The traditional use of medicinal plants to treat sexually transmitted diseases

by

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Chapter 1

Introduction

1.1 Background

Plants have been used traditionally as a source of treatment for various ailments for many years throughout the world among all races. Because of the fear of diseases and death every cultural group has responded by developing a medicinal system making use of natural products to cure various ailments, but undoubtably plants play a major role (Ellis, 1986). Some of these traditions and medicinal practices may seem strange and magical, others appear rational and sensible, but all of them are attempts to overcome illness and suffering, and enhance quality of life (Fnimh, 2001).

In most traditional African societies there are individuals such as herbalists, spiritualists, traditional practitioners etc. who have the responsibility of providing relief from different diseases. It is often argued that traditional healers operate close to the people and that they are very helpful in many rural communities where modern medicine is not readily available (Cunningham, 1988). In the past, traditional healers were considered to be more effective against psychosocial illnesses. It is now known that traditional healers use a vast array of medicinal plants for infections such as colds, coughs and inflammatory diseases (Shale *et al.*, 1999).

Many traditional healers still use the plants in their crude form (herbal remedies), although western technologies have transformed some plant products into more palatable forms like tablets, capsules and syrups. Extracts from some of the medicinal plants being used by traditional healers have been found to contain properties that inhibit the growth of bacteria, viruses and other microbes (Ndubani & Hojer, 1999).

The recognition and validation of traditional medicine and the search for plant derived drugs could lead to new strategies to control various diseases. It is necessary to obtain more scientific information concerning the efficacy and safety of remedies in use. With such information we will be able to evaluate the position and effectiveness of traditional medicine (Gessler *et al.*, 1994).

1.1.1. Medicinal plants industry

The global herbal market and industry have been growing rapidly in recent years and therefore, today medicinal plants are of utmost importance (WHO, 1987). Medicinal plants have become the focus of intense study recently in terms of discovering new drugs and as to whether their traditional uses are supported by pharmacological effects (Rabe & Van Staden, 1997). Medicinal plants are an integral component of research developments in the pharmaceutical industry. Such research focuses on the isolation and direct use of active medicinal constituents of plants, semi-synthetic drugs and pharmacologically active compounds. As a result the industry has invested vast resources into screening the active constituents of medicinal plants from all over the world (Fnimh, 2001). About half of the world's 25 best selling pharmaceuticals in 1991 originated from natural source materials (Table 1.1)

Of the 250 000 species of higher plants known to exist on earth, only a relative handful have been thoroughly studied for all aspects of their potential therapeutic value in medicine. About 25% of the prescribed drugs used today has been yielded from the plant kingdom (Balick, 1990). Bioassay-guided fractionation of plant extracts linked to chromatographic separation techniques can lead to isolation of biological active molecules. Plants investigated for their medicinal properties has yielded many useful compounds such as quinones, flavonoids, alkaloids, glycoproteins, terpenoids, lectins, coumarins, glycosides and aglycones. This has resulted in the discovery of a number of modern drugs (Table 1.2). Among the successful clinical agents derived from plants are benzodiazepines for insomnia and anxiety attacks, atenolol for the treatment of hypertension and salbutamol for asthma (Phillipson, 2001).

Table 1.1. The world's 25 best selling pharmaceuticals in 1991 (Kinghorn& Balandrin 1992)

Position 1991	Product	Therapeutic Class	Sales $m
1	Ranitidine	H2 antagonist	3,032
2	[a]Enalapril	ACE inhibitor	1,745
3	[a]Captopril	ACE inhibitor	1,580
4	[a]Diclofenac	NSAID	1,185
5	Atenolol	β-antagonist	1,180
6	Nifedipine	Ca^{2+}	1,120
7	Cimetidine	H_2 antagonist	1,097
8	[a]Mevinolin	HMGCoA-R inhibitor	1,090
9	[a]Naproxen	NSAID	954
10	[a]Cefaclor	β-lactam antibiotic	935
11	Diltiazem	Ca^{2+} antagonist	912
12	Fluoxetine	5HT reuptake inhibitor	910
13	Ciprofloxacin	Quinolone	904
14	Amlodipine	Ca^{2+}	896
15	[a]Amoxycillin/ clavulanic acid	β-lactam antibiotic	892
16	Acyclovir	Anti-herpetic	887
17	[a]Ceftriaxone	β-lactam antibiotic	870
18	Omeprazole	H^+ pump inhibitor	775
19	Terfenadine	Anti-histamine	768
20	[a]Salbutamol	β_2-agonist	757
21	[a]Cyclosporin	Immunosuppressive	695
22	[a]Piroxicam	NSAID	680
23	Famotidine	H_2 antagonist	595
24	Alprazolam	Benzodiazepine	595
25	[a]Oestrogens	HRT	569

[a]Natural product derived

The practice of traditional medicine is widespread throughout the whole world. In China about 40% of the total medicinal consumption is attributed to traditional tribal medicines and it is estimated that in the mid-90s, receipts of more than US $ 2.5 billion have resulted from the sales of herbal medicines in Thailand. The same applies to Japan where herbal medicinal preparations are more in demand than mainstream pharmaceutical products (Lemma, 1991). The industrial uses of medicinal plants range from traditional medicines, health foods and pharmaceuticals. The world market for plant-derived chemicals, for example: pharmaceuticals, fragrances and flavours etc. alone exceeds several billion dollars per year. The annual global trade of medicinal plants is estimated to be US $ 800 million.

Africa is known to be a rich source of medicinal plants and some pharmaceutical agents have been produced from local plant species. The notable examples are as follows.

Table: 1.2 Plants which are exported from Africa for medicinal purposes (Sofowora, 1982).

Plant Species (part used)	Source area	Constituents
Allanblackia floribunda (fruit)	Ivory Coast	Fats
Ancistrocladus korupensis (whole plant)	Ghana Cameroon	Michaelamines A Michaelamines B
Corynanthe pachyceras (bark)	Ghana	Yohimbe Corynanthine
Dennentia tripetala (fruit)	Ghana	Essential oils
Griffonia simplicifolia (seed)	Ivory Coast Cameroon	BS11 lectin
Hunteria eburnea (bark)	Ghana	Eburine
Jateorhiza palmata (root)	Tanzania	Palmatine Colombamine
Pausinystalia yohimbe (bark)	Cameroon	Yohimbine
Pentadesma butryacea (fruit)	Ivory Coast	Fats
Physostigma venenosum (fruit)	Ivory Coast Ghana	Physostigmine

1.1.2 Future of traditional medicine

Despite the increasing use of medicinal plants, their future is being threatened by complacency concerning their conservation. Each year large numbers of medicinal plants are destroyed through over-exploitation by herbalists (medicinal plants traders) and also through land conversion to agriculture (Balick, 1990). In developing countries reserves of herbs and stocks of medicinal plants are diminishing and in danger of extinction as a result of growing trade demands for cheaper healthcare products in preference to more expensive target-specific drugs and biopharmaceuticals. The result of growth-exploitation, environmentally unfriendly harvesting techniques, loss of growth habitats and unmonitored trade of medicinal plants have put the future of traditional medicine under threat (Hoareau and Da Silva, 1999). Scientific validation of traditional medicinal plants is essential in order to benefit humankind. Many medical scientists still find it hard to accept that natural medicines can be as good as chemical cures in treating illness. The increase in scientific research on traditional medicine will probably change this thinking (Fnimh, 2001)

1.1.3. Traditional medicine and sexually transmitted diseases

Herbal remedies play a fundamental role in the traditional medicine in rural areas of South Africa. More and more people utilize traditional medicine for their major primary health care needs (Sindambiwe *et al*, 1999). Sexually transmitted diseases (STD's) seem to be a major public health problem, which is confirmed by data from different health related bodies. This data is not accurate because traditional healers treat many cases of STD's and they do not keep records. There is a high occurrence of STD's in general these days, and STD's like gonorrhea and syphilis are prevalent in Africa. In many countries it is fairly established that STD's, especially those that are untreated, play a role in the transmission of HIV. It is known that STD's such as gonorrhea provoke a lymphocyte response, which might increase the amount of virus transmitted from a HIV infected

person. The gonorrheal pus discharge may also play a role in carrying HIV into the bloodstream (Green, 1992). Widespread misuse of antibiotics has led to the emergence of new strains of gonorrhea that are resistant to penicillin, and therefore difficult and expensive to treat.

Low costs and privacy are not the only enticements of traditional healers, there is a strong belief in the efficacy of traditional medicines. Traditional medicine is believed to be more effective because it cures the root cause of infection. Western medicine addresses merely the signs and symptoms. It is also believed that the traditional medicines cleanse all the dirt left after the modern treatment, therefore some patients from hospitals go back to traditional healers (Moss *et al*, 1999 and Halsey, 1999). In research done in Zambia it was found that some people do not like to go to government centers and hospitals because of lack of privacy, long queues, being examined by a member of the opposite sex, high medical fees, demand of the attendance of the partner before given the treatment etc. (Msiska *et al*, 1997).

Traditional healers view themselves as knowledgeable and more competent to treat many illnesses that could be classified as STD's. Recent studies have focused on medicinal plants that are mostly used traditionally to treat STD's (gonorrhea and syphilis). Many healers claim success with conditions scientifically classifiable as syphilis and gonorrhea after only one or two treatments (Ndubani, 1997). These were also confirmed by interviewing their patients.

Like western medicine, traditional medicine for STD's may have very serious consequences, e.g. overdose and toxicity. All over South Africa traditional medicine is being used to treat STD's, but this study focuses on plants that are used by the Vhavenda peoples in the Limpopo province. Herpes, gonorrhea and syphilis are among the diseases that are treated by Venda traditional practitioners since many teenagers suffer from them. Some of the plants which they use are also used by other cultures to treat the same ailments, for example various parts of *Senna petersiana* are used in other African countries to treat STD's (Palgrave, 1977).

1.1.3.1. Gonorrhea

Gonorrhea is caused by *Neisseria gonorrhea*, a bacterium that grows and multiplies in moist, warm areas of the body such as the cervix, urethra, mouth, or rectum. This bacterium is most commonly spread during genital contact, but it can also be passed from the genitals of one partner to the throat of the other during oral sex. It can also be passed from an infected mother to her newborn infant during delivery. The early symptoms include a painful or burning sensation when urinating or an abnormal genital discharge. If untreated it can spread to the bloodstream and infect the joints, heart valves or the brain (Stanier *et al.*, 1976).

1.1.3.2. Syphilis

Treponema pallidum is the causative agent of syphilis. It is transmitted through the mucus membranes, usually of the genital area, mouth, or the anus of the sexual partner. In addition to sexual contact, it may also be transmitted through skin lesions on other parts of the body (Stanier *et al.*, 1976). It has three stages of symptoms and the early signs are sores, which may appear on the genitals or inside the mouth. Like gonorrhea, if untreated, it may cause serious health problems, such as blindness, paralysis, brain damage or death (Norris and Edmondson, 1986).

1.1.3.3. Herpes simplex

There are two types of herpes simplex virus (HSV-1 and HSV-2). The type 1 virus mainly causes cold sores and the type 2 virus causes genital sores which are transmitted through sexual contact with an infected person. Clinical studies are now ongoing to attempt to control it, since there is no cure. HSV-2 is the principal agent of chronic remittent genital herpes. Worldwide, only 10-20% of genital herpes are HSV-1. Previous studies indicated that HSV-1 is responsible for a significant proportion or even the majority of first clinical episodes of genital herpes in young women (Lippelt *et al*, 2002).

1.2 Objectives of the study

- Identification of plants which are being used to treat sexually transmitted diseases (STD' s) in South Africa.
- Investigation of *in vitro* antibacterial activity of plants against five Gram-negative and five Gram-positive bacteria.
- Testing *in vitro* antiviral activity
- Evaluate cytotoxicity of the plants
- Isolation and identification of active compounds from the plants with the highest activity
- Investigation of antimicrobial activity of the isolated compounds

1.3 Scope of the dissertation

The distribution, medicinal uses and chemistry of the plants selected for this study are described in Chapter 2. Chapter 3 reports on the antibacterial activity of six selected species. Cytotoxicity and antiviral activity of extracts of three selected species with the best antibacterial activity is described in Chapter 4. The isolation and identification of active compounds is described in Chapter 5. Chapter 6 focuses on antibacterial activity of the isolated compounds. Chapter 7 describes the cytotoxicity and antiviral activity of the isolated compounds. Chapter 8 deals with the general discussion and conclusion of the entire study. Finally Chapter 9 summarizes the whole project and the importance of traditional medicine.

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Chapter 2

Plants selected, descriptions, medicinal uses and chemistry

2.1 Introduction

Venda is situated in the far northern region of the Limpopo Province, South Africa. It is in one of those areas where traditional healing is still very much alive and functioning. It is true that the Venda people have for ages depended on the natural environment for their health and survival. Almost all medicines used by Venda people are derived from plants and to a lesser extent also from animals (Mabogo, 1990). This study focuses on some of the plants that are used traditionally to treat sexually transmitted diseases (STD's) in this area.

General interviews and information on the plants used traditionally in the treatment of STD's (mostly herpes, gonorrhea and syphilis) was collected from the traditional healers and local people. The information was obtained from rural dwellers of the following villages: Duthuni, Tshidzivhe, Mukumbani and Tshilapfene; all located within the district of Thohoyandou in Venda. As a result 20 plants were identified, but only six species which are mostly used by the interviewed healers were collected and investigated. Information on the parts of the plants used, preparation methods as well as personal experience from some of the patients was also noted. The plants were identified initially using local names and their proper identification was done at the herbarium of the University of Pretoria. Some of the plants collected were frequently used by different healers to treat the same ailment which might substantiate their reliability (Arnold and Gulumian, 1984). This chapter gives a brief description on the medicinal uses and chemistry of the plants collected.

2.2. Plants selected

2.2.1. *Senna petersiana* (Bolle)

Senna petersiana belongs to the family Caesalpinaceae and is a shrub or tree that can grow up to 7 m in height, 7-12 leaflets per leaf, dark green above and pale green below; rachis with conspicuous stalked gland between all or most pairs of leaflets; flowers in large, loose, branched sprays, yellow; pods flattened, green (Fig. 2.1).

a b

Figure 2.1: (a) Fruit, (b) flowers of *Senna petersiana* (Van Wyk & Van Wyk, 1997)

2.2.1.1. Distribution

This tropical species is widespread in eastern Africa, extending from Ethiopia and Sudan, southwards to Zimbabwe, Mozambique and the northern parts of South Africa.

2.2.1.2. Medicinal uses

Various parts are used medicinally, the pods are used in preparing a fermented beverage, and are relished by birds. In eastern Africa the plant is used for stomach complaints. In tropical and southern Africa it is used as purgative as well as a remedy for gonorrhea (Watt & Breyer-Brandwijk, 1962).

2.2.1.3. Chemistry

The roots, leaves and fruits of other *Senna* species are known to contain large amounts of anthraquinones (Figs. 2.2 and 2.3).

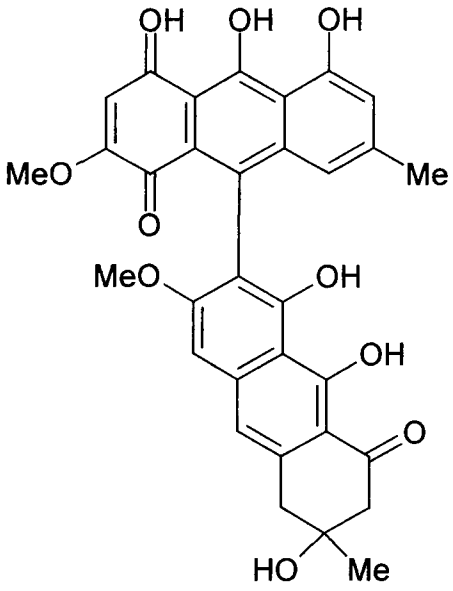
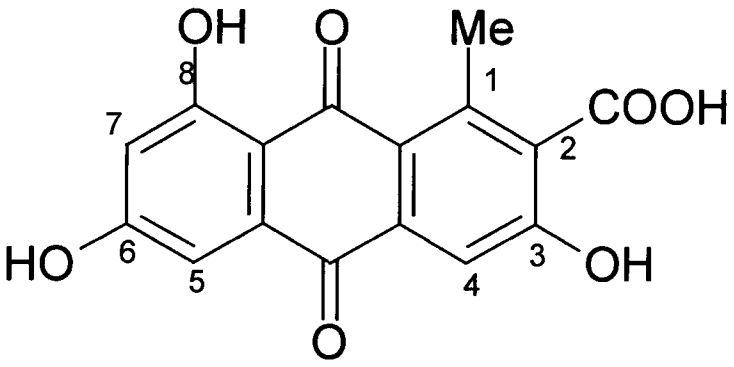


Figure 2.2 Structure of presengulone (anthraquinone) isolated from *Senna* sp.



