
Chapter 6

Evaluation of different extracts from *Pelargonium sidoides*

6.1 Introduction

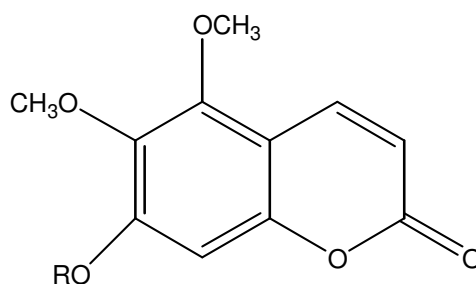
As mentioned on Chapter 2, the plants belonging to the genus *Pelargonium* have yielded different secondary metabolites, e.g. coumarins (Figure 6.1 (a – e), and tannins (Figure 6.2), in addition to glycosides. Many of these compounds are known to possess medicinal properties.

Latté *et al.*, (2000), described the isolation of coumarins, such as umckalin, 7-*O*-methyl ether, 7-acetoxy-5,6-dimethoxycoumarin, 6,8-dihydroxy-7-methoxycoumarin, 6,8-dihydroxy-5,7-tetramethoxycoumarin, artelin and three unique coumarin sulfates from *P. sidoides*. Furthermore, the highly oxygenated coumarins; fraxinol, isofraxetin and fraxidin were found to be associated with 8-hydroxy-5,6,7,-trimethoxycoumarin as representatives of *P. reniforme*. Kayser and Kolodziej (1997), investigated the highly oxygenated coumarins, fraxinol, isofraxetin and fraxidin, together with a unique trimethoxy coumarin found in *P. reniforme* for antibacterial activity. Scopoletin and 6,7,8-trihydroxycoumarin are found in both species. Most of the coumarins found in these two *Pelargonium* species contain a methoxy function at the C7 position and an OH group at either the C6 or C8 positions; functionality that is responsible for their antibacterial activity. Gallic acid and its methyl ester present in large amounts in *P. sidoides* and in its active extracts, were identified as the prominent immunomodulatory principle for this herbal medicine umckaloabo (Latté *et al.*, 2000). The German chemist

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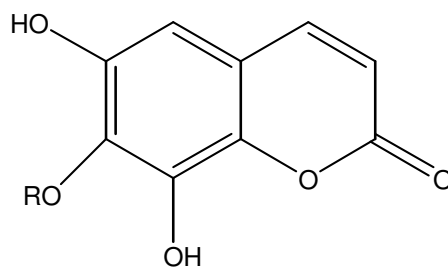
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Richard Willstätter, elucidated pelargoniidin from *Pelargonium* species in 1915. Latté *et al.*, 2002, first described *O*-galloyl-C-glycosylflavones associated with non-galloylated parent analogues and the flavonoid pattern of the roots and aerial parts of *P. reniforme* and isolated the unique series of C-2''- acylated C-glycosylflavones extended by the discovery of the C-8-glucosyl derivatives 2''-*O*-galloyl-vitexin and 2''-*O*-galloylorientin and their C-6 analogues 2''-*O*-galloylisovitexin and 2''-*O*-galloylisoorientin.



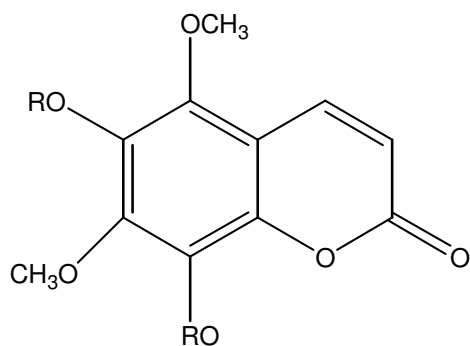
(a)

1. **R = CH₃ (5,6,7-Trimethoxy coumarin)**
2. **R = H (6-Hydroxy-5,7-dimethoxy coumarin, umckalin)**
3. **R = COCH₃ (7-Acetoxy-5,6-dimethoxy coumarin)**



(b)

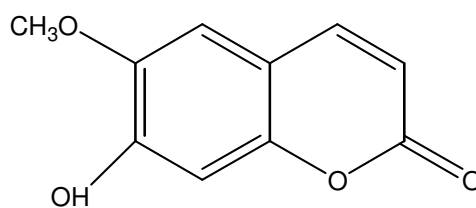
4. **R = CH₃ (6,8-Dihydroxy-7-methoxy coumarin)**
5. **R = H (6,7,8-Trihydroxy coumarin)**



(c)

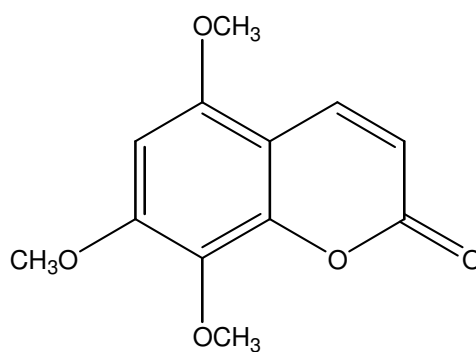
6. R = CH₃ (5,6,7,8-Tetramethoxy coumarin, artelin)

7. R = H (6,8-Dihydroxy-5,7-dimethoxy coumarin)



(d)

8. 7-Hydroxy-6-methoxy coumarin, (scopoletin)



(e)

9. 5,7,8-Trihydroxy coumarin

Figure 6.1 Coumarins isolated from *P. sidoides*

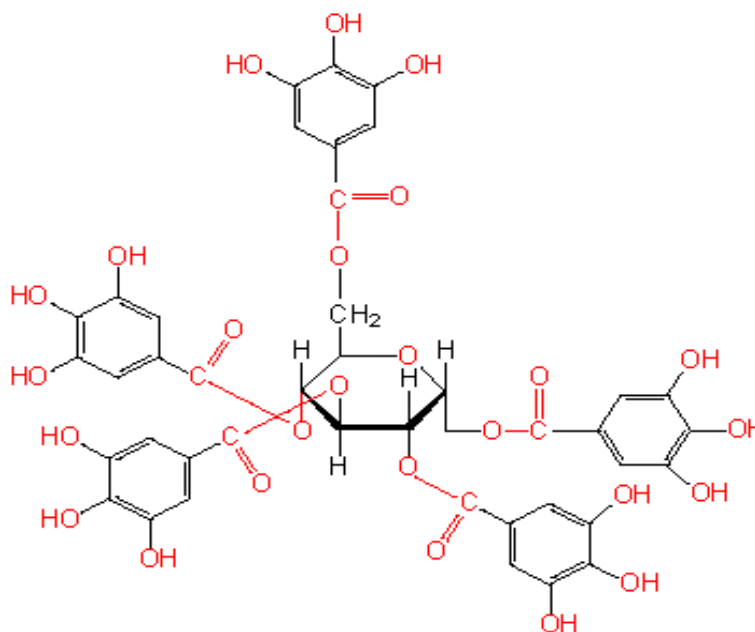


Figure 6.2 Tannins isolated from *P. reniforme*

In our investigation, the roots of *P. sidoides* were found to have antimicrobial activity against Gram-negative bacterial species *H. influenza*, *M. catarrhalis* and *S. pneumonia* and fungal pathogens *A. niger*, *F. oxysporum* and *R. stolonifer*. It has been documented in a book review that ‘Umckalin’, a coumarin is responsible for antituberculosis activity (Bladt, 1977).

Since bioactive compounds such as coumarins, flavanoids, tannins, etc. have been found to be present in the roots of both *P. reniforme* and *P. sidoides*, an attempt was made to isolate the active compounds from the roots of *P. sidoides*. Bioassay guided fractionation of the roots of *P. sidoides* was done using five Gram-positive, four Gram-negative bacterial species and *M. tuberculosis*.

6.2 Materials and Methods

6.2.1 Preparation of plant extracts

Dried and powdered roots of *P. sidoides* (500g) were extracted with 100% chloroform, and 70% acetone. The aqueous acetonetic extract was sequentially extracted with ethyl acetate and butanol. The fractions were filtered and concentrated with rotary evaporator to dryness at reduced pressure. All fractions were dissolved in dimethyl sulphoxide (DMSO) to a concentration of 100.0 mg/ml.

