The traditional use of medicinal plants to treat erectile dysfunction and the isolation of their bioactive compounds

by

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1.1. Background

Medicinal plants have been used over many centuries, however, many people still rely on them even in this sophisticated world of our present time. They have always been considered important and effective for the treatment of many different diseases in Africa and other parts of the world. There has been a time where some people regarded traditional medicine as an uncivilized type of medicine, forgetting that 25.0% of modern medicine contains ingredients derived from herbal plants (Adimoelja, 2000). Many people have survived due to the use of medicinal plants. The majority of traditional medicines are directed towards the curing and prevention of diseases, rather than the treatment of symptoms (De Smet, 1998).

Root, stem, leaves and fruit extracts from the same medicinal plant can be used to treat different diseases. People from different countries also use the same medicinal plants for the treatment of different diseases. It has been reported that traditional healers from Togo use the roots of the herb *Securidaca longependuncula*ta to prevent and treat snakebite, whilst in Tanzania, it is used for the treatment of headaches (Neuwinger, 1996). This might be explained by the fact that habitat, seasons and weather conditions play a significant role in the chemical composition of a plant.

There are many incurable diseases at present for which drugs are needed urgently. Erectile failure is a common problem affecting men (Licht, 1998) who cannot afford Viagra. Those affected may experience minimal or moderate erection or be permanently flaccid. Historically, the prevalence of erectile dysfunction (ED) has been difficult to estimate due to the fact that it is not life threatening, patients often do

not seek treatment and literature terminology for the condition has been confusing (Melman & Gingell, 1999). However, it is estimated that at least one in every ten men is affected by erectile failure. It has a significant negative impact on quality of life (Singer *et al.*, 1994). Although erectile dysfunction affects men, it also influences the quality of life of their partners.

Until recently, psychological factors was seen as the sole cause of ED. According to urologists, ED is not manifested in the man's head only; it is also a physiological condition in the blood vessels as well (Friedman, 1997). As Leonardo wrote in his notebook, "the penis has the mind of its own, often the man is asleep and it is awake, and many times the man is awake and it is asleep" (Friedman, 1997). The Massachusetts Male Aging Study indicated that factors such as age, medical disease, lipids, medication, cigarette smoking and psychological indices are associated with ED. The study conducted in the US indicated that ED affected 52.0% of the male population between the age of 40 and 70 years (Friedman, 1997 and Viagra Datasheet).

In order to combat the problem of erectile dysfunction, Vhavenda traditional practitioners have their own way of treating people with this condition. There are a number of medicinal plants that traditional healers in the area use to treat erectile failure. Some of the plants used are *Securidaca longependunculata, Wrightia natalensis* and *Rhoisiccus tridentata.* To date, very little is known about the chemical composition of these medicinal plants.

Results from other studies showed that some plants have biological activity against ED. According to Kim *et al.*, (1998), extracts from *Panax ginseng* relaxes the rabbit corpus cavenorsal smooth muscle which is an indication of erectile function. The roots of *S. longependunculata* were studied by Mouzou *et al.*, (1999) to investigate its effect on rat skeletal muscle cells. The results of this study clearly demonstrate the existence of an agonistic effect of skeletal muscle myoballs. In addition the study provides some interesting results concerning the possible action on the dihydropyridine receptor acting as voltage-sensor.

The plant kingdom represents an enormous reservoir of biologically active molecules and so far only a small fraction of plants with medicinal activity have been assayed. Natural products and their derivatives represent well over 50.0% of all drugs used in modern medicine in the world (Van Wyk *et al.*, 1997). More than half of the world's 25 best selling pharmaceuticals owe their origin to natural sources (Adimoelja, 2000). Africans were treating diseases like erectile failure using medicinal plants during times when no medicine was available from hospitals. In their effort to combat the problem, scientists recently developed the orally administered drug, Viagra. This drug was found accidentally like many other medicines, such as penicillin. Viagra did not lower blood pressure as well as they had hoped, but it did improve erection, something they had not anticipated at all (Friedman, 1997). It has proven to be effective in the treatment of erectile dysfunction of different etiologies (Licht, 1999). Although there are a number of drugs used for ED, Viagra received an amazingly world wide publicity.

Today, medicinal plants enjoy great potential for export and it must be noted that the majority of plant resources originate from African countries (Dladla, 2001). South Africa alone has more than 3000 plant species that are used for medicinal purposes. It was estimated that with millions of consumers using about 20 000 tons of indigenous plants a year, the trade in muti products could be worth more than R270 million a year in South Africa (Dladla, 2001).

1.2. Objectives of the study

The main objectives of this research project are as follows:

- Identifying the plants used traditionally in Venda (South Africa) to treat male erectile dysfunction;
- Developing a reliable and fast bioassay to measure smooth muscle relaxation;
- Testing extracts for smooth muscle relaxation;
- Testing *in vitro* antibacterial activity of extracts;
- Investigating the effects of extracts on motility parameters of sperm and
- Isolating and identifying the active compound(s) from the selected medicinal plants.

1.3. Scope of the dissertation

The thesis is composed of seven chapters. Chapter 2 deals with the plants used during the study and their medicinal uses. Their traditional use to treat ED is also described. The effect of extracts on rabbit cavernosal smooth muscle from the penis is described

in Chapter 3. Chapter 4 describes the antibacterial activity of the extracts and Chapter 5 describes the effect of extracts on sperm parameters. The isolation and identification of the active and some non-active compounds is described in Chapter 6. Chapter 7 describes the biological activity of the isolated compounds on the rabbit cavernosal smooth muscle and their antibacterial activity. Chapter 8 comprises of the general discussion and conclusions of the research. The summary of the dissertation is given in Chapter 9 and lastly, Chapter 10 deals with the acknowledgements.

1.4. References.

Adimoelja A (2000) Phytochemicals and the breakthrough of traditional herbs in the management of sexual dysfunction. International Journal of Andrology 23, Suppl. 2: 82- 84.

Ayesu ES (1978) Medicinal plants of West Africa. Reference publication, Inc. United States of America.

De Smet PGM (1998) Traditional pharmacology and medicine in Africa. Journal of Ethnopharmacology 63(1,2): 1- 180.

Dladla S (2001) Muti trade and boom. Sowetan 29 July 2001.

Friedman D (1997) Are you man enough to handle four-hour erection. Esquire. September: 105- 109.

Torssell KBG (1997) National product chemistry. 2nd edition. Apotekarsocieteten. Swedish Pharmaceutical society. Stockholm.

Licht MR (1998) Sildenafil (Viagra) for treating male erectile dysfunction. Cleveland Clinic Journal of Medicine 65 (6): 301- 304.

Licht MR (1999) Use of oral sildenafil (Viagra) in the treatment of erectile dysfunction. Comparative Theraphy 25 (2): 90- 94.

Melman A, Gingell JC (1999) The epidemiology and pathophysiology of erectile dysfunction. Journal of Urology 161: 5- 11.

Montorsi F (1999) Efficacy and safety of fixed-dose oral sildenafil in the treatment of erectile dysfunction of various etiologies. Journal of Urology 53 (5): 1011- 1018.

Mouzou AP, Bulteau L, Raymond G (1999) The effects of *Securidaca longependunculata* root extract on ionic currents and contraction of cultured rat skeletal muscle cells. Journal of Ethnopharmacology 65: 157- 164.

Neuwinger HD (1996) African ethnobotany. Poisons and drugs. Chapman & Hall, London.

Viagra Datasheet. In the interest of Men's Sexual Health. Pfizer Laboratories (Pty) Ltd.

Singer C, William MDJ, Weiner MD (1994) Sexual dysfunction. Futura publishing company, Inc, New York.

Van Wyk B-E, Van Oudtshoorn B, Gericke N (1997) Medicinal plants of South Africa. Briza Publications, Pretoria.

CHAPTER 2

Plants used in this study and their medicinal uses

Contents

2.1. Introduction

Three indigenous, plants *Securidaca longependunculata* (Mpesu), *Wrightia natalensis* (Musunzi) and *Rhoicissus tridentata* (Mutumbula) have been used traditionally by Vhavenda people to treat erectile dysfunction for centuries.

S. longependunculata (Fresen) belongs to the subclass Rosidae, order Polygalales and family Polygalaceae. It is a family of about 80 genera and more than 950 species with a cosmopolitan distribution mostly in moist warm and temperate regions, primarily in the northern hemisphere (Leistner, 2000).

W. natalensis (Stapf), falls under the subclass Asteridae, order Gentianales and Apocynaceae family. It is a family of about 480 genera and 4800 species of almost cosmopolitan distribution, mostly in tropical, warm (especially Africa), and a few temperate regions. Of those, 90 genera and ± 696 species are indigenous to our region (Leistner, 2000).

Rhoicissus tridentata (Wild & R. B. Drumm) belongs to the subclass Rosidae, order Polygalales and family Vitaceae. It is a family of about 14 genera in the world and ± 1000 species distributed in the tropical to temperate regions of both hemispheres (Leistner, 2000).

2.1.1. *Securidaca longependuncula*

2.1.1.1. Distribution

The species of the genus are found in Africa, America and Asia. Only one species (*S. longependuncula*) grows in the southern African region in countries such as Namibia, Malawi, Zimbabwe, Zambia and South Africa. In South Africa the species occurs (Fig 1) only in the Gauteng-, Mpumalanga-, North-West- and Limpopo provinces (Leistner, 2000).

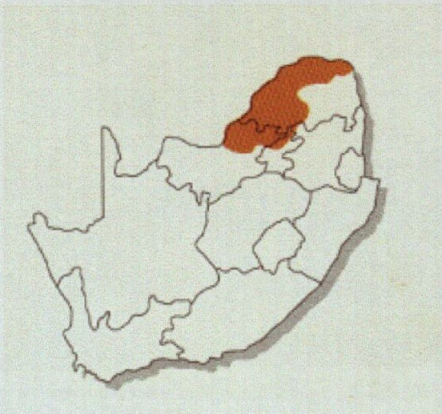


Figure 1: The South Africa distribution of *Securidaca longependuncula* (Van Wyk *et al.*, 1997).

2.1.1.2. Botanical description

It is one of the most beautiful flowering trees (or shrubs) of up to 10 m in height, with a pale gray smooth bark and a thin trunk. It has erect branches and stiff erect leaves, but sometimes with branchlets. Its leaves are variable in size and shape, grey-green, more or less hairless and about 30 mm long. Flowers are pink to purple in colour, produced with new leaves during summer (Palgrave, 1977). The fruit is more or less

round in shape with a single membranous wing of up to 4 mm long. They are puplish green when young but become pale brown when mature (Fig 2).

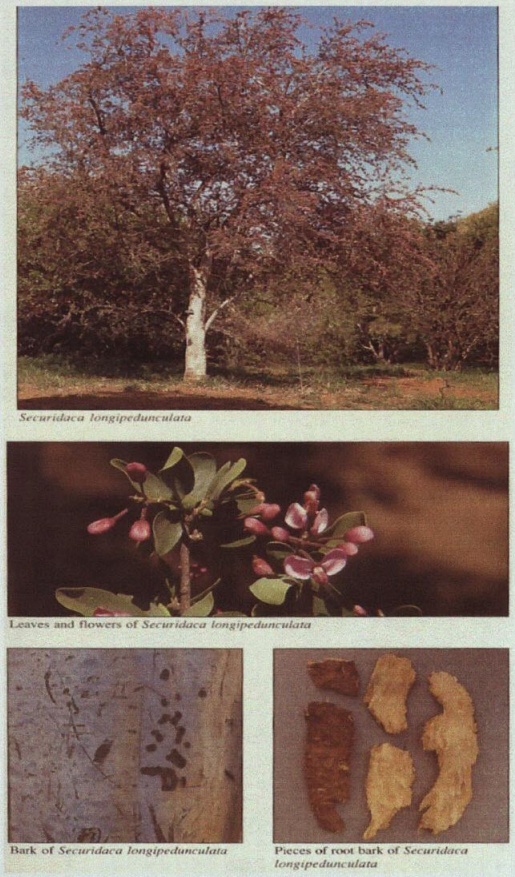


Fig 2: The leaves, flowers, stem bark and root bark of *Securidaca longependunculata* (Van Wyk *et al.*, 1997).

2.1.1.3. Medicinal value

S. longependunculata is a legendary medicinal and poisonous plant in Africa. It has been reported that it killed women in Zambia after they have inserted the root powder into their vaginas (Palgrave, 1977). Neuwinger (1996) reported that the roots are less poisonous than the stem bark. The plant is also used orally to cure different diseases. It is known to treat headache, malaria, impotence, inflammation, rheumatism, venereal diseases, snakebite, ulcers, backache, pneumonia, tuberculosis, sleeping

sickness, toothache, coughs, colds, fever and is used as an antidote for poisons (Neuwinger, 1996).

2.1.1.4. Plant chemistry

Very little is known about the secondary plant constituents of the genus *Securidaca*, although species such as *S. longependuculata* is widely used in Africa and other areas in the world (Hamburger *et al.*, 1985, Neuwinger, 1996). Some of the compounds isolated from *S. longependuculata* are shown in Figure 3 (Galeffi *et al.*, 1990, Van Wyk *et al.*, 1997).

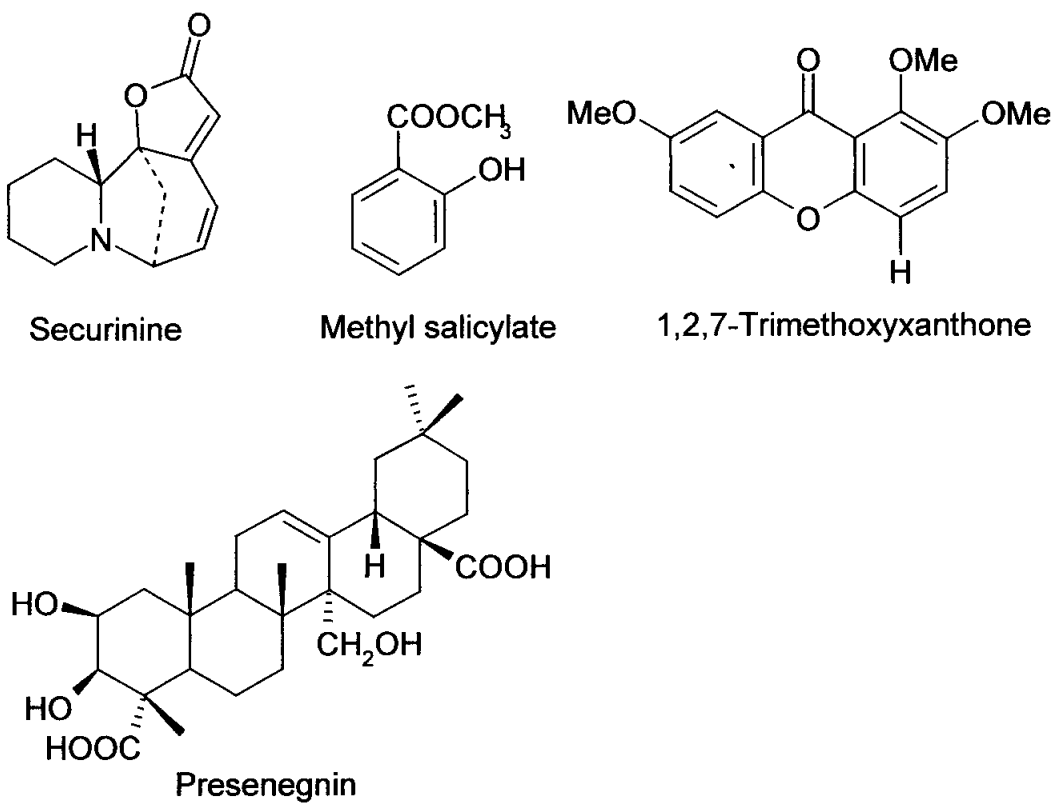


Fig 3: Compounds isolated from *Securidaca longependuculata* root bark.

2.1.2. *Wrightia natalensis.*

2.1.2.1. Distribution

Wrightia natalensis belongs to the tribe Wrightieae of the subfamily Apocynoideae. This genus consist of 23 species in the world, of which two are found in Africa and one (*W. natalensis*) grows in South Africa and Swaziland. In our country, it only occurs in the Limpopo-, Mpumalanga - and KwaZulu-Natal Provinces (Fig 4) (Leistner, 2000). It occurs in hot and arid regions, bushveld, sand forest and frequently on hillsides (Van Wyk & Van Wyk, 1997).

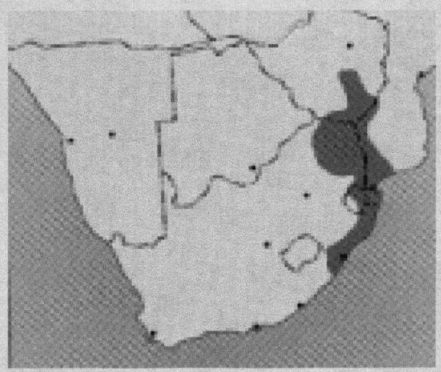


Fig 4: The Southern African distribution of *Wrightia natalensis* (Van Wyk & Van Wyk, 1997).

2.1.2.2. Botanical description

It is a shrub or small tree of up to 8 m in height, but occasionally reaches 15 m. It has a whitish milky latex. The bark is slightly furrowed or fissured. It has light clear green, opposite, simple, lanceolate leaves of about 15-20 mm wide. The small flowers are found in clusters towards the end of the branches, scented and creamy or pale

yellow in colour. The young, long, thin and dehiscent fruits are found in pairs and are dark green with white dots, becoming pale brown when mature (Fig 5) (Palgrave, 1977, Van Wyk & Van Wyk, 1997).

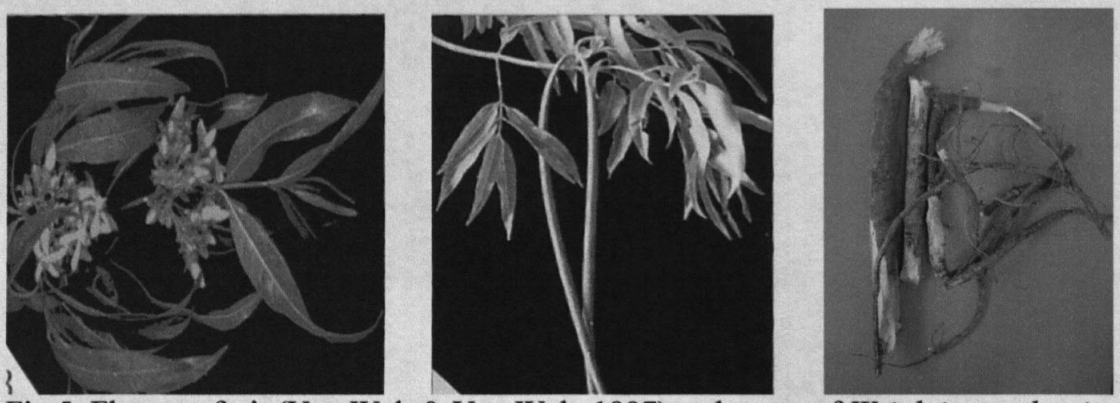


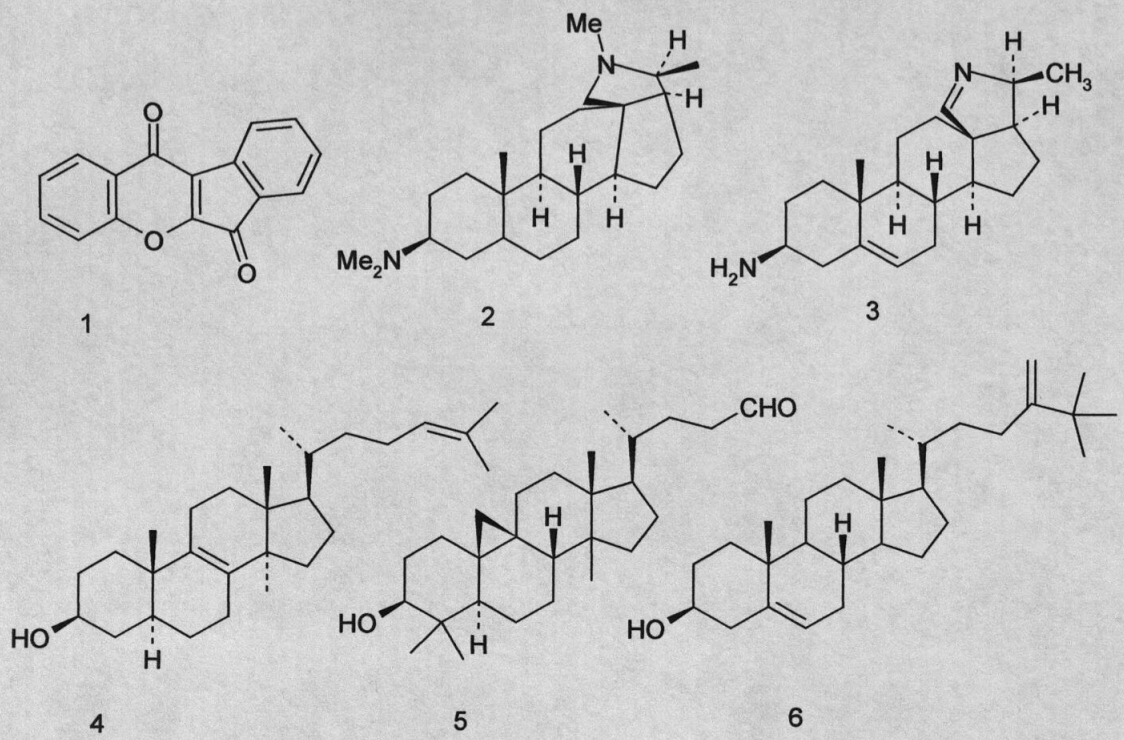
Fig 5: Flowers, fruit (Van Wyk & Van Wyk, 1997) and roots of *Wrightia natalensis*

2.1.2.3. Medicinal value

The species is known to be used to treat impotence and malaria (Galeffi *et al.*, 1990)

2.1.2.4. Plant chemistry

The family contains many alkaloid compounds (Van Wyk & Van Wyk, 1997), but to date, no compounds have been isolated from the species *W. natalensis.* Only compounds (Fig 6) from the related species, *W. tomentosa* and *W. tinctoria* were isolated and identified (Teresa *et al.*, 1987).