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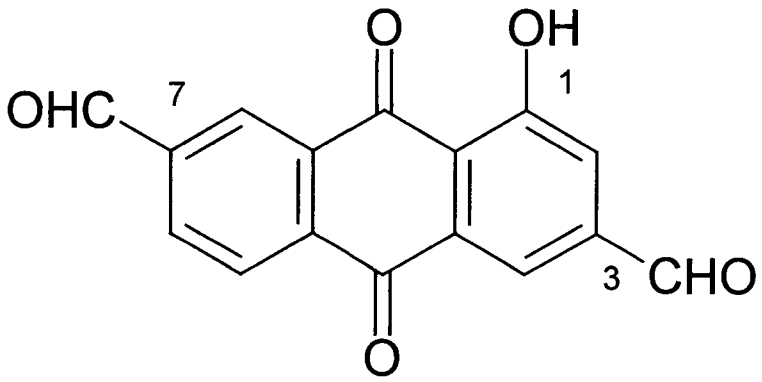
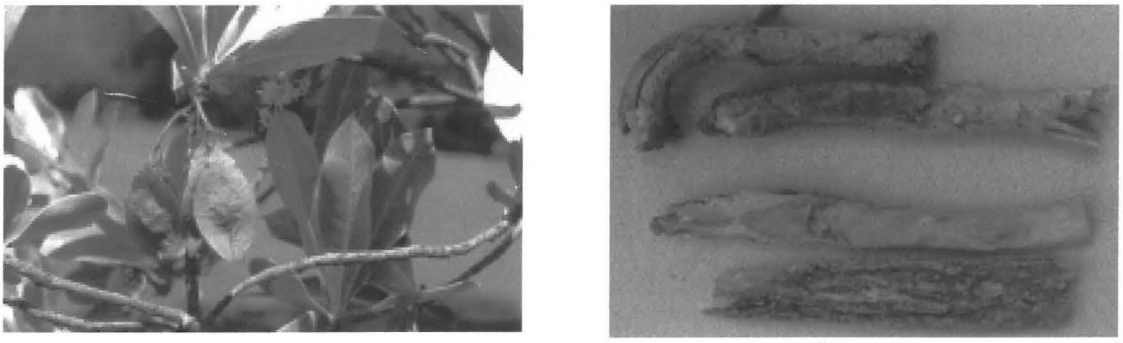


b

Figure 2.3 Anthraquinones isolated from *Senna* species (a) 3,6,8 – trihydroxy -1-methylanthraquinone -2- carboxylic acid, (b) 1-hydroxy-3,7- diformylanthraquinone

2.2.2. *Terminalia sericea* (Burch)

Terminalia sericea belongs to the family Combretaceace. It is a small to medium -sized deciduous tree, with a rounded to flattish crown, its foliage is silvery grey, rarely green; occurring in bushveld. The bark is grey to pale brown. The flowers are small, cream to pale yellow with an unpleasant smell. The fruits are about 30 mm long (Fig. 2.4), with two broad papery wings (15-45 mm) surrounding the thickened central part (Van Wyk & Gericke, 2000).



a b

Figure 2.4 *Terminalia sericea* (a) aerial part (b) roots

2.2.2.1. Distribution

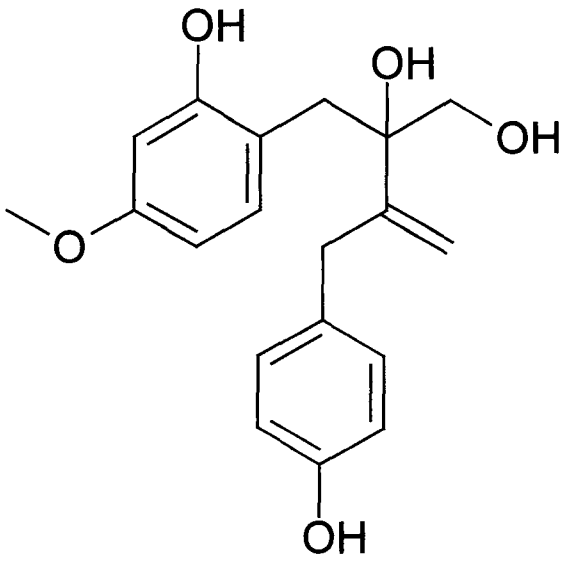
The tree is characteristic of sandy savanna areas in the northern parts of South Africa and is widely distributed in other countries of Africa such as Namibia, Zimbabwe, Botswana and Angola (Neuwinger, 1994).

2.2.2.2. Medicinal uses

The root decoctions are used by Tswana people of South Africa as a remedy for stomach disorders and diarrhoea. In Tanzania *T. sericea* have medical applications against various bacterial infections, such as gonorrhoea and syphilis and against symptoms like diarrhoea, hypertension and even cancer (Fyhrquist, 2002).

2.2.2.3. Chemistry

Terminalia species are known to produce tannins and several pentacyclic triterpenoids have been isolated (Fig. 2.5). A glucoside, nerifolin, has also been isolated from various parts of *T. sericea* and this has been found to have an effect on the heart and pulse rate (Van Wyk *et al.*, 1997).



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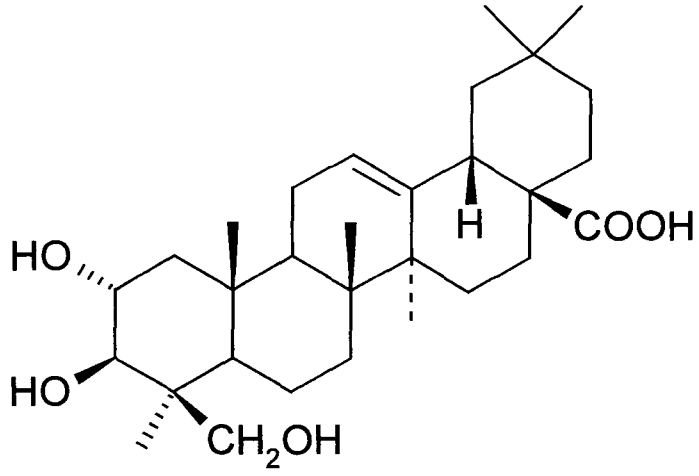
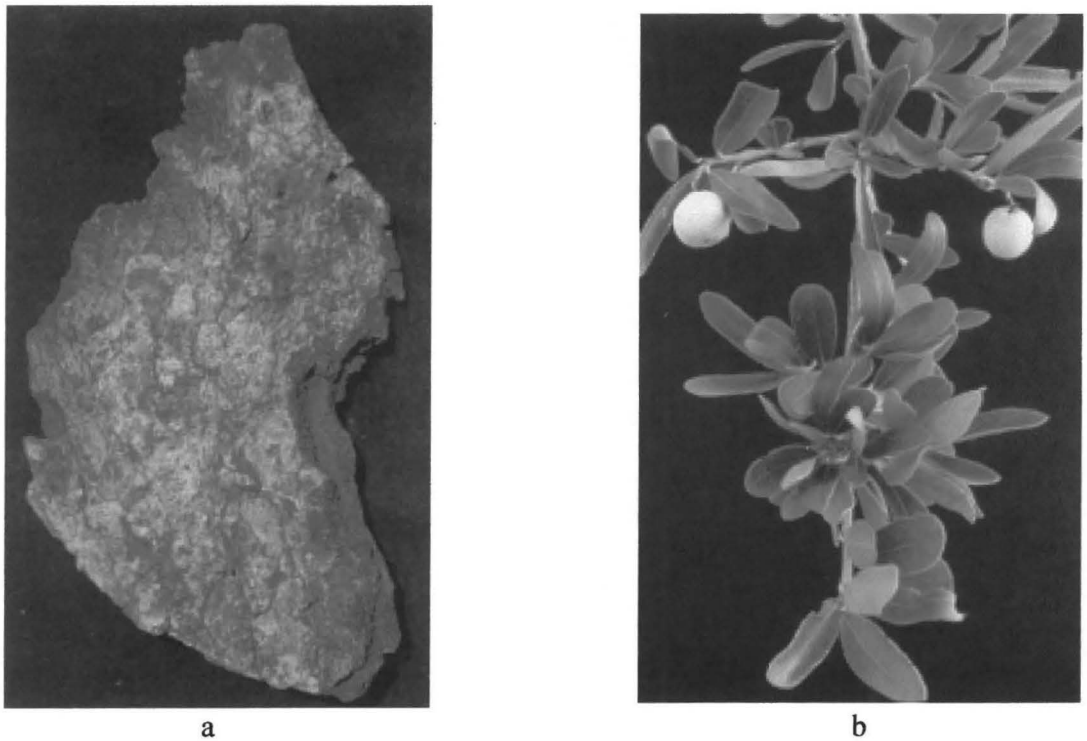


b

Figure 2.5 Compounds isolated from *Terminalia sericea* (a) thannilignan, (b) sericic acid

2.2.3. *Cassine transvaalenis* (Burtt Davy) Codd

Cassine transvaalenis belongs to the family Celastraceae. It is a shrub or small tree (about 5 m high) occurring in forest and quite often on rocky outcrops in mountainous regions. The bark is generally smooth and has a grey colour. Leaves often clustered on reduced lateral shoots, oblong in shape, about 50 mm long and 20 mm wide. The leaf margin is sometimes toothed. The flowers are greenish in colour and produce oblong, yellow to dark orange, berry-like fruits, which are edible (Fig. 2.6).



a b

Figure 2.6 (a) Bark and (b) branches of *Cassine transvaalenis* (Palgrave, 1977).

2.2.3.1 Distribution

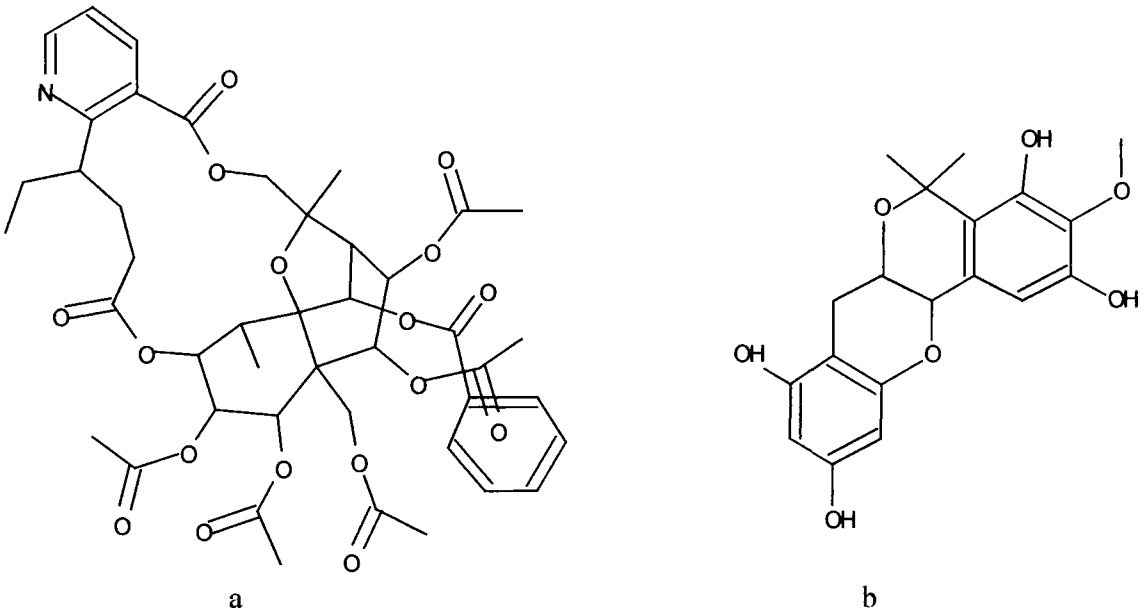
The species is widely distributed in the north-eastern parts of South Africa. It also occurs along the coastal parts of KwaZulu-Natal and in Mpumalanga, Gauteng and the Limpopo province (Mabberley, 1981).

2.2.3.2. Medicinal uses

The bark is extensively used for cleaning the stomach and used as an enema for stomach ache, diarrhoea and fever (Mabberley, 1981).

2.2.3.3. Chemistry

Cassine species are rich in gallotannins and proanthocyanidin and a few other phenolic compounds like alaeocyanidin have been isolated from this species (Fig. 2.7).

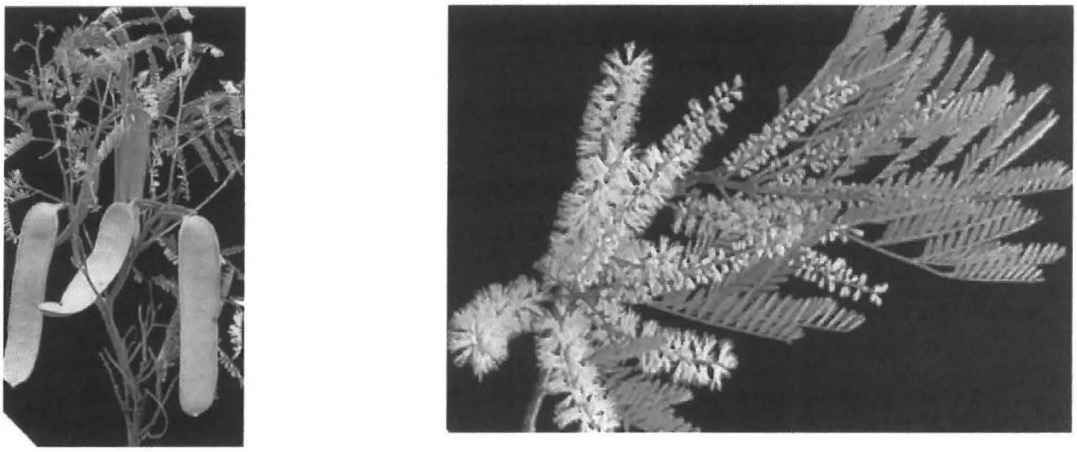


a b

Figure 2.7 Compounds isolated from *C. transvaalenis* (a) cassinine (b) elaeocyanidin

2.2.4. *Elephantorrhiza burkei* (Benth)

Elephantorrhiza burkei belongs to the family Fabaceae and is a multistemmed deciduous shrub or small tree with feathery foliage (Fig. 2.8). The leaves are blue-green, with 4-8 pairs of pinnae. Flowers are pale yellow, becoming dark with age. Pods are flat, up to 19 x 2.5 to 4 cm, dark brown to reddish brown and splitting in a characteristic manner (Van Wyk & Van Wyk, 1997).



a b

Figure 2.8 (a) Fruits and (b) flowers of *Elephantorrhiza burkei* (Van Wyk *et al*, 1997)

2.2.4.1. Distribution

E. burkei is widely distributed in central Africa and also occurs in southern Africa.

2.2.4.2. Medicinal uses

Various parts of this tree is used in African medicine. The seeds are used to relieve severe cases of abdominal pain and as a remedy for toothache. The smoke of a twig is inhaled to cure headaches (Palgrave, 1977).

2.2.4.3. Chemistry

Plants that belong to this genus usually contain saponins (Fig. 2.9). There is not much published information about the compounds isolated from this plant.

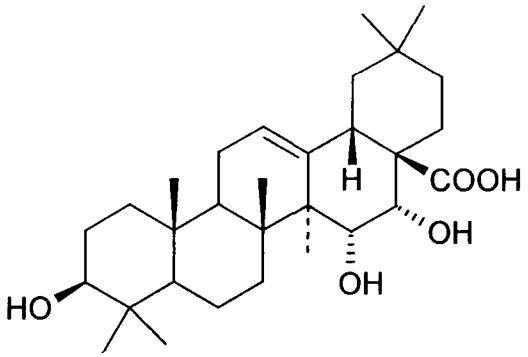


Figure 2.9 Entagenic acid from *Elephantorrhiza burkei*

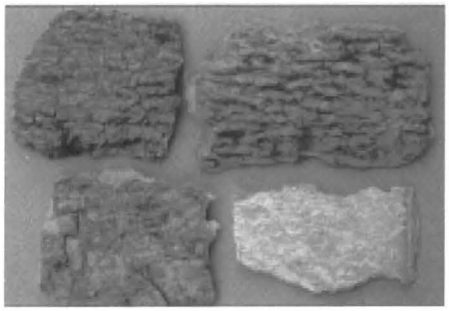
2.2.5. *Rauvolfia caffra* (Sonder)

Rauvolfia caffra is also called the quinone tree and belongs to the family Apocynaceae. It can reach a height of 20 m. Mature trees have a spreading crown with pale yellowish-brown to grey bark, which is soft and cocky (Fig. 2.10).

The leaves are oblong in shape and occur in groups of three to five on the branches. The flowers are white, small and produce egg-shaped berries, which are bright green (Palgrave, 1977).

a

b



c

Figure 2.10 *Rauvolfia caffra* (a) tree, (b) fruit and (c) bark (Van Wyk &Van Wyk, 1997)

2.2.5.1. Distribution

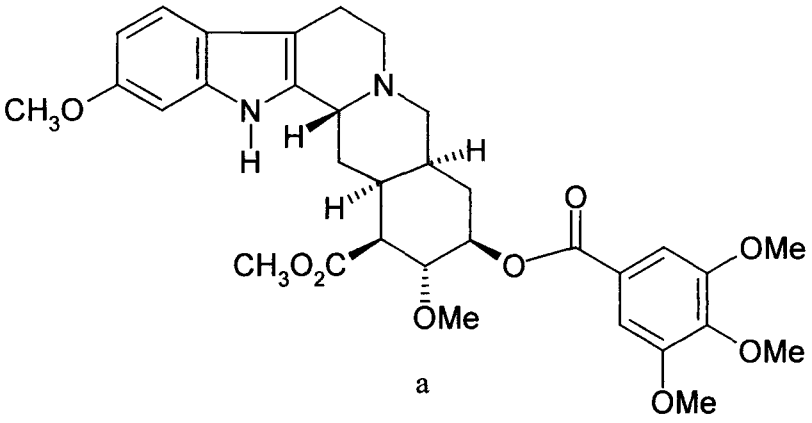
It occurs in the eastern parts of South Africa, and further north into east Africa (Webb *et al.*, 1988).

2.2.5.2. Medicinal uses

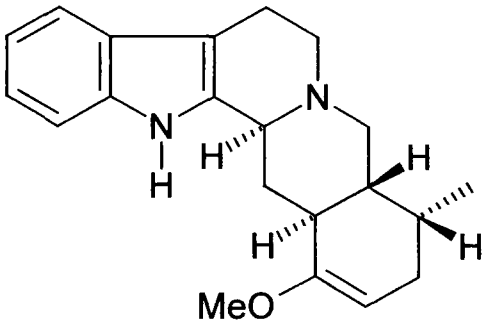
The bark is known to treat malaria, fevers, insomnia and hysteria. Some Africans use the latex as a remedy for infant diarrhea and many other ailments such as skin disorders (Van Wyk *et al.*, 1997).