6.3 Bacteria

Nine bacterial species (Table 6.1) were obtained from the Department of Microbiology and Plant Pathology, University of Pretoria. Each organism was maintained on nutrient agar slant, and was recovered for testing by growing them in nutrient broth (No. 2, Biolab) for 48 hours at 37 °C. Before streaking, each culture was diluted 1:10 with fresh sterile nutrient broth (Dilika and Meyer, 1996; Lall and Meyer, 2000). H37Rv, American Type Culture Collection, MD, USA 27294 experimental strain of *M. tuberculosis*, was used to investigate the activity of the plant extracts and bacterial inoculum was prepared as mentioned in Chapter 5, section 5.3.

6.4 Antibacterial assay

The antibacterial assay activity of chloroform, acetone, ethyl acetate, butanol and water fractions against Gram-positive and Gram-negative bacteria were determined by incorporating various amounts of the extracts into petri dishes containing the culture media. Different fractions of the plant extract were introduced by adding autoclaved

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nutrient agar (BIOLAB), swirled carefully until the agar began to set into sterile petri dishes. The organisms were streaked in radial patterns on agar plates containing plant extracts in laminar flow cabinet (Figure 6.3), petri dishes were sealed, incubated at 37 °C and observed after 24 hours (Mitscher *et al.*, 1972). The fractions were tested at 5.0; 2.5; 1.0 and 0.5 mg/ml concentrations. Two blank petri dishes containing only nutrient agar and two containing nutrient agar and 1% acetone without plant extracts served as controls. In addition plates containing streptomycin sulfate at concentration of 200, 100, 50 and 10 µg/ml served as positive controls. The MIC values were regarded as the lowest concentrations of the extracts that did not permit any visible growth after 24 hour of incubation at 37 °C (Mitscher *et al.*, 1972). Each treatment was replicated three times.



Figure 6.3 Bacterial streaking in laminar flow cabinet

6.5 Antituberculosis assay

Chloroform, acetone, ethyl acetate, butanol and water fractions of the roots of *P. sidoides* were investigated for antimycobacterial activity at concentrations ranging from 5.0 to 2.5 mg/ml as described in Chapter 5, section 5.2.2.1.

6.6 Results and Discussion

6.6.1 Antibacterial and antituberculosis bioassays of extracts of *P. sidoides*

The antibacterial bioassays of extracts of *P. sidoides* against the Gram-positive and Gram-negative bacteria species showed that butanol extract was the most significant one as compared to other extracts, inhibiting the growth of *B. cereus*, *B. pumilus*, *B. subtilis*, *S. aureus* and *E. coli* at concentrations ranging from 1.0 mg/ml to 2.5 mg/ml. In addition, ethyl acetate extract was found to be active at 1.0 to 5.0 mg/ml against five Gram-positive bacteria (*B. cereus*; *B. pumilus*; *B. subtilis*; *S. aureus* and *E. faecalis*) and one Gram-negative bacteria, (*P. aeruginosa*) (Table 6.1). The acetonic fraction was found to be active only against *B. cereus* at 0.5 mg/ml. Chloroform and water fractions inhibited only a few Gram-positive bacteria at concentration ranging from 1.0 to 5.0 mg/ml. The reference antibiotic, streptomycin sulfate inhibited the growth of all the bacterial species tested in this study at 0.01 mg/ml except *Pseudomonas aeruginosa* and *Serratia marcescens* which were inhibited at 0.05 and 0.1 mg/ml respectively. The antituberculosis assay of extracts was interpreted on day five or six when the control vials (V2) reached a GI value of 30 or more. Chloroform, ethyl acetate and butanol fractions from the roots of *P. sidoides* showed inhibitory activity at 5.0 and 2.5 mg/ml against the drug-sensitive strain of *M. tuberculosis*. The acetone and water fractions did not show

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inhibition on *M. tuberculosis* (Table 6.2). Similar to the results obtained by Fabry *et al.*, 1998 where methanol extracts of *Entada abyssinica*, *Terminalia spinosa*, *Harrisonia abyssinica*, *Ximenia caffra*, *Azadirachta indica* and *Spilanthes mauritiana* were found to be inactive against *M. tuberculosis* at concentrations ranging from 2.0 – 0.5 mg/ml.

Kayser and Kolodziej (1997), tested acetone root extract at concentrations ranging from 5.0 to 7.5 mg/ml. All extracts exhibited a fairly high antibacterial effect against the spectrum of microorganisms. The highest activities consistently resided in the water extracts, with pronounced effects against *E. coli*, *K. pneumonia*, β -hemolytic streptococcus, *S. aureus*, *P. aeruginosa* and *P. mirabilis* with minimum inhibitory concentration (MIC) of 0.62 mg/ml and 1.25 mg/ml for *S. pneumonia* and *H. influenza*.

In our study, the ethyl acetate and butanol extracts were found to exhibit similar, but less antibacterial potencies at MIC's of 1.0 and 2.0 mg/ml, respectively. However, various other species of *Pelargoniums* such as *P. tomentosum*, *P. odoratissimum*, *P. denticulatum* have been found to possess good antibacterial activity against Gram-positive bacterial species such as *S. aureus*, *P. vulgaris*, *B. cereus*, and *S. epidermidis* at the concentration of 1.25 mg/ml (Lis-Balchin *et al.*, 1998a). In another study Lis-Balchin *et al.*, 1998b, found that the essential oil from *P. filcifolium* had good inhibitory activity against *Listeria innocua*, a Gram-positive bacteria.

In this study, the Gram-positive and *M. tuberculosis* bacteria appeared to be more susceptible to the inhibitory effect of extracts of ethyl acetate and butanol fractions of *P. sidoides* than the Gram-negative bacteria. The negative results obtained against Gram-negative bacteria were not surprising as, in general, these bacteria are more resistant than Gram-positive ones (Rabe and van Staden, 1997). The greater resistance of Gram-negative bacteria to plant extracts has been documented previously. Previous studies suggested that the difference in the cell wall structure between Gram-positive and Gram-negative bacteria might be the reason. The Gram-negative bacteria have an outer

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membrane acting as a barrier to many environmental substances, including antibiotics (Palombo and Semple, 2001). Accordingly, our findings slightly corresponded to the previous reports by Kayser and Kolodziej (1997) on antibacterial properties found on the fractionated ethyl acetate and butanol root fractions of *P. sidoides*; exhibiting an MIC of 1.25 mg/ml against same bacteria tested, *E. coli* and *S. aureus*. Similar observations were made by Kuhnt *et al.*, (1994), Afolayan and Meyer (1995); Lall and Meyer (2000) and Saxena *et al.*, (1996), while studying the antibacterial activity of *Hyptis verticillata*, *Helichrysum aureonites*, *Euclea natalensis* and *Masdevallia uniflora*.

Table 6.1 Antibacterial activity (MIC^a mg/ml) of *P. sidoides* extracts

Bacterial species	Gram + / -	Samples					
		CHCl ₃	Me ₂ CO	EtOAc	BuOH	H ₂ O	Streptomycin Sulphate
<i>Bacillus cereus</i>	+	1.0	0.5	1.0	1.0	5.0	0.01
<i>Bacillus pumilus</i>	+	5.0	na ^b	1.0	1.0	5.0	0.01
<i>Bacillus subtilis</i>	+	1.0	na	1.0	1.0	5.0	0.01
<i>Staphylococcus aureus</i>	+	na	na	5.0	2.5	1.0	0.01
<i>Enterococcus faecalis</i>	+	1.0	na	2.5	na	1.0	0.01
<i>Enterobacter cloacae</i>	-	na	na	na	na	na	0.01
<i>Escherichia coli</i>	-	na	na	na	1.0	na	0.01
<i>Pseudomonas aeruginosa</i>	-	na	na	2.5	na	na	0.05
<i>Serratia marcescens</i>	-	na	na	na	5.0	na	0.1

^a Minimum inhibitory concentration.

^b Not active at the highest concentration tested.

