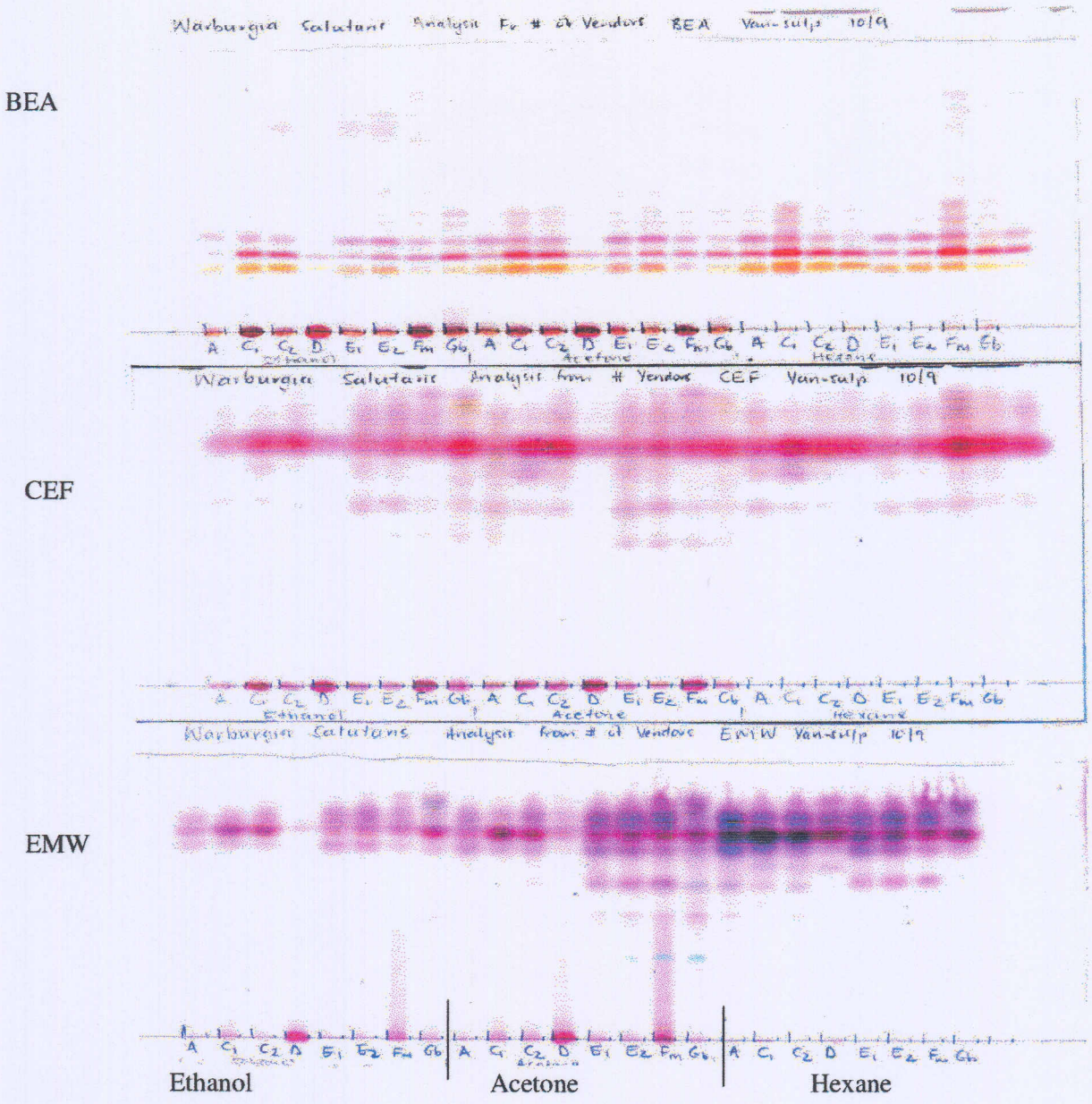

Figure 5.12 TLC profile of *Warburgia salutaris* species from different vendors (D, E, F and G) extracted with ethanol, acetone and hexane, sprayed with vanillin sulphuric acid spray reagent. Key: BEA: benzene, ethanol and ammonium separation system; CEF: chloroform, ethyl acetate and formic acid separation system and EMW: ethyl acetate, methanol and water separation system.

5.3 Conclusion

The TLC technique is not be suitable for differentiation between closely related species. Therefore, it can only be used to identify species belonging to different families and genera. It was indicated that similarities between closely related species may cause uncertainties when identifying these species by TLC, however, we need to expand this investigation by determining the environmental influence on the chemical profile. It is known that genetic mutations increases genetic diversity, however, the geographic influences may also affect the genotype of plant species over a period of time.

The environment has little effect on chemical composition of plants except if treated with insecticide. However, the genetic variation of a plant is more important. It has been shown here that TLC can be used in the identification of plant species sold in the market. However, it is still not known whether there is substitution or adulteration of popular plant medicines in traditional medicine markets. The next stage of this study is to determine the extent to which this happens. It would appear from the research conducted that the environment affects chemical composition in plants (N versus A). The extent to which this is important is not possible to ascertain. Genetic variation may be more important.

TLC fingerprints of *Warburgia salutaris* and *Peltophorum africanum* indicate that planar chromatography can also be used to analyze traditional material for adulteration assays.

The difference in extracts for *Warburgia salutaris* samples received from Vendor D could be an indication of contamination or factor of age. This chapter has indicated that TLC method can be used to determine the magnitude of adulteration, however, it cannot be used to differentiate closely related species. The next chapter will make used of TLC to fingerprint over-exploited medicinal plants.