2.2.5.3. Chemistry

R. caffra is rich in alkaloids (Fig. 2.11) and the bark is known to contain reserpine.



a



b

Figure 2.11 Compounds isolated from *R. caffra* (a) reserpine (b) ajmalicine

2.2.6. *Anredera cordifolia* (Ten)

Anredera cordifolia belongs to the family Basellaceae, is native of South America and naturalized in South Africa (Mabberley, 1981). It is a creeper with fleshy, subsessile leaves and fragrant white flowers. The plant produces tubers both underground on the roots and on the nodes of stems. It is known to be invasive because of its aerial tubers, which can reproduce.

a

b

Figure 2.12 *Anredera cordifolia* (a) leaves and flowers (b) tubers

2.2.6.1. Distribution

A. cordifolia occurs in South America and it has been naturalized in many countries around the world.

2.2.6.2. Medicinal uses

It is used to treat gonorrhea and syphilis by the Venda people of the Limpopo province, South Africa. The tubercles of *A. leptostachys* are used in Cuba for the treatment of the pain and inflammation (Tornos *et al.*, 1999).

2.2.6.3. Chemistry

In preliminary phytochemical tests carried out by Weniger *et al.*, 1986 on different extracts from *A. leptostachys* , steroids, terpenoids, phenols and saponins were found.

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Chapter 3

Antibacterial activity of medicinal plants used in the treatment of sexually transmitted diseases

Short communication*

Antibacterial activity of medicinal plants used in the treatment of sexually transmitted diseases

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Abstract

Water and chloroform extracts of six ethnobotanically selected medicinal plants used traditionally to treat sexual transmitted diseases (STD's) were investigated for their antibacterial activity. Plants used in this study were *Anredera cordifolia, Cassia petersiana, Cassine transvaalensis, Elephantorrhiza burkei, Terminalia sericea,* and *Rauvolfia caffra.* The agar dilution method was used to determine the minimum inhibitory concentration (MIC) of the extracts against four Gram-positive and six Gram-negative bacteria species. The majority of the antibacterial activity was present in the water extracts rather than the chloroform extracts. The Gram-positive bacteria appeared to be more susceptible to the inhibitory effect of the extracts than the Gram-negative ones.

Key words: **Traditional medicine; herbalists; antibacterial; sexual transmitted diseases**

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3.1. Introduction

Traditional medicine usually lacks scientific proof of efficacy, which does not necessarily mean the medicines used are not valuable, but only that more scientific work is needed to investigate its validity (Mabogo, 1990). Low costs and accessibility are not the only advantages of traditional healers and herbalists; there is also a strong belief in the efficacy of traditional medicine. Plants have long provided mankind with herbal remedies for many infectious diseases and even today, they continue to play a major role in primary health as therapeutic remedies in developing countries (Sokmen *et al.*, 1999).

It is known that traditional healers are using indigenous medicinal plants to treat many illnesses including sexually transmitted diseases (STD's) (Ndubani, 1997). In many African countries STD's are a major public health problem and there is a high prevalence rate of diseases, such as gonorrhea and syphilis. Most cases are never reported to the official medical health authorities, but instead are presented to traditional healers (Green, 1992). Both traditional healers and their patients seem to believe that STD's are treated ineffectively by Western medicine (Berger, 1995 and Gadhi, 1999). Even some of those who go to a hospital may come back to the traditional healers to "cleanse" the system after treatment by Western medicine (Moss *et al*, 1999).

Plant–derived medicine have been part of traditional health care around the world for thousands of years, and there is an increasing interest in plants as sources to fight microbial diseases (Palombo, 2001). Plants contains numerous biologically active compounds, many of which have been shown to have antimicrobial activity (Lopez *et al*, 2001, Karaman, 2001). The search for biologically active extracts based on traditionally used plants is still relevent due to the appearence of microbial resistance of many antibiotics and the occurrence of fatal opportunistic infections. Ethnobatanical data have proved to be useful in the search for new antimicrobial agents and many of these compound have been isolated from medicinal plants (Penna *et al.*, 2001).

The purpose of this study was to investigate the antibacterial activity of medicinal plants used to treat STD's. The selection of plants for evaluation was based on ethnobotanical data. At present very little is known about the activity of South African plant species that are used in the treatment of STD's (Ndubani, 1999).

3.2. Materials and methods

3.1.2. Plant material

The roots of six ethnobotanically selected plants used traditionally for the treatment of STD's were collected from Venda in the Limpopo Province (South Africa). The following plants were used: *Anredera cordifolia, Senna petersiana, Elephantorrhiza burkei, Terminalia sericea,* and *Rauvolfia caffra*. Traditional healers and a review of the available literature assisted in gathering information about these medicinal plants. Voucher specimens (E.T 33, 34, 35, 36, 37 and 38) were prepared and identified at the H.G.W.J. Schweikerdt Herbarium, University of Pretoria.

3.2.2. Preparation of extracts

Chloroform extracts were prepared by stirring the powdered roots (30 g) in 300 ml chloroform for 2 hours. The extracts were filtered and dried with a rotary vacuum evaporation apparatus at reduced pressure. The residue was later dissolved in 1% DMSO to a concentration of 100 mg/ml.

The water extracts were obtained by boiling 20 g of powdered root under reflux in 400 ml distilled water for 3 to 4 hours. The extracts were filtered and concentrated to dryness under reduced pressure. The residue was later dissolved in 1% DMSO to a concentration of 100 mg/ml. The water extracts of *A.cordifolia* could not be filtered due to its viscous nature, and was centrifuged for ten minutes at 10000 rpm and then concentrated to dryness.

3.2.3. Bacteria

Ten bacteria were obtained from the Department of Microbiology and Plant Pathology, University of Pretoria and tested for susceptibility in this study. The bacteria were maintained as described by Meyer & Dilika (1996) and Lall & Meyer (2000).

3.2.4. Antibacterial assays

Four Gram-positive and six Gram-negative bacteria (Table 1) were tested against extracts of the above-mentioned plants. Each plant was tested at three different concentrations, 1, 20 and 50 mg/ml by determining their MIC's in the standard petri dish *in vitro* bioassays. MIC values were taken as the lowest concentration of extract that completely inhibited bacterial growth after 24 hours of incubation at 37 ^{o} C. Water extracts were sterilized before the bioassay by filtering through 0.22 µm syringe fitted filters. The extracts were added to the autoclaved culture media to a total volume of 20 ml, shaken and more or less similar amounts were poured into three sterile petri dishes and swirled until the agar set. The bacteria were then streaked in radial patterns on the agar plates (see Fig 3.1). Each treatment was replicated three times, and as solvent controls, the culture medium with 1% DMSO was added into the petri dishes. All the petri dishes were then incubated at 37^{o}C and observed after 24 hours (Mitscher *et al*, 1972).

Figure 3.1 Agar dilution method

3.3. Results and discussion

The water extracts of six ethnobotanically selected plants exhibited a broad antibacterial activity (Table 3.1). The chloroform extract of only *A. cordifolia* inhibited the growth of bacteria. Both water and chloroform extracts of *A. cordifolia* inhibited ninety percent of the bacteria. The Gram-positive bacteria appeared to be more susceptible to the inhibitory effect of the extracts (water and chloroform) than Gram-negative ones. The negative results obtained against Gram-negative bacteria were not surprising as in general, these bacteria are more resistant than Gram-positive ones (Rabe, 1997). The greater resistance of Gram-negetive bacteria to plant extracts has been documented previously. Previous studies have suggested that the differences in cell wall structure between Gram-positive and Gram-negetive bacteria might be the reason. The Gram-negetive bacteria have an outer membrane acting as a barrier to many environmental substances, including antibiotics (Palombo & Semple, 2001).

The water extracts of five of the six plants tested, showed activity against *B. pumilus*, *B. subtilis* and *Staphylococcus aureus* respectively. Water extracts from *S. petersiana* showed a significant antibacterial activity by inhibiting the growth of all the Gram-positive and two Gram-negative bacteria at a relatively low MIC. The water extract of the roots of *T. sericea* had a low MIC value (1mg/ml) against *Bacillus pumilus*. The reasons for the moderate MIC-values of the other extracts could be that the extracts are mixtures of a large number of compounds, and they might supress the biological activities of each other, or that the active compound (s) is present in very low concentrations (Fyhrquist *et al*, 2002)

In general, the plant species studied proved to have some antibacterial activity. This also reinforces the use of these plants traditionally for treatment of various diseases including STD's (Shale, 1999). Further studies are in progress to do antibacterial assays with specific bacteria that cause STD's and also to investigate the antiviral activity of these plants.

Table 3.1
Antibacterial activity (MIC[a] mg/ml) of ethnobotanically selected South African medicinal plants used in the treatment of sexually transmitted diseases.

Bacteria species	Gram + / –	Plant species											
		S. petersiana		*T. sericea*		*A. cordifolia*		*C. transvaalensis*		*E. burkei*		*R. caffra*	
		H_2O[b]	$CHCl_3$[c]	H_2O	$CHCl_3$	H_2O	$CHCl_3$	H_2O	$CHCl_3$	H_2O	$CHCl_3$	H_2O	$CHCl_3$
Baccilus cereus	+	20	na[d]	20	na	na	na	50	na	na	na	na	na
B. pumilus	+	20	na	1	na	50	60	50	na	60	na	na	na
B. subtilis	+	20	na	20	na	60	60	20	na	60	na	na	na
Staphylococcus aureus	+	20	na	20	na	60	60	20	na	60	na	na	na
Enterobacter cloacae	–	20	na	na	na	50	50	na	na	na	na	50	na
Escherichia coli	–	na	na	na	na	60	60	na	na	na	na	na	na
Klebsiella pneumonia	–	na	na	na	na	60	60	na	na	na	na	na	na
Pseudomonas aeruginosa	–	na	na	na	na	60	60	na	na	na	na	na	na
Serratia marcescens	–	20	na	na	na	60	60	na	na	na	na	na	na
Enterobacter aerogenes	–	na	na	na	na	60	60	na	na	na	na	na	na

[a] MIC: Minimum inhibitory concentration

[b] H_2O : water extract

[c] $CHCl_3$: chloroform extract

[d] na: not active

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Chapter 4

Cytotoxicity and antiviral activity of *Senna petersiana*, *Anredera cordifolia* and *Terminalia sericea*

4.1. Introduction

Ethnopharmacological screenings of medicinal plants around the world has led to the selection of several extracts active against herpes virus (HSV-1, Fig. 4.1). Some of the compounds isolated were found to have an inhibitory activity against the replication of HSV viruses, (Lall, 2001).

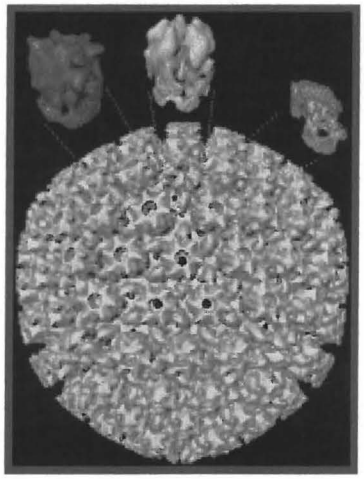

Figure 4.1. Herpes simplex virus type 1 (Zhou *et al.*, 1994)

Cytotoxicity testing on cell cultures has been a controversial issue, because the metabolism of foreign compounds (toxication/detoxication) differs from cell to cell, e.g. pH toxicity testing, especially when animal cell cultures are used, may not be a reliable tool to evaluate toxicological risks for humans (Schramm & Teichmann, 1979). However some researchers have showed comparative effects of *in vitro* and *in vivo* cytotoxicity

assays. Studies on the cytotoxicity of plant extracts are useful in order to evaluate the toxicological risks. Some plants extracts might be very toxic as they contain many different compounds; therefore it is necessary to evaluate cytotoxicity of both crude extracts and isolated compounds. In a study done by Loy *et al.* (2001) it was found that the basic extract had strong cytotoxicity, whereas the acid extract showed lower cytotoxicity. Furthermore, this fraction showed good antibacterial activity.

It is necessary to obtain more scientific information concerning the efficacy and safety of the remedies in use, because many people in third world countries still depend on herbal remedies for the treatment of various ailments (Prozesky *et al.*, 2001). In this study, we examined the cytotoxicity and antiviral effect of plants that are used in the treatment of sexually transmitted diseases. The extracts were tested against HSV-1 on primary vervet monkey kidney (VK) cells.

4.2 Materials and methods

4.2.1. Plant material

Water extracts of *S. petersiana*, *A. cordifolia* and *T. sericea* were prepared as described in Chapter 3 (section 3.2.2.). The residues were dissolved in 1% DMSO to make a final concentration of 100 mg/ml which were diluted with Eagle's minimum essential medium (MEM) (Highveld biological (Pty) Ltd., Kelvin, South Africa), to obtain the desired concentrations.

4.2.2. Cell cultures

The standard cell culturing techniques as described by Grist *et al.* (1979) were used to evaluate the toxicity of plant extracts against vervet monkey kidney (VK) cells. The monolayers of VK cells were prepared by seeding 96-well microtitre trays with 10^5 cells/ml. The cells were propagated in Eagle's MEM, supplemented with 10% heat inactivated (56° C for 30 minutes) fetal calf serum (FCS) (Delta Bioproducts, Kempton Park, South Africa) containing 100 µg/ml penicillin and 100 µg/ml streptomycin. Cell cultures were incubated in a humified CO_2 atmosphere (4 % CO_2 / 96 % filtered air) at 37° C.

Multi-layered cells formed in the tissue culture plates which adhered to the bottom surface. In order to loosen the cells, the plates were rinsed three times with 10% phosphate buffer saline (PBS), and then 3 ml of 0.1 % trypsin EDTA was added onto them. Plates were incubated at 37^0 C for 5 minutes. Fresh maintenance medium (MM) was added to the tissue culture plates and the content was transferred to a test tube, which was then centrifuged for 5 minutes at 300 rpm. The MM differed to the propagation medium (MEM), only because it contained 1-2 % FCS. After centrifugation the cells settled at the bottom of the test tube and the supernatant was discarded. The cells were then mixed with fresh MEM. 100 µl of the freshly mixed cells in MEM was transferred to microtitre plates and incubated at 37^0 C for 24 hours for the formation of monolayers cells (Meyer *et al.*, 1996).

4.2.3 Cytotoxicity assay

Monolayers of VK cells were prepared separately in 96-well microtitre trays by seeding each with 200 µl of 10^5 cells/ml in 10% MEM. Doubling dilutions of all the extracts (*A. cordifolia*, *S. pertesiana* and *T. sericea*) from the concentration of 3.125 mg/ml to 0.008 mg/ml were prepared in MM. The extracts were tested for cytotoxicity by exposing the monolayer of the cell cultures to 200 µl of dilutions of extracts at 37^0 C. Monolayers of cells exposed to MM, without the addition of the extracts were used as controls. The cells were incubated for a period of seven days at 30^0 C and were monitored daily.

The morphology of the cells was inspected and observed for microscopically detectable alteration, i.e. loss of monolayer, rounding, shrinking of cells, granulations, and vacuolization in the cytoplasm. Results were expressed as the dose that inhibits 50% cell growth after the incubation period (ID_{50}).

4.2.4. Virus stock

Stock suspensions of clinical isolates of HSV-1 were prepared in the appropriate cell cultures. The viral titre of each suspension was determined before dilution in MM for use at a final concentration of 100 TCID 50/ml (50 % tissue culture infectious doses/ml).

4.2.5. Antiviral assay

A. cordifolia extract was tested for antiviral activity at a concentration of 1.560 mg/ml whereas both *T. sericea* and *S. petersiana* were tested at 0.024 mg/ml since these are the non toxic concentrations of the extracts. The final concentration of the virus in the assay was 100 TCID 50/ml. 200 µl of the different dilutions of extract solution and 200 µl of the viral suspension, both diluted in MM, were added simultaneously to monolayers of each cell culture in microtitre wells. As positive controls, cells were infected with the same concentration of virus but without the addition of plant extracts, and as negative control; only MM was added to the cells. Cells were examined daily for a period of a week, by light microscopy, for the appearance of a cytopathic effect (CPE). The absence of CPE at a specific concentration of extract at the same time that the corresponding positive control showed CPE was considered to be indicative of antiviral activity.

4.2.5.1 The 3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide (MTT) staining method

In the MTT staining method described by Mossman (1983), the tetrazolium ring is cleaved and it is reduced to blue formazan, which is then solubilised, by isopropanol. This result in a colour change, the intensity of which reflects the enzymatic activity the living cells (Hussain *et a.l.*, 1993). A description of the method follows:

- 100 mg of MTT powder was dissolved completely in 200ml of phosphate buffer saline (PBS). The solution was filtered and sterilized and kept in the dark until used, as MTT is light sensitive.
- MTT stain was added according to the volume in the microtitre plate wells, 20 µl of MTT mixture was added to each microtitre well and incubated for 4 hours at 37^0C.
- The microtitre plates were centrifuged at 2000 rpm for 10 minutes.
- Supernatant was carefully removed.
- 150 µl of PBS was added to each microtitre well.
- The plates were centrifuged for the second time at 2000 rpm for 10 minutes and the supernatant was removed.
- 100 µl of 0.2 % of DMSO were added to all wells and the plates were shaken for 1h.
- The absorbance was read by a spectrometer at wavelength of 540 nm using a reference wavelength of 620 nm.

4.3. Results and discussion

The concentrations of the plant extracts at which 75 % of the vervet monkey kidney (VK) cells were alive until the seventh day was considered as the highest concentration which is non toxic to the cell (ID_{50}). Table 4.1 contains the (ID_{50}) values of the extracts tested. The non-toxic concentration of the *Anredera cordifolia* extract tested against VK cell culture was found at 1.560 mg/ml. Both *Senna peteriana* and *Terminalia sericea* extracts showed a significant toxicity against VK cells exhibiting ID_{50} values of 0.024 mg/ml.

