

CHAPTER 1

PHYTOTHERAPY AND BENIGN PROSTATIC HYPERPLASIA

1.1 INTRODUCTION

1.1.1 History

Hypoxis hemerocallidea extracts were used traditionally as folk medicine to treat symptoms of cancers, testicular tumours, prostate hypertrophy and urinary disease (Albrecht et al., 1995b) for centuries. A South African, Mr. Roelof Wilke Liebenberg, took note of the African potato in the 1950's when his uncle's prostate cancer was cured after being treated with African potato tea. He made contact with Dr. Scheffel in Germany, who in turn persuaded an urologist, Dr. Ebbinghouse, to treat patients with benign prostatic hyperplasia (BPH) at the Kreis Krankenhaus with *H. hemerocallidea* extracts. Meanwhile Mr. Liebenberg requested a phytochemist from the University of the Witwatersrand, Prof. Karl Pegel, to isolate and identify the active compound in the African potato. Pegel and his assistant extracted a fatty substance which was identified as β -sitosterol. The German clinical trials were successful and in 1974 a patented remedy, Harzol[®] (10 mg β -sitosterol and 0.1 mg β -sitosterolin per capsule), was released. A few years later the South African equivalent, Moducare[®]/Sterinol[™] (20 mg β -sitosterol and 0.2 mg β -sitosterolin per capsule), was released in South Africa (Vanderhaeghe & Bouic, 1999).

1.1.2 Aim of chapter

In this chapter, literature background on phytosterols and their use in immune modulation; hypercholesterolaemia, and specifically, indications in treating benign prostatic hyperplasia (BPH), is discussed. Three BPH phytotherapeutic plants *Hypoxis hemerocallidea*, *Prunus africana* and *Serenoa repens*, are also considered in terms of their mechanisms of action, the extraction processes, their general compositions, recommended daily therapeutic dosages, efficacy and side effects.

1.2 STEROLS: PHYTOSTEROLS VERSUS CHOLESTEROL

Steroids are members of a large class of lipid compounds called terpenes, which are biogenically derived from the same parent compound, isoprene (C_5H_8). Steroids contain, or are derived from, the perhydro-1,2-cyclopentenophenanthrene ring system (Kroschwitz & Howe-Grant, 1997).

Phytosterols are steroid alcohols (plant fats) similar to the major animal steroid alcohol (sterol), cholesterol (5-cholesten-3 β -ol). β -Sitosterol (24 β -ethyl-5-cholesten-3 β -ol) differs from cholesterol only by an extra ethyl group in its side chain (Bouic et al., 1997). Plant and animal sterols have very distinct biological effects although they are both essential cell membrane components, and phytosterol esters have a similar fatty acid transport and storage function as cholesterol esters (Pegel, 1997). Phytosterols are synthesised in plants whereas humans and animals obtain them through diet (Bouic & Lamprecht, 1999). However, the human absorption of β -sitosterol is 5% or less of the daily intake in comparison with the 50% absorption of cholesterol (Salen et al., 1970). The molecular mechanism regulating the amount of cholesterol retained in the body and selectively excluding other dietary sterols, is poorly understood, but is probably genetically determined (Platel et al., 1998). Sterols never exist on their own in nature, but are always found in combination with their glucosides, which are called sterolins. Thus a sterolin is a sterol with a sugar attached to it. All plants, including fruits; vegetables, seeds, and nuts contain sterols and sterolins (Vanderhaeghe & Bouic, 1999). Approximately 80% of the total phytosterol content of higher plants is composed of β -sitosterol, with about 1% in its glucosidated form (Bouic et al., 1997). Other important phytosterols are campesterol (24 α -methyl-5-cholesten-3 β -ol) and stigmasterol (3 β -hydroxy-24-ethyl-5,22-cholestadiene). See Figure 1.1 for the chemical structures of the sterols and Figure 1.2 for that of β -sitosterolin.

It is important to note that the term “ β -sitosterol” on a product usually represents a defined extract of phytosterols with β -sitosterol as the main component. Therefore, even though Harzol[®]'s container states that every capsule contains 10 mg β -sitosterol and 0.1 mg β -sitosterolin, it in fact contains mainly β -sitosterol and smaller amounts of campesterol, stigmasterol and other sterols along with their glucosides (Berges et al., 1995).