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Table 6.2 Antituberculosis activity of the root extracts against the sensitive strain (H37Rv) of *M. tuberculosis* as determined by the radiometric method. Δ GI value (mean \pm SD) of the control vial was 20 ± 1.4 for the sensitive strain

Samples	MIC^a (mg/ml)	ΔGI^b
<i>Pelargonium sidoides</i> (chloroform)	5.0 (S ^c)	1.5 ± 0.7
<i>P. sidoides</i> (70% acetone)	5.0 (N ^d)	37.5 ± 7.4
<i>P. sidoides</i> (ethyl acetate)	2.5 (S)	0.0 ± 0.0
<i>P. sidoides</i> (butanol)	2.5 (S)	0.5 ± 0.7
<i>P. sidoides</i> (water)	5.0 (N)	376.0 ± 9.97
Streptomycin	0.004 (S)	5.0 ± 0.0
Ethambutol	0.006 (S)	0.33 ± 0.0
Rifampicin	0.0002 (S)	0.0 ± 0.0
Isoniazid	0.002 (S)	4.0 ± 0.0

^a Minimum inhibitory concentration.

^b Growth Index.

^c Susceptible.

^d Not active at highest concentration tested.

6.7 Conclusion

The Gram-positive bacteria (*B. cereus*; *B. pumilus*; *B. subtilis*; *S. aureus* and *E. faecalis*) appeared to be more susceptible to the extracts obtained from *Pelargonium sidoides* than the Gram-negative ones. Chloroform, ethyl acetate and butanol extracts of *P. sidoides* were found to be active against *M. tuberculosis* at 5.0 mg/ml.

Coumarins that have been isolated from the butanol extract of *P. sidoides* previously by other researchers were found to have antibacterial properties (Kayser and Kolodziej, 1997). An attempt was therefore made to isolate compounds from the butanol extract of the plant. A number of different chromatography techniques were used, but we could not succeed to purify compounds from this extract. Due to the lack of enough plant material, further analysis with respect to purification and identification of pure compounds was carried out using a different approach from the one used as mentioned in this chapter.

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Bioassay guided fractionation of *Pelargonium sidoides*

7.1 Introduction

Plants belonging to the genus *Pelargonium* have yielded essential oils, tannins, flavonoids, phenols and coumarins. When it comes to the economic importance of these plants, some of the species have aromatic oils that are used in perfumes, cosmetics, and as insect repellent. The oil of geranium, widely used in perfumery and cosmetics, is stable and blends well with other fragrances. Dried leaves are used in sachets and potpourris (Simon *et al.*, 1984). Leaves of geranium are also used in herbal teas and the oil is used in baked goods and fruit desserts. The geranium of florists comes from many annual and perennial geranium species that vary in fragrance, growth habit and leaf and flower color. The scented geraniums are extensively used in flower gardens and as potted herbs (Lis-Balchin, 2002).

Kayser and Kolodziej (1995), reported the isolation of highly oxygenated coumarins from acetone root extracts of *P. sidoides*. Latté *et al.*, (2000), analysed and compared the coumarin patterns of *P. sidoides* and *P. reniforme*, forming the origin of the herbal medicine 'umckaloabo'. In the search of the underlying active principle(s) of medicinally used *Pelargonium* species for the treatment of respiratory infections, the antibacterial activity of *P. sidoides* and *P. reniforme* constituents were evaluated by Kayser and Kolodziej (1995), followed by a systematic study on the antifungal effects of tannins and related compounds by Latté and Kolodziej (2000b).

In this study, we report the bioassay-guided fractionation of *P. sidoides* and the isolation of compounds. ‘Umckaloabo’ has been reported to be used to treat tuberculosis. However, the active principles of *P. sidoides* have not been scientifically validated for their cure against tuberculosis. In this chapter, we have investigated the efficiency of isolated compounds from *P. sidoides* against *M. tuberculosis*.

7.2 Materials and Methods

7.2.1 Preparation of plant extracts

Fresh roots of *P. sidoides* (1 kg) were extracted with 100% ethanol. The extract was filtered and concentrated with rotary evaporator to dryness at reduced pressure and dissolved in methanol. Aqueous methanol extract was introduced to Di-ion column using water and acetone as eluent.

Fractions obtained were combined using 100% acetone and 75% acetone and sequentially extracted with ethyl acetate and butanol. Antituberculosis of all the extracts was conducted against *M. tuberculosis* as mentioned in Chapter 5, section 5.2.2.1.

7.2.2 Sephadex LH-20 and silica gel column separation

Since the butanol extract showed antituberculosis activity against *M. tuberculosis* (Table 7.1), therefore, it was selected for further work. Schematic representation of the purification steps for the isolation of the compounds is illustrated in figure 7.1. The butanol fraction (15 g) was dissolved in methanol and subjected to a sephadex column using methanol as eluant (Figure 7.2). The fractions (500 ml) were spotted on TLC plates of silica gel 60 F₂₅₄ using 10% methanol in ethyl acetate (10 mL) and hexane: ethyl acetate (7:3) as eluents and analysed under UV spectrum (Figure 7.3). Similar fractions

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were pooled together and dried, which resulted in three main fractions. The content of the combined dried fractions (Figure 7.1) were subjected to a sephadex column eluted with 100% methanol. Similar fractions were combined and concentrated to dryness, which resulted in four fractions. Fractions (Figure 7.1) were subjected to silica gel column eluted with chloroform and methanol in order of increasing polarity (5 – 50%). Similar fractions were combined together based on TLC profile.

Fractions II b₁₂ (Figure 7.1) were subjected to preparative TLC of silica gel and eluted with hexane: ethyl acetate (7:3). The semipure and pure compounds (Figure 7.4), were scraped off from the preparative plate and eluted with ethyl acetate, filtered and concentrated to dryness. The samples were then sent to NMR for analysis.

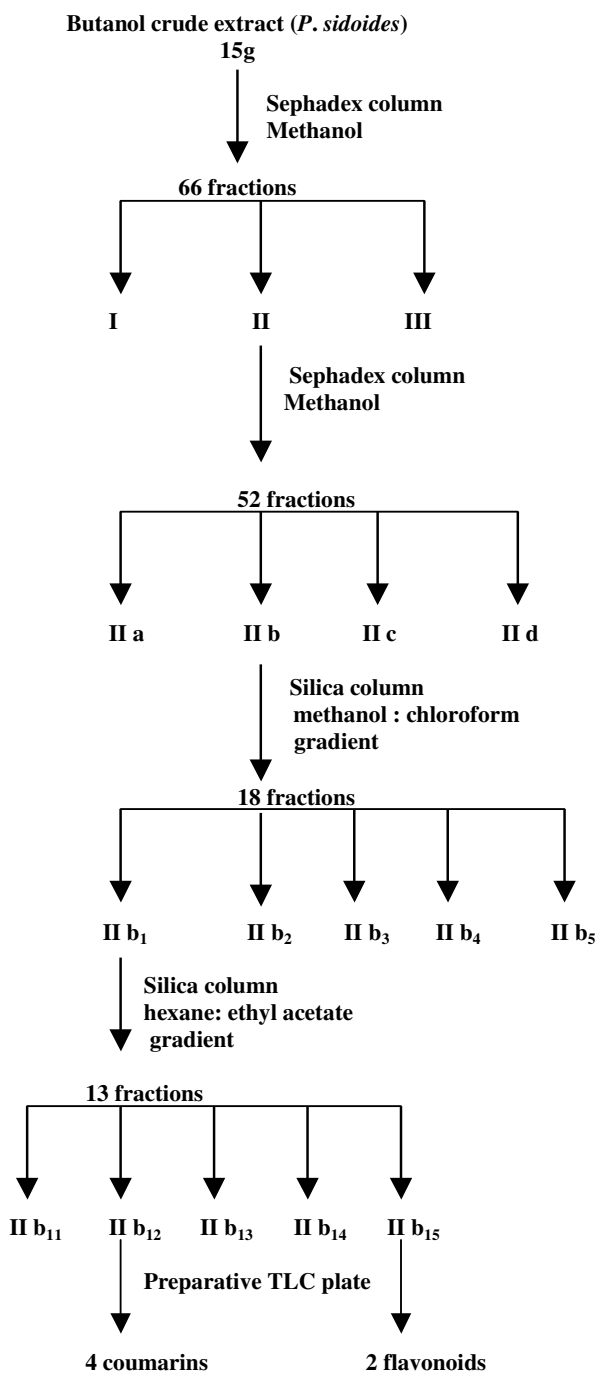
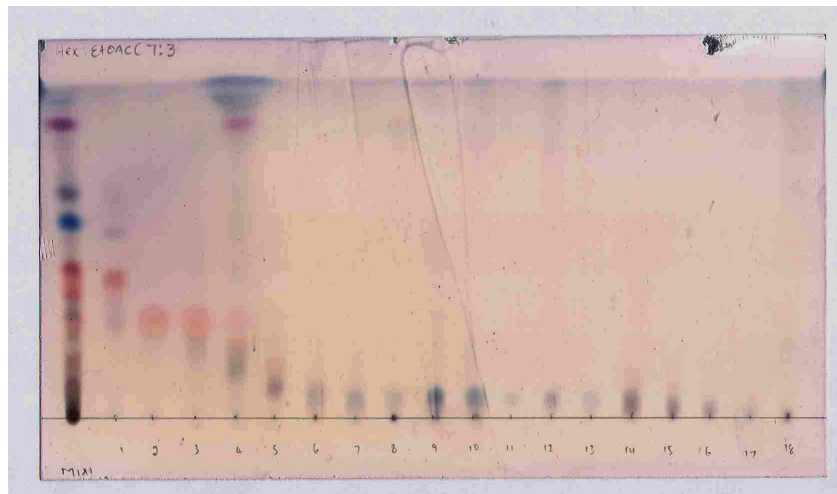