The cytotoxicity as determined in this study on the extracts, is not a clear indication of toxicity of the active compounds (antimicrobial). It is therefore possible that the active compounds in these plants might have less cytotoxicity. The active compounds will probably have a lower dose, and further investigation is needed on this aspect.

In the assay to assess the possible antiviral properties of the extract, *T. sericea* (Table 4.4) and *A. cordifolia* (Table 4.2) extracts were found not to inhibit HSV-1. Only *S. petersiana* extract showed some antiviral activity at the concentration of 0.024 mg/ml, which is the highest non toxic concentration (Table 4.3). There was only 20 % reduction of the virus after the sixth day and 10 % reduction after the seventh day of the experiment. Antiviral activity is expressed as the reduction of virus infectivity of the tissue by 50 % (50 % culture infective dose per ml or TCID 50/ml) (Sindambiwe *et al*, 1999). The reason for this low activity might be that the active compound is in a very low concentration present in the extract or that it is toxic, since we could not test the antiviral activity at the concentration above 0.024 mg/ml. The isolated active compound will probably increase the activity against HSV-1.

Table 4.1 ID_{50}[a] (mg/ml) values of three plant extracts on monkey kidney cells

Plants tested for cytotoxicity	ID_{50} (mg/ml)
Anredera cordifolia	1.560
Senna petersiana	0.024
Terminalia sericea	0.024

[a] Dose that inhibits 50% of cell growth after the incubation period

Table 4.2 Dose response pattern of herpes simplex virus type-1 on VK cells to *Anredera cordifolia* extract

Concentration of *A. cordifolia* (mg/ml)	Percentage cytopathic effect post infection						
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Positive control	25	80	90	90	95	100	100
Negative control	0	0	0	0	0	0	0
1.56	25	90	90	90	100	100	100
0.78	25	80	90	90	100	100	100
0.39	25	80	90	90	100	100	100
0.195	25	80	90	90	100	100	100
0.098	25	80	90	90	100	100	100
0.049	25	80	90	90	100	100	100

Table 4.3 Dose response pattern of herpes simplex virus type-1 on VK cells to *Senna petersiana* extract

Concentration of *S. petersiana* (mg/ml)	Percentage cytopathic effect post infection						
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Positive control	50	80	90	90	100	100	100
Negative control	0	0	0	0	0	0	0
0.024	50	50	75	80	80	80	90
0.0122	50	80	90	90	100	100	100
0.0061	50	80	90	90	100	100	100
0.003	50	80	90	90	100	100	100
0.0015	50	80	90	90	100	100	100
0.0008	50	80	90	90	100	100	100

Table 4.4 Dose response pattern of herpes simplex virus type-1 on VK cells to *Terminalia sericea* extract

Concentration of *T. sericea* (mg/ml)	Percentage cytopathic effect post infection						
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Positive control	25	75	90	90	100	100	100
Negative control	0	0	0	0	0	0	0
0.024	0	50	80	90	100	100	100
0.0122	10	75	90	90	100	100	100
0.0061	15	75	90	90	100	100	100
0.003	25	75	90	90	100	100	100
0.0015	25	75	90	90	100	100	100
0.0008	25	75	90	90	100	100	100

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Chapter 5

Bioassay guided isolation and chemical structure of antibacterial compounds from *Senna petersiana*

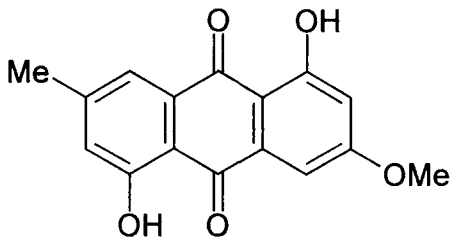
5.1 Introduction

As mentioned before (Chapter 2) the plants belonging to the genus *Senna* (formerly known as *Cassia*) have yielded a large number of polyketide-derived anthraquinones, bianthraquinones and flavonoids in addition to glycosides and many of these compounds are known to possess important medicinal properties. Kazmi *et al.,* (1994) described the isolation of 1,5-dihydroxy-3-methoxy-7-methyl-anthraquinone (Fig. 5.1) and a new anthraquinone from *Senna italica* which had antimicrobial and antitumor activity. From the roots of *S. marginata*, Singh and Singh, (1987) isolated 1,3-dihydroxy-6,8-dimethoxy-2-methyl anthraquinone , 3-O-rhamnosyl- (1→ 6)-glucopyranoside and 1,3,5,8-tetrahydroxy-2-methyl anthraquinone 3-O-glucoside. Bianthraquinones like floribundone and anhydrophlegmic has been previously isolated by Alemayehu & Abegaz, (1996) (Fig. 5.2).

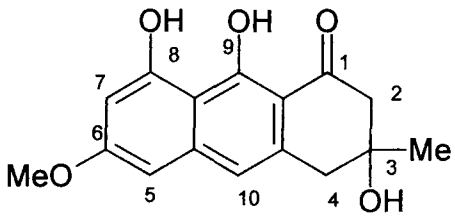
Torsachrysone, physicion, floribundone-1 and a new bianthraquinone, 9-(physcion 7-yl)-5,10-dihydroxy-2-methyl-7-methoxy-1,4-anthraquinone, named isosengulone (Fig. 5.3), are reported to have been isolated from seeds of *S. multiglanduloasa* (Alemayehu & Abegaz 1996) .

Kitanaka and Takido (1992) reported the isolation of two novel flavonoids, demethyltorosaflavones C and D from aerial parts of *S. nomane*. During the investigation of antibacterial activity of plant extracts (Chapter 3, section 3.3) it was found that the roots of *S. petersiana*, exhibited high activity against bacteria.

Preliminary antibacterial tests on TLC plates (direct bioassay method) showed that the leaves, roots and seeds of *S. petersiana* had significant antibacterial activity. It was therefore decided to study the phytochemistry of the roots and seeds of this species. The roots, leaves and seeds of *S. petersiana* have been used in tropical and southern Africa as a remedy for gonorrhoea, syphilis and for skin diseases. Although a literature survey showed the presence of anthraquinones and flavonoids, not many compounds have been isolated from this plant.

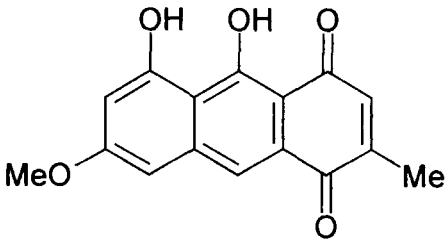


1,5-Dihydroxy-3-methoxy-7-methyl-anthraquinone

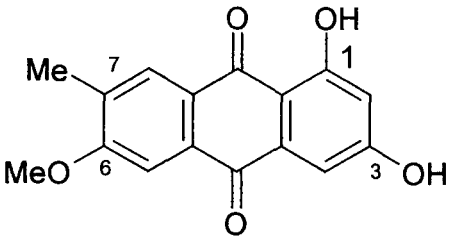


torosachrysone

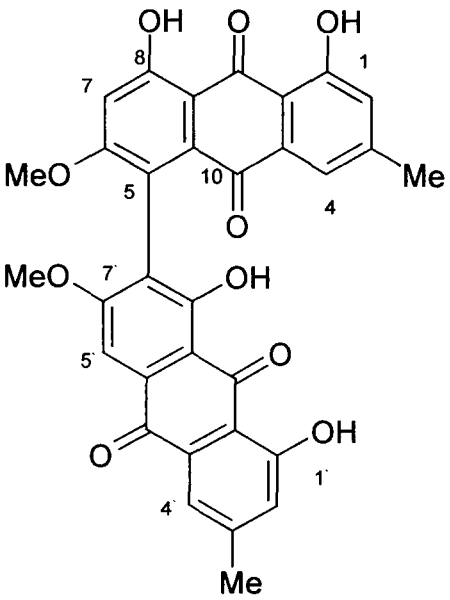
Figure 5.1 An anthraquinone isolated from *Senna italica* and torosachrysone isolated from *S. multiglandulosa*



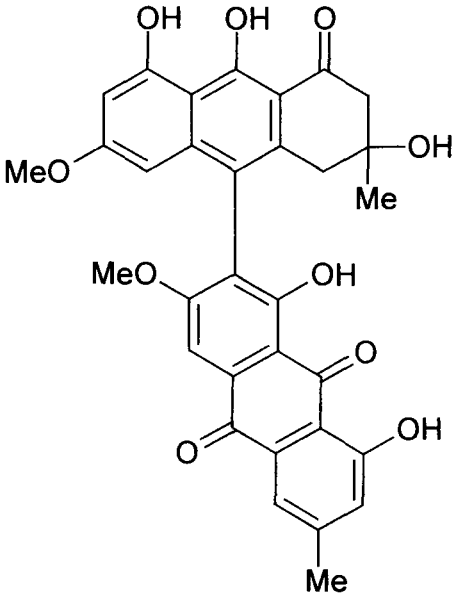
8,9 - dihydroxy - 6 - methoxy - 3 - methylanthraquinone



1,3 - dihydroxy-6 - methoxy - 7 - methylanthraquinone

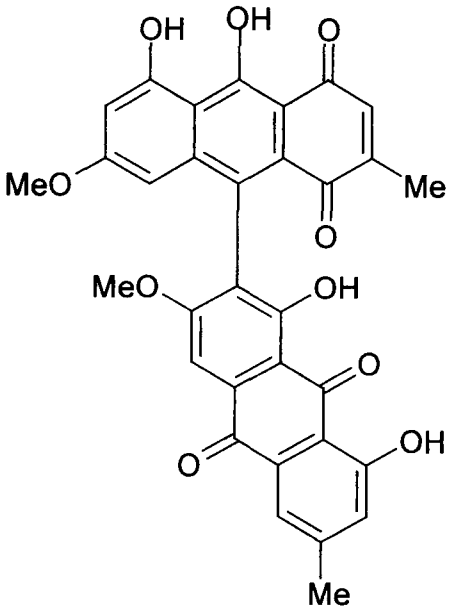


Floribundone

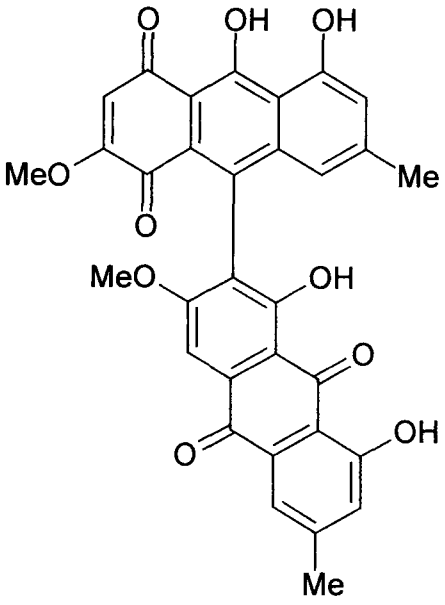


Anhydrophlegmic

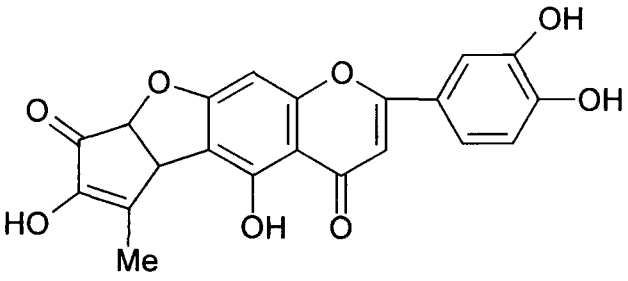
Figure 5.2 Anthraquinones and bianthraquinones from *Senna* species



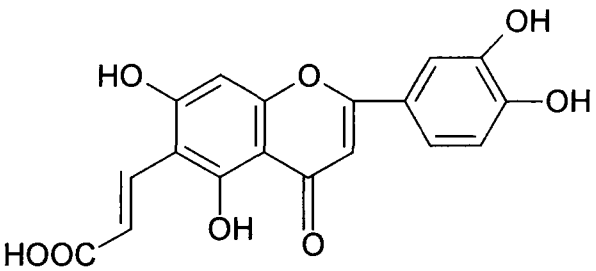
Isosengulone



Sengulone



Demethyltorosaflavone C



Demethyltorosaflavone D

Figure 5.3 Bianthraquinones and flavonoids from *Senna* species.

5.2 Materials and methods

5.2.1 Plant material

S. petersiana roots and seeds were collected along the roads of Thohoyandou in Venda (Limpopo Province). Voucher specimens were prepared and identified at the H.G.W.J. Schweikerdt Herbarium, University of Pretoria.

5.2.2. Compounds isolated from the roots

The dried powered roots of *S. petersiana* (600 g) were homogenized in acetone for seven days. The extract was filtered and dried in a rotary evaporator and about 4 g of crude extract was obtained which was dissolved in 20 ml ethyl acetate. 2 ml of crude extract was injected in a flash chromatography column and eluted with chloroform : methanol, first 9:1 (500 ml), and later it was changed to 4:1 (250 ml). The column was finally washed with pure methanol. 35 fractions were collected and developed on TLC plates in order to identify the fractions with similar composition. They were then combined to yield a total of 8 fractions. The structures of the compounds seemed to be unstable, and it was decided to acetylate them.

5.2.2.1. Acetylation

Fractions 4, 5 and 6 were mixed in a round bottom flask, pyridine and acetyl chloride was dissolved in the flask for acetylation to take place. The solution was then concentrated and dissolved in ethyl acetate. After acetylation the compounds remained unstable and it was decided to methylate them.

5.2.2.2. Methylation

The methylation procedure was done as follows:

- The dried powdered root bark of *S. petersiana* (600 g) was homogenized in acetone for seven days;
- The extracts were filtered and dried in a round bottom flask on a rotavapor;
- 20 grams of potassium hydroxide weighed in 1 litre an Erlenmeyer flask and 200ml of distilled water was added;
- The flask was put inside an ice bucket and stirred until the temperature of the solution became zero;
- Diethyl ether and diazomethane was added to Erlenmeyer flask and stirred until it dissolved;
- The solution was transferred into a separating funnel;
- The bottom colourless layer was discarded and the upper (yellow) transferred into a round bottom flask and kept at room temperature for 48 hours for the reaction to take place;
- The whole procedure was done in a fume hood.

5.2.2.3. Final purification

After methylation the sample was dried under vacuum and then dissolved in ethyl acetate. TLC plates were developed in hexane ethyl acetate (1:1), hexane ethyl acetate (8:2) an in 100 % ethyl acetate. The best result was obtained in hexane : ethyl acetate (8:2).

Six grams of methylated crude extract was subjected to a Sephadex LH 20 column and eluted with methanol. Similar fractions were combined, the active one was resubjected to the Sephadex column and gave two fractions. The active fraction was again resubjected to the Sephadex column eluted with methanol and gave two fractions. One of the resulted fractions was resubjected to Sephadex column chromatography which resulted in 3 fractions. The first fraction was subjected to silica column chromatography and eluted with a gradient of 9:1, 7:2 and 6:4 (hexane: ethyl acetate). Six fractions, that seemed to be pure or almost pure were collected and analysed by NMR.

5.2.3. Compounds isolated from the seeds

Since a pure compound could not be isolated from the roots of *S. petersiana*, we also study the phytochemitry of the seeds, which also showed high antibacterial activity by direct bioassays on TLC plates.

5.2.3.1. Extraction and isolation.

The fresh seeds (1.5 kg) were crushed and soaked in ethanol for 3 days. The extract was filtered and dried in a rotary evaporator. The residue was dissolved in methanolic water and ethyl acetate was then added in a separating flask. After solvent-solvent extraction, the ethyl acetate fraction (200 g) was removed from the methanolic fraction and subjected to silica gel column chromatography. The column was eluted with a gradient of

solvent (hexane : ethyl acetate 7:3, hexane : ethyl acetate 1:1, hexane : ethyl acetate 3:7 and 100 % ethyl acetate). From seven collected fractions, fraction 6 was resubjected to the silica column and eluted with hexane-ethyl acetate (7:3), similar fractions were combined (monitored by TLC) and yielded a pure compound (20 mg).

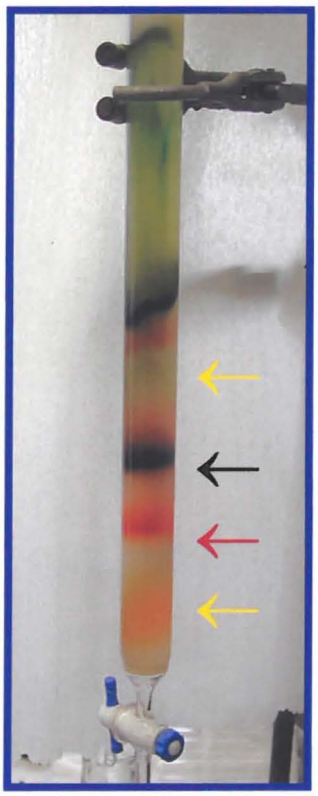


Figure 5.4. Separated bands (arrows) after subjecting extract to silica gel column chromatography

5.3. Results and discussion

The results of the extensive chromatographic and NMR investigation showed the instability of the compound mixtures of the *Senna petersiana* roots. As a result we could not isolate any of the constituents of the roots (Fig. 5.5) because the compounds degraded on silica gel during the chromatographic process. Acetylation and methylation of the crude extract were also tried and gave the same results. All the spectral data indicated compounds with a basic anthraquinone structure (Figs. 5.6, 5.7 and 5.8).