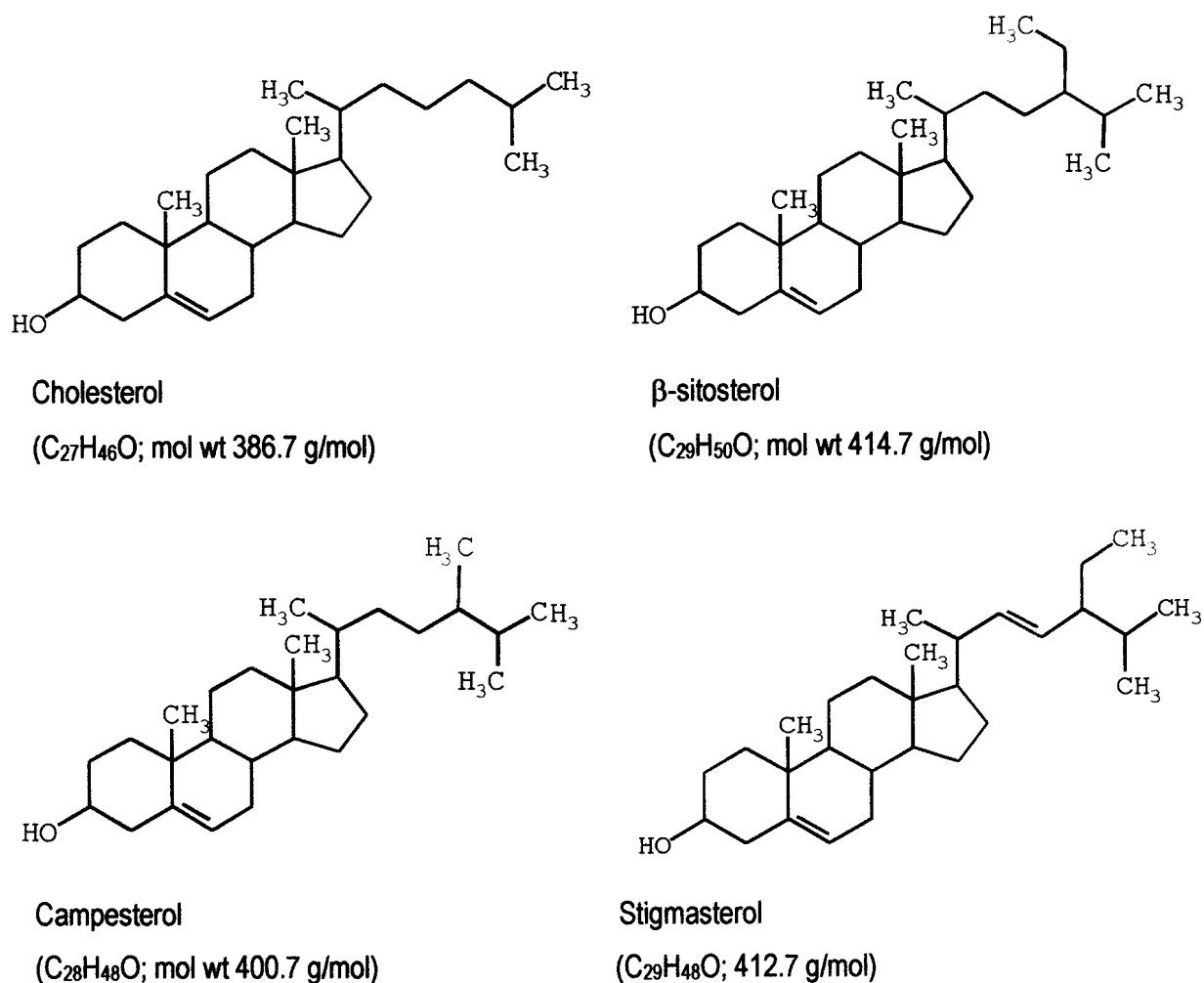


Figure 1.1: The chemical structures, molecular formulae and weights of cholesterol, β -sitosterol, campesterol and stigmasterol. The red groups indicate the differences from cholesterol.