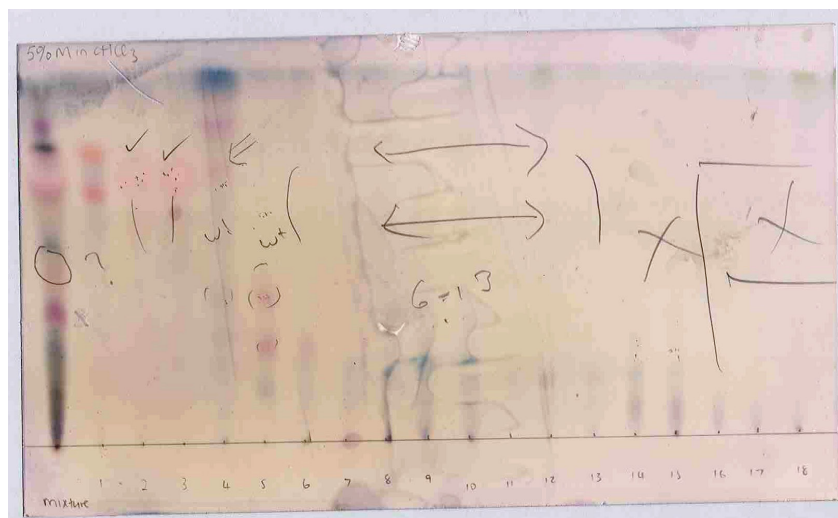
Figure 7.1 Schematic representation of the purification steps for the isolation of the compounds from the roots of *P. sidoides*



Figure 7.2 Sephadex column chromatography of butanol fraction obtained from the fresh roots of *P. sidoides*



(a)



(b)

Figure 7.3 TLC plates of fractions obtained from chromatographic separation of butanol extract of *P. sidoides*

Solvent systems: (a) hexane: ethyl acetate (7:3)

(b) 10% methanol in ethyl acetate

Detection : Vanillin in H₂SO₄

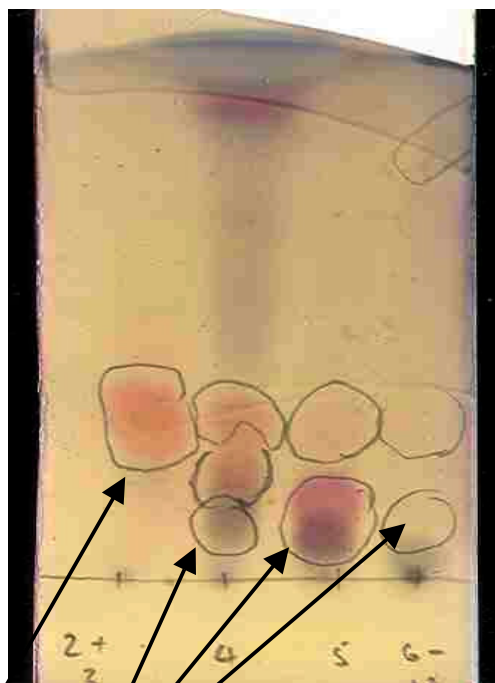


Figure 7.4 Pure and semipure compounds obtained from *P. sidoides*
Solvent systems: 10% methanol in ethyl acetate
Detection : Vanillin in H₂SO₄

7.3 Results and Discussion

7.3.1. Antituberculosis results

The antituberculosis assay of extracts was interpreted on day five or six when the control vials (V2) reached a GI value of 30 or more. Ethanol and butanol extracts from roots of *P. sidoides* showed inhibitory activity at 2.5 mg/ml against *M. tuberculosis*. The fractions obtained from the butanol extract (100, 75, 50 and 25% acetone), did not show inhibition on *M. tuberculosis*. Activity of the standard antituberculosis drug (isoniazid), used as a positive control was stronger than those of the extracts and fractions (Table 7.1). Our results are in agreement with previous experiments on ethanol extract (Chapter 5, section 5.3.1), and butanol extract (Chapter 6, section 6.6.1).

Table 7.1 Antituberculosis activity of the fractions obtained from the chromatographic separation of butanol extract of *Pelargonium sidoides* against the sensitive strain (H37Rv) of *M. tuberculosis* as determined by the radiometric method. Δ GI value (mean \pm SD) of the control vial was 36 ± 8.5 for the sensitive strain

Samples	MIC ^a (mg/ml)	Δ GI ^b
<i>Pelargonium sidoides</i> (ethanol)	2.5 (S ^c)	34.0 ± 5.7
<i>P. sidoides</i> (ethyl acetate)	na ^d	40.5 ± 14.8
<i>P. sidoides</i> (butanol)	2.5 (S)	5.5 ± 2.1
<i>P. sidoides</i> (100% acetone)	na	165 ± 46.6
<i>P. sidoides</i> (75% acetone)	na	199.5 ± 30.4
<i>P. sidoides</i> (50% acetone)	na	322.5 ± 77.0
<i>P. sidoides</i> (25% acetone)	na	375.5 ± 51.6
Isoniazid	0.002 (S)	1.0 ± 0.0

^a Minimum inhibitory concentration.

^b Growth Index.

^c Susceptible.

^d Not active at highest concentration tested (2.5 mg/ml).

7.3.2 Identification of the isolated compounds

The compounds were identified as coumarins (umckalin, scopoletin, 6,8-Dihydroxy-5,7-dimethoxy-2H-benzopyran-2-one and 6,8-Dihydroxy-7-methoxy-2H-benzopyran-2-one; and flavonoids (catechin and epigallocatechin), (Figure 7.5) by comparing their ¹H-NMR and ¹³C-NMR spectral data (Figure 7.6 and 7.7) with the published article of highly oxygenated coumarins (Kayser and Kolodziej, 1995). Coumarins constitute a major category of secondary metabolites that are widely distributed in the plant kingdom. They are characterized by a variety of oxygenated patterns on the benzopyrone nucleus and display an array of biochemical and pharmacological actions (Murray, 1982).

Previous researchers such as Kayser and Kolodziej revealed the presence of coumarins in *P. reniforme* and *P. sidoides*. The popular herbal medicine, Umckaloabo originates from these two plant species (Kayser and Kolodziej, 1998).