Compounds isolated from *W. tomentosa*

1. Benz (b) indeno (1,2-e) pyran-6, 11-dione.
2. N, N-Dimethylcon-5-enin-3β-amine, 9cl.
3. 23-Norcona-5, 18 (22)- dienin-3β-amine, 9cl.

Compounds isolated from *W. tinctoria*

4. 14-Methylcholesta-8, 24-dien-3-ol
5. 3-Hydroxy-4, 4, 14-trimethyl-9, 19-cyclocholan-24-al, 9cl.
6. 25-Methyl-5, 24 (28)-ergostadien-3-ol, 9cl.

Fig 6: Compounds isolated from the root bark ***W. tomentosa*** and ***W. tinctoria***

2.1.3. *Rhoicissus tridentata*

2.1.3.1. Distribution

Rhoicissus is a genus with about 10 species occuring in Africa of which 7 species are found in the eastern part of southern Africa. These 7 species are only absent in

Namibia, Botswana and the Northern Cape province (Fig 7) (Leistner, 2000). The plant is found in a variety of habitats (Van Wyk *et al.*, 1997).

Fig 7: The South African distribution of *Rhoicissus tridentata* (Van Wyk *et al.*, 1997).

2.1.3.2. Botanical description

R. tridentata is a climber with tendrils and the branches spreading outwards from a thick woody base. Sometimes the plant is a shrub of 2-3 m in height. The leaves are wedge-shaped, dark green in color with three leaflets. It is hairless above and with or without hairs below, reddish in autumn. The flowers are small, greenish yellow in colour with a small stalk (Van Wyk *et al.*, 1997). Fruits are fleshy and edible with a sour taste, almost spherical, red when young becoming black when mature (Fig 8).

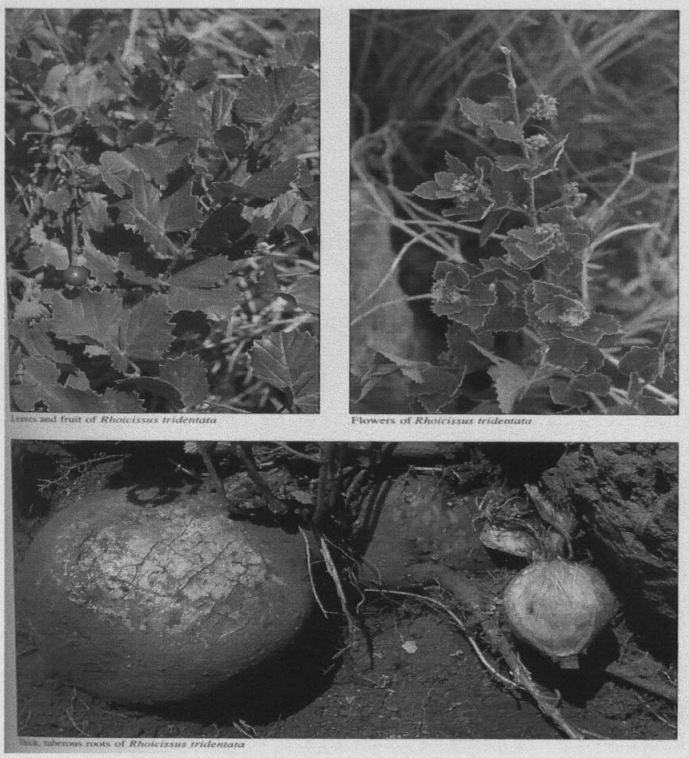


Fig 8: The leaves, fruit, flowers and root tubers of *R. tridentata* (Van Wyk *et al.*, 1997)

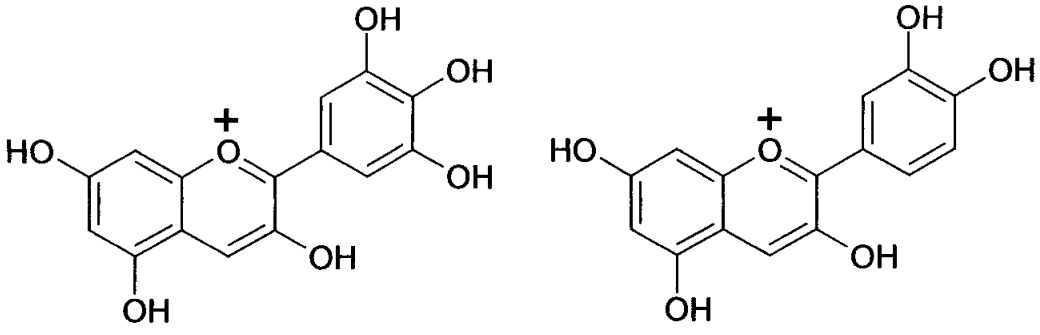
2.1.3.3. Medicinal value

The plant is used as a remedy for a number of diseases. It has been used as an anthelmic, enema, to relieve painful menstruation, to facilitate childbirth, to induce delayed labour, impotence, stomach ailments, dysmennorhoea, kidney and bladder complaints (Galeffi *et al.*, 1990).

2.1.3.4. Plant chemistry

There are no reports of compounds isolated from the species *R. tridentata*. Numerous phenolic compounds have been isolated and identified from the related *Vitis riparia*. Polyphenols, anthocyanins and proanthocyanidins were obtained from this species.

Delphinidin and cyanidine are both examples of anthocyanins (Fig 9) (Van Wyk *et al.*, 1997) isolated from *V. riparia.*



Delphinidin Cyanidin

Fig 9: Compounds isolated from *Vitis riparia*

2.2. References

Galeffi G, Federici E, Msonthi JD, Marini-Bettolo GB, Nnicoletti M (1990) New xanthones from *Ectiadiopsis oblongifolia* and *Securidaca longependunculata.* Fitoterapia LXI (1): 79- 82.

Hamburger M, Gupta M, Hostetmann K (1985) Flavonol glycosides from *Securidaca diversifolia*. Phytochemistry 24 (11): 2689- 2692.

Leistner OA (ed.) (2000). Seed plants of Southern Africa: Families and genera. *Strelitzia* 10. National Botanical Institute, Pretoria.

Neuwinger HD (1996) African ethnobotany. Poisons and drugs. Chapman & Hall, London.

Palgrave KC (1977) Trees of Southern Africa. Struik Publishers (Pty) Ltd, Cape Town.

Teresa J de P, Urones JG, Marcos IS, Basabe P, SexmeroCuadrado M[a]J, Fernandez Moro (1987) Triterpenes from *Euphorbia broteri*. Phytochemistry 26: 1767-1776.

Van Wyk B, Van Wyk P (1997) A field guide to trees of Southern Africa. Struik Publishers (Pty) Ltd, Cape Town.

Van Wyk B-E, Van Oudtshoorn B, Gericke N (1997) Medicinal plants of South Africa. Briza Publications, Pretoria.

CHAPTER 3

In vitro smooth muscle relaxation activity of plants used in the treatment of male erectile dysfunction

Contents

3.1. Introduction

Diseases and medical conditions like erectile dysfunction have been treated with plants long before orthodox medicine was available on this continent. Erectile dysfunction is a medical condition characterized by the inability to achieve or maintain erection sufficient to satisfactory sexual function (Montorsi *et al.*, 1999). Penile erection occurs through the relaxation of the corpus cavernosal smooth muscle induced by neurotransmitters released from the cavernorsal nerve and endothelial cells (Kim *et al.*, 1998). There has been a considerable interest in the use of medicinal plant extracts for the treatment of this ailment. Despite all advances in modern and orthodox medicine, traditional medicine still plays a significant role in the lives of many people, especially in Venda (a region in the Limpopo province of South Africa) (Mabogo, 1990).

A number of herbal plants such as *Securidaca longependunculata, Wrightia natalensis* and *Rhoicissus tridentata* occurring in Venda are being used to treat erectile dysfunction. Furthermore, these plants are traditionally used to treat many other ailments. For example, *S. longependunculata* is also used to treat sexually transmitted diseases, or as blood purifier. However, there have been no scientifically proven reports concerning the *in vitro* activity against erectile dysfunction of these species. This study was conducted to investigate whether extracts of these plants have a direct relaxing effect on the corpus cavernosum smooth muscle.

According to Godschalk *et al.* (1997), the normal male sexual function has three principal components: libido, erectile rigidity and orgasm. This study concentrates

only on the erectile rigidity, which relies on the interaction of psychological function, hypothalamic-pituitary-testicular function, autonomic and somatic nervous system function, penile arterial inflow and the veno-occlusive mechanism.

In the veno-occlusive mechanism, the penile artery is the main blood supply to the penis. This artery is divided into the dorsal and the cavernosal (profunda) arteries of the penis (Fig 3). The helicine arterioles are the end-arterioles of the cavernosal arteries and empty into the lacunar spaces within the corpus cavernosa. These spaces are formed by the smooth muscle trabeculae. These muscles must relax in order for blood to fill the lacunar spaces. Blood from these spaces drain into the short emissary veins which in turn drain into the deep dorsal veins and finally into the pudendal vein (Jonas *et al.*, 1991, Godschalk *et al.*, 1997).

When there is erotic stimulation, the parasympathetic activity increases, the NO/cGMP mechanism becomes activated and then produces cGMP. The smooth muscle is induced, penile arterial resistance is decreased and arterial inflow into the lacunar spaces is increased. As the blood fills the lacunar spaces, the sutunical venules are compressed between the expanding corpus cavernosa and the non-elastic tunica albuginea, trapping blood within the penis. When the arterial inflow increases and the venous outflow decreases, the pressure within the penis increases and the penis becomes erect (Godschalk *et al.*, 1997) (Fig 4).

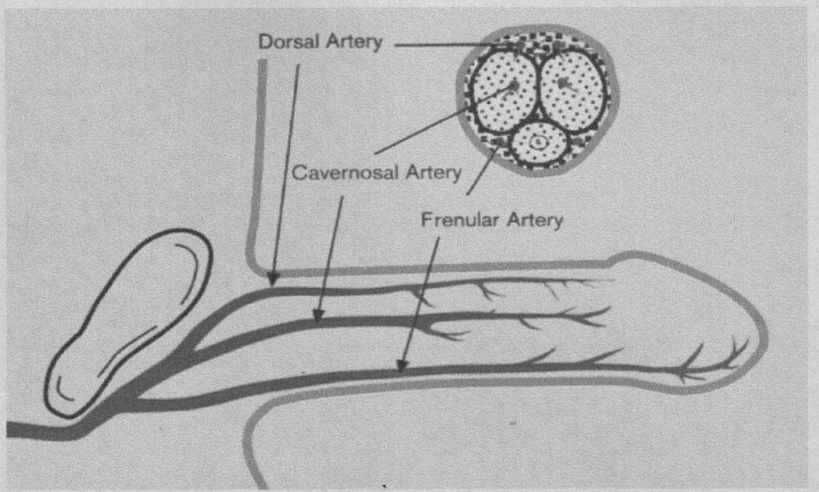


Fig 3: The mechanism of vascular supply of blood to the penis (Godschalk *et al.*, 1997)

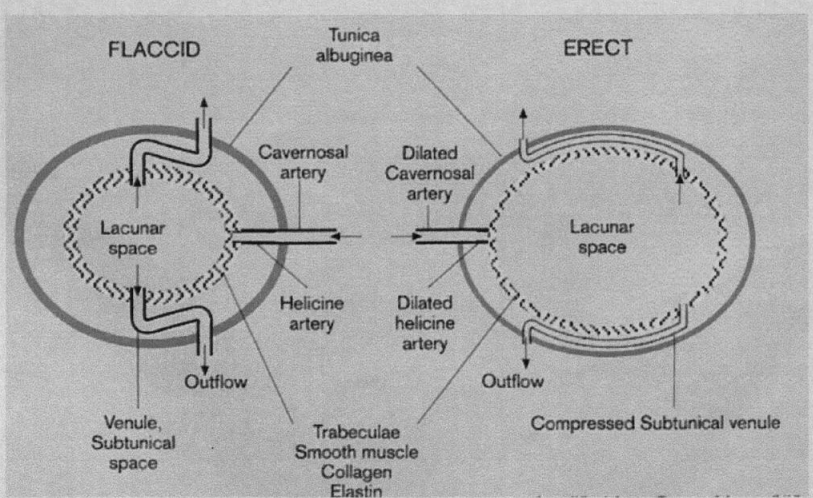


Fig 4: The veno-occlusive mechanism (Godschalk *et al.*, 1997)

3.2. Materials and methods

3.2.1. Collection of plant material

The root bark of *S. longepedunculata, W. natalensis* and *R. tridentata* were collected in Venda (South Africa) during June 1999. Plant selection were based on a ethnobotanical literature review (Mabogo, 1990; Galeffi *et al.*, 1990) and in consultation with a few traditional healers. Voucher specimens (NCR. 16, 17 & 18 respectively) were deposited and identified at the H.G.W.J. Schweickerdt Herbarium (PRU), University of Pretoria.

3.2.2. Preparation of extracts

Chloroform extracts of root bark of *S. longepedunculata* (40.6 g), *W. natalensis* (55.6 g) and *R. tridentata* (12.7 g) were prepared by homogenizing the powdered root bark in chloroform (3l) and stirring under reflux for 12 hours. The extracts were filtered and concentrated to dryness under reduced pressure at 37°C. The resultant residue was later dissolved in 1.0% dimethylsulfoxid (DMSO) to concentrations of 100, 50.0 and 1.0 mg/ml.

Ethanol extracts of root bark of *S. longepedunculata* (39.9 g), *W. natalensis* (51.8 g) and *R. tridentata* (12.6 g) were prepared through homogenizing the powdered root bark in ethanol (3l) and stirring for 12 hours. The extracts were filtered and

concentrated to dryness under reduced pressure at about 39°C. The resultant residue was later dissolved in 1.0% DMSO to concentrations of 100, 50.0 and 1.0 mg/ml.

3.2.3. Preparation of the corpus cavernosal smooth muscle

Male white New Zealand rabbits (mass 2.0 - 2.3 kg) were used for the experiment. The entire penis was kept in Krebs-phosphate buffered saline aerated with 95.0% O_2 and 5.0% CO_2 (Kim *et al.*, 1998). One or two strips of the corpus cavernosum smooth muscle (12 mm long and 1-2 mm thick) were dissected from each penis. The strips were mounted in an organ-bath chamber containing Krebs solution with the following composition: NaCl = 7.01 g/l, KCl = 0.34 g/l, KH_2PO_4 = 0.1 g/l, $NaHCO_3$ = 1.99 g/l, $CaCl_2$ = 0.2 g/l, $MgSO_4$ = 0.3 g/l and glucose = 1.8 g/l.

3.2.4. Preparation of Viagra

Commercially available Viagra (0.6 μg/ml in water) (Fig 1) was used as the positive control in this experiment.

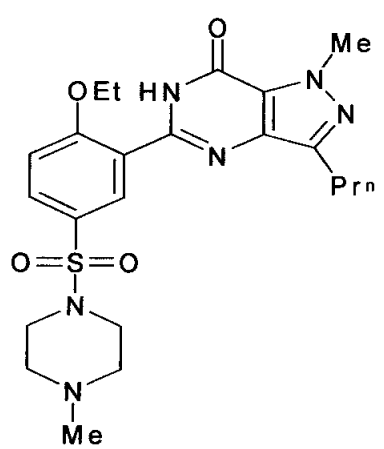


Fig 1: The chemical structure of Viagra

3.2.5. Determination of smooth muscle relaxation

A strip of rabbit corpus cavernosum smooth muscle (12 mm long and 1-2 mm thick) was mounted in a perfusion bath, with one end tied to the inside bottom of the perfusion bath and the other end to a thin wire connected to a Harvard isotonic force transducer for isotonic tension measurements (Fig 2). Changes in isotonic tension were recorded on a chart polygraph on a Harvard isotonic recorder. The corpus cavernosum muscle was perfused with 2 ml Krebs-PSS buffered saline and oxygenated with 95% O_2 and 5% CO_2 for 5 min to ascertain a stable baseline recording. This was followed by perfusion with 2 ml of $CaCl_2$-PSS (17.8 mg/ml) for muscle contraction was achieved. Baseline tension was set at the point of maximal contraction following the addition of calcium chloride into the experimental bath. $CaCl_2$-PSS was washed out after the maximal contraction. The Krebs-PSS (2 ml) was added after 5 minutes to cover the strips and 0.3 ml of extract (1.0, 50.0 and 100 mg/ml) were added after a stable baseline was obtained. The final extract concentration analyzed in the perfusion bath was 13.0 and 6.5 mg/ml. The extracts were administered through superfusion of the corpus cavernosum smooth muscle in a temperature controlled perfusion bath. The same procedure was repeated for the positive control, Viagra with the final concentration of $3.2x10^{-5}$ mg/ml and 1.0% DMSO was used as negative control. A frequency of 12 Hz was used for the stimulation of the corpus cavernosum smooth muscle relaxation during the experiment.

Fig 2: Experimental bath, Harvard isotonic force transducer and recorder.

3.2.6. Statistical analysis

The relaxation from the baseline was calculated as an average percentage of three replicates at the final concentrations of 13.0 and 6.5 mg/ml respectively. The significance of differences among groups were determined using the Student's t-test, with differences considered significant at $P<0.01$ (Heaton *et al.*, 1991, Calabro *et al.*, 1996, Keaton and Clark, 1997).

3.3. Results

S. longependunculata, W. natalensis and *R. tridentata* extracts (chloroform and ethanol) stimulated dose-dependent relaxation (Table 1) in the corpus cavenosum smooth muscle at concentrations of 13.0 and 6.5 mg/ml. The stimulation of relaxation was between 0 – 60 seconds after the application of the extracts. An Harvard isotonic transducer elicited relaxation of strips of corpus cavenosum smooth muscle in a frequency-dependant manner and the relaxation was significantly higher at 13.0 mg/ml of the *S. longependunculata* chloroform extract ($P<0.01$; n=3; Table 1) than the other two species. For both chloroform and ethanol extracts, the relaxation stimulated was significantly lower at the concentration of 6.5 mg/ml than at 13.0 mg/ml, except for the chloroform extract of *W. natalensis* which stimulated only 26% of relaxation. At a concentration of 13.0 mg/ml the relaxation induced was significantly higher than that of the $3.2x10^{-5}$ mg/ml of Viagra (positive control), whilst the later induced relaxation of the corpus cavernosum smooth muscle much better than the plant extract at 6.5 mg/ml. It was also found that the 1.0% of DMSO (negative control) used to dissolve the extracts, acted against the activity of the extracts by being responsible for 20.0% of contraction.

Table 1: Averages of relaxation % of the pre-contracted rabbit corpus cavernorsal smooth muscle.

Plant name	**Average % relaxation (Standard deviation)**					
	Chloroform Extracts		**Ethanol Extracts**		**Viagra**	**DMSO**
	13.0 mg/ml	6.5 mg/ml	13.0 mg/ml	6.5 mg/ml	$3.2x10^{-5}$ mg/ml	1.0%
W. natalensis	26.0 (5.7)	18.0 (0.9)	63.0[a] (13.1)	16.0 (4.3)	-	-
S. longependunculata	66.7[a] (12.8)	16.7 (4.7)	55.0[a] (7.1)	17.6 (6.1)	-	-
R. tridentata	30.7[a] (3.3)	1.7 (2.4)	43.0[a] (9.4)	15.0 (0.0)	-	-
	-	-	-	-	21.0 (5.7)	20.0 (4.1)[b]

[a] Significant difference compared to Viagra at $P< 0.01$

[b] Contraction

3.4. Discussion

The results obtained in this study are similar to that of the study conducted by Kim *et al.* (1998) where *Panax ginseng* relaxed the rabbit corpus cavernosum smooth muscle. According to Kim *et al.* (1998), the relaxation was immediate upon the application of the extracts. Based on the results it is postulated that these extracts can facilitate the flow of blood into the penis. This has been suggested since the extract relaxes corpus carvenosal smooth muscle. Erection can not take place if the corpus cavenosum smooth muscle is contracted. The contraction of the corpus carvenosum smooth muscle, similar to all smooth muscle contraction, is mediated by both translocations of calcium from extracellular sources and the release from intracellular sites especially the sarcoplasmic reticulum (Levin. *et al.*, 1997). Although there is a strong relationship between relaxation of the corpus cavenosum smooth muscle and sexual function (Keaton & Clark, 1997), it is also well known that there are many different factors that contribute to the deterioration of sexual function. It is reported that factors such as aging, diseases, some medications and cigarette smoking disturb the sexual function especially in older people who are exposed to these for a long time (Colabro *et al.*, 1996)

This study verified that some of the extracts analysed in this study, as well as Viagra, could relax the corpus cavenosum smooth muscle, which should lead to erection following the inflow of blood into the penis. It was also shown that the results could have been much better if 1.0% DMSO did not contract the muscle.