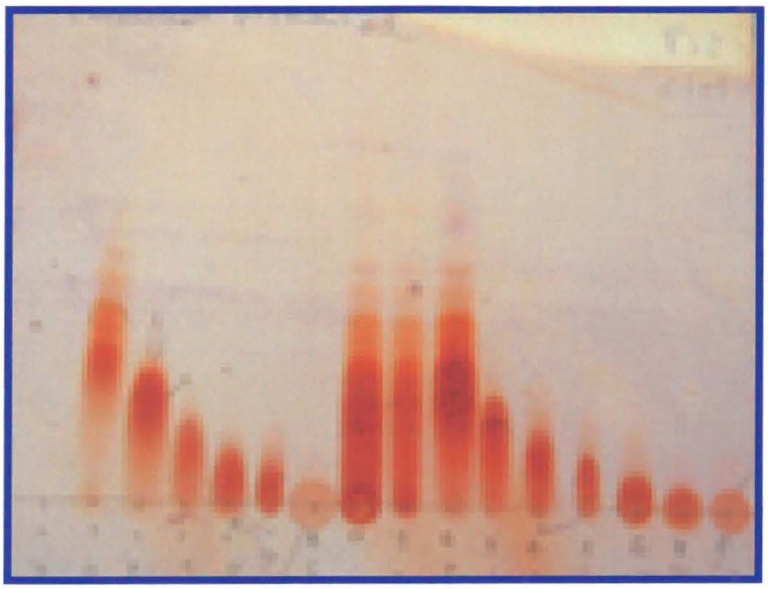
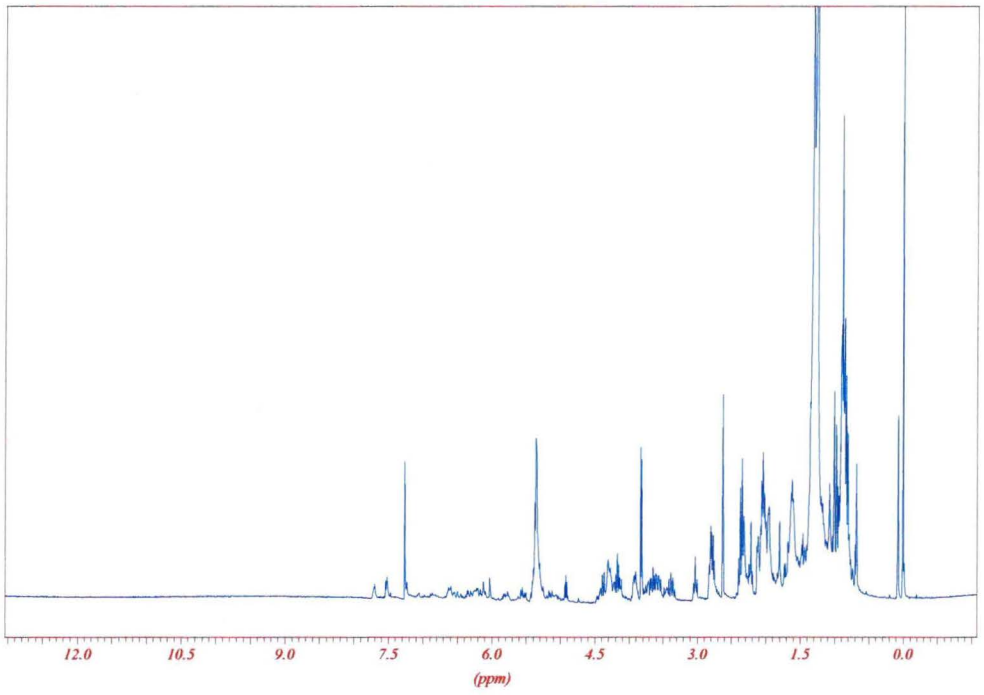
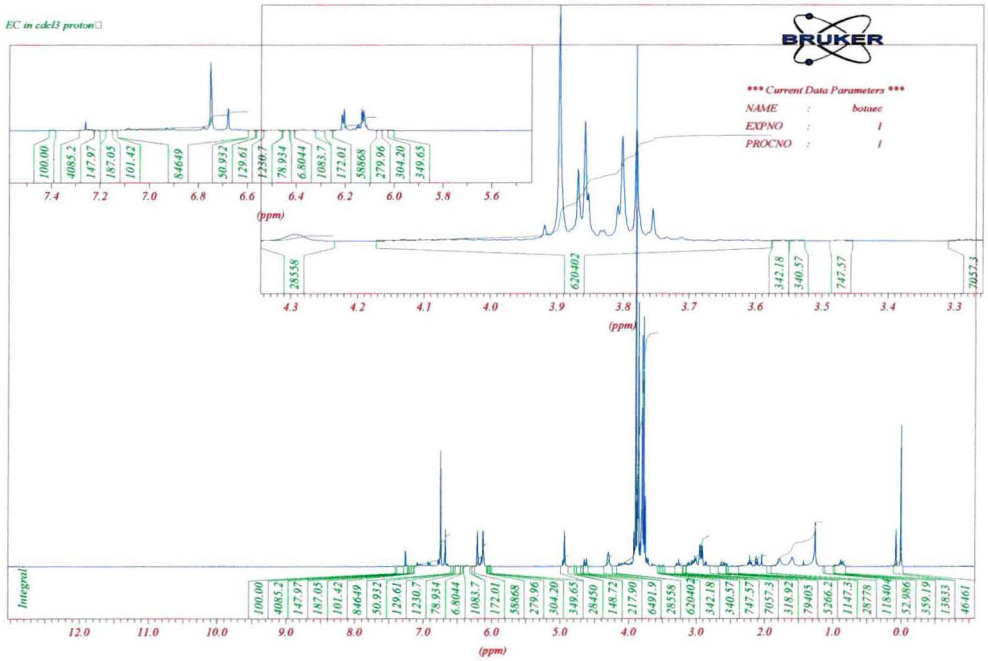


Figure 5.5. TLC plate of fractions obtained after subjecting the crude extract to a Sephadex column.

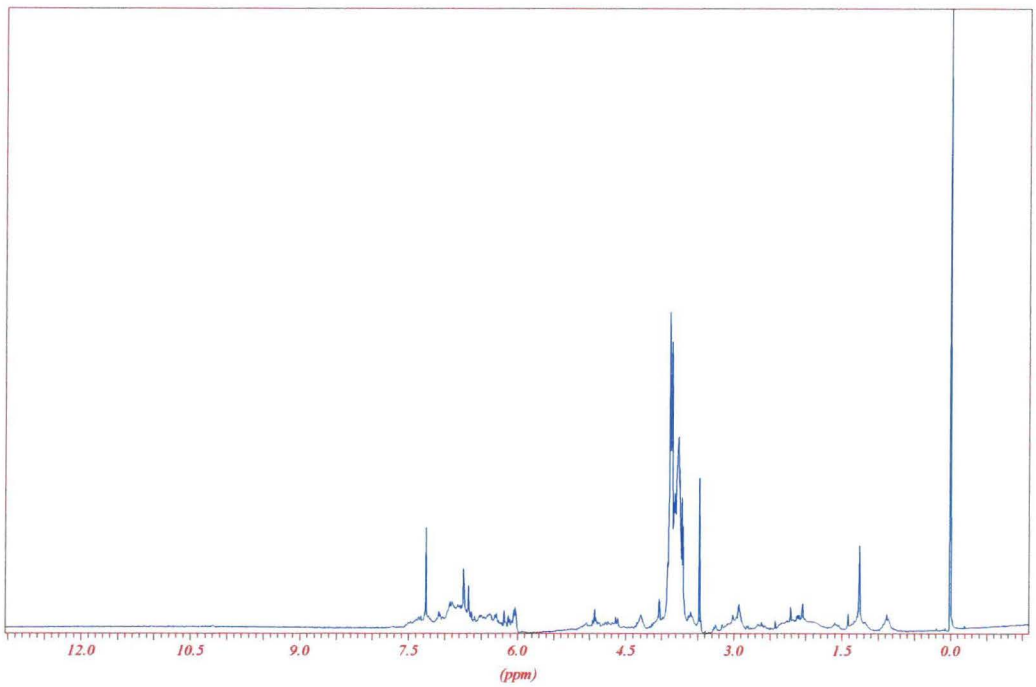


a

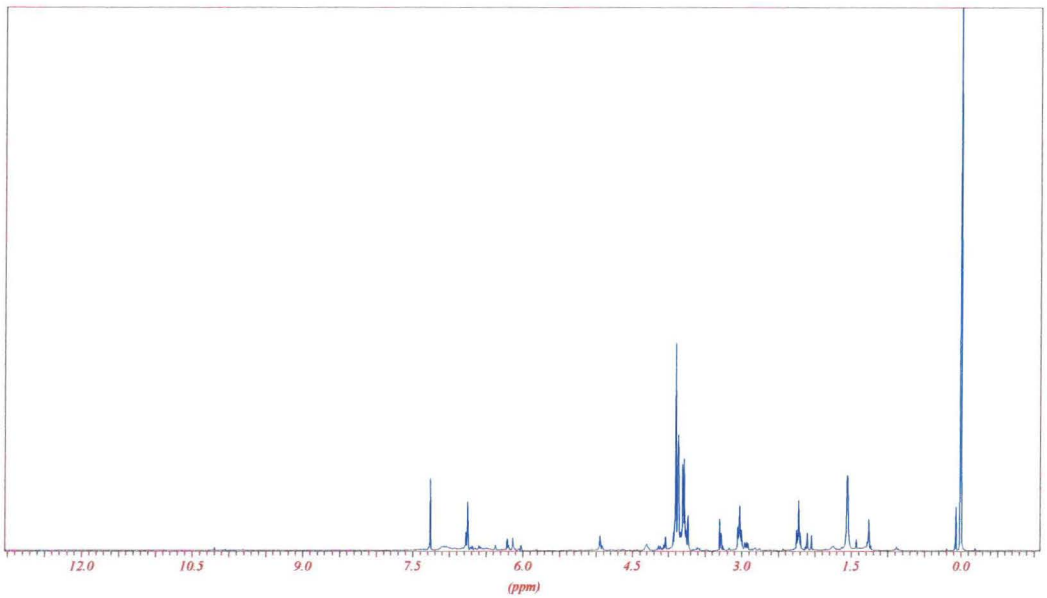


b

Figure 5.6. Two ^{1}H NMR spectrum of Sephadex fractions (a and b)



a



b

Figure 5.7. Two ^{1}H NMR spectrum of Sephadex acetylated (a) and methylated (b) fractions.

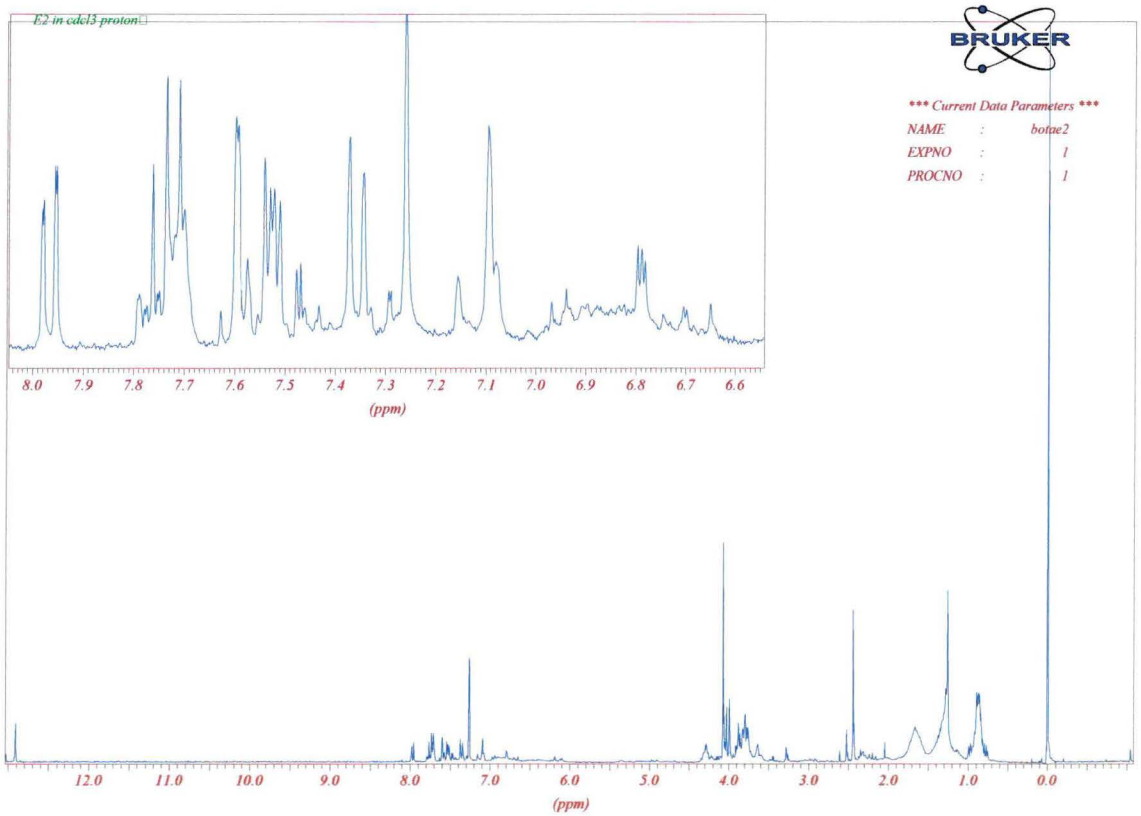


Figure 5.8 ^{1}H NMR spectrum of a Sephadex methylated fraction.

The phytochemical studies of the seeds of *S. petersiana* resulted in the isolation of a pure compound, which proved to be a flavonoid. The identification of the isolated compound (Fig. 5.9) was done by different physicochemical methods, e.g. mp, UV, MS, NMR including ^{1}H, ^{13}C, HMQC and HMBC .

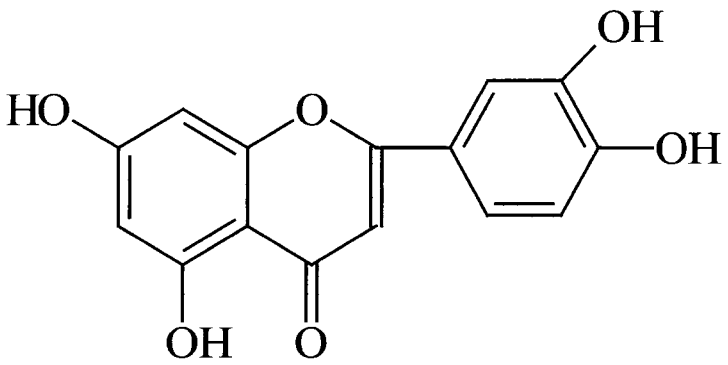
NMR spectra of the compound showed two doublets (Fig. 5.10) with *meta* coupling at 6.18, (^{1}H, J=2.0 Hz) and 6.39 (^{1}H, *d*, J=2.0 Hz) characteristics of H-6 and H-8 respectively of ring A of flavonoids, signals at *d* 6.85 (^{1}H, *d*, J=8.4Hz), 7.49 (^{1}H, *d*, J=2.2Hz) and 7.70 (^{1}H, *dd*, J=2.2, 8.4 Hz), were characteristics of a 3,4-disubstituted B ring, a singlet at δ 4.12 (^{1}H, *s*, J=2.2 Hz) assigned to the H-3 proton (Harborne, 1986, Harborne & Mabry, 1982, Mabry *et al*, 1970). All of the above data indicated that the compound is luteolin, a widespread flavonoid of higher plants.

C^{13} – NMR (Fig. 5.11): 181.9 (C-4) , 164.2 (C-7) , 164.1 (C-2) , 161.5 (C-5), 157.6 (C-9), 149.7 (C-4′) , 145.8 (C-3′) , 121.9 (C-1′) , 119.2 (C-6′) , 116.2 (C-5′) , 113.5 (C-2′) , 104.0 (C-10) , 103.3 (C-3) , 99.0 (C-6) , 94.1 (C-8).

These findings were also supported by 2D spectra of HMQC and HMBC (Figures: 5.12 and 5.13) and finally by comparing the spectral data with published data (Mabry *et al.*, 1970).

The compound isolated, has a molecular formula $C_{15}H_{10}O_6$ based on its MS spectra. UV spectra (Figures 5.14 –5.19) showed absorption peaks in MeOH (λ_{max}, 242sh, 253, 267,

291sh, 349) which suggested a flavone type for the isolated compound, *ortho* dihydroxy systems indicated by the shift produced by the addition of $AlCl_3$ (λ_{max} , 274, 300sh, 328, 426), the addition of HCl to $AlCl_3$ (λ_{max}, 266sh, 275, 294sh, 355, 385) indicated the presence of a 5-OH (Harborne, 1986).