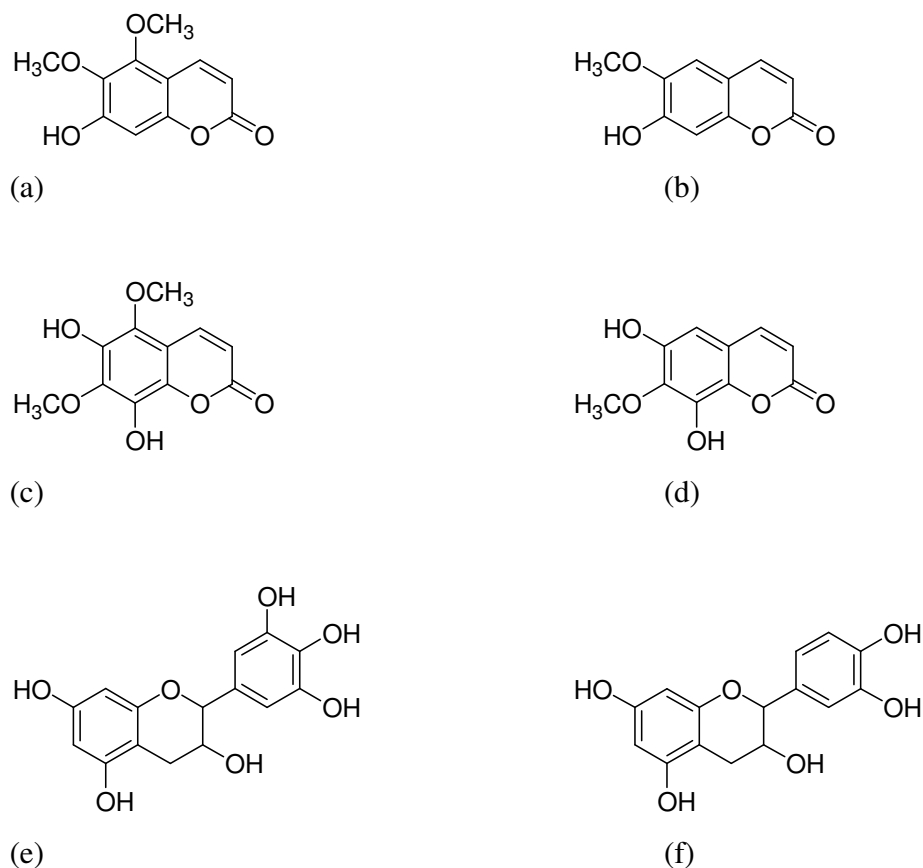


Figure 7.5 Coumarins and flavonoids isolated from butanol extract of *P. sidoides*

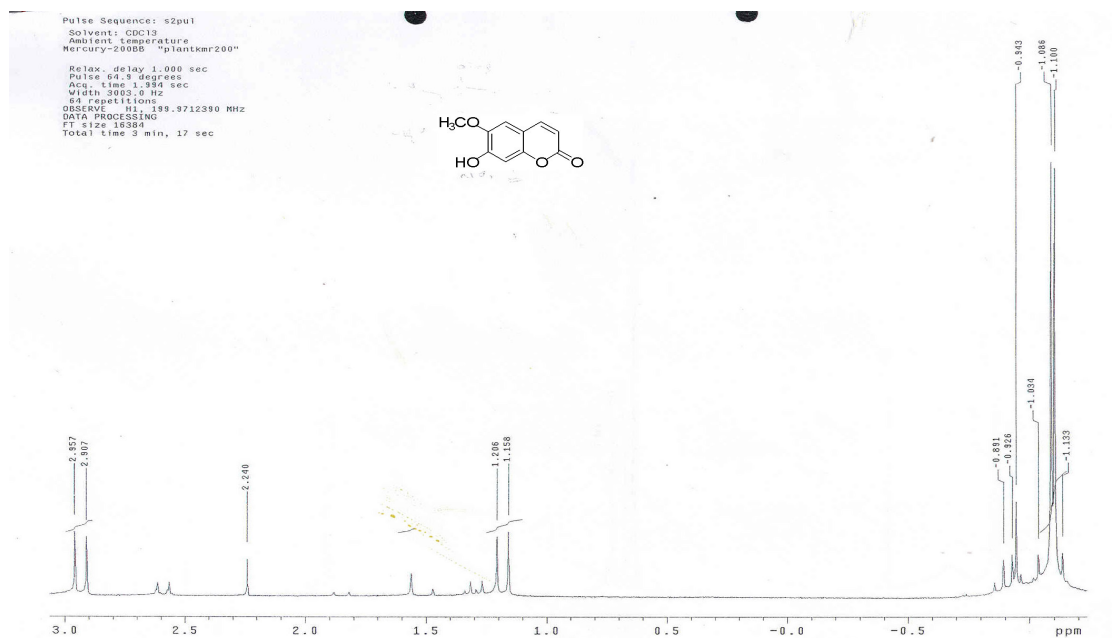
- (a) 6-Hydroxy-5,7-dimethoxy-2H-benzopyran-2-one (umckalin)
- (b) 7-Hydroxy-6-methoxy-2H-benzopyran-2-one (scopoletin)
- (c) 6,8-Dihydroxy-5,7-dimethoxy-2H-benzopyran-2-one
- (d) 6,8-Dihydroxy-7-methoxy-2H-benzopyran-2-one
- (e) Epigallocatechin
- (f) Catechin

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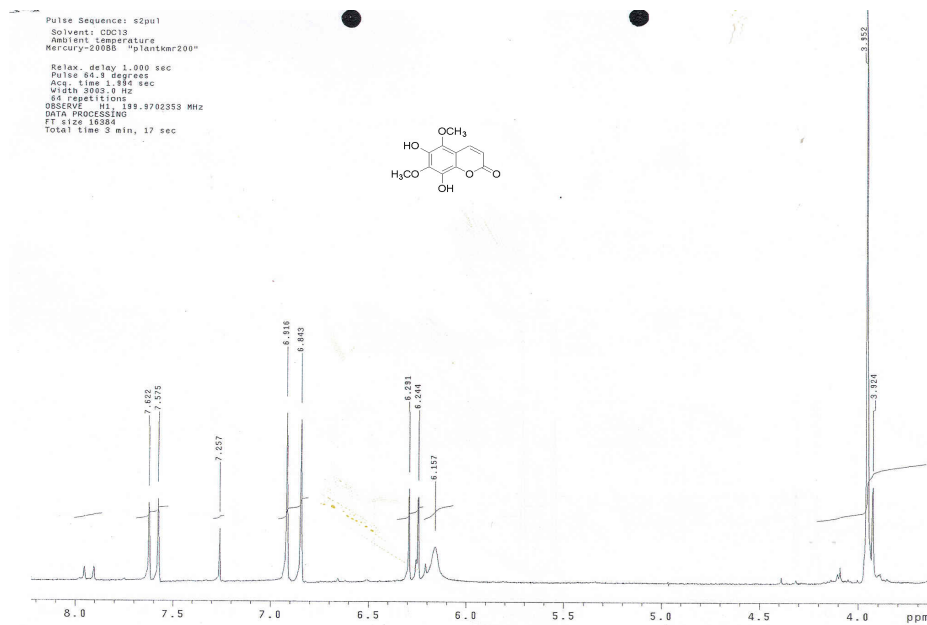
(a) 6-Hydroxy-5,7-dimethoxy-2H-benzopyran-2-one (umckalin)