3.5. Acknowledgements

We acknowledge the National Research Foundation for their financial contribution. Thanks also to Mr. Dick Carr's family for the free supply of the corpus cavenosum smooth muscles. We also thank Billy Cloete for the help he provided us during the experiment.

3.6. References

Colabro A, Italiano G, Pescatori ES, Marin A, Gaetano O, Abatangelo G, Abatangelo G, Pagano F (1996) Physiological aging and penile erectile function: A study in the rat. European Urology 29: 240- 244.

Godschalk MF, Sison A, Mulligan T (1997) Management of Erectile Dysfunction by the Geriatrician. Journal of American Geriatric Society 45: 1240- 1246

Heaton JPW, Varrin SJ, Morales A (1991) The characterization of a Bio-Assay of erectile function in a Rat model. Journal of Urology 145: 1099-1102.

Jonas U, Thon WF, Stief. CG (1991) Erectile dysfunction. Springer-verlag Berlin Heidelburg. Germany.

Keaton AK, Clark JT (1997) Effects of Angiotensin 11 on sexual function, Blood pressure, and Fluid intake are differentially affected by AT-1 Receptor Blockade. Physiology and Behavior 64 (3): 339- 346.

Kim HJ, Woo DS, Lee G, Kim JJ (1998) The relaxation effects of ginseng saponin in rabbit corporal smooth muscle. British Journal of Urology 82: 744-748.

Mabogo DEN (1990) The ethnobotany of Vhavenda. MSc Thesis. University of Pretoria, Pretoria.

Levin RM, Hypolite JA, Broderik G, Broderik A (1997) Evidence for a role of intracellular- calcium release in Nitric Oxide- Stimulated relaxation of the rabbit corpus cavenorsum. Journal of Andrology 3: 246-249.

Montorsi F, McDermott TED, Morgan R, Olsson A, Scholtz A, Kirkeby HJ, Osterloh IH (1999) Efficacy and safety of fixed-dose oral sildenafil in the treatment of erectile dysfunction of various etiologies. Journal of Urology 53(5): 1011-1018.

CHAPTER 4

Antibacterial activity of crude extracts from *Securidaca longependunculata, Wrightia natalensis and Rhoicissus tridentata*

Contents

4.1. Introduction

There are thousands of plants used traditionally to treat various ailments that have been shown to contain bioactive compounds. Medicinal plants continue to play a significant role in primary health care as therapeutic remedies in African countries (Sokemen *et al.*, 1999). Despite being neglected largely by western people, medicinal plants remain the source of many drugs for pharmaceutical companies globally.

In South Africa, the demand for medicinal plants is exceeding supply because of population growth, unemployment and increasing levels of urbanization. People use detrimental practices such as the uprooting of plants and over-exploitation. Many of the valuable species will soon no longer be found in the wild. As a result, botanists and other scientists are now concerned about the cultivation, harvesting and conservation of medicinal plants.

This study was aimed at investigating three ethnobotanical selected Venda plants names for their antibacterial activity through agar dilution methods. In Chapter 3 the effect of these plants on the treatment of erectile dysfunction were investigated. However, very few scientific work has been done on the antibacterial activity of these herbal plants (Lin et al., 1999)

4.2. Materials and methods

4.2.1. Collection of plant material

Root bark of *S. longependunculata, W. natalensis* and *R. tridentata* were collected from Venda, a region in the Limpopo Province of South Africa. Plants were selected based on a literature review and consultation with a few traditional healers and plant herbalists. Voucher specimens (NCR 16, 17 and 18) were deposited at the H.G.W.J. Schweickerdt Herbarium (PRU), University of Pretoria.

4.2.2. Preparation of extracts

4.2.2.1. Chloroform extracts

The chloroform extracts of *S. longependunculata* (51.0 g), *W. natalensis* (43.2 g) and *R. tridentata* (38.1 g) were prepared by stirring the powdered root bark in 1.5l of chloroform for about 24 hrs under reflux. These extracts were then filtered and concentrated to dryness at reduced pressure. The resultant residue was later dissolved in 1.0% DMSO to a concentration of 100.0 mg/ml.

4.2.2.2. Ethanol extracts

The ethanol extacts of *S. longependunculata* (50.1 g), *W. natalensis* (41.4 g) and *R. tridentata* (39.3 g) were prepared by stirring the powdered root bark in 1.5l of ethanol for about 24 hrs under reflux. These extracts were then filtered and concentrated to dryness at reduced pressure. The resultant residue was later dissolved in 1.0% DMSO to a concentration of 100 mg/ml.

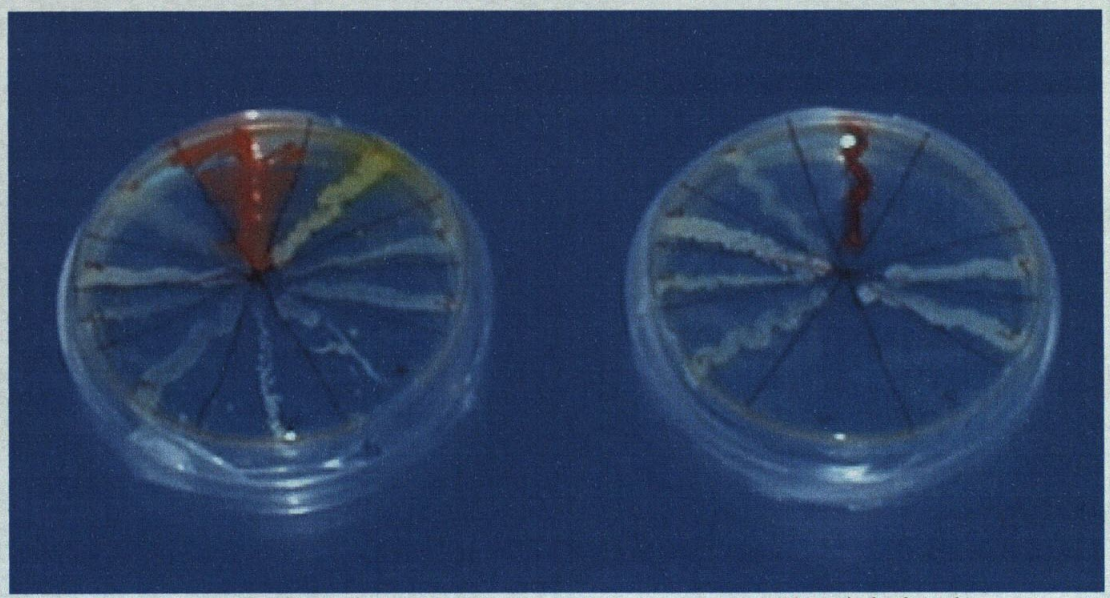
4.2.3. Bacteria

The following bacteria were tested for susceptibility and were obtained from the Department of Microbiology and Plant Pathology, University of Pretoria: Gram-positive: *Bacillus cereus, B. pumilus, B. subtilis, Staphylococcus aureus* and Gram-negative: *Enterobacter cloacae, Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa, Serratia marcescens* and *Enterobacter aerogenes*. Each organism was maintained on nutrient agar slant and was recovered for testing by growth in nutrient broth (No. 2, Biolab) for 48 hrs at 37°C. Before streaking, each culture was diluted 1:10 with fresh sterile nutrient broth (Meyer & Dilika 1996).

4.2.4. Antibacterial activity

Plants extracts were sterilized by filtering through 0.22 μm syringe fitted filters and then added to autoclaved nutrient agar (Biolab). Before congealing, 10.0 ml of nutrient agar

medium containing the plant extract was added aseptically to each petri dish and swirled carefully until the agar began to set. The organisms were streaked in radical patterns on agar plates containing plant extracts, incubated at 37°C (Fig 1) and observed after 24h (Mitscher *et al.*, 1972). Complete inhibition of bacterial growth was expected for an extract to be declared active. The extracts were tested at 50.0, 20.0 and 1.0 mg/ml and three replicates were used for treatments. A blank plate containing only nutrients agar and another containing nutrient agar and 1.0% DMSO were used as a controls (Meyer & Dilika 1996).



Control 50 mg/ml ethanol extracts

Fig 1: Effect of ethanol extracts of *Securidaca longependunculata* on bacteria at 50 mg/ml.

4.2.5. Direct bioassay

For direct bioassays on thin layer chromatography (TLC) plates, 20.0 µl chloroform extract (100 mg/ml) were applied to silica gel (Merck) and developed with chloroform-ethylacetate-hexane (4:2:1). *B. cereus* was cultured in sterile nutrient broth for 24 hrs and centrifuged at 3000 rpm for 20 minutes, the supernatant discarded and the sedimented bacteria resuspended in fresh sterile nutrient broth to an absorbance of 0.84 at 560 nm with a spectrophotometer (Meyer & Dilika, 1996). A fine spray was then used to transfer the bacterial suspension onto the TLC plates. These plates were then dried until they appeared translucent and incubated at 37 °C for 24 hrs in humid conditions. After 24 hrs the plates were sprayed with an aqueous solution of 0.02 g/ml p-iodonitrotetrazolium violet. The plates were then reincubated at 37°C for a few hours. Each experiment was repeated 3 times. The inhibition of the growth of bacteria could be seen as a white spots on a reddish-brown background (Fig 2).

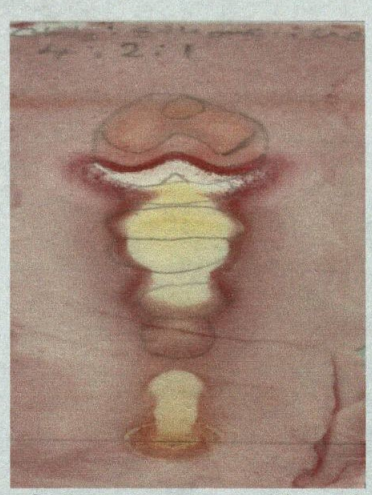


Fig 2: Inhibition zones of *B. cereus* resulting from chloroform extracts of *S. longependunculata* root bark after development in chloroform-ethylacetate-hexane (4:2:1).

4.3. Results and discussion

The results are shown in Table 2. All the plants showed remarkable activity against nearly all the bacteria tested in this study. Chloroform extracts showed better activity than ethanol extracts as it completely inhibited all Gram-positive bacteria. Almost all extracts inhibited the growth of *B. cereus* and *S. aureus* bacteria. Although Gram-negative bacteria are usually more resistant than Gram-positive bacteria, *E. cloacae* were completely inhibited by all extracts (Kudi *et al.*, 1999). This was not the case in studies by Rabe and Van Staden (1997), Meyer and Lall (2000) where all Gram-negative bacteria were exhibited. In general, the chloroform extracts of *S. longependunculata* and *W. natalensis* showed significant activity on Gram-negative bacteria as well as Gram-positive bacteria.

More than five zones of bacterial growth inhibition (Fig 2) appeared as a white spots on a reddish-brown background of the TLC plate. Isolation and identification of the bioactive compounds are discussed in Chapter 6. UV light could detect almost all of these bioactive compounds at 366 nm.

Table 2: Antibacterial activity expressed as minimum inhibition concentration (MIC) values of different extracts.

Bacteria	**Gram +/-**	**MIC (mg/ml)**					
		S. longependunculata		***W. natalensis***		***R. tridentata***	
		Chloroform	**Ethanol**	**Chloroform**	**Ethanol**	**Chloroform**	**Ethanol**
B.cereus	+	20	50	1	50	50	na
B.pumilus	+	20	na	50	na	50	na
B.subtilis	+	20	na	50	na	50	na
S.aureus	+	20	50	1	na	50	50
E.cloacae	-	20	50	1	20	50	50
E. coli	-	20	na	50	na	na	50
K. pneumonia	-	20	na	50	na	na	50
P. aeruginosa	-	na	na	50	na	na	na
S. marcescens	-	20	na	50	50	na	na
E. aerogenes	-	20	na	na	na	na	na

na: not active

4.4. Acknowledgements

The National Research Foundation (NRF) of South Africa is thanked for their financial support. We also thank the Department of Microbiology and Plant Pathology for the supply of microorganisms.

4.5. References

Kudi AC, Umoh JU, Eduvie LO, Gefu J (1999) Screening of some Nigerian medicinal plants for antibacterial activity. Journal of Ethnopharmacology 67 (2): 225- 228.

Meyer JJM, Dilika F (1996) Antibacterial activity of *Helichrysum pendunculatum* used in circumcision rites. Journal of Ethnopharmacology 53: 51- 54

Lall N, Meyer JJM (2000) Antibacterial activity of water and acetone extracts of the roots of *Euclea natalensis*. Journal of Ethnopharmacology 72: 313- 316.

Lin J, Opoku A.R, Geheeb-Keller M, Hutchings A.D, Terblanche S.E, Jager A. K, Staden J (1999) Preliminary screening of some traditional zulu medicinal plants for anti-inflammatory and anti-microbial activities. Journal of Ethnopharmacology 68: 267-274.

Mitscher LA, Leu R, Bathala MS, Wu W, Beal JL (1972) Antimicrobial agents from higher plants. 1. Introduction, rationale and methodology. Lloydia 35 (2):152-166.

Rabe T, Van Staden J (1997) Antibacterial activity of South African plants used for medicinal purposes. Journal of Ethnopharmacology 56: 81- 87.

Sonkemen A, Jones BM, Erturk M (1999) The *In vitro* antibacterial activity of Turkish medicinal plants. Journal of Ethnopharmacology 67 (1): 79- 86.

CHAPTER 5

In vitro effect of *Securidaca longependunculata* extracts on sperm parameters

Contents

5.1. Introduction

In response to modern, sophisticated, chemical medication, there has been a renewed interest in medicinal plants, which are being shown to be both effective and safe (Adimoelja, 2000). Though many practitioners of modern medicine dismiss traditional healers as quakery, it is well known that plants are an important source of medicine and there is now a growing scientific need to investigate their effectiveness and safety. Although it seems implausible to many people, it is a known fact that herbal plants can treat incurable medical ailments within a very short period. It is for these reasons that scientists have developed a renewed interest into studies on extraction, isolation and structure elucidation of their active biological components.

Aproximately 80% of the black population in South Africa live in rural areas and depend on traditional medicine to treat their common diseases (Netshiluvhi, 1999). There has been a number of herbal plants used for the treatment of sexually related problems i.e. infertility, impotence and sexual transmitted diseases. Very few scientific studies have been conducted on herbal plants, which are used in the treatment of infertility and impotence, compared to that of the sexual transmitted diseases. Ondrizek *et al.* (1999) reported the effect of extracts of *Hypericum perforatum*, *Serenoa repens, Sabal serrulatum* and *Ginkgo biloba* on sperms cells. The results indicated that at a concentration of 0.6 mg/ml motility was inhibited within an hour of incubation by *Hypericum perforatum* and after 24 hrs of incubation by *Serenoa repens* (9.0 mg/ml). Kim *et al.* (1998) conducted a study on the effect of *Panax ginseng* extracts on rabbit corpus cavernosum smooth muscle. The results of this study indicated the relaxation of

the smooth muscle, which is associated with erection after the application of 0.5-8.0 mg/ml of *Panax ginseng* extracts.

Although there was no scientific proof of their pharmacological properties in the past, V0havenda traditional healers have used reliable herbal plants like *S. longependunculata* to treat erectile dysfunction. Apart from this use, many African tribes use it as a general remedy for several other ailments such as coughs, colds, fever, backache, toothache, sleeping sickness, venereal disease, malaria, impotence, inflammation, rheumatism, snakebite, tuberculosis, ulcers and pneumonia, (Galeffi, *et al.* 1990). Palgrave (1977) reported that the plant is also used as a contraceptive.

Studies on the effects of *S. longepenunculata* extracts on sperm cells are lacking. It is an undisputed fact that sperm motility plays a significant role during the process of fertilization more than any other factor (Zheng & Zheng, 1996). The amalgamation of the sperm and egg membranes is also important for spermatozoa to carry out fertilization, as it requires high membrane integrity and the vitality of membranes. If either membrane is damaged, fertilization will be impaired (Zheng & Zheng, 1996). The aim of this study was to analyze the effect on parameters of sperm cells incubated in the presence of different concentrations (1.0-10.0 mg/ml) of extracts of this plant.

5.2. Materials and methods

5.2.1. Collection of plant material

The root bark of *S. longependunculata* was collected in Venda (South Africa). A voucher specimen (NCR.16) was deposited and identified at the H.G.W.J. Schweickerdt Herbarium (PRU), University of Pretoria.

5.2.2. Preparation of extract

An ethanol extract of *S. longependunculata* was prepared by homogenizing 38.3 g of the powdered root bark in ethanol and extracting it for 24 hours. The extract was filtered and concentrated to dryness under reduced pressure at 37°C. The resultant residue was later dissolved in 9.8 g Ham's F10 medium; 0.2452 g Ca-lactate; 0.05 g penicillin-G; 2.1060 g sodium bicarbonate and Sabax water to the concentrations of 10.0, 6.5, 2.5 and 1.0 mg/ml.

5.2.3. Sperm processing and determination of motility

Fresh sperm was obtained from a single donor to avoid interdonor variability. Washing of sperm was done in replicate. It consisted of layering the semen on top of a discontinuous two-layer colloid gradient (e.g. Pure Sperm- Nidacon) in a 13.5 ml centrifuge tube. The

bottom layer consisted of 1.0 ml of 80.0% Pure Sperm in Ham's F10, whilst the next layer consisting of 1.0 ml of 40.0% Pure Sperm in Ham's F10 and 1.0 ml of semen on top. The gradients were centrifuged at 300 g for 20 minutes. The supernatant was removed from each tube and the pellet resuspended in 3.0 ml sperm washing medium (Ham's F10 supplemented with 10.0% maternal serum). The tubes were then centrifuged at the same speed for 10 minutes. The supernatant was removed and washed again with 3.0 ml 10.0% Ham's F10 at 300 g for 10 minutes. The final pellet was resuspended in 1.0 ml of 10.0% Ham's F10 (control), or 1.0 ml of medium supplemented with the herbal extract to make final concentrations of 1.0, 2.5, 6.5 and 10.0 mg/ml. The sperm was then incubated at 37°C, under 5.0% CO_2 in air (WHO, 1999) with test tube tops loosened (for gaseous exchange) for 48 hrs. Motility analysis as described in the WHO (1999) manual was performed after 0, 4, 24, and 48 hrs of incubation. The Eosin-Nigrosin stain and the HOS tests were performed after 24 and 48 hrs of incubation to determine sperm vitality and membrane integrity.

5.2.4. Sperm vitality determination

5.2.4.1. Eosin-Nigrosin test

The Eosin-Nigrosin test was conducted because it is the least expensive and simplest technique used for sperm vitality assessment, yet reliable. One drop (11.5 µl) of washed sperm was mixed with two drops (23.0 µl) of 1.0% Eosin Y. Three drops (34.5 µl) of 10.0% Nigrosin solution were then added after 30 seconds. A drop of sperm-Eosin-

Nigrosin mixture was placed on a microscope slide and smeared. The smeared microscope slide was then air dried and examined under an oil immersion (100x magnification) light microscope. At least 100 sperm cells were counted each time. The proportion of live spermatozoa was determined; using this staining technique based on the principle that dead cells with damaged plasma membranes will stain red. The live sperm remain unstained and whitish in colour (WHO, 1999).

5.2.4.2. Hypo-Osmotic Swelling (HOS) test

The HOS test is the most appropriate technique for investigating the membrane integrity and viability of human sperm (WHO, 1999). The test is based on the principle that fluid is transported across the sperm tail membrane under hypo-osmotic conditions until equilibrium is reached. Due to this influx of fluid, the tail expands and coils into different characteristic patterns, considered the HOS response. If the cells are damaged, fluids pass through the membrane, but no accumulation of fluids takes place and consequently no tail curling occurs (Hossain *et al.*, 1999). When more than 60.0% of the spermatozoa undergo a tail swelling the HOS test of a specimen is considered normal, whilst if less than 50.0% of the spermatozoa show sperm tail swelling the specimen is considered abnormal (Mortimer, 1994; WHO, 1999).

In this method, 1.0 ml of swelling solution was warmed in a closed 6.0 ml tube at 37 °C for about 5 minutes. 0.1 ml of washed sperm was added and mixed gently with a pipette

and kept at 37°C for at least 30 minutes. The solution was mixed again, a drop (11.5 µl) of the solution was transferred to a clean microscope slide and mounted with a 24 x 24 mm^2 coverslip. At least 100 sperm cells were examined with a phase-contrast microscope at a 400x magnification.

5.2.5. Osmolarity determination

The osmolarity of the prepared medium was in the order of 310 mOsmol/kg and needed to be adjusted to 280 mOsmol/kg. The osmometer was calibrated while the ingredient was dissolved completely.

5.2.6. Statistical analysis

The effect on the sperm parameters was calculated as an average percentage of three replicates at the concentrations of 10.0, 6.5, 2.5 and 1.0 mg/ml. The significant differences among groups were determined using the Student's t-test, with differences considered significant at $p< 0.05$ (Heaton *et al.*, 1991, Calabro *et al.*, 1996, Keaton and Clark, 1997).

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5.3. Results

5.3.1. Effect of extracts on sperm motility

S. longependunculata extracts at the concentration of 1.0 mg/ml did not affect the sperm motility. However, sperm motility was inhibited from 4 hrs at 2.5 mg/ml and 0 hrs at 6.5 and 10.0 mg/ml as the concentration increased. The sperm motility was already zero at a concentration of 10.0 mg/ml and near to zero at the concentration of 6.5 mg/ml (Table 1).