Luteolin

Figure 5.9. Structure of luteolin isolated from *Senna petersiana*

H-spectra

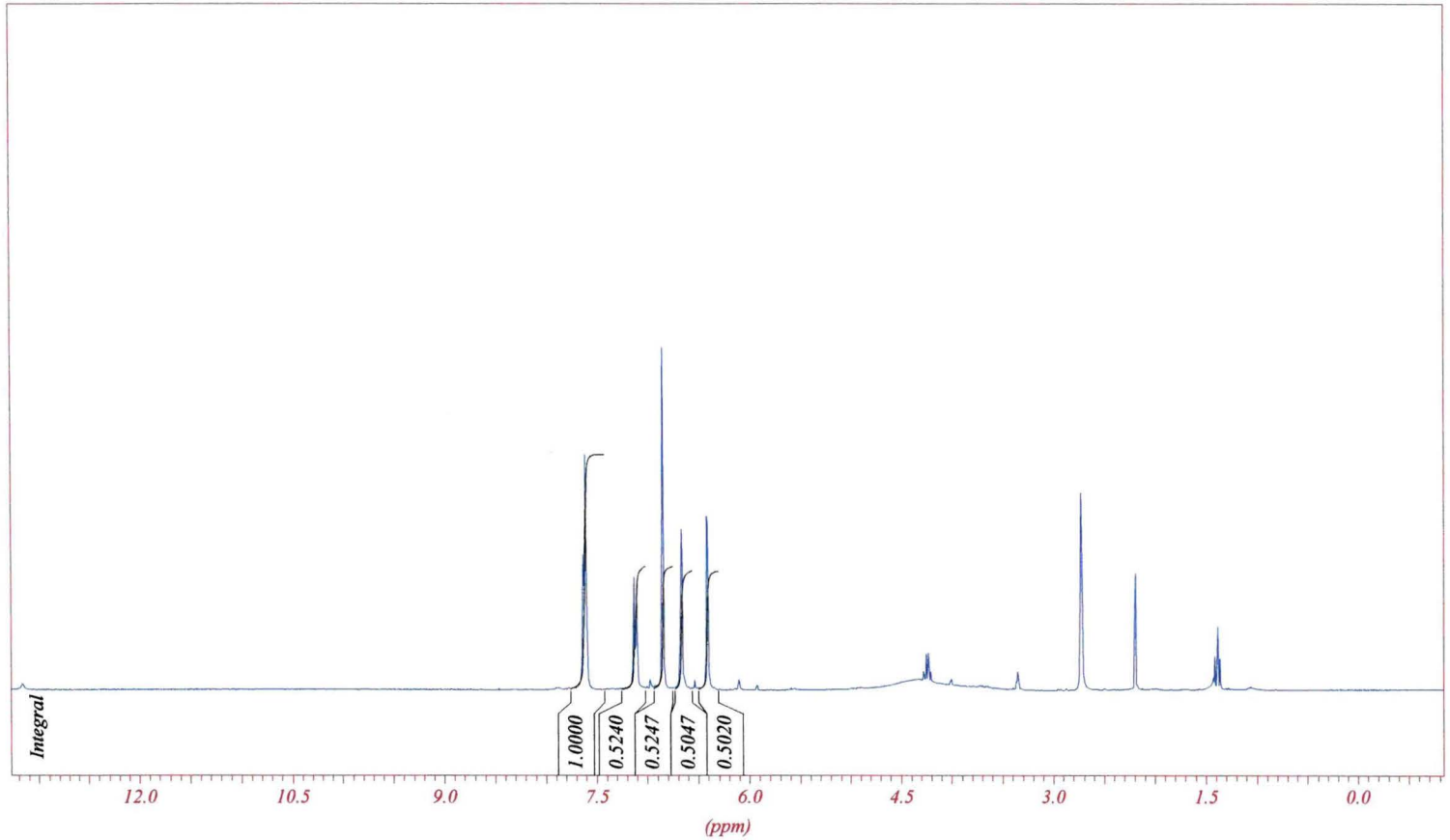


Figure 5.10. ^{1}H NMR spectrum of luteolin isolated from the seeds of *Senna petersiana*

C-Spectra

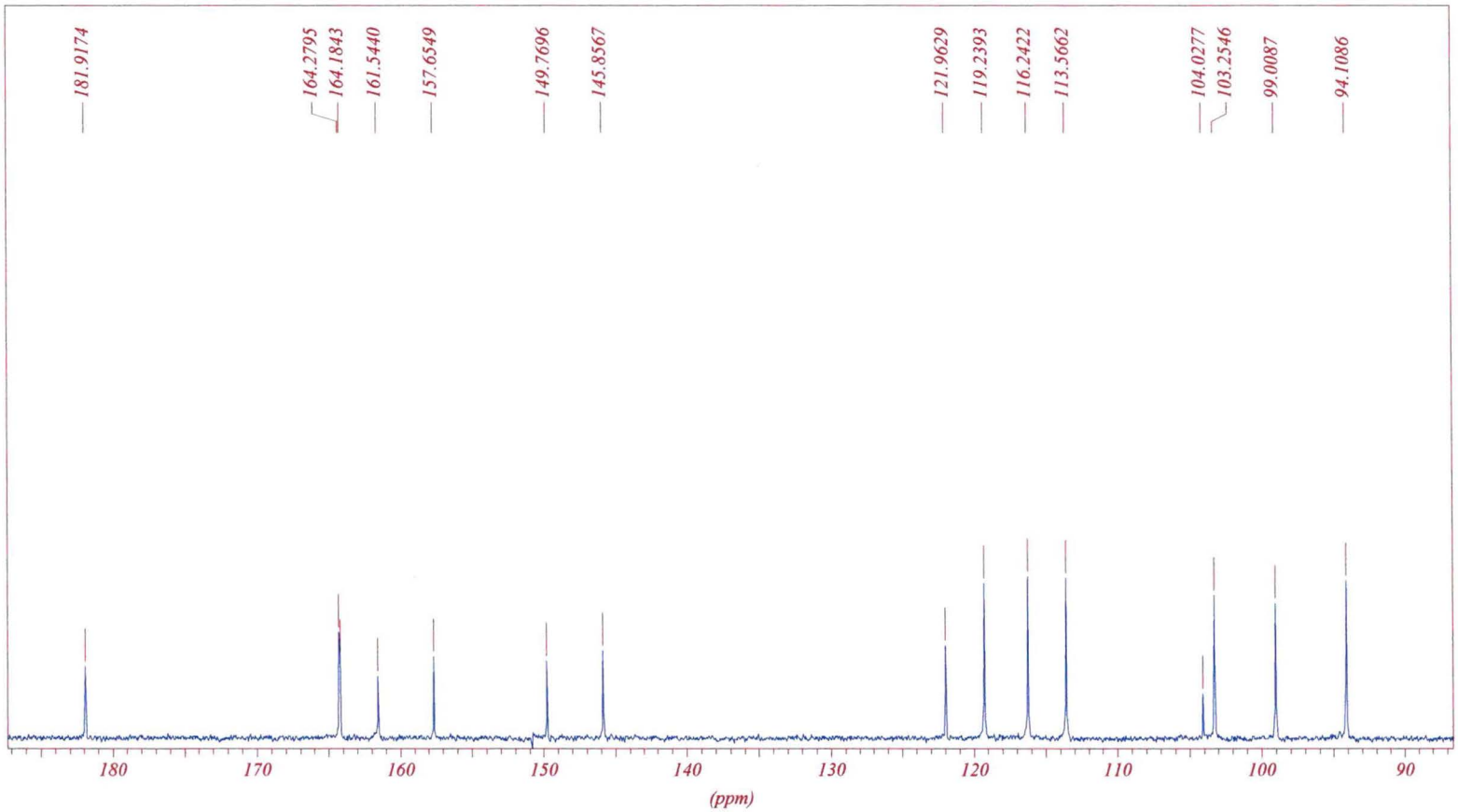


Figure 5.11. ^{13}C NMR spectrum of luteolin isolated from the seeds of *Senna petersiana*

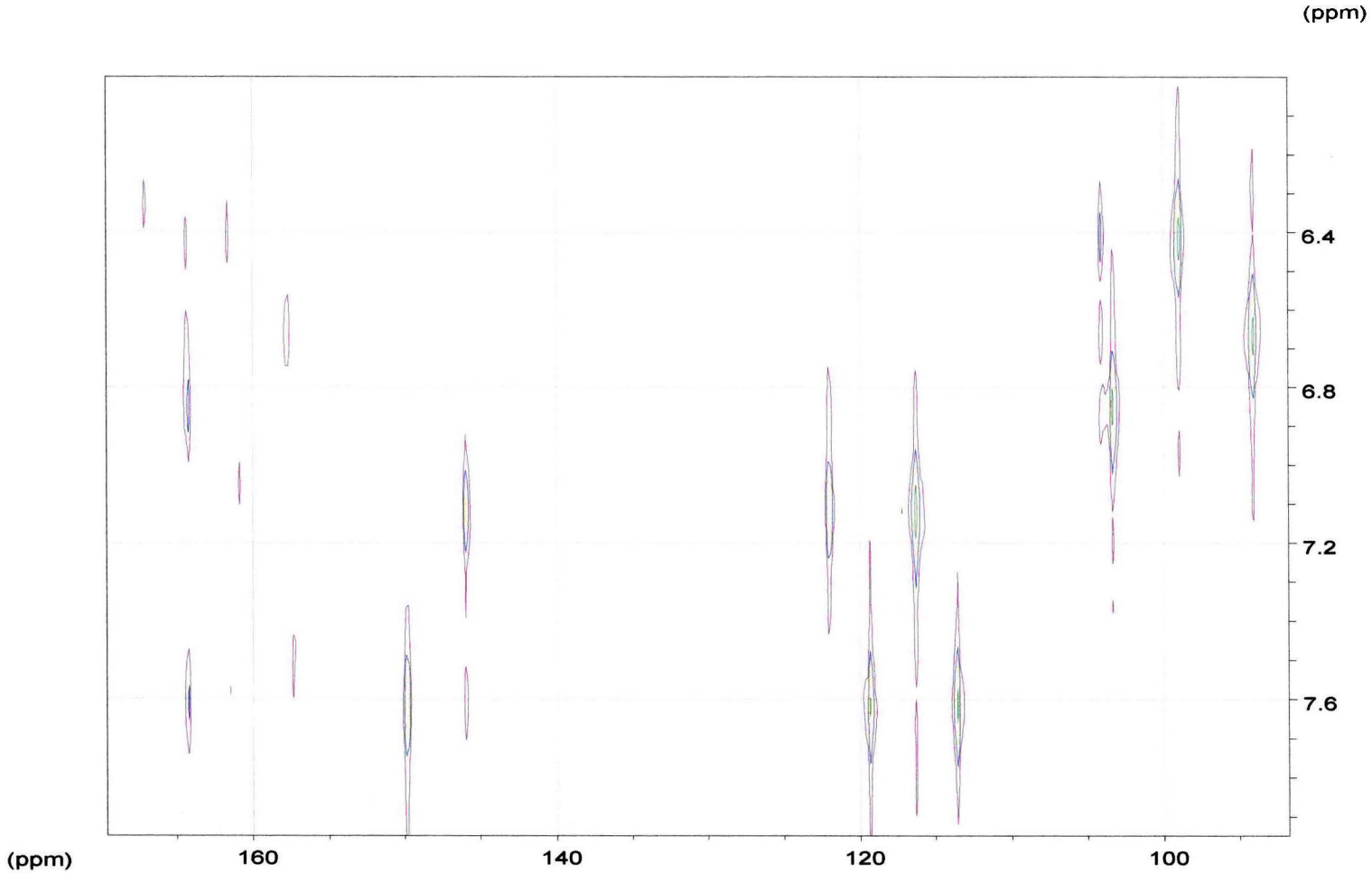


Figure 5.12. HMBC spectrum of luteolin isolated from the seeds of *Senna petersiana*

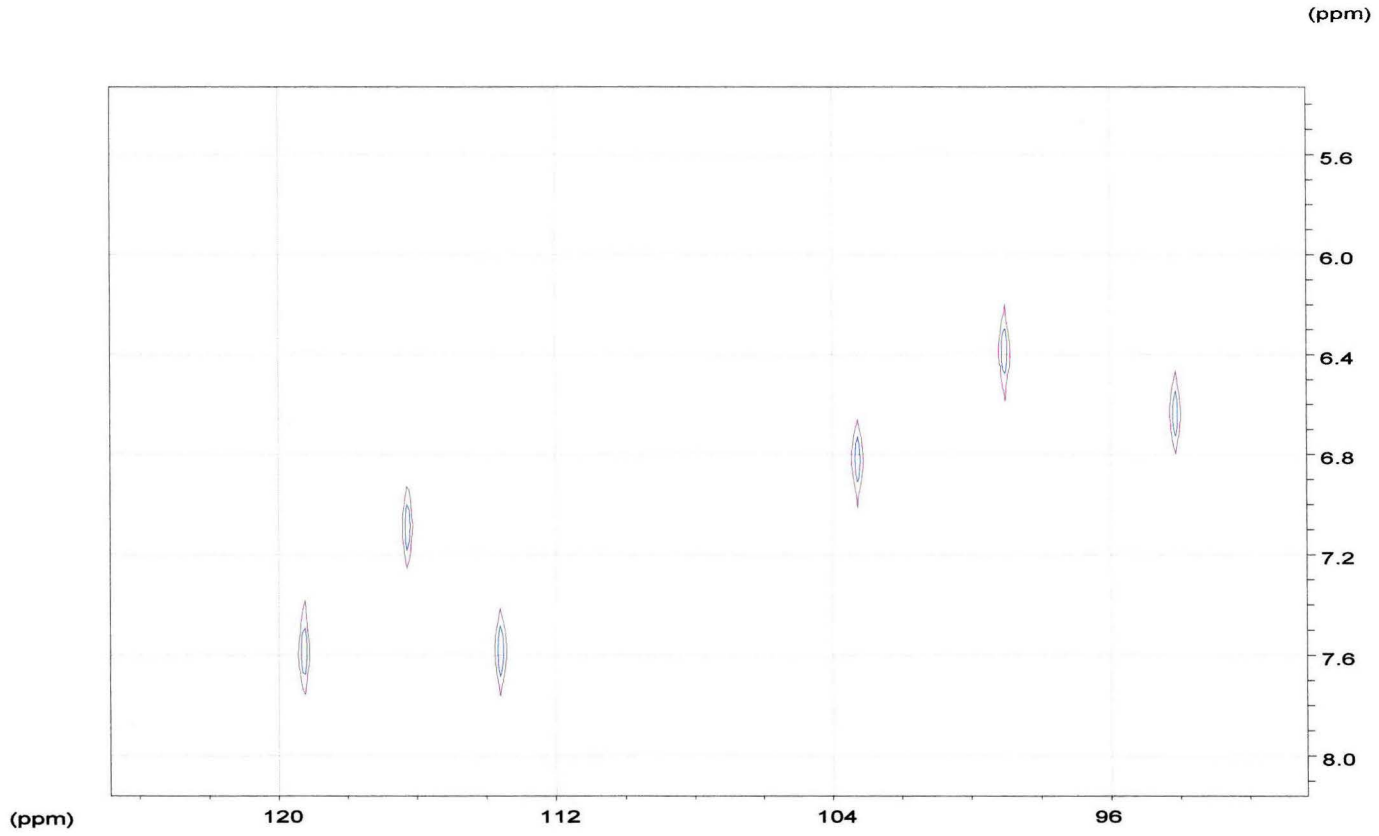


Figure 5.13. HMBQ spectrum of luteolin isolated from the seeds of *Senna petersiana*.

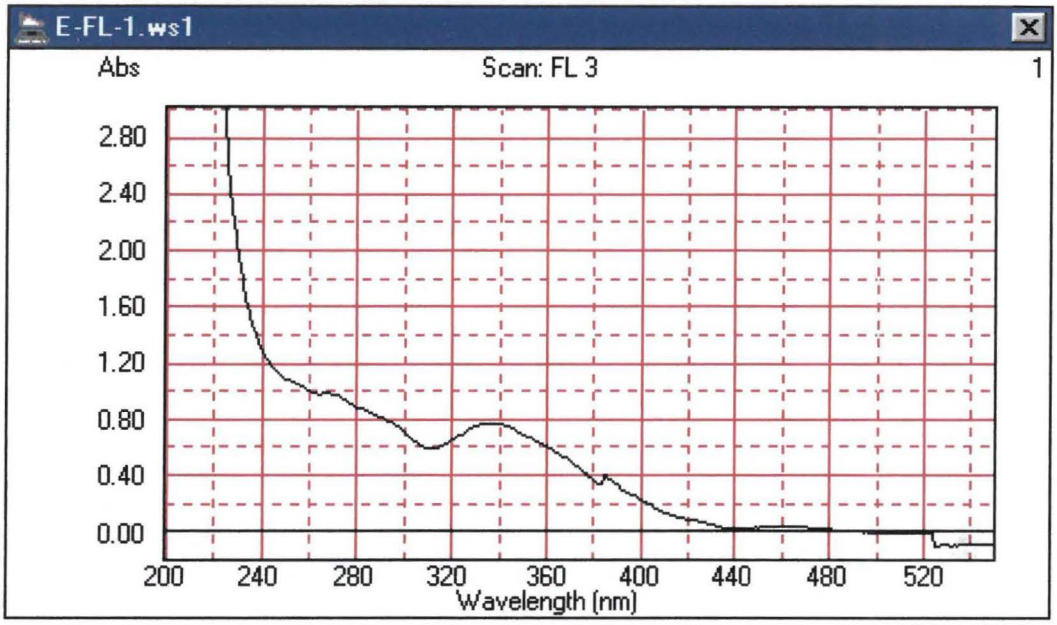


Figure 5.14. UV spectrum (FL3) of luteolin isolated from the seeds of *Senna petersiana*