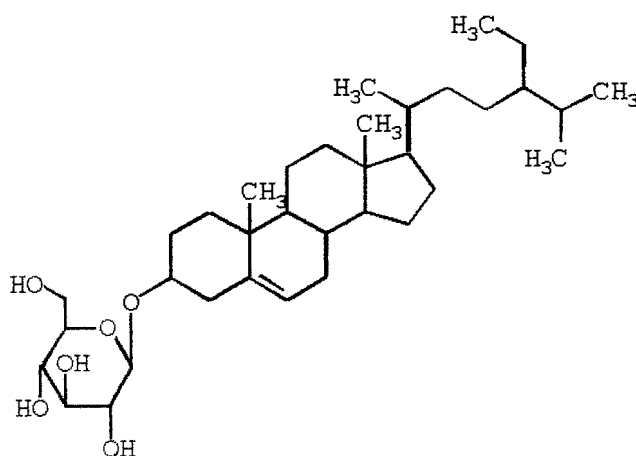


Figure 1.2: Chemical structure of β -sitosterolin ($C_{35}H_{60}O_6$; mol wt 576.9 g/mol)
(β -sitosteryl β -D-glucoside)

1.3 THERAPEUTIC INDICATIONS OF PHYTOSTEROLS

The three major indications for phytosterols are immune modulation (Bouic et al., 1999), hypercholesterolaemia (Nguyen, 1999) and benign prostatic hyperplasia (Wilt et al., 1998), although, research on β -sitosterol also revealed antihyperglycemic and insulin-releasing effects (Ivorra et al., 1988), anti-inflammatory (similar to cortisone)- and antipyretic (similar to aspirin, but not analgesic) effects (Gupta et al., 1980).

1.3.1 Immuno-modulation – Reinstating the balance in the immune system

1.3.1.1 The immune response

T-cells (thymus lymphoid cells) are divided into T-helper cells (CD4+), T-suppressor cells (CD8+) as well as cytotoxic T-cells (CD8+), all of which mediate cellular immunity. B-cells (bone marrow lymphoid cells) form antibodies and mediate humoral immunity. The T-cells recognise antigens e.g. pathogens, and release chemical substances (cytokines) to regulate the immune system – stimulation or suppression. T-helper cells (CD4+) are divided into type 1 (T_H1) and type 2 (T_H2) cells (Ingraham & Ingraham, 1995).

T_H1 releases interleukin 2 (IL2) and gamma interferon (IFN- γ), that bind to, and activate, the (CD8+) cytotoxic T-cells (T_c) to become killer T-cells. These killer cells attack and remove intracellular pathogens (Vanderhaeghe & Bouic, 1999) e.g. viruses and *Mycobacterium tuberculosis*, cancerous cells and unfortunately, donor transplant cells (Ingraham & Ingraham, 1995).

T_H2 secretes interleukin 4 (IL4), IL6 and IL10, that stimulate B-lymphocyte differentiation into antibody forming cells (forming immunoglobulins e.g. IgE, IgG etc). Other important immune factors are phagocytes (granulocytes and macrophages) and natural killer cells. The natural killer cells form the first line of defence against pathogens and cancer cells by releasing toxic enzymes and do not need activation by T-cells (Vanderhaeghe & Bouic, 1999).

Too high levels of IL4 promote allergic responses, and too high levels of IL6 are associated with auto-immune disorders, inflammatory and allergic conditions. In health there is a delicate balance between T_H1 and T_H2 which cross-regulate each other's activity. Cortisol, the stress hormone,

enhances T_H2 activity and increases the release of IL6 which promotes inflammation and destruction of body tissues. DHEA (dehydroepiandrosterone) antagonizes cortisol and enhances T_H1 activity which balances the immune system (Vanderhaeghe & Bouic, 1999).