Table 1: The influence of herbal extracts on the progressive motility expressed as % at a concentration range of 1.0-10.0 mg/ml

Concentration	**Number of hours during incubation**			
	0 hrs	**4 hrs**	**24 hrs**	**48 hrs**
1.0 mg/ml	87.7 (2.0)	80.9 (3.0)	73.9 (3.6)	64.0 (5.4)
2.5 mg/ml	82.2 (6.6)	64.6 (4.0)	40.0 (2.9)	5.1 (1.0)
6.5 mg/ml	60.1 (7.0)	1.8 (0.4)	0.0 (0.0)	0.0 (0.0)
10.0 mg/ml	47.0 (3.5)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Control	90.8 (3.1)	86.4 (2.0)	77.9 (3.0)	70.0 (4.0)

5.3.2. Effect of extracts on sperm vitality

According to the Eosin-Nigrosin test (Table 2), where the control indicates that around 50.0% of the spermatozoa were alive after 24 and 48 hrs of incubation, only 36.0% of spermatozoa were alive at 1.0 mg/ml. When the concentration was increased to 2.5, 6.5 and 10 mg/ml nearly 100% of spermatozoa were dead.

Table 2: The vitality of sperm (%) determined with the Eosin-Nigrosin test after 24 and 48 hrs of exposure to different concentrations of herbal extracts

Concentration	**Vitality (%) of sperm**	
	24 hrs	**48 hrs**
1.0 mg/ml	37.17 (1.6)	36.76 (1.3)
2.5 mg/ml	4.89 (0.2)	2.28 (0.6)
6.5 mg/ml	0.0 (0.0)	0.0 (0.0)
10.0 mg/ml	0.0 (0.0)	0.0 (0.0)
Control	51.11 (5.0)	50.0 (1.9)

5.3.3. Effect of extracts on sperm membrane integrity

The sperm membrane integrity seems to be affected by the herbal extracts as the concentration increased (Table 3). The highest decrease in membrane integrity was noted

at the concentration of 6.5 and 10.0 mg/ml where only 9.0-13.0% of sperm cells were HOS positive. As in the case of sperm vitality and motility the concentration of 1.0 mg/ml remains harmless on sperm membrane integrity.

Table 3: The results of the HOS tests expressed as % after 24 and 48 hrs of exposure to different concentrations of herbal extracts

Concentration	**% Membrane integrity**	
	24 hrs	**48 hrs**
1.0 mg/ml	70.2 (2.3)	57.6 (0.7)
2.5 mg/ml	31.7 (2.1)	14.1 (1.4)
6.5 mg/ml	13.6 (2.0)	12.5 (1.5)
10.0 mg/ml	11.0 (1.2)	9.9 (0.2)
Control	72.7 (1.2)	68.7 (1.7)

5.3.4. Effect of osmolarity on sperm cells

When assessing the osmolarity of the sperm cells using the Friedman test (Table 4), there was no significant difference noted between the progressive motility of the spermatozoa in relation to medium osmolarity over time. This indicates that the results obtained were influenced by increases in the concentrations of extracts.

Table 4: The influence of osmolarity on the progressive motility expressed as % value of spermatozoa over 48 hrs.

Concentration	Number of hours during incubation			
	0 hrs	**4 hrs**	**24 hrs**	**48 hrs**
283 mOsm/kg	75.7 (2.6)	74.1 (3.2)	60.7 (3.5)	35.3 (3.4)
312 mOsm/kg	75.7 (5.0)	73.0 (3.6)	61.6 (5.0)	36.7 (4.5)
317 mOsm/kg	75.5 (3.5)	74.2 (3.2)	57.2 (3.8)	30.5 (4.3)
440 mOsm/kg	72.5 (4.5)	71.2 (3.1)	53.2 (4.1)	32.6 (6.1)
Control	77.6 (3.1)	76.1 (3.7)	64.2 (5.0)	39.9 (3.2)

5.4. Discussion

It is known that fertilization is principally dependent on sperm motility and membrane integrity. Fertilization will be impaired if the sperm motility and membrane integrity are damaged (Zheng & Zheng, 1996). The present study demonstrates a potent inhibitory effect on sperm motility, vitality and membrane integrity in the presence of extracts at concentrations higher than 1.0 mg/ml. The results on sperm motility are comparable to the study conducted by Ondrizek *et al.* (1999) where *Hypericum perforatum* extracts (0.6 mg/ml) inhibited the sperm motility. Although the mechanism involved in the inhibition of sperm motility is unknown, it is clear that with no or poor sperm motility, vitality and membrane integrity, fertilization will be impaired. The fact that the extracts of *S.*

longependunculata affected several sperm parameters supports the use of this species as a contraceptive as reported by Palgrave (1977).

In contrast to this study, *Hypericum perfratum* extracts did not significantly effect sperm motility when tested over 48 hrs. At a concentration of 0.6, 1.2 and 9.0 mg/ml, studies by Ondrizek *et al.* (1999) on *Hypericum perfratum*, *Echinacea species*, *Ginkgo biloba* and *Serenoa repens* respectively showed a decrease in motility at 24 hrs or more of incubation whilst *S. longependunculata* extracts inhibited motility between 0-4 hrs of incubation. At high concentrations of *S. longependunculata* extracts, the membrane integrity decreased significantly to 9.0-13.0% whilst the control was 70.0% and more. The amount of sperm cells alive at 6.5 mg/ml and 10.0 mg/ml were 0.0% after 24 hrs compared to 48.0% of the control.

The inhibitory effect was caused by the extracts rather than changes in the pH as the osmolarity of the control showed no significant difference at a similar pH of 6.5. In general, the results showed that *S. longependunculata* extracts did not have an effect at the lower concentration, while at higher concentrations the sperm was affected. The results obtained here were *in vitro* and suggests that the low concentration might not affect the sperm *in vivo*. It is possible that the compound responsible in relaxing the rabbit corpus cavernosal smooth muscle might not be the one affecting the sperm cells as the extracts contain many different conmpounds. The investigation of the effect of the isolated compounds on sperm was not in the scope of this study, though it will be investigated in the near future.

Although sperm motility, vitality and membrane integrity were affected at high concentrations of *S. longipendunculata* extracts, preliminary results suggest that the DNA integrity (CMA_3) of the sperm cells was not affected at any concentrations used. This interesting result was not part of this study and will be further investigated in future.

5.5. Acknowledgements

Acknowledgements are due to Dr C. Huyser, Miss T.G. Raidani and Mrs S.P. Mdlalose from the Reproduction Biology Laboratory of the Department of Obstetrics and Gynaecology at the University of Pretoria and the Pretoria Academic Hospital.

4.6. References

Adimoelja A (2000) Phytochemicals and the breakthrough of traditional herbs in the management of sexual dysfunction. International Journal of Andrology 23, Supplement 2: 82- 84.

Colabro A, Italiano G, Pescatori ES, Marin A, Gaetano O, Abatangelo G, Abatangelo G, Pagano F (1996) Physiological aging and penile erectile function: A study in the rat. European Urology 29: 240- 244.

Galeffi G, Federici E, Msonthi JD, Marini-Bettolo GB, Nicoletti M (1990) New Xanthones from *Ectiadiopsis oblongifolia* and *Securidaca longipendunculata.* Fitoterapia LXI (1): 79- 81.

Heaton JPW, Varrin SJ, Morales A (1991) The characterization of a Bio-Assay of erectile function in a Rat model. Journal of Urology 145: 1099- 1102.

Hossain AM, Selukar R, Barik S (1999) Different effect of common laboratory treatments on hypo-osmotic swelling responses of human spermatozoa. Journal of Assisted Reproduction and Genetics 16: 30- 34.

Keaton AK, Clark JT (1997) Effects of Angiotensin 11 on sexual function, Blood pressure, and Fluid intake are differentially affected by AT-1 Receptor Blockade. Physiology and Behavior 64 (3): 339- 346.

Kim HJ, Woo DS, Lee G, Kim JJ (1998) The relaxation effects of ginseng saponin in rabbit corporal smooth muscle: is it a nitric oxide donor? British Journal of Urology 82: 744- 748.

Mortimer D (1994) Practical Laboratory Andrology: Chapter 3:Semen analysis. Oxford University Press. 43- 87.

Netshiluvhi TR, (1999) Demand , propagation and seedling establishment of selected medicinal trees. S.A. Journal of Botany 65 (5& 6): 331- 338.

Palgrave KC (1977) Trees of Southern Africa. Struik publishers (Pty) Ltd, Cape Town.

Ondrizek RR, Chan PJ, Patton WC, King A (1999) Inhibition of human sperm motility by specific herbs used in alternative medicine. Journal of Assisted Reproduction and Genetics 16: 87- 91.

World Health Organization (1999) WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction. Cambridge University Press.

Zheng R-L, Zheng H (1996) Effects of ferulic acid on fertile and asthenozoospermic infertile human sperm motility, viability, lipid peroxidation, and cyclic nucleotides. Free Radical Biology & Medicine 22 (4): 581- 586.

CHAPTER 6

Xanthones isolated from *Securidaca longependuncula*.

Contents

6.1. Introduction

Although species such as *Securidaca longependuncula*ta are widely used in African folk medicine, very little is known about the chemical constituents of these plants (Hamburger *et al.*, 1985). The species was selected for isolation of compounds because it showed the most activity on smooth muscle relaxation (Chapter 3). A short review on the secondary metabolites isolated from the genus *Securidaca*, follows.

Compounds isolated from the root bark. Prista and Alves (1958) isolated one of the major components, methyl salicylate (Fig 1a) from *S. longependuncula*ta. According to Moers (1966), the plant contains the same sapogenin as found in the folk medicine plant, *Polygala senega*, senegin. Delaude (1971) isolated the same aglycones from both *S. longependuncula*ta and *P. senega*, senegenin, senegenic acid (Fig 1b) and de(hydrochloro)senegenin as well as the sugars glucose, fructose, rhamnose, galactose and arabinose.

Dugan *et al.*, (1964) isolated presenegenin (Fig 1c) from *S. longependuncula*ta. On treatment with ethanolic HCl it is converted quantitavely to a mixture of senegenin and senegenic acid (polygalic acid). In the root it is present in the free form as well as glycosides. A new xanthone 1,7-dimethoxy-2-hydroxyxanthone (Fig 1d) was isolated by Geleffi *et al.*, (1990) from the roots collected in Malawi.

Costa *et al.*, (1992) reported psychoactive ergot alkaloids in the methanolic extract of the roots. Elymoclvine (Fig 1e) and dehydroelymoclavine were identified along with a novel ergoline, alkaloid A (Fig 1f). The presence of other alkaloids with an ergoline

skeleton was identified by Scandola *et al.* (1994). Such alkaloids are very rarely found in higher plants. Mahmood *et al.*, (1993) extracted 3,4,5-tri-o-caffeoyl-quinic acid (Fig 2a), 4,5-di-0- caffeic acid and sinapic acid from the roots.

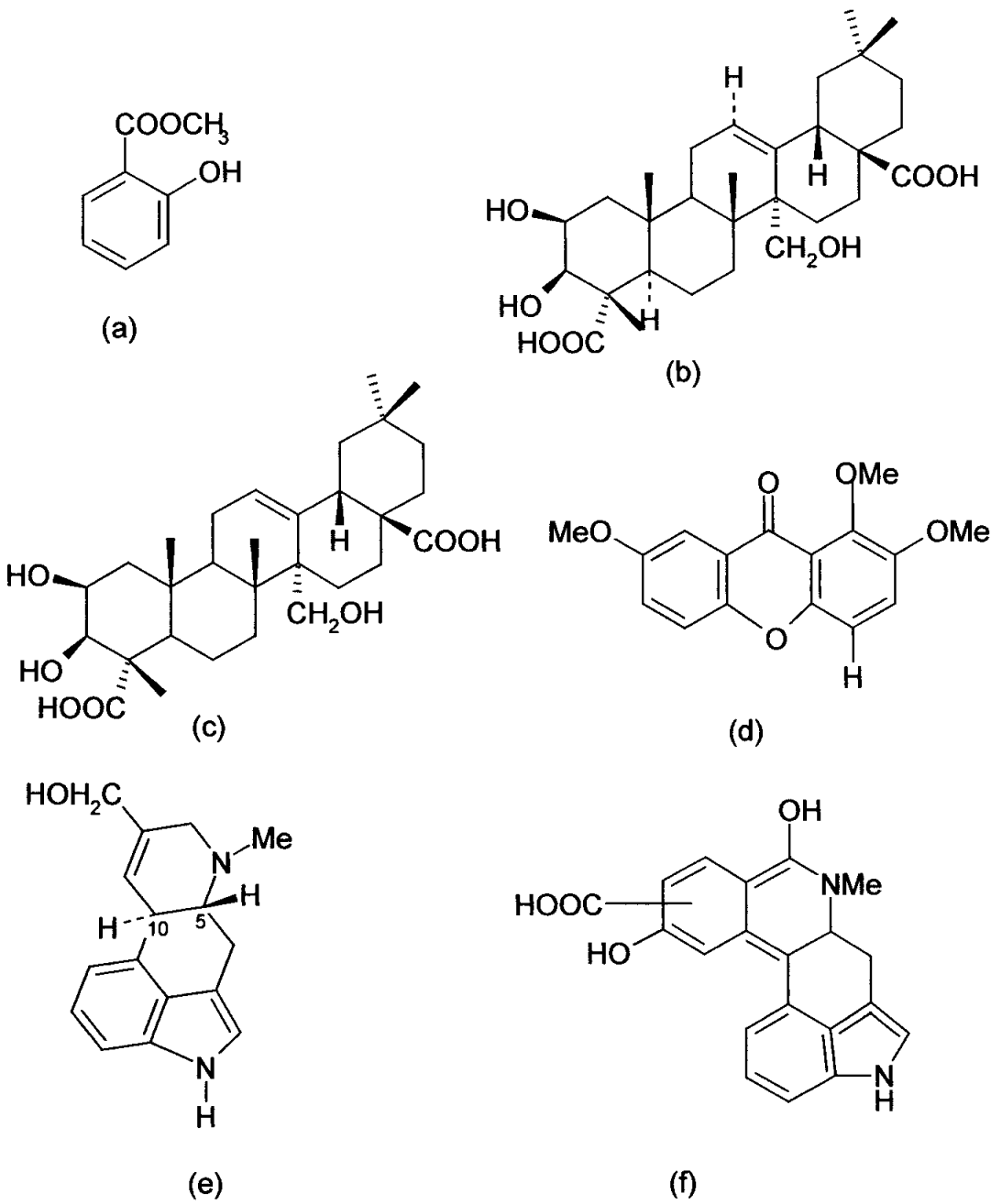

Fig 1: Compounds isolated from *S. longependunculata* root and stem bark (a) methyl salicylate; (b) senegenic acid; (c) presenegenin; (d) 1,7-dimethoxy-2-

hydroxyxanthone; (e) elymoclvine and (f) alkaloid A.

Compounds isolated from the stem bark. Kogan *et al.*, (1970) isolated an alkaloid, which proved to be the very toxic securinine (Fig 2b), previously extracted from the Euphorbiaceae species, *Securinega suffruticosa*. This remarkable alkaloid is a tricyclic decahydro-1,10-methano-pyrido(1,2-a)-azepin system fused with an α,β-unsaturated butenolide ring which is common in cardenolides (Horii *et al.*, 1967). Biogenetically, it is derived from the amino acids tyrosine and lysine (piperidine ring). It is the first alkaloid derived from lysine with the nitrogen part of the two rings (Sankawa *et al.*, 1974).

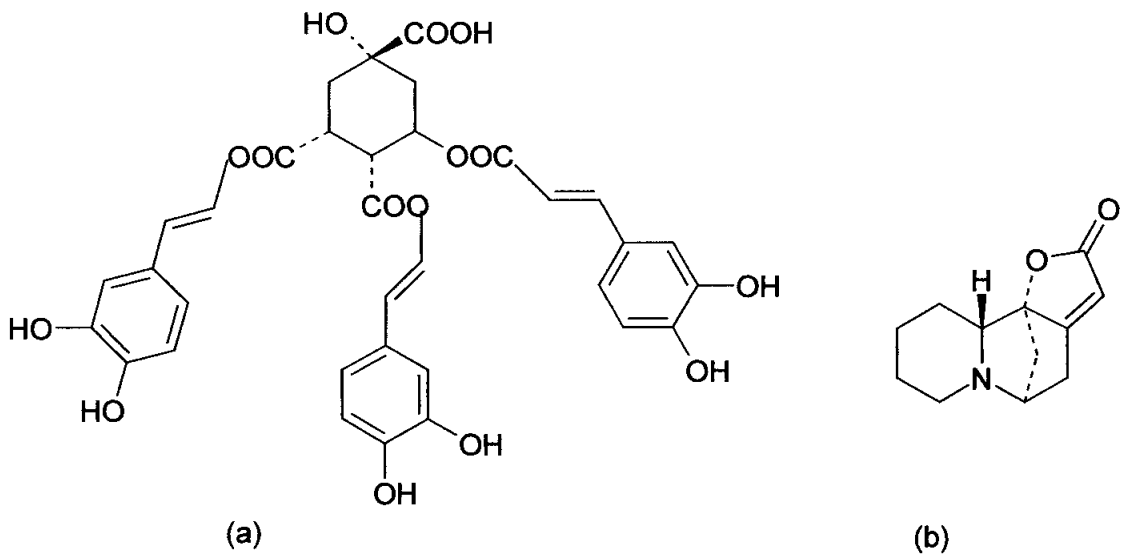

Fig 2: Compounds isolated from *S. longependunculata* root and stem bark (a) 3,4,5-tri-o-caffeoyl-quinic acid and (b) securinine.

Compounds isolated from the seeds. Smith *et al.*, (1979) showed that the seed oil is a rich source of fatty acids (conjugated hydroxy dieonic fatty acids) and tricylglycerols (acetotriacylglycerols) of unusual structure.

Compounds isolated from the leaves. Odebiyi (1978) reported that the leaves are very rich in saponines. Kwamwendo *et al.*, (1985) screened the leaves in Malawi for phytochemical constituents and showed the presence of saponines, tannins, anthroquinones, sterols and terpenes; alkaloids and flavonoids were absent.

6.2. Materials and methods

6.2.1. Collection of plant material

The root bark of *S. longependunculata* was collected from Venda (South Africa). Voucher specimen (NCR. 16) was deposited and identified at the H.G.W.J. Schweickerdt Herbarium (PRU), University of Pretoria.

6.2.2. Extraction and purification of chemical constituents

Dry root bark (634 g) of *S. longependunculata* was extracted in acetone by homogenizing the plant material using a fire protected blender and leaving it in the dark, at room temperature for 8 days. The extraction process was repeated twice. The extract was filtered and concentrated to dryness under reduced pressure at 37°C yielding 11 g. The total extract was subjected to column chromatography using silica gel 60 (Merck, 7724, 500 g), and eluted with the following solvent percentages of *n*-hexane in ethyl acetate 10.0%, 20.0%, 40.0%, 60.0%, 80.0%, 100%, followed by 10.0% methanol in ethyl acetate and finally 100% methanol. 46 fractions were collected, and reduced to 7 subfractions based on the TLC results. The TLC plates

were developed using chloroform: methanol (8:2).

Fraction 3 was resubjected to column chromatography using a silica gel column. The column was eluted with *n*-hexane:ethyl acetate 8:2. 6 subfractions were obtained. Further purification of fraction 4, was affected by using Sephadex LH-20 column chromatography. The column was packed by using 15.0 g of Sephadex LH 20 and dissolving fraction 4 (48.0 mg) in methanol. The column was eluted with methanol (100%) and 18 fractions were obtained. Subfractions 4 - 14 were combined (46.0 mg) and seemed to contain a pure compound. The same procedure was followed for fractions 2 (8.0 mg), 3 (23.0 mg) and finally 6 (21.0 mg), which yielded four major pure compounds 1-4, which were further analyzed by different physicochemical methods to determine their chemical structures. The isolation of these compounds was based on bioassay-guided fractionation.

6.3. Results

Column chromatography of the acetone extract of the fresh root bark of *S. longependunculata* yielded four xanthones (1-4), two of them (3 and 4) are new compounds.

6.3.1. Compound 1

Compound **1**(Fig 3) was obtained as yellow crystals, mp 173-175°C. The molecular formula was assigned as $C_{15}H_{12}O_5$ on the basis of MS (m/z 272). The UV spectra (in MeOH) (Fig 4) showed absorption at 239, 265, 320 nm, no change observed upon the addition of NaOAc (Fig 5), indicating the absence of hydroxyl groups on positions 3 and 6. In NMR spectra, **1** showed signals of three protons at δ 7.2, *d*, (*J* = 9.31 Hz), 7.30, *dd*, (*J* = 9.31, 3.11 Hz) and 7.66, *d*, (*J* = 3.11 Hz), forming ABM system characteristics of B ring substituted at positions C-6 or C-7 (Nguyen & Harrison, 1998; Inuma *et al.*, 1996; Bashir *et al.*, 1991), two signals of aromatic protons *ortho* coupled at δ 7.39, 7.36, each *d*, (*J* = 9.31 Hz), indicating, disubstituted A ring system at 1, 2 or 3, 4 or 1, 4 positions. 3 and 4 position was excluded by the fact that, UV absorption in NaOAc showed no change, and the ^{13}C values of one methoxyl group (δ_c 60.83, *O*-disubsitiuted) (Bashir *et al.*, 1991) excluded 1, 4 position.

^{1}H (Fig 6), ^{13}C (Fig 7), HMQC (Fig 8) and HMBC (Fig 9) data are only compatible with 2-hydroxy-1,7-dimethoxyxanthone (1), furthermore, the data obtained are in agreement with the same compound isolated before from the same source (Galeffi *et al.*, 1990).