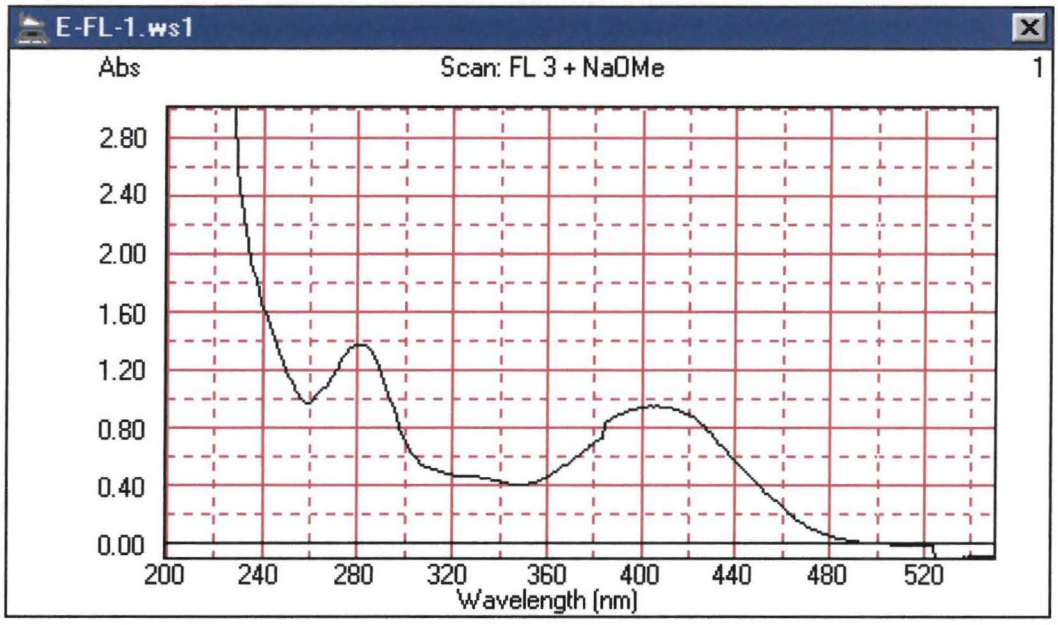


Figure 5.15. UV spectrum (FL 3 + NaOMe) of luteolin isolated from the seeds of *Senna petersiana*

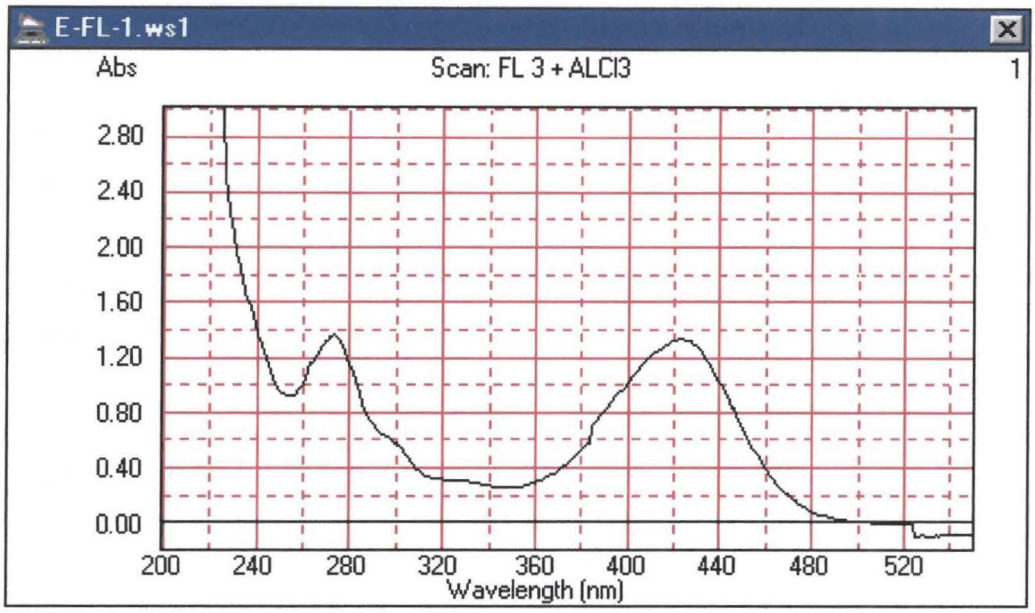


Figure 5.16. UV spectrum (FL3+ AlCI3) of luteolin isolated from the seeds of *Senna petersiana*

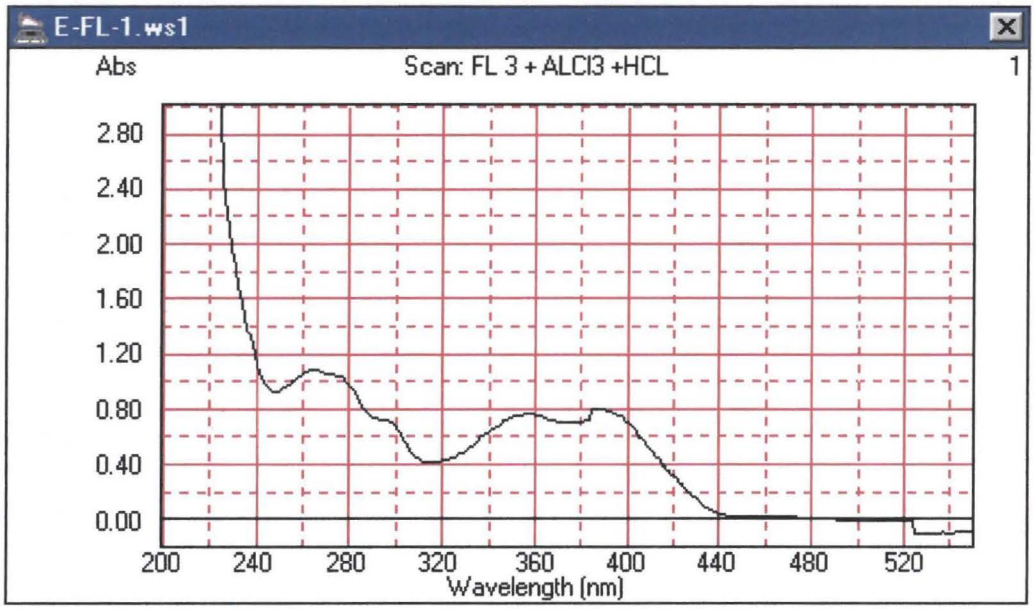


Figure 5.17. UV spectrum (FL3 + AlCI3 + HCl) of luteolin isolated from the seeds of *Senna petersiana*

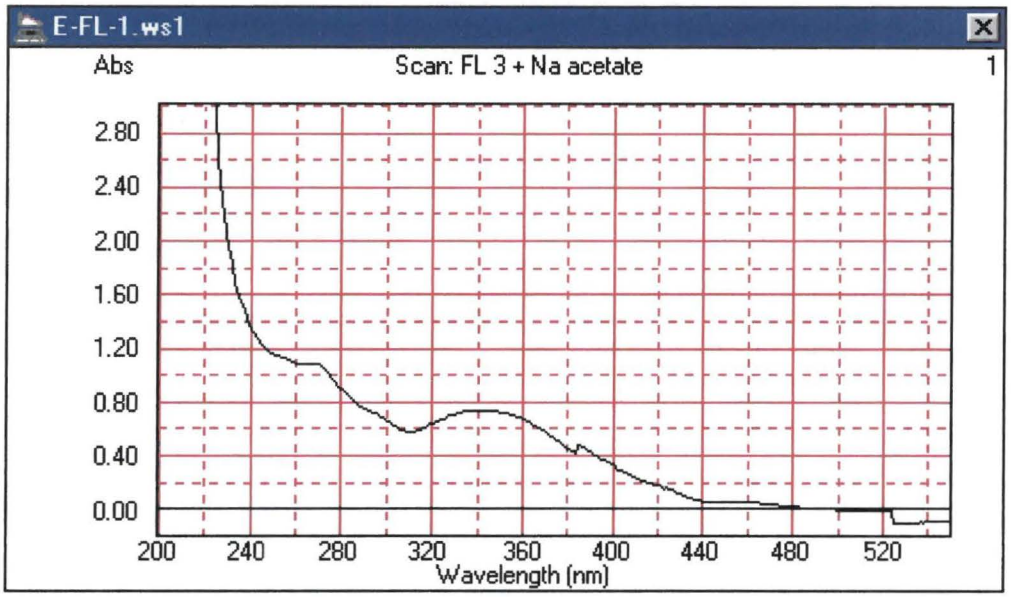


Figure 5.18. UV spectrum (FL3 + Na acetate) of luteolin isolated from the seeds of *Senna petersiana*

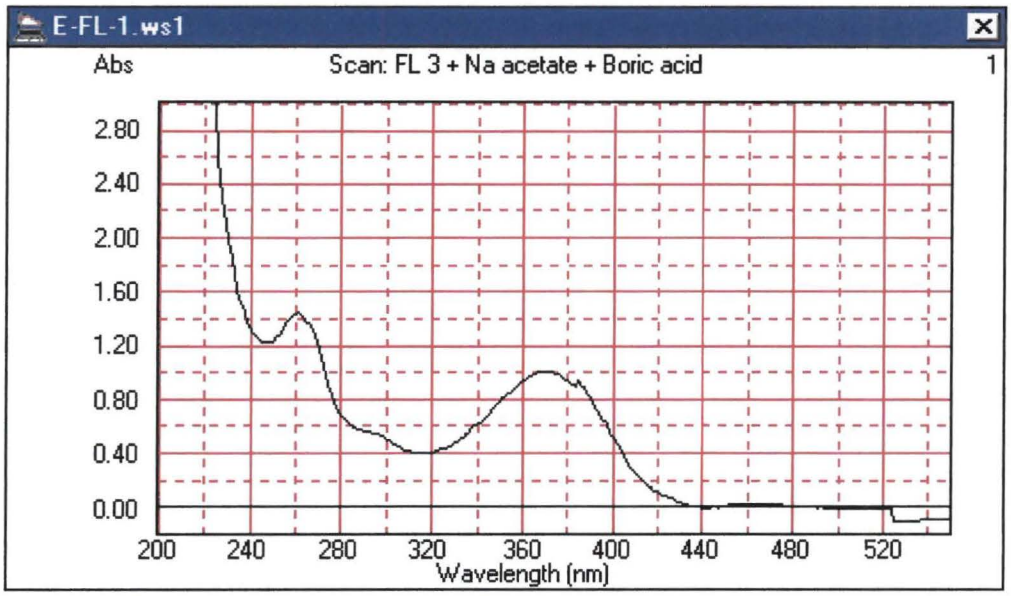


Figure: 5.19. UV spectrum (FL3 + Na acetate + boric acid) of luteolin isolated from the seeds of *Senna petersiana*

5.4. References

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Chapter 6

Antibacterial activity of luteolin isolated from *Senna petersiana*

6.1. Introduction

Plants belonging to the genus *Senna* have yielded a large number of anthraquinones and flavonoids, which have important medicinal properties (Messana *et al.*, 1991, Singh, 1987 and Kazmi *et al.,* 1994). In general, from the plants studied by Ali *et al,* (1999) *Senna* species proved to be more biologically active than other studied plants (Palgrave, 1977). The plants tested in previous studies showed significant growth inhibition against both bacteria and fungi.

Kitanaka and Takido (1992), reported the isolation of luteolin from the seeds of *S. nomame*. In this study we report the antibacterial activity of luteolin isolated from the seeds of *S. petersiana*. The roots, leaves and seeds of *S. petersiana* have been used in tropical and southern Africa as a remedy for gonorrhea, syphilis and skin diseases. Although a literature survey showed the presence of some compounds, not many individual constituents had been isolated from this plant and luteolin have not previously been isolated from *S. petersiana* (Shale *et al* 1999, and Singh & Singh 1987).

6.2. Materials and methods

6.2.1. Plant material

The seeds of *S. petersiana* were collected along Thohoyandou roads as described in Chapter 5 (5.4.1).

6.2.2. Preparation of extracts

Luteolin was isolated from the seeds of *S. Petersiana* as described in Chapter 5 (section 5.5.2)

6.2.3. Bacteria and antibacterial assays

The bacteria were obtained from the Microbiology Department University of Pretoria and the assay was done as described in Chapter 3. Luteolin was tested at the concentrations of 0.01, 0.1 and 1.0 mg/ml against 5 Gram-positive and 6 Gram-negative bacteria.

6.2.4. Direct bioassay

Direct bioassays on TLC plates were done by applying a small spot of 20 µl of luteolin to silica gel F_{254} 60 plates. The plates were developed in hexane : ethyl acetate (1:1) and dried carefully. A 30 hours old *S. aureus* culture in nutrient broth was centrifuged at 1000 rpm for 15 minutes, the supernatant discarded and the pellet was resuspended in fresh nutrient broth. A fine spray was then used to apply the bacterial suspension onto TLC plates (Meyer and Dilika, 1996). These plates were then incubated at 30° C for 20 hours in humid conditions. After incubation, the plates were sprayed with an aqueous solution (colouring reagent) of 2 mg/ml p-iodonitrotetra-zolium violet. The plates were then reincubated at 30° C for 4 hours. Metabolically active bacteria converted the tetrazolium salt into the corresponding intensely coloured formazan and the antibacterial compounds appeared as clear spots against a coloured backround. The experiment was replicated three times.

6.3. Results and discussion

The antibacterial assay of luteolin, the compound isolated from the seeds of *S. petersiana* showed activity against four Gram-positive bacteria tested (Table 6.1).

Table 6.1 Antibacterial activity of luteolin isolated from the seeds of *S. petersiana*

Bacterial species	Gram +/-	MIC[a] (mg/ml)
1. *Bacillus cereus*	+	1.0
2. *B. pumilus*	+	1.0
3. *B. subtilis*	+	na[b]
4. *Streptococcus aureus*	+	1.0
5. *Staphylococcus aureus*	+	1.0
6. *Enterobacter cloacae*	_	na
7. *Escherichia coli*	_	na
8. *Klebsiella pneumoniae*	_	na
9. *Pantoea agglomerans*	_	na
10. *Pseudomonas aeruginosa*	_	na
11. *Serratia marcescence*	_	na

[a] Minimum inhibitory concentration

[b] Not active.

None of the 11 bacteria tested were inhibited at the lowest concentration. Luteolin partially inhibited the growth of *Bacillus cereus* and *Staphylococcus aureus* at the concentration of 0.1 mg/ml.

One clear zone (Fig 6.1) of bacterial growth inhibition was seen on TLC plates sprayed with *S. aureus*. These confirm that the isolated compound has an antibacterial activity and previous phytochemical analyses have shown the presence of antibacterial compounds in some *Senna* species (Kazmi *et al.,* 1994).

Luteolin has been shown to have antibacterial activity against *Enterobacter aerogenes*, *Proteus vulgaris*, *Staphyllococcus aureus* and some other bacteria in previously reported studies (Basile *et al.*, 1999, Sato *et al.*, 2000, Mori *et al.*, 1987). Luteolin has not been previously investigated against five of the bacteria (*Bacillus cereus, B. pumilus*, *Serratia marcescence, Pantoea agglomerans* and *Streptococcus aureus*) tested in this study. Mittra *et al.,* (2000), demonstrated the inhibitory effect of luteolin against the growth of *Leishmania donovani* promastigotes *in vitro*. It arrests cell cycle progression in *L. donovani* promastigotes, leading to apoptosis. Luteolin has also showed antimicrobial, anti-inflammatory and anti-HIV activity (Yamamoto & Ogawa, 2002, Xu *et al.*, 2000).

The results demonstrate the importance of traditional medicine, especially in rural populations where Western medicine is not readily available. The traditional knowledge is a valuable guide in the selection of plants, which can be used to isolate and identify active compounds.

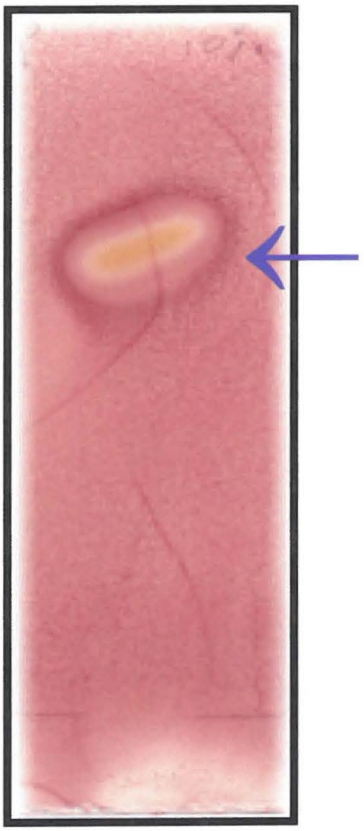

Figure: 6.1 Zone of inhibition (arrow) of *Staphylococcus aureus* produced after chromatography of luteolin on a TLC plate developed in hexane: ethyl acetate (1:1).

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Chapter 7

Cytotoxicity and antiviral activity of luteolin

7.1 Introduction

Flavonoids are a large group of naturally occurring compounds with a low molecular weight and are widely distributed in the plant kingdom (Kaul *et al.*, 1985). Previous papers have reported that flavonoids from the roots of *Scutellaria baicalensis* have an inhibitory effect against human immunodeficiency virus (HIV), human T cell leukemia virus type 1 and promotes mouse skin tumors (Zhou *et al.,* 1997).

Luteolin is a widely distributed flavonoid found in various species of *Senna* (Kitanaka & Takido, 1992), *Viburnum* (Parveen *et al*, 1998) and *Phlomis* (Bucar *et al*, 1998). It has shown some antibacterial (Cottiglia *et al.*, 2001), antiinflammatory (Fnimh, 2001) and even antiviral (against herpes simplex) activity. Studies on *Vitex agnus-castus* has reported cytotoxicity activity of four glycoside attached luteolin against lympocyte leukemia cells (Hirobe *et al.*, 1997)

The purpose of this study was to investigate the effect of *Senna petersiana* extract and luteolin isolated on viral replication, viral binding to the host cell and viral inactivation, since previously studies reported the effect of luteolin on viral replication only (Wleklik, 1988).

7.2 Materials and methods

Luteolin was isolated from the seeds of *S. petersiana* as described in Chapter 5

7.2.1. Cytotoxicity

The cytotoxicity assay of luteolin was done as described in Chapter 4 (section, 4.2.3).

7.2.2. Antiviral assay

Luteolin and plant extract were tested against herpes simplex virus type 1. One ml of luteolin or extract was added to 9 ml of serum free MEM (0.1 %) and filtered through 0.22 µm filters to make a total stock of 1000 µg/ml. Doubling dilutions were made from 500 µg/ml to 3.9 µg/ml.