Table 1.1: The dichotomy of T-helper cells based on their defining cytokine profiles and functions (Bouic et al., 1999)

Cell	Cytokine profile	Function of TH subset
T_H1	IL2, IFN- γ	Activation of cytotoxic cells Antagonism of T_H2
T_H2	IL4, IL6, IL10	Activation and maturation of B-cells Antagonism of T_H1

If T_H1 -levels drop (due to cortisol release, as with unrelenting stress, DHEA decrease or chronic viral and bacterial diseases) T_H2 -levels will rise. A humoral, but non-protective immune response, will result and chronic conditions such as cancer, allergies ($\uparrow$ IgE, $\uparrow$ IL6, $\uparrow$ IL4) and auto-immune disorders ($\uparrow$ IL6 - rheumatoid arthritis, lupus erythematosus, pernicious anaemias, Type 1 diabetes mellitus, Crohn's disease *etc.*) can appear (Vanderhaeghe & Bouic, 1999).

1.3.1.2 Immune stimulation and suppression by sterols/sterolins

Both β -sitosterol and β -sitosterolin enhance T-cell proliferation *in vitro* and *in vivo*. However, they increase T_H1 (immuno-stimulation) and, therefore, IL2 and γ -interferon secretion and cytotoxic T-cell activity is augmented, but T_H2 is reduced (immuno-suppression) resulting in reduced IL4, IL6 and IL10 levels, Thus reinstating the balance (immuno-modulation). A mixture of β -sitosterol and β -sitosterolin in a ratio of 100:1 is claimed to be more effective than either sterol or its glucoside alone – synergism (Vanderhaeghe & Bouic, 1999).

Indications:

$\uparrow T_H1$ ($\uparrow$ IL2, $\uparrow$ IFN- γ) – Immuno-stimulation

- Cancer - $\uparrow$ IL2, $\uparrow$ IFN- γ , $\uparrow T_c$ (Vanderhaeghe & Bouic, 1999). - HT-29 colon cancer (Pegel, 1997).
- AIDS - $\uparrow$ IL2, $\uparrow$ IFN- γ - FIV (cats) and HIV (humans) CD4 counts remained stable with no progression of the disease - up to date (36 months). Plasma viral loads also declined as result of

an enhanced cell-mediated immune response. Sterols and sterolins do not have anti-viral properties (Bouic et al., 1999).

- Tuberculosis - ↑eosinophil & ↑lymphocyte counts, faster weight gain (Donald et al., 1997)
- Post-marathon or high intensity exercise immuno-suppression - ↑DHEA, ↑T_H1, IL2 & IFN-γ and ↓cortisol, ↓T_H2, ↓IL6 (Bouic et al., 1999)
- Others: Chronic fatigue syndrome (↑T_C, ↑IL2, ↑IFN-γ),

↓ T_H2 (↓IL4, ↓IL6) – Immuno-suppression

- Rheumatoid arthritis (↓IL6, ↓ B-cell antibody formation) and other auto-immune diseases e.g. Type 1 diabetes mellitus (↓IL6, ↓ B-cell antibody formation, ↑DHEA & ↓cortisol) – can decrease insulin requirement (Vanderhaeghe & Bouic, 1999).
- Allergies (↓IL4, ↓IL6 → ↓IgE) – asthma, allergic eczema, chronic rhinitis and sinusitis, hives and rashes (Vanderhaeghe & Bouic, 1999).
- Hepatitis C – (↓IL6, ↓ B-cell antibody formation) - Reduce attacks on liver cells by antibodies (↑ IL2, ↑IFN-γ) increased protection against viral infection (Vanderhaeghe & Bouic, 1999).

1.3.2 Hypercholesterolaemia

β-sitosterol is absorbed less efficiently (1/10) than cholesterol, but in high dosages (≥10 g/d) it lowers the absorption of cholesterol by 10–20%, and lowers total cholesterol and low density lipoprotein (LDL) levels. Plant sterols appear to decrease the solubility of cholesterol in oil and micellar phases, thus displacing cholesterol from bile salt micelles and interfering with its absorption (Nguyen, 1999). They can, therefore, be used to treat mild cases of hypercholesterolaemia (Pegel, 1997).