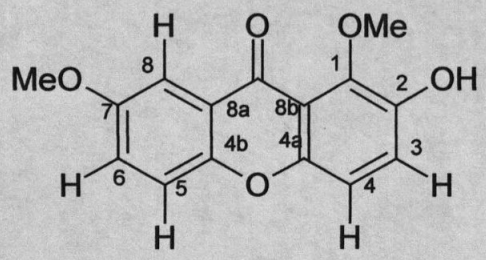

Fig 3: Structure of the compound 1

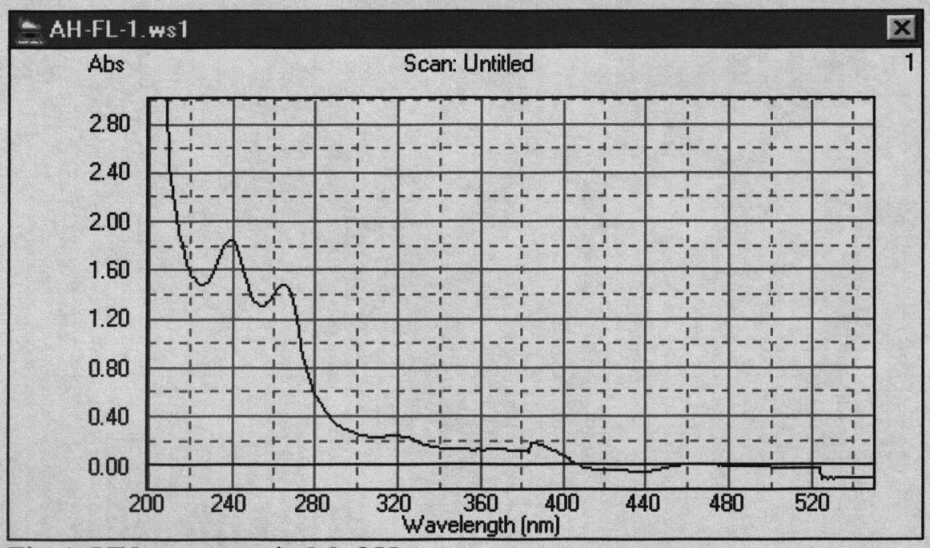

Fig 4: UV spectrum in MeOH

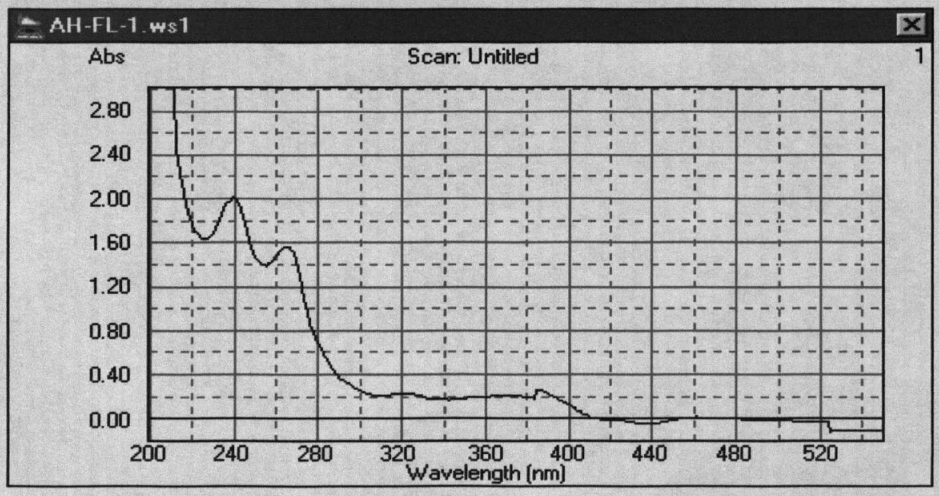

Fig 5: UV spectrum in NaOAc

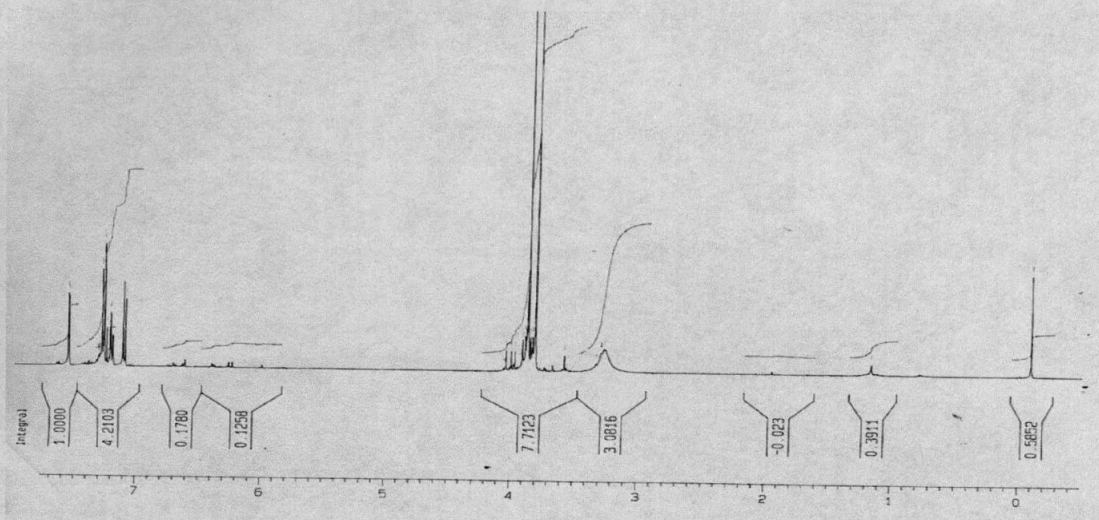

Fig 6: Proton spectrum

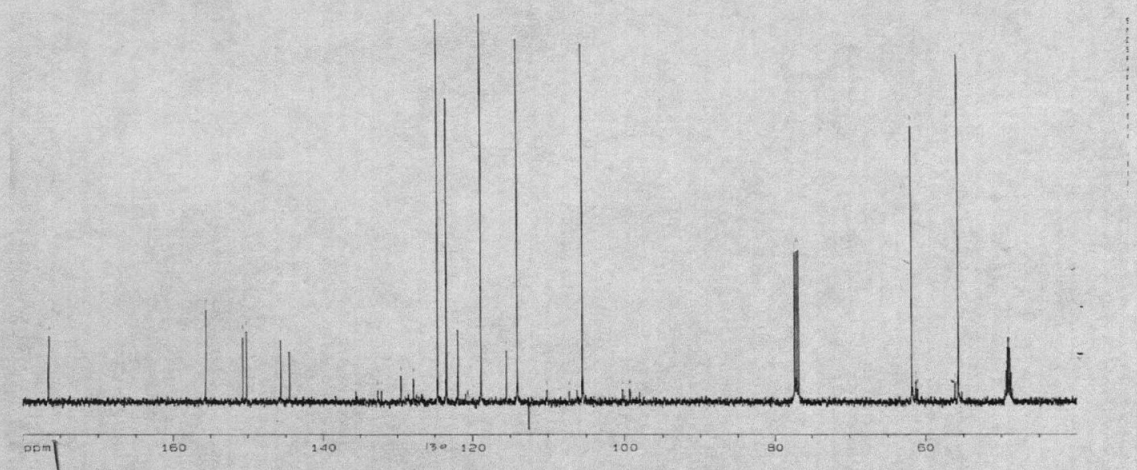

Fig 7: ^{13}C spectrum

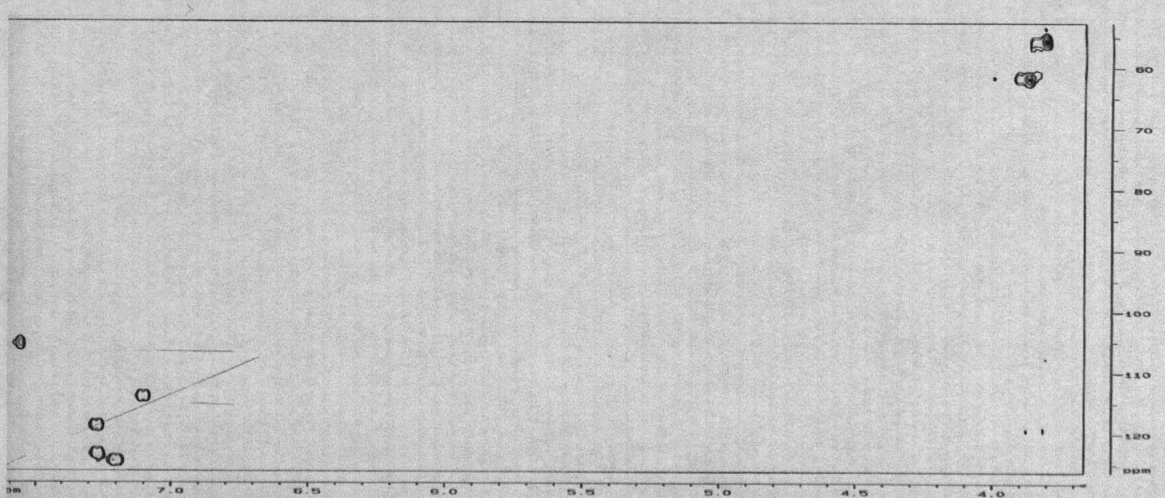

Fig 8: HMQC spectrum

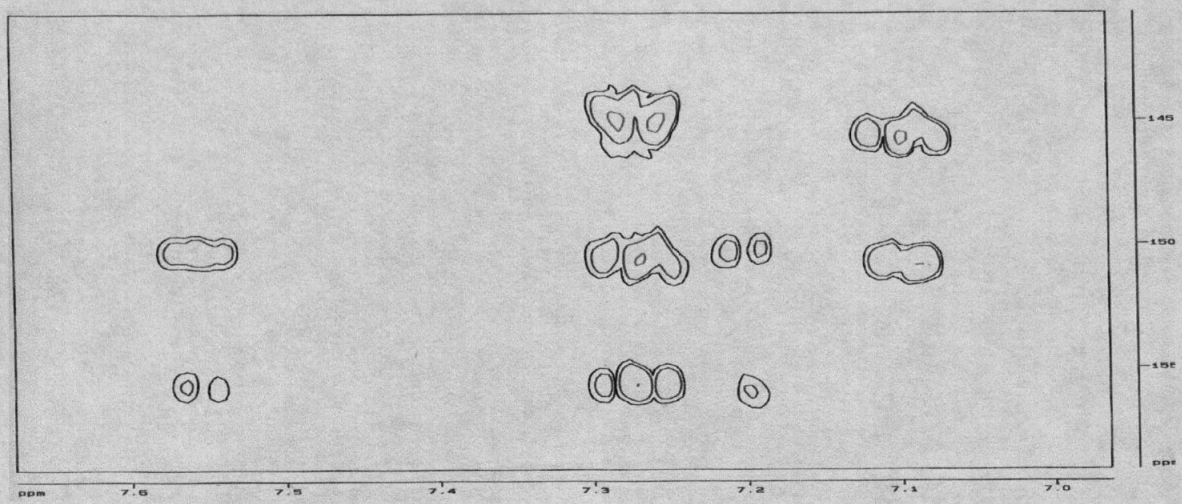

Fig 9: HMBC spectrum

6.3.2. Compound 2

Compound 2 (Fig 10) was isolated as yellow crystals mp 224-26°C, and indicated a molecular formula of $C_{14}H_{10}O_5$ deduced from MS (M+258.0). The UV spectra of 2 showed absorption bands (in MeOH) (Fig 11) at 237, 265, 322(sh) nm, strong bathochromic shift obtained with $AlCl_3$ (Fig 12) which was not affected by HCl, indicating the presence of *peri*-hydroxyl groups in 1 and/or 8, no change occured with NaOAc (Fig 13), indicating the absence of acidic hydroxyls. NMR spectra of 2 showed one proton signal δ 6.62, *d*, *ortho* coupled with a proton resonating at δ 7.15 (*d*, J = 8.8Hz), suggesting 1-4 disubstituted system for ring A, signals of three protons forming ABM system δ 7.23 (*dd*, J = 9.05, 3.47 Hz), 7.42 (*d*, J = 9.05 Hz) and 7.45 (*d*, J = 3.47 Hz) suggested 6 or 7 monosubstituted system of ring B. A signal at δ 12.3 indicated the presence of one *peri*-hydroxyl group.

The ^{1}H and ^{13}C data are in agreement with the data published of 1, 4-dihydroxy-7-methoxyxanthone (2) isolated before from *Visamia guaramirangae* (Guttiferae) (Martson, *et al.*, 1993). Furthermore other spectroscopic data of HMQC (Fig 16) and HMBC (Fig 17) with ^{1}H and ^{13}C of 2 showed that the chemical structure should be as shown below.