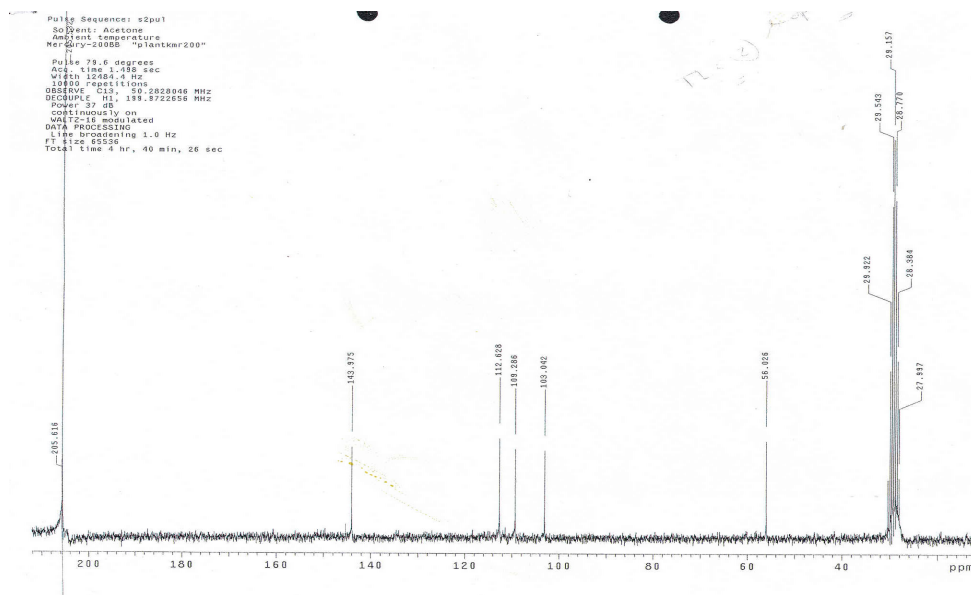
(b) 7-Hydroxy-6-methoxy-2H-benzopyran-2-one (scopoletin)

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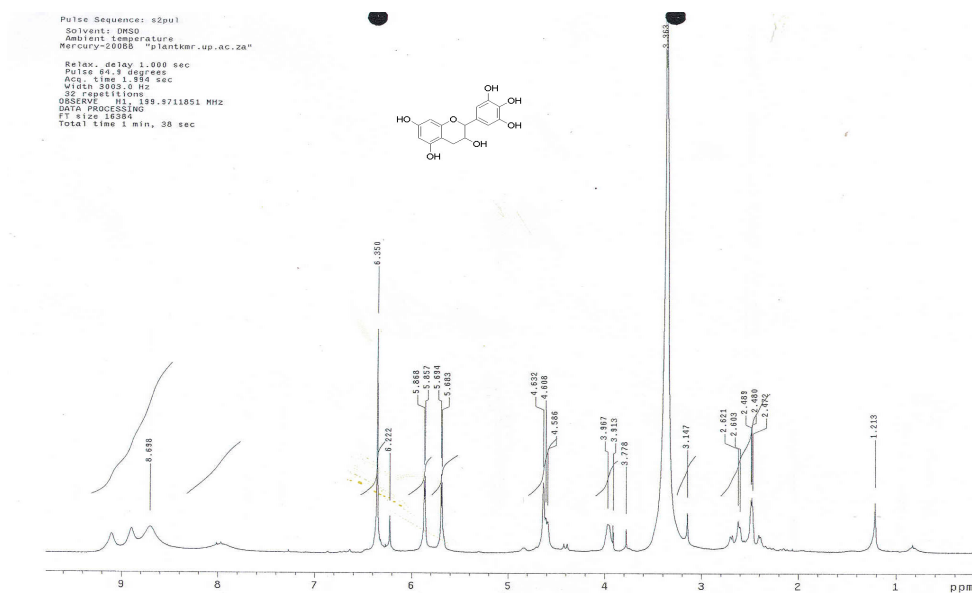
(c) 6,8-Dihydroxy-5,7-dimethoxy-2H-benzopyran-2-one



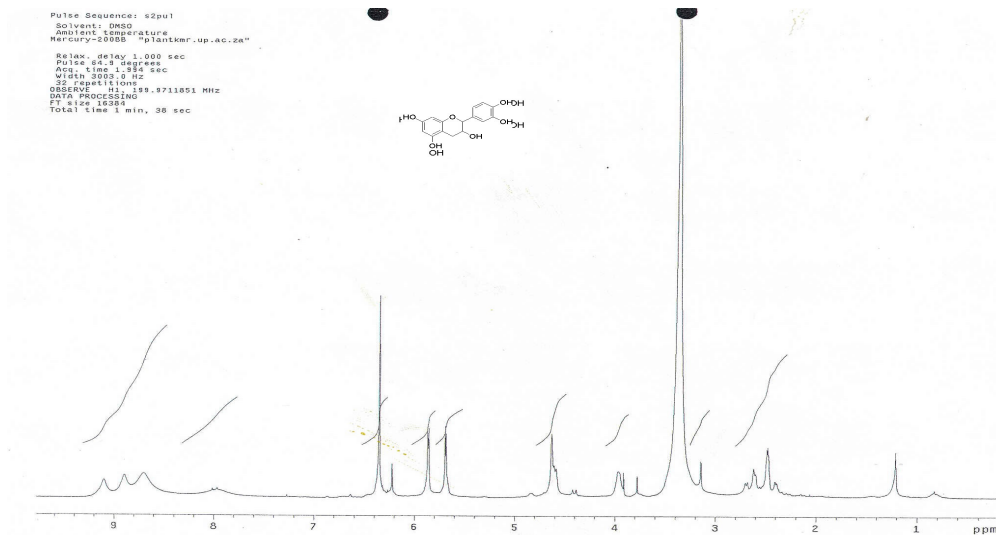
(d) 6,8-Dihydroxy-7-methoxy-2H-benzopyran-2-one

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(e) Epigallocatechin

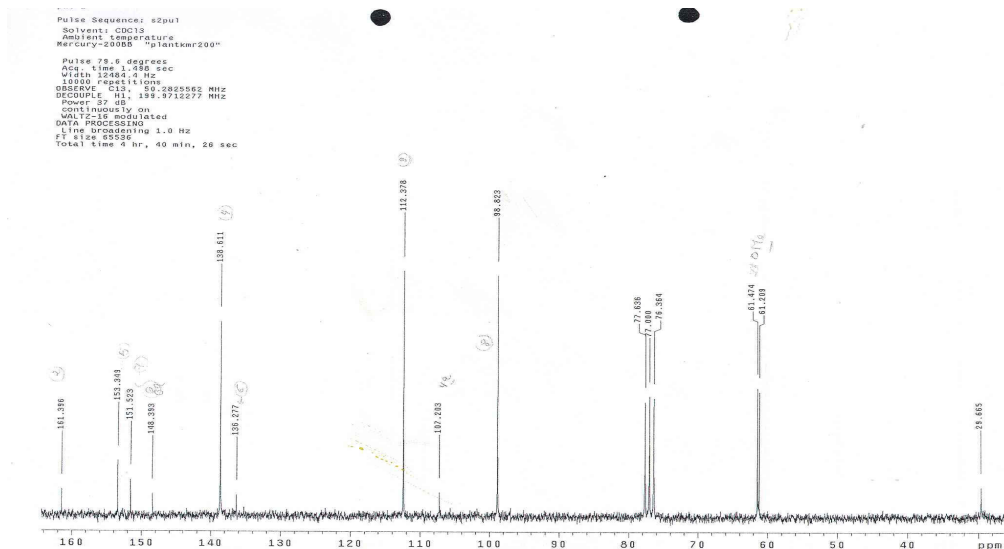


(f) Catechin

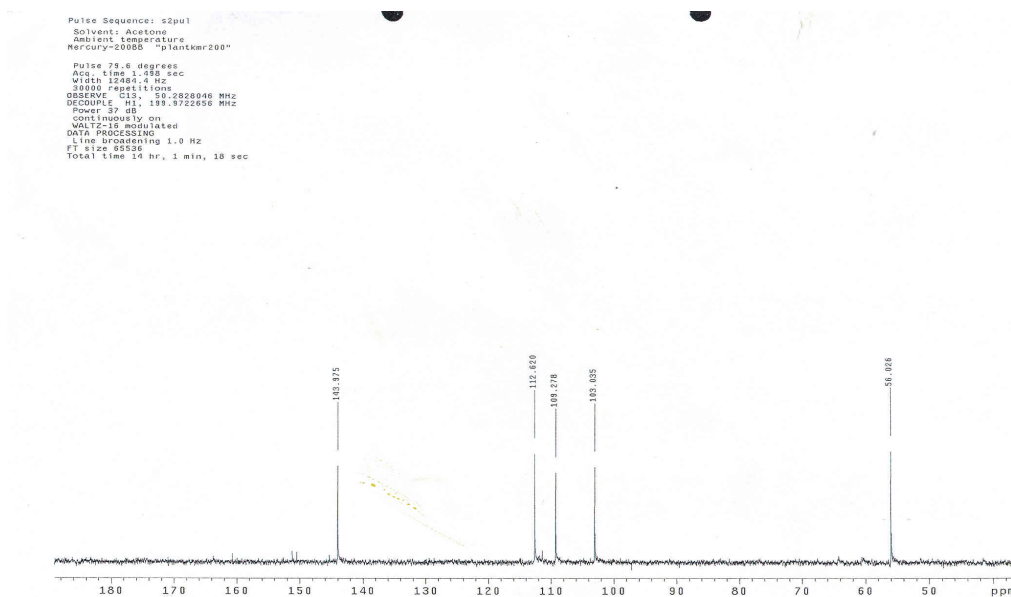
Figure 7.6 ¹H-NMR spectrums of coumarins and flavonoids isolated from butanol extract of *P. sidoides*