However saturated derivatives of plant sterols, called plant stanols (Δ4 double bond absent), can reduce serum cholesterol at lower doses (2-3 g esterified sitostanol lowers LDL cholesterol by 10-15%). They reduce total cholesterol and LDL, but not high-density lipoprotein (HDL) or triglyceride (TG) levels. These plant stanols are virtually unabsorbable and remain in the intestinal lumen where they can interfere continuously and more efficiently with micellar solubility of cholesterol. The most common dietary plant stanol is sitostanol, the saturated derivative of sitosterol. It occurs naturally in wood pulp, tall oil and in lesser amounts in soybean oil. The Western daily diet contains 100-300 mg

plant sterols and 20-50 mg plant stanols. In 1995 the Finnish introduced plant stanol esters (PSE) into margarine as dietary adjuncts, to lower cholesterol (Nguyen, 1999).

Sitosterolaemia (phytosterolaemia) is a rare, autosomal, recessively inherited disorder characterised by premature coronary artery disease, skin xanthomas and increased plasma plant sterols, stanols and cholesterol. Affected individuals show an increased absorption of both cholesterol and sitosterol from the diet, which distinguishes it from homozygous familial hypercholesterolaemia. Furthermore, the excretion of both cholesterol and phytosterols into the bile is also greatly impaired. The molecular defect in sitosterolaemia has not been identified, but it is probably determined genetically (Platel et al., 1998). The role of the increased serum phytosterol levels in the resulting premature coronary artery disease and skin xanthomas has not been elucidated.

1.3.3 Benign prostatic hyperplasia

Benign prostatic hyperplasia (BPH) is a common medical condition in older men affecting as many as 40% of men aged 70 years and older (Wilt et al., 1998). Symptoms associated with the disease include urgency, frequency, hesitancy, weak stream and incomplete bladder voiding (Segars, 1999). In the United States of America, treatment exceeds \$2 billion in costs; accounts for 1.7 million physician office visits, and results in more than 100 000 prostatectomies annually (Wilt et al., 1998).

1.3.3.1 Allopathic treatment

Western medicines are seen as those opposing the so-called alternative, complementary or unconventional medicines (including phytotherapy). These western medicines have to be critically evaluated for safety, efficacy etc. and approved by the American Food and Drug Administration or related council (the Medicines Control Council in South Africa) before production or marketing. Alternative medicines on the other hand are regarded as dietary supplements and regulated under the 1994 Dietary Supplement Health and Education Act in the USA (Food supplements, Cosmetics and Disinfectants Act 54 of 1972, in South Africa) (Marks & Tyler, 1999). Though it seems as if certain of these herbal medicines, especially those for which medicinal claims are made, will have to be registered under the Medicines and Related Substances Control Act 101 of 1965, in South Africa in the future.

Transurethral resection of the prostate in men with symptoms of obstruction is the standard treatment (Berges et al., 1995). The most commonly used western medicines for the treatment of BPH are α_1 -adrenergic antagonists such as prazosin, terazosin, doxazosin and trimazosin, as well

(QoL); increases the peak urinary flow rate ($Q_{\max}$) and decreases the post void residual urinary volume (PVR). (Klippel et al, 1997). These results are comparable with those achieved by α_1 -blockers or 5- α -reductase inhibitors, but in contrast to finasteride, β -sitosterol does not reduce prostate size. Adverse effects, such as dizziness, decreased blood pressure, tachycardia etc. associated with α_1 -blockers (Berges et al., 1995), and erectile dysfunction, ejaculatory disturbance or altered libido associated with finasteride (Marks & Tyler, 1999), are absent with β -sitosterol (Berges et al., 1995).

However, the problem of drug therapy (allopathic or phytotherapeutic) in the treatment of BPH is complicated by the fact that the natural history of the untreated disease is one of fluctuating symptoms and spontaneous improvements (Kadow & Abrams, 1986). It is well known that placebo effects occur in pharmacological therapies in general and particularly in patients with BPH who wish to avoid operative intervention; responses of up to 40% or more have been reported (Klippel et al., 1997). Ideally, BPH treatment and follow-up should be for years rather than months and placebo control is essential (Kadow & Abrams, 1986).

1.3.3.3 Phytosterols' mechanism of action in BPH

It is speculated that the activity of phytosterols can be ascribed to enzymatic effects (inhibition of 5 α -reductase leading to a reduction in dihydrotestosterone levels) or to decreased binding of dihydrotestosterone within the prostate (Van Wyk et al., 1997). Other possible mechanisms of action are competition with androgen precursors and inhibition of prostaglandin biosynthesis (anti-inflammatory) by the phytosterols in the extract. The pentacyclic terpenes exhibit anti-inflammatory activity by inhibiting glucosyl transferase and β -glucuronidase enzymes involved in the depolymerisation of proteoglycans in the connective tissues (Shulz et al., 1991).