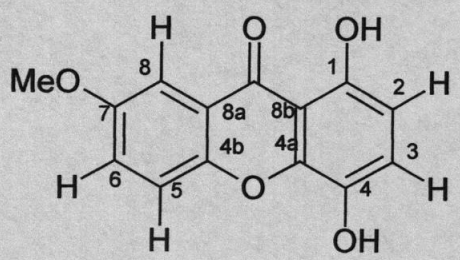

Fig 10: Structure of the compound 2

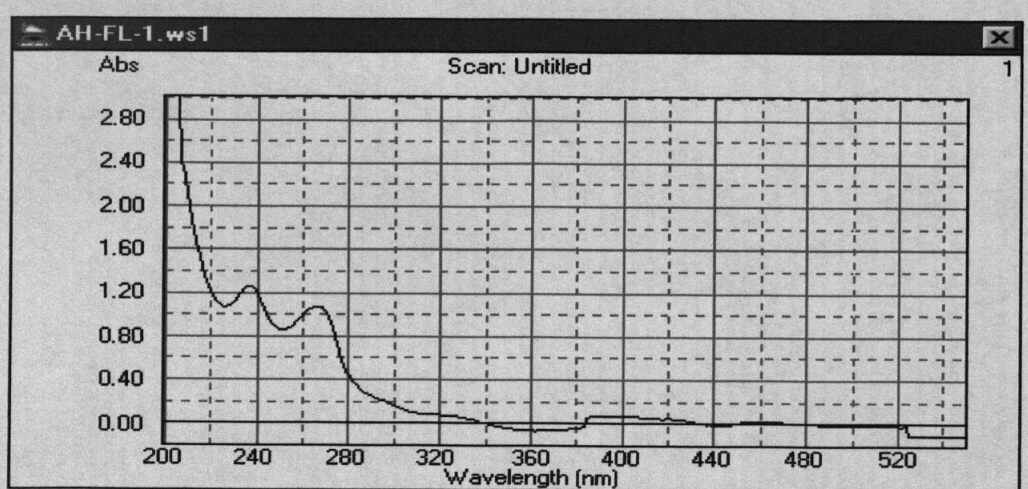

Fig 11: UV spectrum in MeOH

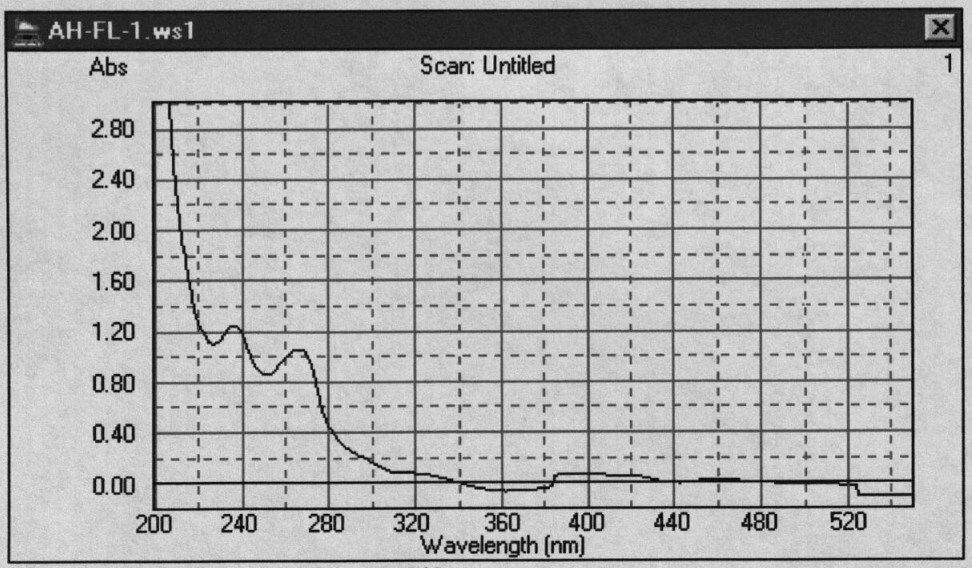

Fig 12: UV spectrum with ALCl3

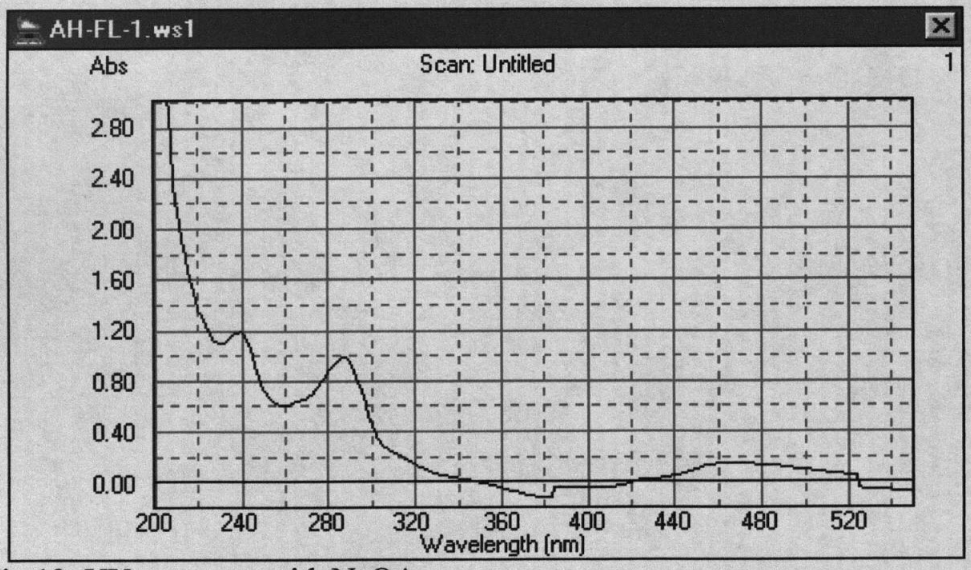

Fig 13: UV spectrum with NaOAc

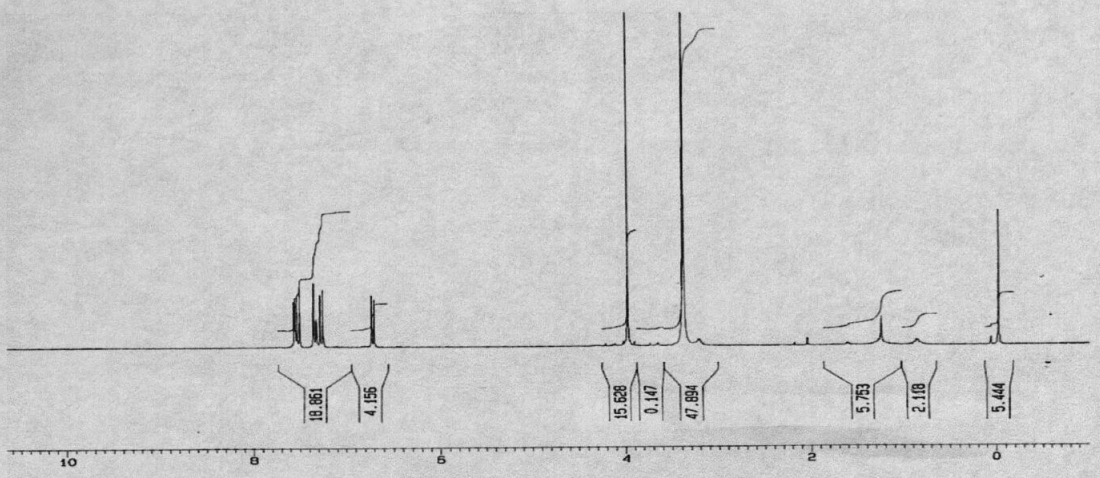

Fig 14a: Proton spectrum

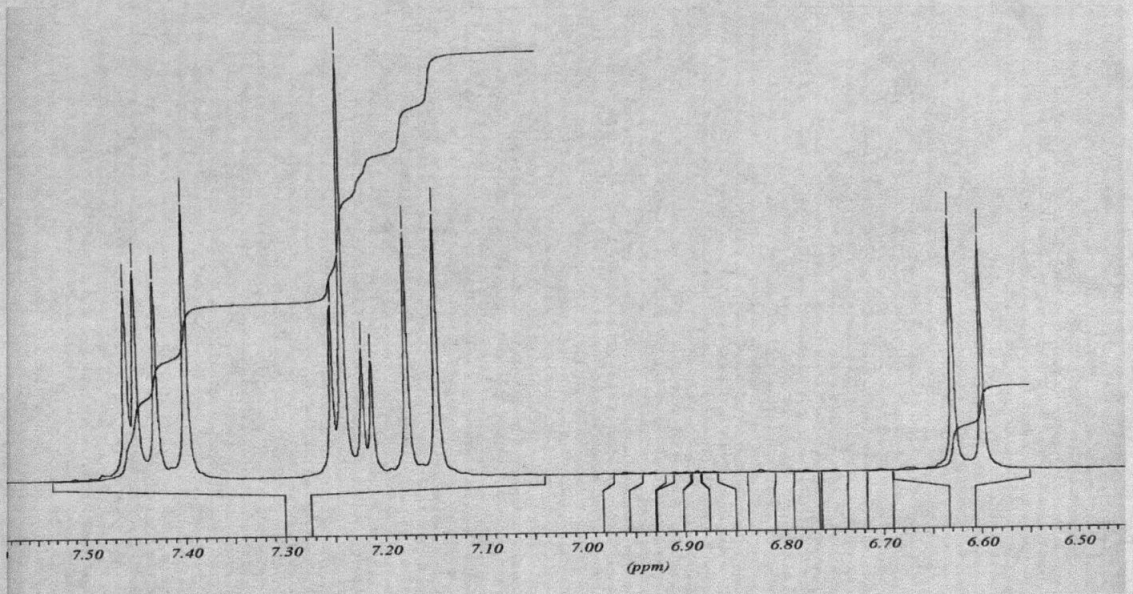

Fig 14b: Proton spectrum

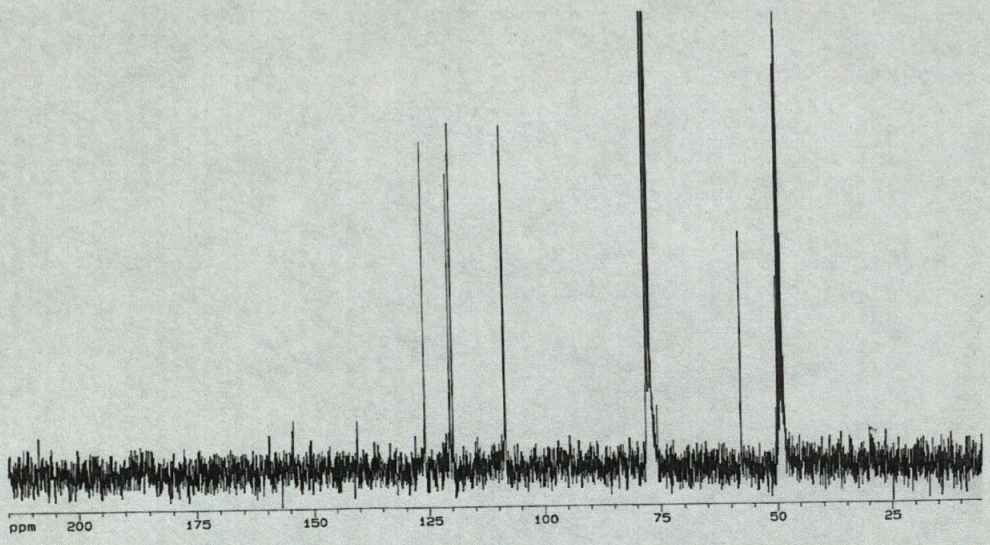

Fig 15: ^{13}C spectrum

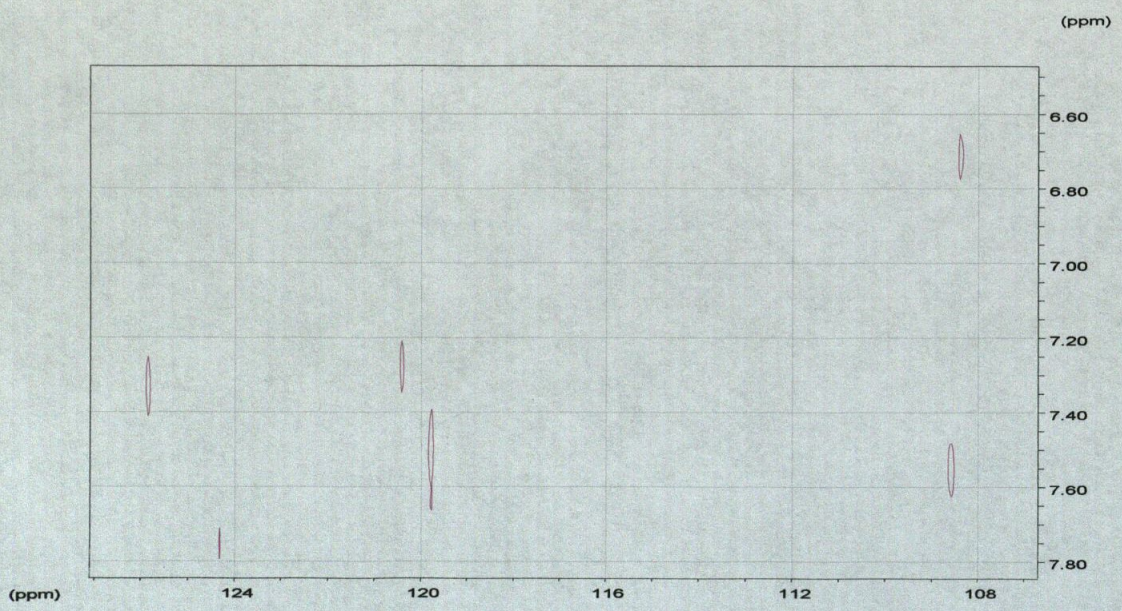

Fig 16: HMQC spectrum

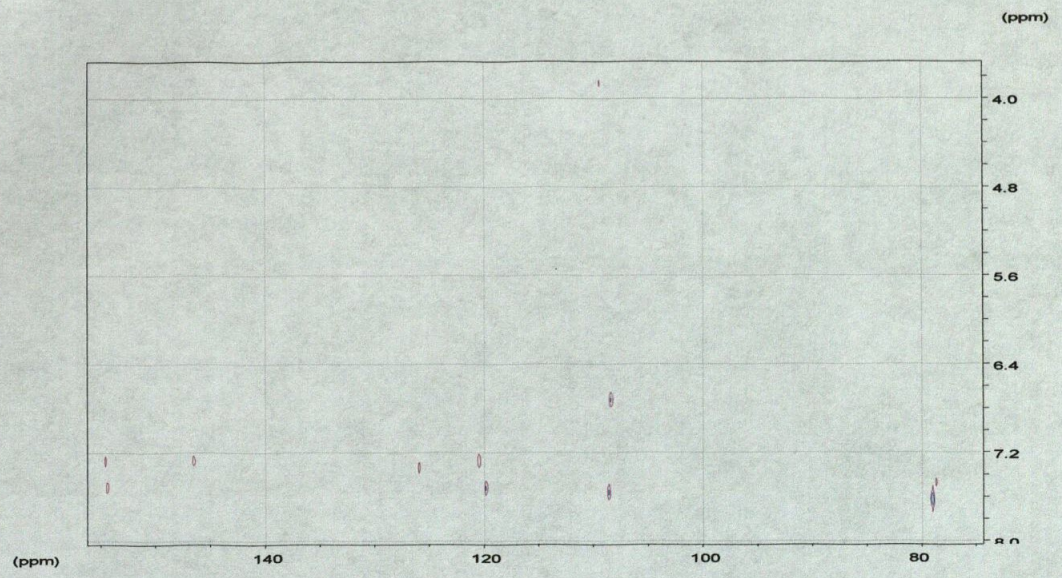

Fig 17. HMBC spectrum

6.3.3. Compound 3.

Compound **3** (Fig 18) was isolated as yellow crystals, mp 152-5°C. Molecular formula $C_{15}H_{12}O_8$ deduced from MS (M+, 310.0), ^1H, ^{13}C and HMQC spectra. UV spectral data showed absorption in MeOH (Fig 19) at 237_{sh}, 258, 330 nm, strong bathochromic shift observed with $AlCl_3$ (Fig 20), which was not affected by the addition of HCl, indicating the presence of *peri*-hydroxyl group(s) at 1 and/or 8. Bathochromic shift in NaOAc (Fig 21) spectra (20 nm), indicated the presence of acidic hydroxyl(s) at 3 and/or 6 position(s).

NMR ^1H spectra (Fig 22) of compound **3** showed, two proton singlets at δ 6.25, 6.45 attached to carbons δ_c 93.1, 98.3 ppm respectively, two three proton singlets, at δ 3.84, 3.88 attached to carbons at δ_c 61.54, 60.73, suggesting two methoxyl groups *O*-

disubstituted. The two signals at 12.1, 11.80 indicated the presence of two *peri*-hydroxyls.

This data suggested that, compound 3 is a *hexa*-oxygenated xanthone, with two positions occupied by free hydroxyls at C-1 and C-8, the other two are methoxylated and the last two positions occupied by another two free hydroxyls group.

HMBC (Fig 23) spectra of 3 showed correlations between the methoxyl proton at δ 3.84 and the C-127.5, the other methoxyl group δ 3.88 with C-130.5. The chemical shift of both carbons suggested the positions 2, 4, 5 or 7 for the methoxyl groups. The bathochromic shift of **3** in NaOAc and the ^{13}C values of the hydroxyls attached carbons (δ_c 157.7, 158.3) suggested that both the 3 and 6 positions are occupied by free hydroxyls group. ^{13}C data of **3** is in a good agreement with 1,3-dihydroxy-2-methoxyxanthone (Ortega *et al.*, 1981), which is closely related to the structure of 3.

The 6,8-dihydroxy-5-methoxyxanthone substitution pattern of ring B were confirmed by the fact that firstly the NMR data of 3 showed no symmetry. Secondly the ^{13}C NMR spectra showed aromatic carbons oxygen substituted at δ_c 130.5 (attached to one methoxy group), 158.8, 152.7, which suggest the presence of 5, 6, 8-trisubstituted ring B. Thirdly the methoxyl carbon at δ_c 60.73 suggested the *O*-disubstituted, which require -OH substitution at C-6. The structure of 3 was confirmed finally by the correlation observed in HMBC and HMQC (Fig 24) spectra.

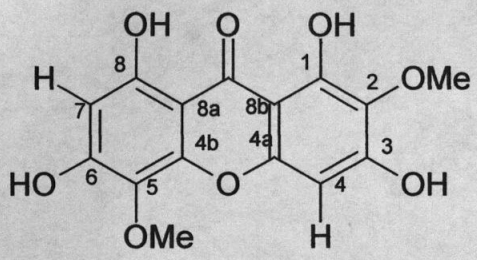

Fig 18: Structure of the compound 3

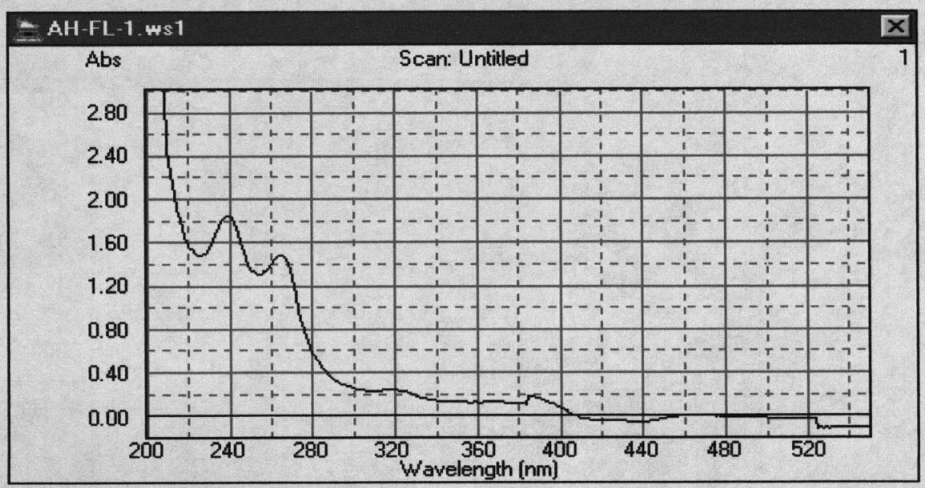

Fig 19: UV spectrum in MeOH

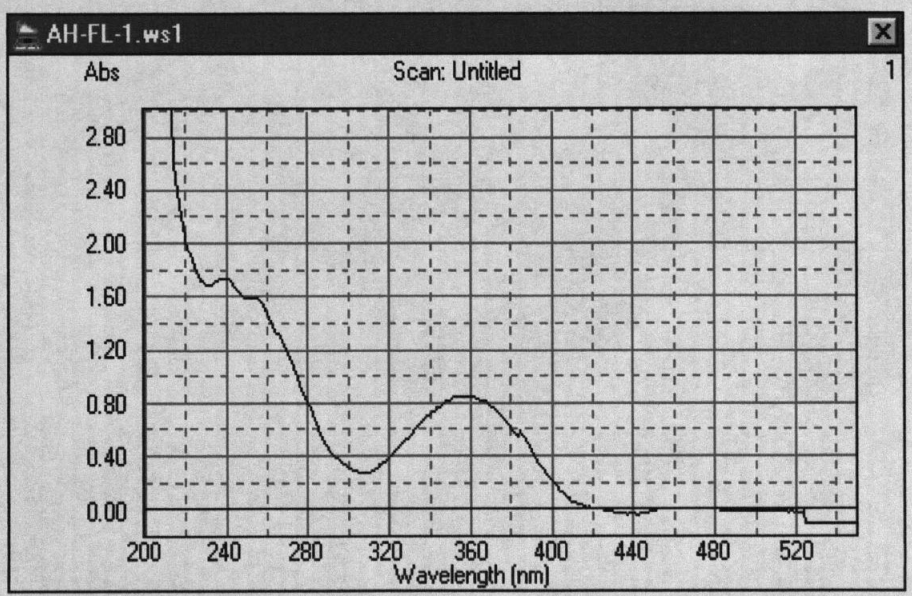

Fig 20: UV spectrum with $AlCl_3$

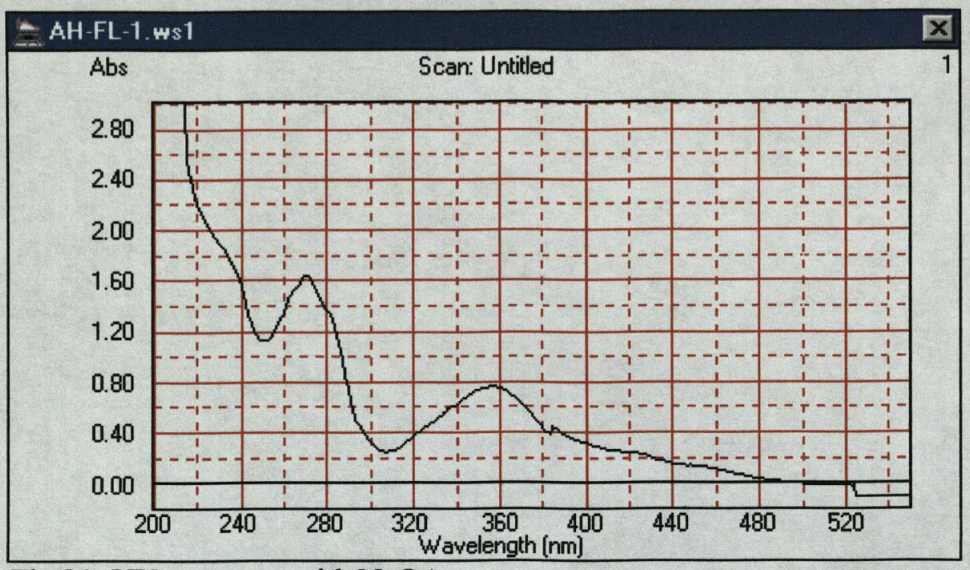

Fig 21: UV spectrum with NaOAc

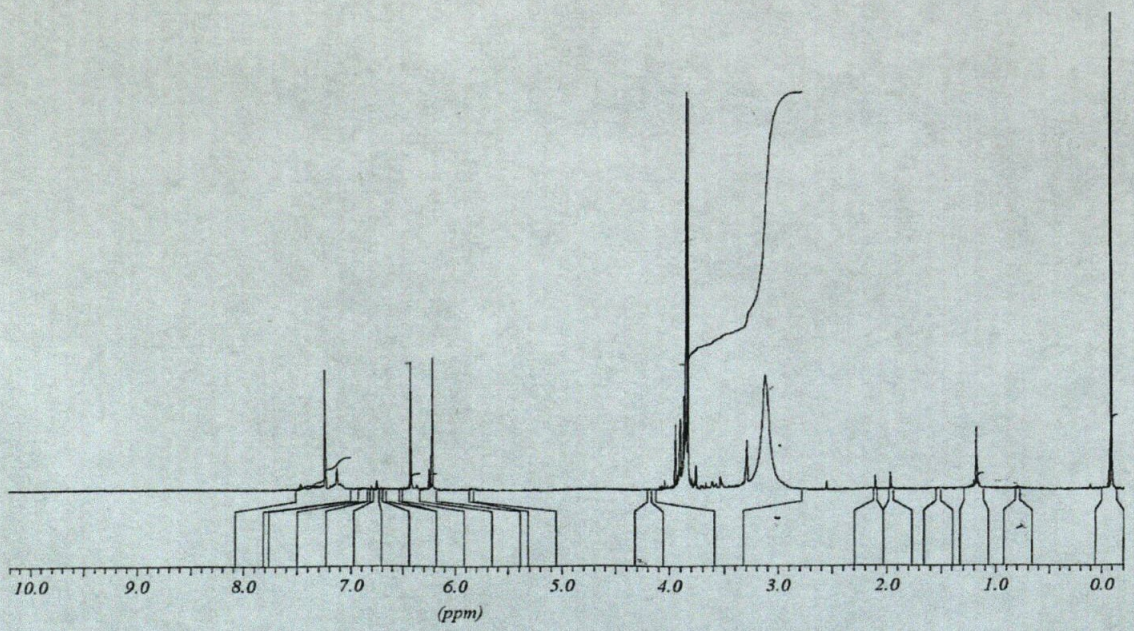

Fig 22: Proton spectrum

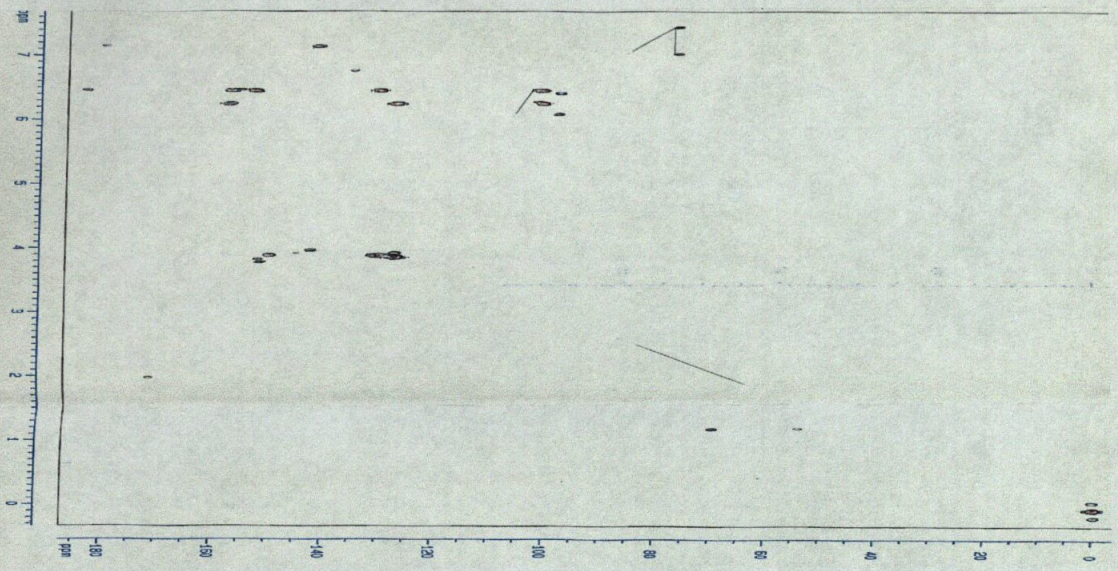

Fig 23: HMBC spectrum

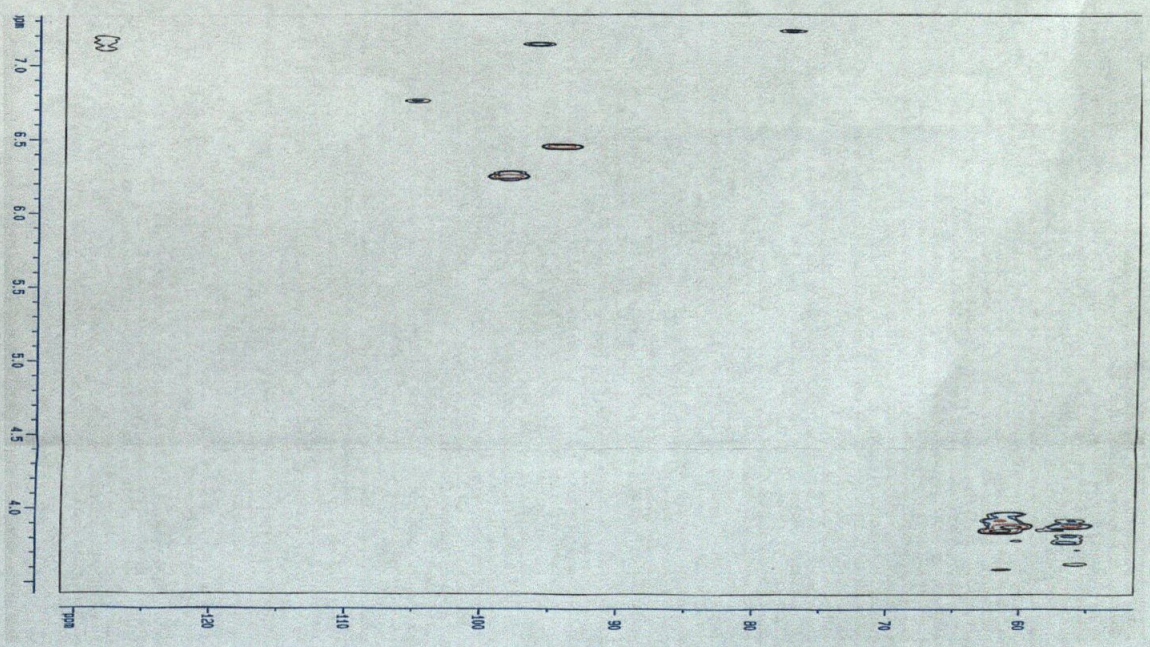

Fig 24: HMQC spectrum

6.3.4. Compound 4

Compound 4 (Fig 25) was isolated as pale yellow crystals, mp 201-204°C, has a molecular formula $C_{17}H_{16}O_9$ deduced from MS. The UV spectra of **4** showed (in

MeOH) (Fig 26) absorptions at 235_{sh}, 265, 282_{sh} and 355. Strong bathochromic shift was observed by addition of $AlCl_3$. Also, the addition of NaOAc (Fig 27) gives a small bathochromic shift. ^{1}H-NMR (Fig 28 a & b) spectra showed one singlet aromatic proton at δ 6.82, four methoxyl signals at δ 4.14, 3.99, 3.97, 3.92, and two *peri*-hydroxyl signals at 11.38, 11.88.