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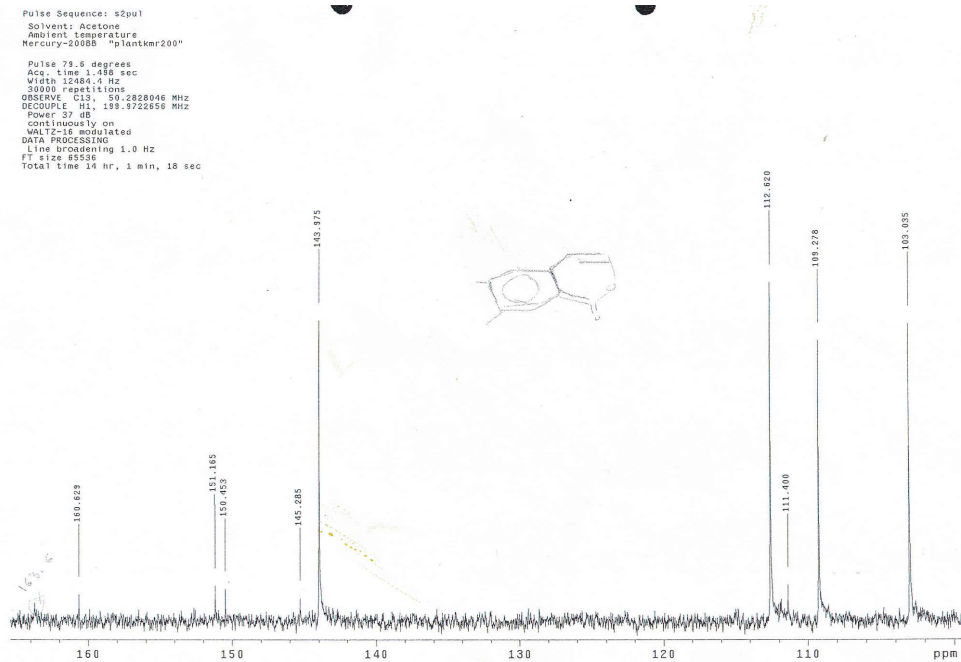
(a) 6-Hydroxy-5, 7-dimethoxy-2H-benzopyran-2-one (umckalin)



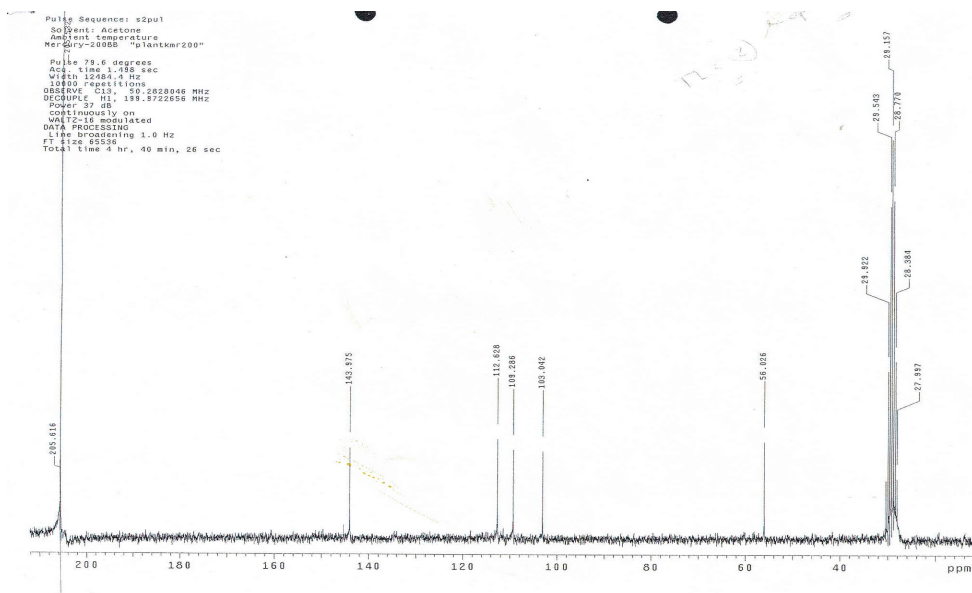
(b) 7-Hydroxy-6-methoxy-2H-benzopyran-2-one (scopoletin)

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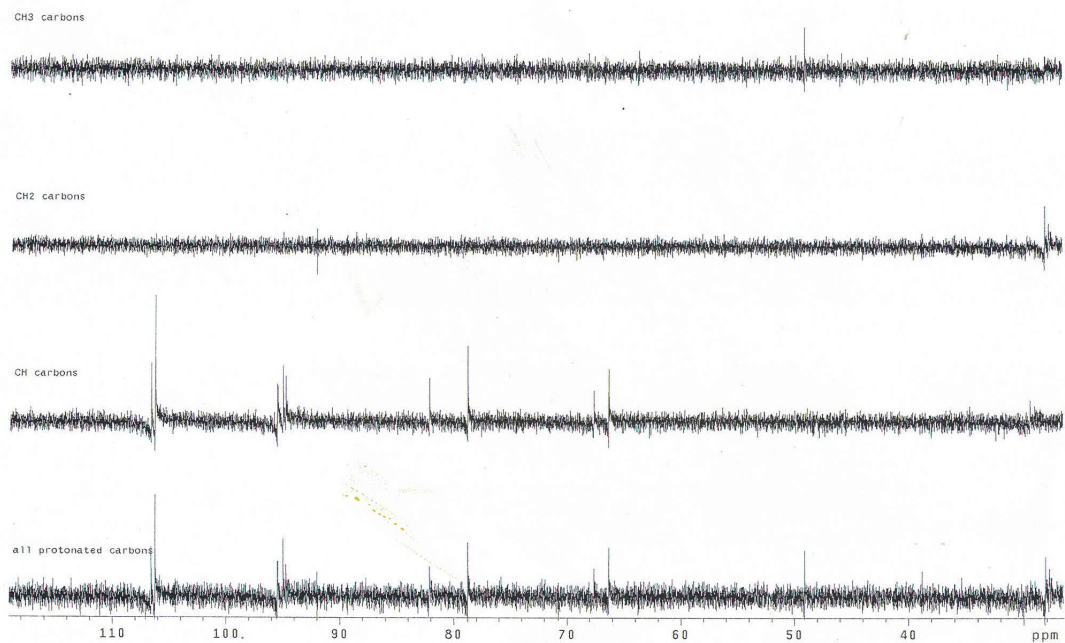
Bioassay guided fractionation of *P. sidoides*