1.3.3.4 β -Sitosterol, β -sitosterolin or combination?

Dr. Ebbinghouse of the Kreis Krankenhaus, Germany, (See 1.1.1 History) experienced that β -sitosterol alone was not as effective as the extract of *H. hemerocallidea* on patients with BPH. Prof. Karl Pegel, who isolated and identified β -sitosterol, realised that sterolglucoside, the sterolin, was possibly better absorbed than the sterol, but that during the process of isolation of the sterol, the sterolin was removed via hydrolysis, yielding sterols and glucose. This was the reason why the β -sitosterol used on Dr. Ebbinghaus's patients did not work as well – it was lacking the “key to the immune-enhancing effect”, the sterolin. Together with Prof. C.B. Rodgers, a product containing

sterols and sterolins was developed and capsules were sent to Dr. Ebbinghaus in Germany. Several clinical trials were performed there, resulting in the release of a patented BPH remedy, Harzol® (10 mg β -sitosterol and 0.1 mg β -sitosterolin per capsule) in 1974 (Vanderhaeghe & Bouic, 1999). However, a double blind, placebo-controlled urodynamic study on 53 patients with proven outflow obstruction, has failed to prove that β -sitosterolin (formula WA184) is superior to a placebo in the treatment of outflow obstruction due to BPH, when administered at a dose of 0.3 mg/day (Kadow & Abrams, 1986). Thus, β -sitosterolin alone is also not effective in treating BPH, but a combination of β -sitosterol and β -sitosterolin has been found to be effective.

1.4 PHYTOSTEROL CONTAINING BPH PHYTOTHERAPEUTICS

Phytotherapy, the use of plant extracts, for treating BPH symptoms, was first described in Egypt in the 15th century BC. Currently phytotherapy is common in Europe and increasing in usage in the western hemisphere (Wilt et al., 1998). The most widely used and thoroughly tested of the BPH phytotherapies is saw palmetto, scientifically known as *Serenoa repens* or *Sabal serrulata* (Marks & Tyler, 1999). There may be some confusion regarding the names used for the particular plants in this study as earlier synonyms may be used in addition to the widely accepted scientific names. Only one of these names will be used. To avoid confusion the recognised name, synonyms, common name, plant part used for extraction and trade name of some plants, often used as BPH phytotherapeutic agents, are tabled in Table 1.2. Besides saw palmetto, other plant extracts that are used for the treatment of lower urinary tract symptoms (LUTS) associated with BPH, include the following: *Hypoxis hemerocallidea* (African potato), *Prunus africana* (African plum), *Urtica dioica* (stinging nettle), *Secale cereale* (rye pollen), *Cucurbita pepo* (pumpkin seeds), *Picea* (spruce) and *Pineas* (pine) (Lowe et al., 1998).

In this chapter three of the above mentioned plants will be considered: *H. hemerocallidea*, *P. africana* and *S. repens*, in terms of their mechanism of action in BPH, the extraction process, their general composition, recommended daily therapeutic dosage in BPH, efficacy and side effects.

Table 1.2: Different names and trade names of some important BPH phytotherapeutic agents (composed from data by Lowe et al., 1998)

Recognised name	Synonym	Common name	Part of plant used	Tradename
<i>Serenoa repens</i>	<i>Sabal serrulata</i>	Saw palmetto	Berries	Permixon® Strogen® etc.
<i>Hypoxis hemerocallidae</i>	<i>Hypoxis rooperi</i>	African potato	Corms (rhizomes)	Harzol®
<i>Prunus africana</i>	<i>Pygeum africanum</i>	African plum/ Red stinkwood	Bark	Tadenan®
<i>Urtica dioica</i>		Stinging nettle	Roots	Prostagutt Forte® (with Saw palmetto)
<i>Secale cereale</i>		Rye pollen	Pollen	Cernilton®
<i>Cucurbita pepo</i>		Pumpkin seed	Seeds	