The ^{1}H- and ^{13}C-NMR (Fig 29) data indicated a *hepta* oxygenated xanthone with only one unsubstituted position. From the seven oxygenated positions, three are free hydroxyls group, two of them located at C-1 and C-8 as indicated from the ^{1}H-NMR spectra and the strong bathochromic shift in $AlCl_3$ spectral UV data.

The UV spectra give bathochromic (10 nm) shift by addition NaOAc, which indicated the presence of an acidic hydroxyl at position 3 or 6. The remaining four oxygenated positions should be methoxylated. ^{13}C data showed chemical shifts at δ_c 61.8, 61.6, 61.2 and 61.1, which indicated four *O*-substituted methoxyls. Ring A is therefore completely substituted with three methoxyl groups at positions 2,3 and 4. According to the ^{13}C data, the fourth methoxyl group should be located at position between the two hydroxyls groups in ring B.

As indicated from the previous data, compound **4** should be 1,6,8-trihydroxy-2,3,4,7-tetramethoxyxanthone (4). The aromatic proton δ 6.82 showed correlations with C-6, C-7, C-4b and C-8a in HMBC spectra. Other data of HMQC (Fig 30) and HMBC (Fig 31) are only compatible with structure 4. Compound 4 is the first example of heptahydroxyxanthone derivative isolated from natural sources.

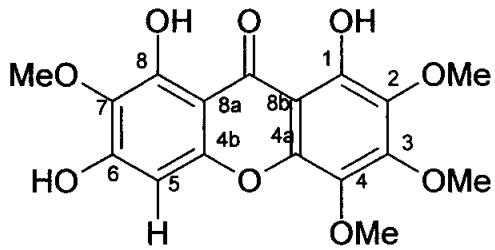

Fig 25: Structure of compound 4.

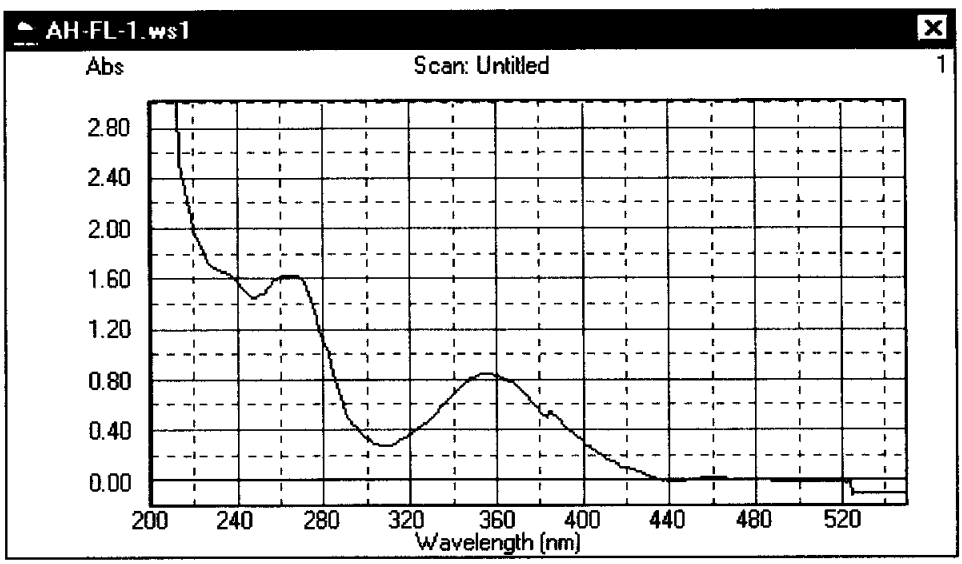

Fig 26: UV spectrum in MeOH

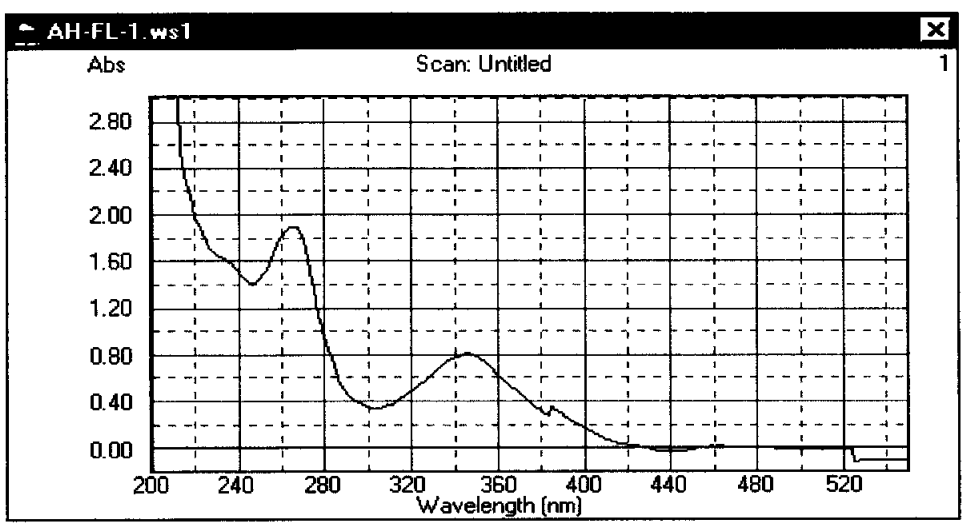

Fig 27: UV spectrum in NaOAc

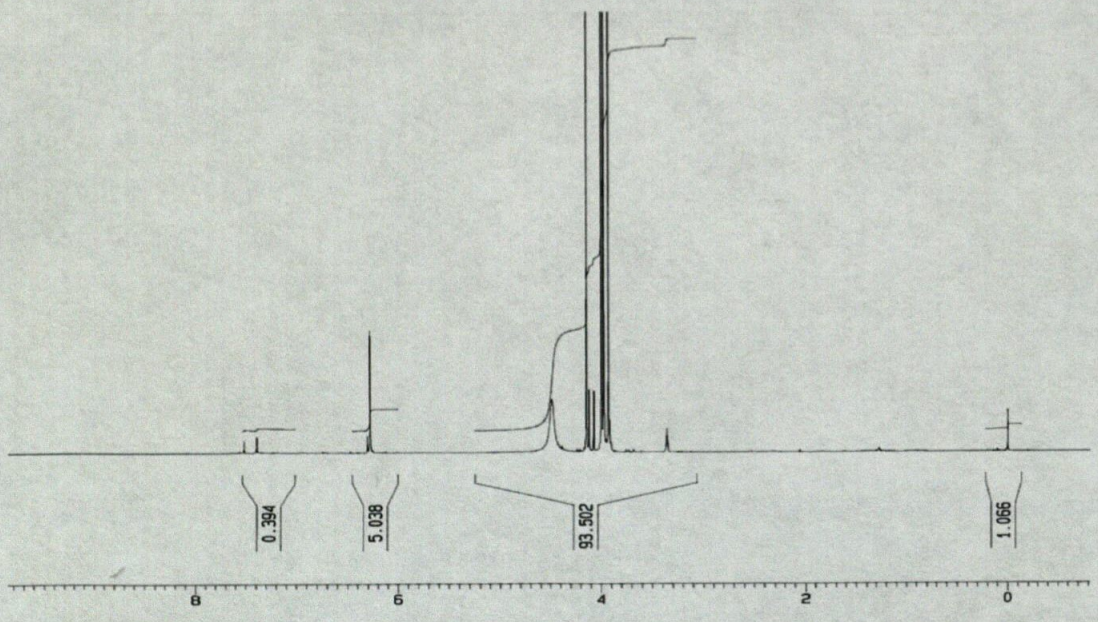

Fig 28a: Proton spectrum

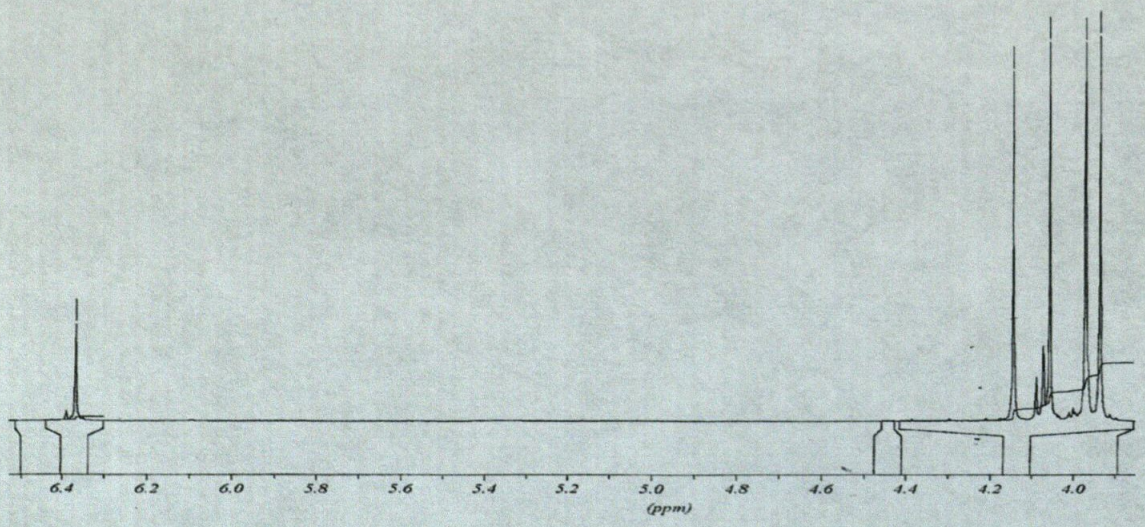

Fig 28b: Proton spectrum

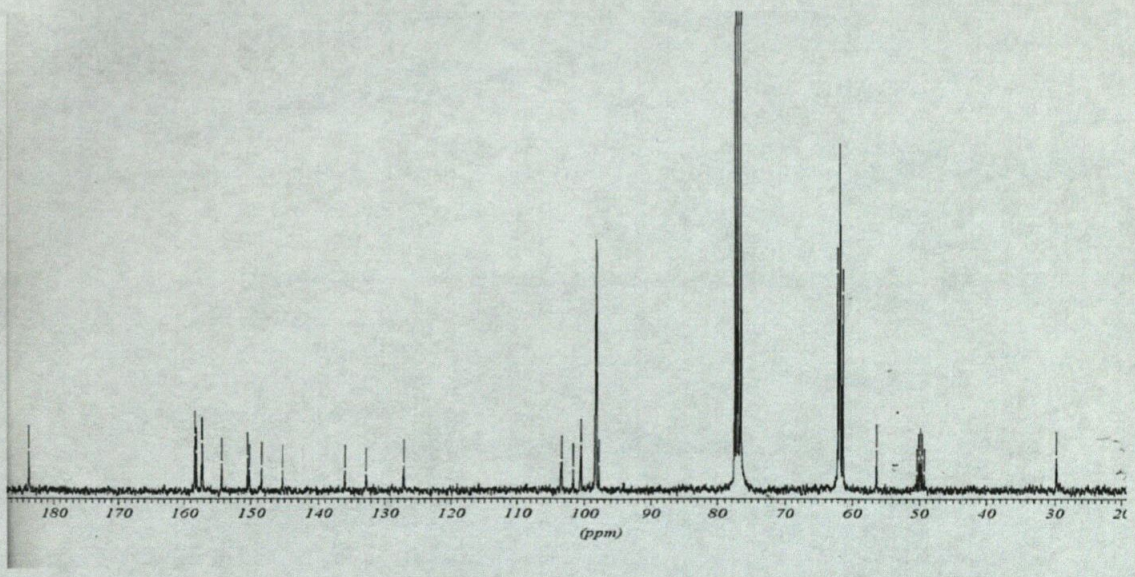

Fig 29: ^{13}C spectrum

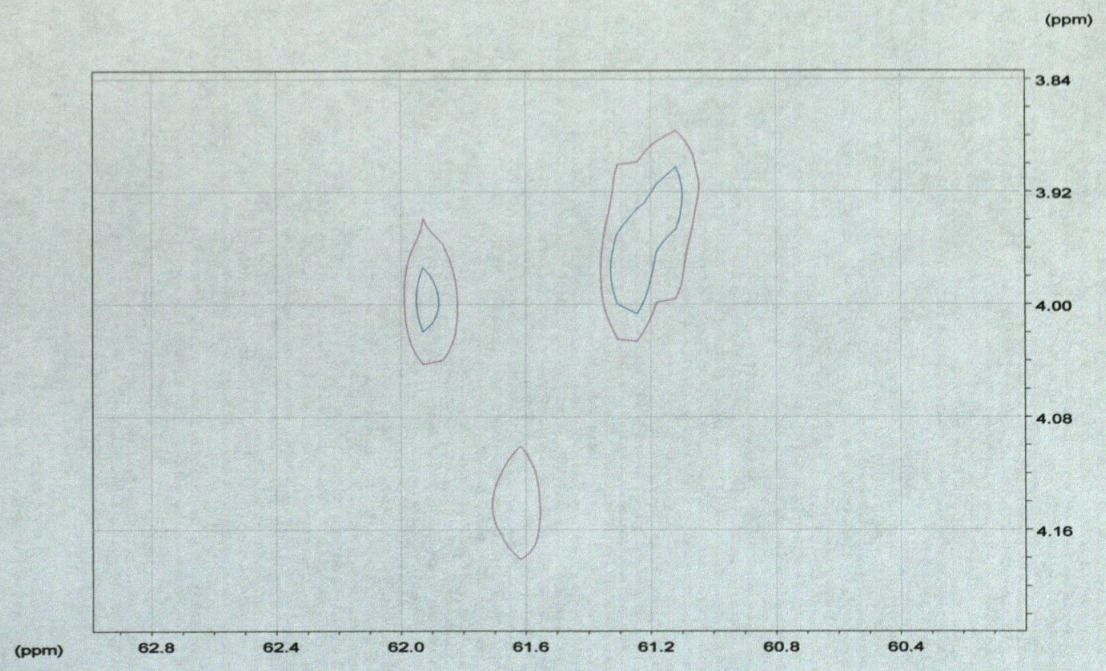

Fig 30: HMQC spectrum

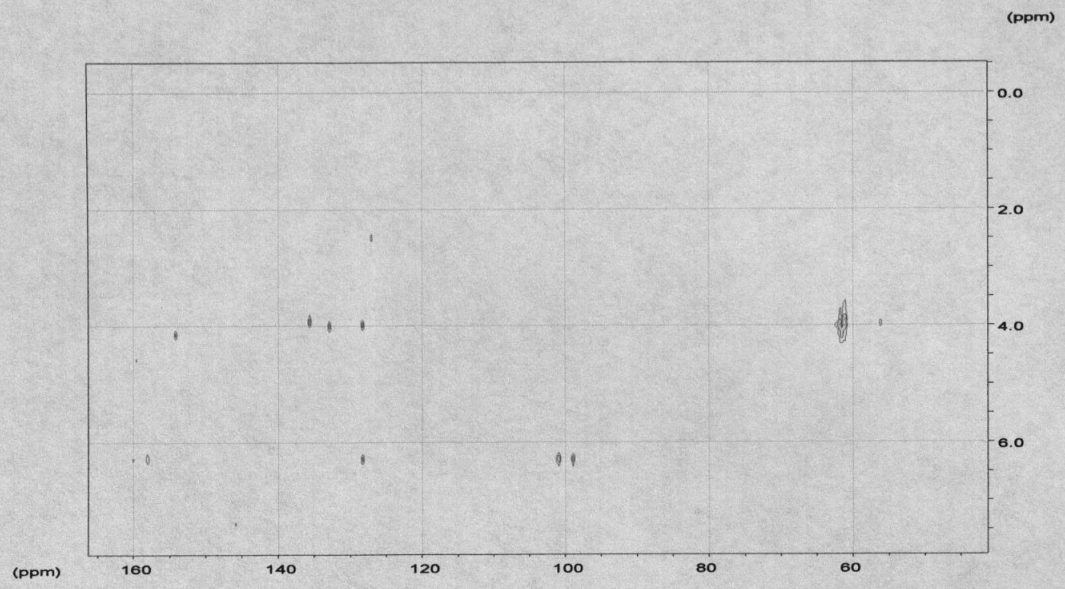

Fig 31: HMBC spectrum

Table 1: ^{1}H and ^{13}C-NMR chemical shifts for compounds 1-4 (δ, ppm, in $CDCl_3$)

No.	Compound 1		Compound 2		Compound 3		Compound 4	
	^{13}C	H, mult, *J*	^{13}C	H, mult, *J*	^{13}C	H, mult, *J*	^{13}C	H, mult, *J*
1	144.5		154.0		149.3		150.0	
2	145.6		108.4	6.62, *d*, 8.79	127.5		132.8	
3	123.4	7.36, *d*, 9.01	119.3	7.15, *d*, 8.79	157.7		154.0	
4	118.8	7.39, *d*, 9.01	140.0		93.1	6.3, *s*	135.6	
4a	150.2†		146.1		153.0		149.8	
4b	150.7†		150.2		148.8		157.9	
5	113.9	7.20, *d*, 9.31	119.8‡	7.42, *d*, 9.06	130.5		98.9	6.36, *s*
6	124.6	7,30, *dd*, 9,31, 3.11	125.8	7.22, *dd*, 9.05, 2.97	158.3		159.8	
7	155.7		153.8		98.3	6.5, *s*	128.3	
8	105.6	7.66, *d*, 3.11	108.6♠	7.45, *d*, 2.97	152.7		145.6	
8a	121.9		119.9‡		101.5		100.9	
8b	115.4		109.0♠		101.3		100.4	
9	175.7		182.3		183.7		183.7	
OMe	61.8	3.86, *s*	57.2	3.87, *s*	61.5	3.84, *s*	61.8	3.99, *s*
	55.64	3.80, *s*			60.7	3.88, *s*	61.6	4.14, *s*
							61.2	3.97, *s*
							61.1	3.92, *s*
OH								

Note: ♠, ‡, † = interchangeable signals.

6.4. Discussion

The compounds isolated in this study were found to be from the same xanthone family and two were new. Compounds 1,4-dihydroxy-7-methoxyxanthone and 1,7-dimethoxy-2-hydroxyxanthone) were previously isolated from *Visamia guaramirangae* (Martson, *et al.*, 1993) and *Securidaca longependuncula*ta (Galeffi *et al.*, 1990); respectively.

Polygalaceae, the plant family to which *S. longependuncula*ta belong, have been reported in the litrature to be one of few higher plant families that are rich in xanthones. Twelve xanthones have previously been isolated form *S. inappendunculata* (Yang *et at.*, 2001). Xanthones have previously been isolated by Massias *et al.* (1981); Parra *et al.* (1984); Ortega *et al.* (1988); Galeffi *et al.* (1990); Nagen *et al.* (1992); Nguyen *et al.* (1998) etc. To date more than 60 kinds of xanthones have been isolated from the genus *Polygala L* all containing three substitutes groups (hydroxyl, methoxyl and methylenedioxyl) unlike the compounds isolated from *S. inappendunculata* (Yong et al., 200b) and *S. longependuncula*ta (Galeffi *et al.*, 1990), as well as those isolated during this study, which have only hydroxyl and methoxyl groups.

In our subsequent investigation of the isolated compounds (Chapter 7) it was shown that three of the isolated compounds (1,7-dimethoxy-2-hydroxyxanthone, 1,3,6,8-tetrahydroxy-2,5-dimethoxyxanthone and 1,6,8-trihydroxy-2,3,4,7-tetramethoxyxanthone) do have activity in the stimulation of erection as well as antibacterial activity. These findings are supported by the results ofSordat-Diserens *et al.*

(1992) who indicated that xanthones does have pharmacological properties such as antibacterial, antifungal, anti-inflammatory and immunomodulatory activity. It is probable that many xanthones have undiscovered biological activity because chemists in the past concentrated only on structure elucidation.

6.5. Acknowledgement

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6.6. References

Bashir A, Hamburger M, Msonthi JD, Hostettmann K (1991) Isoflavones and xanthones from *Polygala virgata*. Phytochemistry 31(1): 309– 311.

Costa C, Bertazzo A, Curcuruto O, Traloli P (1992) Indole alkaloids from the roots of an African plant, *Securidaca longependunculata*. Isolation by column chromatography and preliminary structural characterization by mass spectrometry. Journal of Heterocyclic Chemistry 29: 1641- 1647.

Delude C (1971) Etude comparative des saponines extraite de deux polygalacees africaines, le *Securidaca longependunculata* var. parvifolia et le polygala aciculans Olive. Bulletin Societe Royale des Sciences de Liege 40: 498- 501.