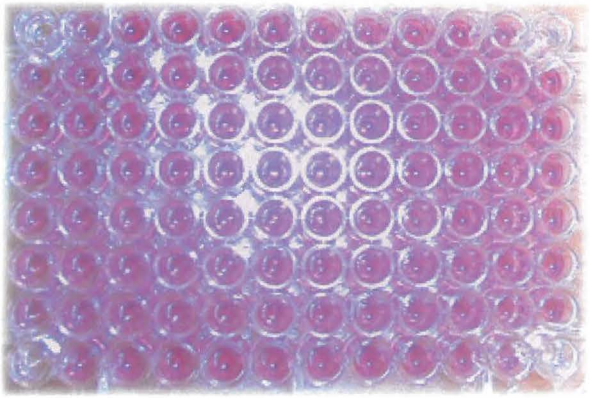

Figure 7.1. A microtitre plate for antiviral assay

7.2.2.1. The influence of the extract and luteolin on viral replication

Monolayers of vervet monkey kidney cells (VK) were prepared in the microtitre plates as described in Chapter 4. The medium was then withdrawn and the cells starved for a hour in serum free MEM at 37^0 C in CO_2. The starvation medium was withdrawn and 100 µl of virus stock (100 TCID50/ml) was added to each of six wells (100 TCID 50 virus/well)

in each row of the plate. For the negative control 100 µl serum free MEM was used. The tray was adsorbed at 37 ^{0} C for an hour and then the virus was withdrawn and the plates were rinsed with serum free MEM. 200 µl of the appropriate dilution of extract/compound was added to each well. The plates were then incubated at 37^0 C in CO_2 and examined daily for seven days.

7.2.2.2. Effect of the extract and luteolin on viral binding to the host cell

Monolayers of VK cells were prepared in the microtitre plates as mentioned in Chapter 4 (section 4.2.2). The medium was then withdrawn and the plates was starved for an hour in serum free MEM at 37 ^{0} C in CO_2. The starvation medium was withdrawn and 100 µl of the appropriate dilution of extract/compound was added to six wells of each row of the microtitre tray. For the positive control 100 µl of serum free MEM was added. Thereafter 100 µl of virus stock (1000 TCID50/ml) was added to all wells except to the negative control. Hundred µl of serum free MEM was added to the cell culture controls. The plates were incubated at 37 ^{0} C under CO_2 and examined daily for seven days.

7.2.2.3. Effect of the extract and luteolin on the inactivation of the virus

Monolayers of cells were prepared and the medium was withdrawn. The plates were starved for an hour in serum free medium at 37 ^{0} C in CO_2. Half a ml of each dilution was added to 0.5 ml of viral suspension. For the positive control 0.5 ml of serum free MEM

was mixed with 0.5 ml of the viral suspension. For the negative control 1ml of serum free MEM was added. The plates were then allowed to stand for an hour at room temperature. Starvation medium was withdrawn and 200 µl of virus/compound or virus/extract mixture was added to all the wells except negative controls. The plates were incubated at 37 ^{0}C and observed daily for cytophathic effect.

7.3. Results and discussion

The highest non-toxic concentration was considered to be the concentrations of luteolin and crude extract at which 75 % of the cultured cells were still alive. The cytotoxicity results are summarized in Table 7.1.

Table 7.1 Percentage of living cells after treatment with luteolin/crude extract for the cytotoxicity assay.

Concentration used (µg/ml)	% of living cells		
	Luteolin	Crude extract	Control
31	84	86	100
62	87	97	100
125	83	97	100
250	86	98	100
500	69	97	100

At the concentration of 500 µg/ml, luteolin showed toxicity since only 69 % of cultured cells were still alive as compared to the controls. From the concentration of 250 µg/ml and below luteolin did not exhibit altered morphology or growth characteristics indicative of a cytotoxic effect, thus suggesting that there is no toxicity. More than 75 % of the culture treated with crude extracts were still alive at the concentrations ranging from 31 to 500 µg/ml. These results demonstrate that there is no toxicity effect of crude extracts on VK cells at all concentrations tested, while luteolin only showed toxicity at 500 µg/ml.

7.3.1. Effect of luteolin and crude extracts on HSV-1 replication and binding to the host cells.

The cythopathic effect in tissue culture plates which had been treated with luteolin and crude extracts was not significantly different from the untreated control plates at all concentrations tested. Exposure of cells to luteolin and crude extracts did not reduce viral binding to the host cells or replication of the virus.

7.3.2. Effect of luteolin and crude extracts on HSV-1 inactivation

In this experiment the cytophathic effect (CPE) on the cells was reduced by 30 % at 62 and 125 µg/ml luteolin, while the crude extract reduced the CPE by more than 60 % at the same concentration (Table 7.2). At the concentration of 250 µg/ml luteolin showed that it can inactivate 50 % of the virus, reducing the CPE by half (Fig. 7.2). The antiviral activity of the crude extract shows that at the concentration of 250 µg/ml the virus is

completely inactivated. Thus we can conclude that the seeds of *S. petersiana* contain other antiviral compounds.

Table 7.2 Percentage of cytopathic effect (CPE) after the cells were treated with luteolin/crude extract in the antiviral assay

Treatment	Concentration (µg/ml) luteolin and extract	Cytopathic effect (%)
Virus control		100
Crude extract	31	70
	62	40
	125	35
	250	0
	500	0
Luteolin	31	85
	62	70
	125	70
	250	50
	500	45

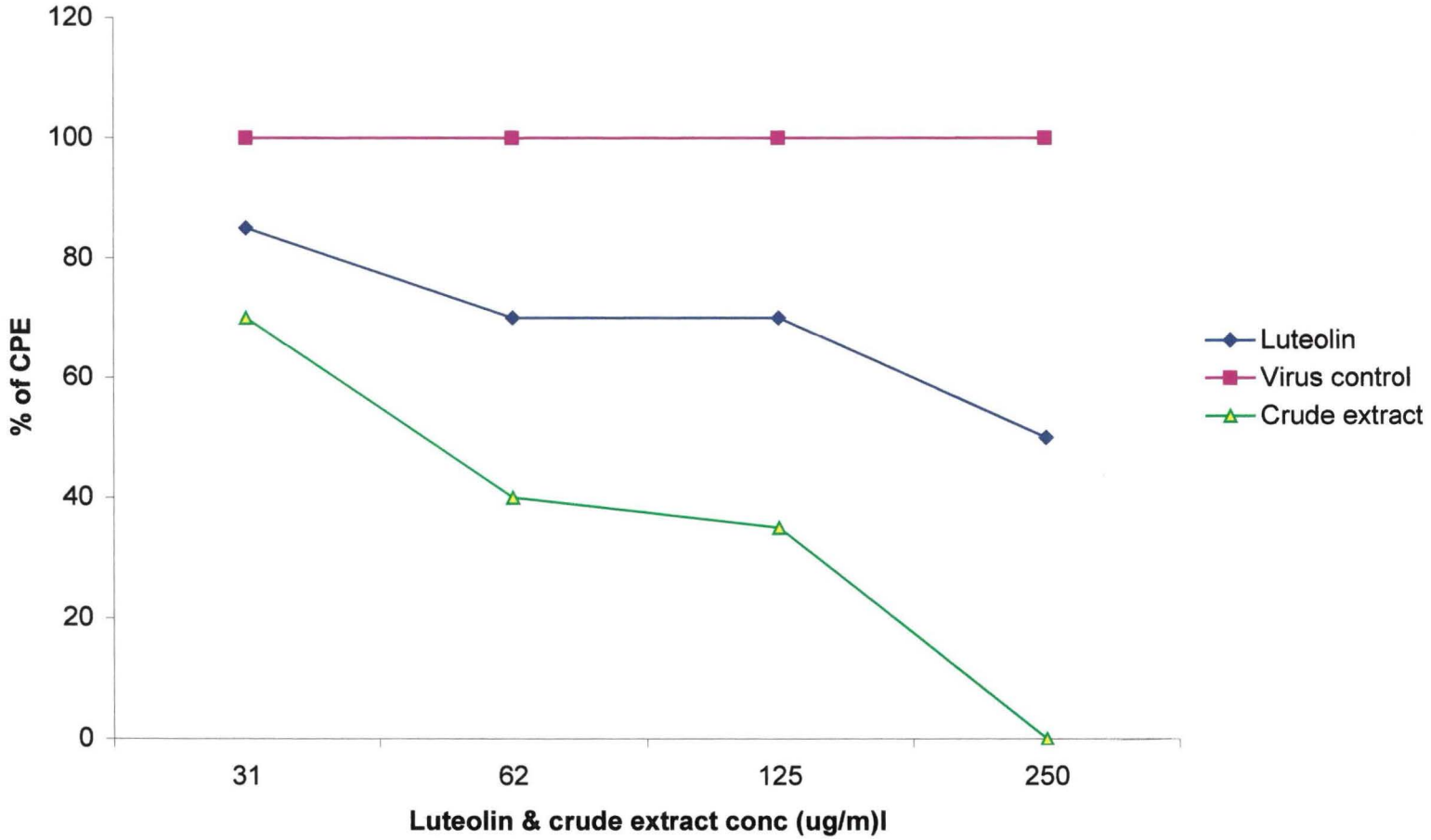

Figure 7.2 Cythophathic reduction effect of the cultured cells after treatment with luteolin and crude extract.

These experiments indicate that like other naturally occuring flavonoids, luteolin possesses antiviral activity, confirming earlier observations by other researchers (Kaul *et al.,* 1985, Wleklik *et al.*, 1988 & Cottiglia *et al.*, 2001). The antiviral activity of flavonoids appears to be associated with non-glycosidic compounds and hydroxylation at the 3-position is apparenty a prerequisite (Harborne, 1986). The reason for luteolin not to have more antiviral activity might be that there is a free C-3. The crude extract had more activity and this shows that there might be other active compounds present in it.

The present results have shown that luteolin can inactivate herpes simplex virus type 1, whereas previous studies only demonstrated the inhibitory effect of luteolin against HSV 1 replication (Wleklik, *et al.,*1988).

It seems that parent compounds with free hydroxyl groups at C-5, C-7, C-3', C-4' and additionally at C-3 have the highest antiviral activity. Wleklik, *et al.,* (1988), reported that substitution of those hydroxyl groups caused decrease of or complete abolition of the antiviral activity of quercetin and luteolin.

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Chapter 8

General discussion

8.1 Introduction

From the earliest times, medicinal plants have played a vital role in primary health care needs. Today we still rely on the curative properties of plants in about 75% of our medicines. It has been estimated that 80 % of people living in developing coutries are almost completely dependent on traditional medicines (Prozesky *et al*, 2001). Plants continue to be used worldwide for the treatment of various diseases and the research of its constituents are of great importance, for it has given rise to many of world's most useful drugs. The ability of herbal medicine to affect body systems depends on its chemical constituents (Fnimh, 2001 & Khan *et al*, 2001).

Plants contain numerous biologically active compounds, many of which have shown antimicrobial activity (Ali *et al*, 1999). The search for new effective antimicrobial agents is necessary due to the antibiotic resistance of bacteria and the occurrence of fatal opportunistic infections associated with AIDS. (Penna *et al.,* 2001). The continuous development of antibiotically resistant strains of microbial pathogens, such as methicillin- resistant *Staphylococcus aureus* (MRSA), penicillin-resistant *Streptococcus pneumoniae* (PRSP) and vacomycin-resistant enterococci (VRE), is a growing problem (Fyhrquist *et al.,* 2002).

Our study has partly validated the scientific use of six plants that are mostly used by Venda peoples of South Africa in the treatment of STD's. Some of these plants are also used in other southern African countries to treat the same ailments. Anthraquinones, flavonoids and glycosides are among some of the compounds that are reported to have been isolated from the plants investigated in our studies. Various parts of plants are used, but the roots are the most commonly used parts to treat ailments.

8.2. Isolation of compounds from the roots and seeds of *Senna petersiana*

In the bioassay-guided isolation of the root extracts of *S. petersiana* we could not isolate a pure compound because of the sensitivity and degradation of compounds on silica gel. Repeated silica gel column chromatography however, resulted in the isolation of a flavonoid, luteolin from the seeds of *S. pertesiana*. One pure yellow fraction was obtained after silica chromatography. The structure of the isolated compound was eluted by spectroscopic methods including ^{1}H, ^{13}C, UV, HMBC and HMBQ experiments. All the data indicated that the compound isolated is luteolin, a flavonoid already found in other higher plants (Harborne, 1982, Harborne, 1986 and Mabry *et al.*, 1970)

8.3. Antibacterial activity of plants extracts and luteolin

The plants selected for this study showed antibacterial activity *in vitro*. This supports the use of traditional medicine in the treatment of sexually transmitted diseases. Six selected plants (water and choroform extracts) were investigated for their antibacterial activity

against ten common bacteria. As determined by the agar dilution method, all six water extracts exhibited a broad antibacterial activity and only the chloroform extract of *Anredera cordifolia* inhibited the growth of bacteria.

The majority of the water extracts were active against Gram-positive bacteria, with only the extract of *Senna petersiana* displaying activity against Gram-negative bacteria. Plants that have showed moderate or low activity (*Elephantorrhiza burkei, Rauvolfia caffra* and *Cassine transvaalensis*) may have active compounds but probably in smaller amounts (Fabry, 1998). It has been rightly said that, the knowledge of the traditional healers and herbalists should not be underestimated or considered inferior to western methods of treatment (Shale *et al*, 1999).

Antibacterial assay also showed that luteolin isolated from the seeds of *S. petersiana* exhibited activity against four Gram-positive bacteria at 1.0 mg/ml. The antibacterial activity of luteolin was futher investigated by direct bioassay on TLC. Significant growth inhibition was observed on TLC plates sprayed with *Staphylococcus aureus*. The activity of the luteolin isolated from the seeds of *S. petersiana* provides preliminary scientific validation for the traditional use of this plant. Syphillis, herpes, gorrnohea, stomach complaits and skin infections are among some of the ailaments, which are traditionally, treated with *S. petersiana* decoctions (Watt & Breyer-Brandwijk, 1962). Our results are also supported by a study done by Tsou *et al.*, (2001), who found that luteolin inhibited the growth and arylamine N-acetyl-transferase (NAT) activity in *Neisseria gonorrhoeae*.

Xu *et al.*, (2000) also indicated moderate inhibitory activity of luteolin against HIV-protease.

8.4. Cytotoxicity and antiviral activity of the plant extracts and luteolin

In the assay to assess the cytotoxicity and antiviral activity of the plant extracts, *Anredera cordifolia* showed no altered morphology of the VK cells (cytotoxic effect) at 1.560 mg/ml concentration, whereas both *Senna petersiana* and *Terminalia sericea* showed no toxicity at 0.024 mg/ml concentrations. Antiviral activity of *A. cordifolia T. sericea S. petersiana* were tested at this non-toxic concentrations and exhibited low or no antiviral activity. Further purification will probably reduce the toxicity and decrease the concentration of its actual effective dose (Meyer *et al.*, 1996).

Antiviral experiments also showed that luteolin does not stop viral replication and binding to the host cell, but it can inactivate 50% of the viruses at the concentration of 250 µg/ml. The antiviral activity of the seed crude extract (*S. petersiana*) showed that at the concentration of 250 µg/ml, the virus was completely inactivated. Preliminary tests of the roots showed low antiviral activity, thus we can suggest that the seeds of *S. petersiana* has more antiviral activity when compared to the roots.

The results from this study support to a certain degree, the traditional medicinal uses of the plants evaluated and reinforce the concept of screening plants as potential sources of bioactive substances.

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Chapter 9

Summary

The traditional use of medicinal plants to treat sexually transmitted diseases

by

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MAGISTER SCIENTIAE (Plant Physiology)

All six plants studied (*Senna petersiana*, *Terminalia sericea*, *Cassine transvaalensis*, *Elephantorrhiza burkei*, *Rauvolfia caffra* and *Anredera cordifolia*) proved to have considerable antibacterial activity. The water extracts of five of the six plants tested, showed activity against *Bacillus pumilis*, *B. subtilis* and *Staphylococcus aureus* respectively. Water extracts from *S. petersiana* showed a significant antibacterial activity by inhibiting all Gram-positive and two Gram-negative bacteria.

A cytotoxicity assay of three plants (*S. petersiana, T. sericea* and *A. cordifolia*) on primary vervet monkey kidney cells showed that *A. cordifolia* was the least cytotoxic extract with an ID_{50} value of 1.560 mg/ml. Both *S. petersian* and *T. sericea* showed an ID_{50} value of 0.024 mg/ml. Cytotoxicity as determined in this study does not necessarily mean that the active compound which can be isolated from these plants will also be toxic.

Antiviral activity of *S. petersiana, T. sericea* and *A. cordifoli* crude extracts were investigated against herpes simplex virus type 1 at the non-toxic concentrations. Both *T. sericea* and *A. cordifoli* extracts showed to be non-active against HSV-1, but *S. petersiana* showed a 20 % reduction in replication of the virus after the sixth day of the experiment.

Because of the sensitivity and instability of compounds in the root extract of *S. petersiana*, it was very difficult to isolate any pure compound. Bioassay–guided fractionation of the seeds of *S. petersiana* resulted in the isolation luteolin. Its structure was identified and confirmed through spectroscopic methods including ^{1}H, ^{13}C, UV, HMBC and HMBQ.

An antibacterial assay of luteolin isolated from the seeds of *S. petersiana* showed activity against *Baccilus cereus*, *B. pumilis*, *Streptococcus aureus* and *Staphylococcus areus* at the concentration of 1 mg/ml.

In the assay to assess the possible antiviral activity of luteolin against herpes simplex type 1 virus, 50 % of the virus was inactivated at the concentration of 250 µg/ml.

The results of this study have shown that it is possible that the extracts studied, can provide humankind with valuable agents of potential use in the treatment of herpes and some bacterial species.

Chapter 10

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