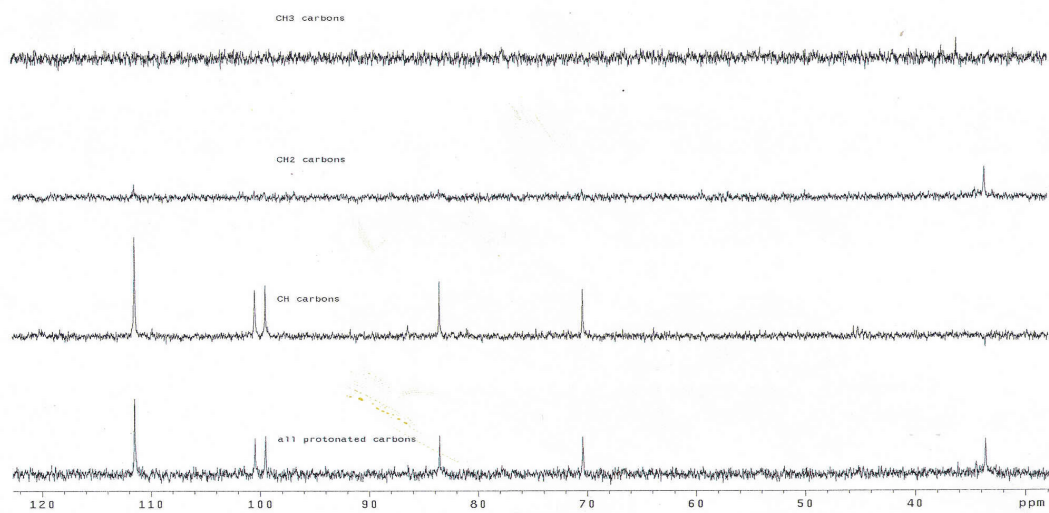
(c) 6,8-Dihydroxy-5,7-dimethoxy-2H-benzopyran-2-one



(d) 6,8-Dihydroxy-7-methoxy-2H-benzopyran-2-one



(e) Epigallocatechin



(f) Catechin

Figure 7.7 ¹³C-NMR spectrums of coumarins and flavonoids isolated from butanol extract of *P. sidoides*

7.4 Conclusion

Bioassay guided isolation of bioactive butanol extract of *P. sidoides* resulted in the isolation of six compounds: 6-Hydroxy-5,7-dimethoxy-2H-benzopyran-2-one, 7-Hydroxy-6-methoxy-2H-benzopyran-2-one, 6,8-Dihydroxy-5,7-dimethoxy-2H-benzopyran-2-one, 6,8-Dihydroxy-7-methoxy-2H-benzopyran-2-one, epigallocatechin and catechin. All compounds isolated in this study have been reported from this plant previously except **compound 6 (epigallocatechin)** is reported here for the first time.

Chapter 8

Evaluations of the isolated compounds

8.1 Introduction

As a medicinal plant, Pelargonium species have traditionally been considered an astringent and used as a folk remedy in the treatment of ulcers. A terpine hydrate synthesized from geraniol is known to be, an effective expectorant. Leaves are reported to have antifungal activity. Scented geranium and oil of geranium are reported to cause contact dermatitis. Geranium is reported to repel insects because of its citronellol content (Lis- Balchin, 2002).

In this study, antituberculosis activity of the isolated compounds from fractions of butanol extract of the roots of *P. sidoides* is reported.

8.2 Bioassay on *M. tuberculosis*

The radiometric respiratory technique with the BACTEC apparatus was used for susceptibility testing of *M. tuberculosis*. Bacterial cultures utilized in this study were grown from specimens received from the Medical Research Council (MRC) in Pretoria. A drug-susceptible strain of *M. tuberculosis*, H37Rv obtained from American Type, MD.USA Culture Collection (ATCC), 27294, was used in the screening procedure. Umckalin, scopoletin, 6,8-Dihydroxy-5, 7-dimethoxy-2H-benzopyran-2-one, 6,8-Dihydroxy-7-methoxy-2H-benzopyran-2-one, epigallocatechin and catechin were each dissolved at 10 mg/ml in DMSO and added to 4mL of BACTEC 12B broth to achieve the final concentrations of 100, 50, 10, 5 and 1.0 µg/ml in triplicates, one with PANTA, two

without PANTA (Becton Dickinson & Company, an antimicrobial supplement). The BACTEC drug susceptibility testing was also done for the primary drug isoniazid at concentration of 0.2 µg/ml respectively against the H37Rv sensitive strain. Preparation of bacterial cultures and the testing procedures were the same as described in Chapter 5, section 5.4.1.

8.3 Results and Discussion

In the present study, fractions and compounds isolated were investigated for antituberculosis activity. Ethanol and butanol fractions from the roots of *P. sidoides* showed inhibitory activity at 2.5 mg/ml against the drug-sensitive strain of *M. tuberculosis*. The ethyl acetate did not show inhibition on *M. tuberculosis*. Coumarins and flavonoids isolated did not show activity at the highest concentration tested (Table 8.1).

Similar results were obtained by researchers; Kolodziej *et al.*, (2003), where inhibition was found on the aqueous acetone root extract of *P. sidoides* against *M. tuberculosis* at a concentration of 12.5 µg/ml, however none of the isolated phenolic compounds or coumarins showed antimycobacterial activity *in vitro*. Scopoletin, umckalin, 5,6,7-trimethoxycoumarin, (+)- catechin, gallic acid and its methyl ester and 6,8-dihydroxy-5,7-dimethoxycoumarin) in both species of *P. reniforme* and *P. sidoides* were evaluated against eight micro organisms including three Gram-positive (*S. aureus*, *S. pneumonia* and β -hemolytic streptococcus) and five Gram-negative (*E. coli*, *K. pneumonia*, *P. mirabilis*, *P. aeruginosa*, *H. influenza*) at MICs of 5 to 7.5 mg/ml. All compounds (scopoletin, 5,6,7- trimethoxycoumarin, gallic acid, gallic acid methyl ester, (+)-catechin and 6.8-dihydroxycoumarin), exhibited more or less pronounced antibacterial activities against Gram-positive and Gram-negative pathogens with the MICs of 0.2 to 2.0 mg/ml. Umckalin and 6.8-dihydroxy-5,7-dimethoxycoumarin showed antibacterial activity with MICs of 0.3 to 0.5 mg/ml. These observations by us and

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Kolodziej *et al.*, (2003), on antimycobacterial activity suggest that the coumarins are not active against *M. tuberculosis*.

Table 8.1 Antituberculosis activity of the compounds against the sensitive strain (H37Rv) of *M. tuberculosis* as determined by the radiometric method. Δ GI value (mean \pm SD) of the control vial was 36 ± 8.5 for the sensitive strain

Samples	MIC ^a (μ g/ml)	Δ GI ^b
6-Hydroxy-5, 7-dimethoxy-2H-benzopyran-2-one (umckalin)	na ^c	178.5 \pm 55.9
7-Hdroxy-6-methoxy-2H-benzopyran-2-one (scopoletin)	na	227.0 \pm 31.1
6,8-Dihydroxy-5, 7-dimethoxy-2H-benzopyran-2-one	na	149.5 \pm 70.0
6,8-Dihydroxy-7-methoxy-2H-benzopyran-2-one	na	16.0 \pm 14.1
Catechin	na	146.0 \pm 12.7
Epigallocatechin	na	145.5 \pm 12.0
Isoniazid	0.2 (S ^d)	1.0 \pm 0.0

^a Minimum inhibitory concentration.

^b Growth Index.

^c Not active at highest concentration tested (100 μ g/ml).

^d Susceptible.

8.4 Conclusion

The coumarins and flavonoids isolated from the roots of *P. sidoides* did not show inhibitory activity against *M. tuberculosis*. As *Mycobacteria* are intracellular pathogens, antimycobacterial activities reported by anecdotal evidences may be due to either direct or indirect effects. In recent studies, chemical constituents and pharmacological studies have demonstrated antibacterial and immunomodulatory activities *in vitro* (Kolodziej *et al.*, 2003). Though the compounds in our study did not show antituberculosis activity, it can be speculated that the anecdotal evidence of tuberculosis-patients could be due to an immunostimulant.

The isolated compounds from *P. sidoides* have not been investigated as yet for intracellular TB activity. It is therefore, recommended that these compounds should be analysed for intracellular activity against *M. tuberculosis* in mice and / or human macrophages.