Dugan JJ, De Mayo P, Starratt AN (1964) Terpenoids. 5. Senegenin: functional groups and part structure. Canadian Journal of Chemistry 42: 491- 501.

Galeffi C, Federici E, Msonthi JD, Marini-Bettolo GB, Nicoletti M (1990) New xanthones from *Extiadiopis oblngifolia* and *Securidaca longependunculata.* Fitoterapia LXI(1): 79- 81.

Hamburger M, Gupta M, Hostetmann K (1985) Flavonol glycosides from *Securidaca diversifolia.* Phytochemistry 24(11): 2689- 2692.

Horii Z, Hanoka M, Yamawaki Y, Tamura Y, Saito S, Shigematsu N, Kotera K, Yoshikawa H, Sato Y, Nakai H, Sugimoto N (1967) The total synthesis of securinine and virosecurinine.Tetrahedron 23(1): 1165- 1174.

Inuma M, Tosa H, Ito I, Tanaka T, Madulid DA (1996) Six xanthones from *Calophyllum nustroindicum.* Phytochemistry 42(4): 1195– 1198.

Kamwendo WY, Chiotha SS, Msonth JD (1985) Screening of plants used traditionally in Schistosomiasis in Malawi. Fitoterapia 56: 229-232.

Kogan VI, Gorbunov VD, Rostotskii BK (1970) Patent U.S.S.R. 277, 796. Chemical Abstracts 74: 91177.

Nagem TJ, Da Silva MG, Da Silveira JC (1992) Clathraxanthone, A 1,2,7-trioxygenated xanthone from *Haploclathra paniculata*. Phytochemistry 31(8): 2913– 2914.

Nguyen LHD, Harrison LJ (1998) Triterpenoid and xanthone constituents of *Cratoxylum cochinchinense*. Phytochemistry 50: 471– 476.

Mahmood N, Moore PS, De Tommasi N, De Simone F, Colman S, Hay AJ, Pizza C (1993) Inhibition of HIV infection by caffeoylquinic acid derivatives. Antiviral Chemical Chemotherapy 4: 235- 240.

Massias M, Carbonnier J, Molho D (1981) Xanthones and C-Glucosylflavones from *Gentana carymbifera*. Phytochemistry 20(7): 1577– 1578.

Martson A, Hamburger M, Sordat-Diserens I, Msonthi JD, Hostettmann K (1993) Xanthones from *Polygala nyikensis*. Phytochemistry 33(4): 809– 812.

Moers A (1966) Journal de Pharmacie de Belgique 21: 347- 362

Odebiyi OO (1978) Preliminary phytochemical and antimicrobial examination of leaves of *Securidaca longependuncula*ta. Niger Journal of Pharmacology 9: 29-30.

Ortega EP, Lopez-Garcia RE, Rabanal RM, Daris V, Valverde S (1988) Two xanthones from *Ixanthus viscosus*. Phytochemistry 27(6): 1912– 1913.

Parra M, Picher MT, Seoane E, Tortajada A (1984) New xanthones isolated from *Centaurium linarifolium*. Journal of Natural Products 47(1): 123– 126.

Prista LN, Correira Alves A (1958) Estudo farmacognostico da *Securidaca longependuncula*ta. Garcia da Orta 6: 131- 147.

Rodriguez S, Wolfender J-L, Odotaya G, Purev O, Hostettmann K (1995) Xanthones, Secoiridoids and Flavonoids from the *Halenia corniculata*. Phytochemistry 40(4): 1265- 1272.

Sankawa, U, Yamasaki, K & Ebizuka, Y, 1974. Biosynthesis of securinine. Incorporation of radioactive Tyrosine, Lysine, and Cadaverine. Tetrahedron Letters 14-26: 1867- 1868.

Scandola M, Games DE, Costa C, Allegri G, Bertazzo A, Curcuruto O, Traldi P (1994) Structural study of alkaloids from *Securidaca longependuncula*ta roots. II.

Isolation and characterization by supercritical fluid chromatography/ mass spectrometry. Journal of Heterocyclic Chemistry 31: 219- 224.

Smith jnr CR, Madrigal RV, Plattner RD (1979) New conjugated hydroxy dieonic fatty acids and acetotriacylglycerols from *Securidaca longependunculata* seed oil. Biochemica et Biophysica Acta 572: 314- 324.

Sordat-Diserens I, Rogers C, Sordat B, Hostettmann K (1992) Prenylated xanthones from *Garcinia livingstone*. Phytochemistry 31(1): 313- 316.

Van Der Sluis WG, Labadie RP (1985) Polyoxygenated xanthones of *Centaurium littorale*. Phytochemistry 24(11): 2601– 2605.

Yang X-D, Xu L-Z, Yang S-L (2001) Xanthones from the stems of *Securidaca inappendunculata*. Phytochemistry 58(8): 1245- 1249.

CHAPTER 7

Biological activity of xanthones isolated from *Securidaca longependunculata*

Contents

7.1. Introduction

Xanthones are secondary metabolites known to occur in a few higher plant families, fungi and lichen (Peres & Nagem, 1997). There is a growing interest in xanthones because of their high taxonomic value within families (Cardona *et al.*, 1990) and their pharmacological properties. Their pharmacological properties include *in vitro* cytotoxicity, *in vivo* antitumour activity, antifungal activity, antibacterial activity, anti-inflammatory properties and immunomodulatory activity (Sordat-Diserens *et al.*, 1992).

In preliminary experiments on crude extracts, *S. longependunculata* exhibited antibacterial activity as well as relaxing rabbit corpus cavernosum smooth muscle. There was no previous report on the pharmacological effects of these xanthones on corpus cavernosum smooth muscle.

7.2. Material and methods

7.2.1. Collection of plant material

The root bark of *S. longependunculata* was collected in Venda (South Africa). Voucher specimen (NCR. 16) was deposited and identified at the H.G.W.J. Schweickerdt Herbarium (PRU), University of Pretoria.

7.2.2. Preparation of extracts

Acetone extracts of *S. longependunculata* and the isolation of xanthones (1,7-dimethoxy-2-hydroxyxanthone (1), 1,4-dihydroxy-7-methoxyxanthone (2), 1,3,6,8-tetrahydroxy-2,5-dimethoxyxanthone (3) and 1,6,8-trihydroxy-2,3,4,7-tetramethoxyxanthone (4)) were done as described in Chapter 6.

7.2.3. Bacteria

The bacteria used was similar as those described in Chapter 4.

7.2.4. Antibacterial testing

The xanthones were tested for their antibacterial activity as described in Chapter 4. The only changes were in the concentrations used.

7.2.5. Measurement of muscle relaxation.

The bioassay was done as described in Chapter 3.

7.3. Results and Discussion

7.3.1. Antibacterial activity

Only 1,3,6,8-tetrahydroxy-2,5-dimethoxyxanthone and 1,6,8-trihydroxy-2,3,4,7-tetramethoxyxanthone (3 and 4) were antibacterial and inhibited the growth of *B. cereus*

and *B. subtilis* at a concentration of 0.1 mg/ml. It therefore seems as though the isolated xanthones have low antibacterial activity.

7.3.2. Smooth muscle relaxation

The results show that compounds 1 and 3 have significant smooth muscle relaxation properties and that the erection stimulation action of the new compound 3 is comparable with that of Viagra (Table 2). Compound 3 relaxed smooth muscle by 97.0%, which is very close to the 100 % of Viagra at the same concentration. In a study by Drewes *et al.* (2002), 5,2'-dihydroxy-[(6","-dimethylpyrano(2",3":4'5')][6"',6"'-dimethylpyrano (2"',3"':7,6)]-isoflavone and 5,7,2'-trihydroxy-6-(3,3-dimethylallyl)-[6",6"-dimethylpyrano(2",3":4',5') isofavone isolated from *Eriosema kraussianum* which stimulated erection by 85.0% and 65.0% respectively at a concentration of 78.0 ng/ml compared to the 97.0% of compound 3 at 18.0 ng/ml. The results of both studies verify to an extent the traditional use of these plants.

The two previously isolated compounds 1 and 2 were not tested for their smooth muscle relaxation until this study. Compound 1 relaxed smooth muscle by 63.0% whilst compound 2 showed no activity at all on smooth muscle relaxation. The new compound 3 and 4 relaxed the smooth muscle by 97.0% and 30.5% respectively with compound 3 showed more potential than compound 4. Further tests are underway to investigate their activity *in vivo* as well as effects on the sperms parameters.

Table 2. Averages of relaxation % of the pre-contracted rabbit corpus cavernosum smooth muscle.

Compound	**Average % relaxation at $1.8x10^{-5}$ mg/ml (standard deviation)**
Compound 1 (Known)	63.0 (5.8)
Compound 2 (Known)	0.0 (0)
Compound 3 (New)	97.0 (5.7)
Compound 4 (New)	30.5 (4.8)
Viagra	100.0 (0)

7.4. References

Cardona ML, Fernandez MI, Pedro JR, Serrano A (1990) Xanthones from *Hypericun reflexum*. Phytochemistry 29: 3003.

Drewes SE, Horn MM, Munro OQ, Dhlamini JTB, Meyer JJM, Rakuambo NC (2002) Pyrana-isoflavones with erectile-dysfuction activity from *Eriosema kraussianum*. Phytochemistry 59(7): 739- 747.

Peres V, Nagem TJ (1997) Trioxygenated naturally occurring xanthones. Phytochemistry 44 (2): 191- 214.

Sordat-Diserens I, Rogers C, Sordat B, Hostettmann K (1992) Prenylated xanthones from *Garcinia livingstonei*. Phytochemistry 31(1): 313- 316.

Levin RM, Hypolite AJ, Broderick GA (1997) Evidence for a role of intracellular-calcium release in nitric oxide-stimulation of the rabbit corpus cavernosum. Journal of Andrology 18 (3): 246- 249.

CHAPTER 8

General discussion and conclusion

Contents

8.1. Introduction

The use of medicinal plants go back to early man (Phillipson, 2001). It has no greater tradition anywhere else than in Africa (Neuwinger, 1996). Plants were once a primary source of all the medicines in the world and they still continue to provide mankind with new remedies (Van Wyk *et al.*, 1997) and drugs. They produce well-known modern medicines such as Asprin, Antropine and Cocaine and there is still room for new drugs in the future.

South Africa alone have an estimated 200 000 traditional healers and up to 60.0% of South Africans consult these healers in addition to using modern medicine (Van Wyk *et al.*, 1997). The use of medicinal plants has increased recently and this might lead to these plants becoming extinct within a few decades. South Africa possesses one of the richest sources of plants (24000 flowering species) in the world with well over 3000 species which are used for medicinal purposes.

Like fruit trees grown in different soils produces different quality of fruit, medicinal plants of the same species also do not consistently produce the same quality and quantity of phytochemicals (Sidik, 1999). This is one of the many reasons why traditional herbs do not give consistent results in medical practices. It was for example reported that extracts of *Tribulus terrestris* grown on different regions in Indonesia do not contain the levels of protodioscin shown in Bulgarian plants (Adimoelja, 2000).

8.2. Plants selected for this study and their traditional uses

Securidaca longependuncula, Wrightia natelensis and *Rhoicissus tridentata* from the families Polygalaceae, Apocynaceae and Vitaceae, respectively, were the plants used in this study. These plants are used traditionally to treat many various diseases such as impotence, malaria, kidney problems, stomach ailments, dysmennorhoea, pneumonia, tuberculosis, fever, coughs and colds. Previously, scientists have isolated many different compounds from these plants and within their genera (Galeffi *et al.*, 1990, Hamburger *et al.*, 1985). These plants were collected based on an ethnobotanical approach as a strategy of plant collection for biologically active ingredients. This strategy employs local people's knowledge about the medicinal uses of plants. Local people together with an ethnobotanist, select the species of plants that are used medicinally in their area. The ethnobotanical strategy involves identifying people who are knowledgeable about the medicinal uses of their flora, and securing their cooperation in identifying the species they use (Balick, 1990).

Another strategy like random collection involves the collection of many plants found in an area. In most cases, only plant species in fruit or flower are collected, as identification of sterile specimens can be time consuming, difficult, or occasionally impossible. Large numbers of species can be collected in this method, depending on the season and the number of fertile plants present in the area. The ethno-directed sampling methods allows the researcher to obtain more leads in a pool of plant sample as compared to a group of plants selected at random (Balick, 1990).

8.3. *In vitro* smooth muscle relaxation activity of plants used in the treatment of male erectile dysfunction

The crude extracts (chloroform and ethanol) of *S. longependunculata, W. natelensis* and *R. tridentata* showed activity against erectile dysfunction. All extracts relaxed the rabbit corpus cavernosum smooth muscle at concentrations of 6.5 – 13.0 mg/ml. The results of these assays showed more rabbit corpus cavernosum smooth muscle relaxation than Viagra at the concentrations tested. These results were similar to that of Kim, *et al.* (1998), who found that 0.5-8.0 mg/ml of *Panax ginseng* extracts relaxed the rabbit corpus cavernosum smooth muscle. In this study the highest relaxation was induced by *S. longependunculata* (chloroform) extracts, which gave 66.7% at the concentration of 13.0 mg/ml. The fact that, the results obtained was from *in vitro* studies suggested that the concentration needed for *in vivo* might be higher.

8.4. Antibacterial activity of crude extracts of *S. longependunculata, W. natalensis* and *R. tridentata*

All the tested plant extracts showed activity against bacteria with the MIC varying from 1-50 mg/ml. The chloroform crude extracts showed more activity than the ethanol extracts as it inhibited all Gram–positive bacteria. All extracts inhibited the Gram-negative bacterium, *E. cloacae* which was suprising since Gram-negative bacteria are not easily inhibited (Kudi *et al.*, 1999). The chloroform extracts of *S. longependunculata* showed activity in more than five zones against *B. cereus* bacteria in direct bioassays on TLC.

8.5. *In vitro* effect of *S. longependuncula*ta extracts on sperm parameters.

Ethanol crude extracts of *S. longependuncula*ta was found to immobilize sperm at a concentration of 2.5-10.0 mg/ml. The same concentration was found to kill nearly 100% of sperm after 24 and 48 hrs while 36.0% were alive at the concentration of 1.0 mg/ml. Membrane integrity were decreased to 9.0 and 13.0% at the concentrations of 6.5 and 10.0 mg/ml respectively. Furthermore, the DNA integrity (Chromatin packaging) of the sperm was not affected at all by the crude extracts. This suggests that, the sperm can still fertilize the egg if it has enough motility.

8.6. Xanthones isolated from *S. longependuncula*ta.

The genus *Securidaca* is known to contain many secondary compounds such as securinine, methyl salicylate, 1,2,7- trimethoxyxanthone, etc. A study by Galeffi *et al.* (1990) lead to the isolation of the new compound 1,7-dimethoxy-2-hydroxyxanthone. In our study, the purification of acetone extracts lead to the isolation of the same compound as well as 1,4-dihydroxy-7-methoxyxanthone and the new compounds 1,3,6,8-tetrahydroxy-2,5-dimethoxyxanthone and 1,6,8-trihydroxy-2,3,4,7-tetramethoxyxanthone which is the first example of a heptahydroxyxanthone derivative isolated from natural sources.

8.7. Biological activity of xanthones isolated from *S. longependunculata*

Three of the compounds isolated relaxed rabbit corpus cavernosum smooth muscle and one of them, 1,3,6,8-tetrahydroxy-2,5-dimethoxyxanthone (97.0% relaxation) showed similar activity to Viagra at the same concentration of 1.8×10^{-5} mg/ml. The other two, 1,7-dimethoxy-2-hydroxyxanthone and 1,6,8-trihydroxy-2,3,4,7-tetramethoxyxanthone relaxed the muscles 63.0% and 30.5% respectively. Two of the compounds, 1,3,6,8-tetrahydroxy-2,5-dimethoxyxanthone and 1,6,8-trihydroxy-2,3,4,7-tetramethoxyxanthone inhibited *Bacillus cereus* and *Bacillus subtilis* respectively at the MIC of 0.1 mg/ml. None of the other, 1,7-dimethoxy-2-hydroxyxanthone and 1,4-dihydroxy-7-methoxyxanthone inhibited any bacteria used in this study. Since the plant extracts had good antibacterial activity, it is possible that these two compounds do not have antibacterial activity.

8.8. References

Adimoelja A (2000) Phytochemicals and the breakthrough of traditional herbs in the management of sexual dysfunctions. International Journal of Andrology 23, Suppl.2: 82- 84.

Balick MJ (1990) Ethnobotany and the identification of therapeutic agents from the rainforest. Bioactive compounds from plants. Wiley. Chichester. Ciba Foundation Symposium 154: 22- 39

Galeffi G, Federici E, Msonthi JD, Marini-bettolo GB, Nnicoletti M (1990) New xanthone from *Ectiadiopsis oblongifolia* and *Securidaca longependunculata.* Fitoterapia LXI (1): 79- 82.

Hamburger M; Gupta M, Hostetmann K (1985) Flavonol glycosides from *Securidaca diversifolia.* Phytochemistry 24 (110): 2689- 1174.

Kim HJ, Woo DS Lee G Kim JJ (1998) The relaxation effects of ginseng saponin I rabbit coporal smooth muscle: is it a nitric oxide donor? British Journal of Urology 82: 744- 748.

Kudi AC, Umoh JU, Eduvie LO, Gefu J (1999) Screening of some Nigerian medicinal plants for antibacterial activity. Journal of Ethnoparmacology 67 (2): 225-228.

Neuwinger HD (1996) African ethnobotany. Poisons and drugs. Chapman and Hall, Germany.

Phillipson JD (2001) Phytochemistry and medicinal plants. Phytochemistry 56: 237-243.

Sidik (1999) Ethnopharmacognosy and photochemical aphrodisiac. Published in Indonesia. Seminar on 'Botanical Aphrodisiac' 'Untag' University of Jakarta. Indonesia. November 11.

Van Wyk B-E, Van Oudtshoon B, Gericke N (1997) Medicinal plants of South Africa. Briza Publications, Pretoria.

CHAPTER 9

Summary

CHAPTER 9

Summary

by

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People in the Northern Province (South Africa) especially the Vhavenda still depend heavily on herbal plants for their primary health care. This is also true for both rich and poor people around the world. In the treatment of male erectile dysfunction the species *Securidaca longependuncula*ta (Polygalaceae), *Wrightia natalensis* (Apocynaceae) and *Rhoicissus tridentata* (Vitaceae) are the most common herbal plants used by the Vhavenda. Although *S. longependuncula*ta and *W. natalensis* are not easily found in the wild, traditional healers strive to obtain them, as the demand is high, due to their many medicinal uses.

The traditional claim that *S. longependunculata, W. natalensis* and *R. tridentata* can treat erectile dysfunction was verified to some extent in this study since both plants relaxed rabbit corpus cavernosum smooth muscle *in vitro* at the concentrations of 6.5-13.0 mg/ml. Although, all of the herbal extracts showed activity, *S. longependunculata* was the most active plant and this led to the investigation into its active compounds.

The root extracts of *S. longependunculata*, *W. natalensis* and *R. tridentata* showed antibacterial activity against some bacteria including the Gram-negative bacteria *Enterobacter cloaceae* and *Escherichia coli*. The inhibition of the bacteria occurred at concentrations from 1.0-50.0 mg/ml. Antibacterial activity was also shown by *S. longependunculata* chloroform extracts on *Bacillus cereus* through direct bioassays where five active compounds were observed.

Ethanol extracts of *S. longependunculata* were tested for their effects on sperm parameters. The results showed that at low concentrations (1.0-2.5 mg/ml) sperm motility, vitality and membrane integrity were not affected, whilst at higher concentrations (6.5-10.0 mg/ml) they were affected. It was also found that although the extract affected sperm parameters, the chromatin packaging of treated sperm was not affected. Therefore it is possible that at high concentrations the extracts can be used both for erectile dysfunction treatment as well as a contraceptive. The deterioration of sperm motility, vitality and membrane integrity may be due to the presence of other compounds in the extracts. Further tests on isolated compounds should however, be carried out.

Four compounds, two previously isolated (1,7-dihydroxy-2-methoxyxanthone and 1,4-dihydroxy-7-dimethoxyxanthone) and two new compounds (1,3,6,8-tetrahydroxy-2,5-dimethoxyxanthone and 1,6,8-trihydroxy-2,3,4,7-tetramethoxyxanthone) were isolated from *S. longependunculata* using silica gel and thin layer chromatography. All the compounds were tested for smooth muscle relaxation and antibacterial activity. In future, the compounds will be tested for their effects on sperm parameters as well.

When tested for erectile dysfunction activity, three compounds (1,7-dihydroxy-2-methoxyxanthone, 1,3,6,8-tetrahydroxy-2,5-dimethoxyxanthone and 1,6,8-trihydroxy-2,3,4,7-tetramethoxyxanthone) showed tremendous activity on the relaxation of smooth muscle. The other compound (1,4-dihydroxy-7-dimethoxyxanthone) showed no sign of relaxing the corpus cavernosal smooth muscle. When tested for their antibacterial effect, only 1,3,6,8-tetrahydroxy-2,5-dimethoxyxanthone and 1,6,8-trihydroxy-2,3,4,7-tetramethoxyxanthone inhibited *Bacillus subtilis* and *Staphylococcus aureus* at the minimum inhibitory concentration of 0.1 mg/ml.

This study opened up an opportunity to explore the extraordinary plants and culture diversity of South Africa to the possible benefit of mankind. However, people need to harvest these plants carefully to avoid the extinction of valuable plants.

CHAPTER 10

Acknowledgements

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Acknowledgements

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