1.4.1 *Hypoxis hemerocallidea* (African potato)

1.4.1.1 General information

The family, Hypoxidaceae, includes fifty *Hypoxis* species (Arnold & De Wet, 1993) of which *H. hemerocallidea* (previously also known by the name *H. rooperi*) is probably the most important in terms of medicinal use. The plants are herbaceous perennials with long, strap-shaped leaves and yellow, star-shaped flowers. The species can be distinguished by the size, shape and orientation of the leaves and the size and shape of the flowers (Van Wyk et al., 1997). They have tuberous rhizomes or corms (up to 10 cm in diameter and length and up to 2 kg in weight) and abundant adventitious roots that enable them to survive under high-stress conditions. Members of Hypoxidaceae are found mainly in the southern hemisphere, especially in Africa (Albrecht et al., 1995b).

Traditional uses in folk medicine have been reported to include treatment of cancers, testicular tumours, prostate hypertrophy and urinary disease (Albrecht et al., 1995b).

In Germany a lipophilic extract of *H. hemerocallidæ* is marketed as a phytomedicine Harzol® and has been used for the treatment of prostate hypertrophy since 1974 (Pegel, 1997). Harzol® contains β -sitosterol and its glucoside in the ratio 10:0.1 per capsule. In South Africa, the dried rhizomes are marketed (Prostamin®) for the preparation of an aqueous potion for the same purpose (Nicoletti et al., 1996). Another German β -sitosterol preparation, Azuprostat®, contains 65 mg of a chemically defined extract of phytosterols, with β -sitosterol as the main component, per capsule. These β -sitosterols are derived from different plants, for example, species of *Pinus*, *Picea* or *Hypoxis* spp. (Klippel et al., 1997).

1.4.1.2 General composition

From the rhizomes of *H. obtusa*, a norlignan diglucoside named hypoxoside was isolated and later the occurrence of hypoxoside in several *Hypoxis* spp. was reported (Nicoletti et al., 1996). Hypoxoside is the trivial name for (E)-1, 5-bis (4'- β -D-glucopyranosyloxy-3'-hydroxyphenyl) pent-4-en-1-yne. It is a pale yellow, water-soluble, crystalline compound which is readily converted to the more lipophilic aglucone rooperol by beta-glucosidase action (Albrecht et al., 1995b). See Figure 1.4.

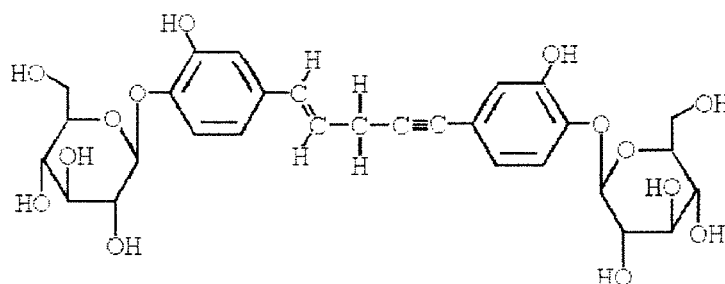


Figure 1.4: Chemical structure of hypoxoside ($C_{29}H_{34}O_{14}$; mol wt 606.6g/mol)

Albrecht et al. (1995a), analysed a standardised *Hypoxis* extract, supplied by ESSENTIAL STEROLIN PRODUCTS in capsule form, each capsule containing 200 mg plant extract. Routine HPLC analyses assured uniform quality of each batch of plant extract within the limits as shown in Table 1.3:

Table 1.3: Composition and limits of a standardized *Hypoxis* plant extract detected by HPLC analyses (composed from data by Albrecht et al., 1995a)

Component	Percentage of contents
Hypoxoside	50-55%
β -sitosterol	8-12%
β -sitosterolin	0.2 – 0.3%
Complex carbohydrates with traces of reducing sugars, amino acids, tannins and flavonoids (not detectable by HPLC)	33-42%
Alkaloids	0%

1.4.1.3 Mechanism of action

The activity of the drug against prostate adenoma and prostatic hypertrophy is ascribed mainly to phytosterol glucosides, mainly β -sitosterol. Anti-cancer (Vanderhaeghe & Bouic, 1999; Pegel, 1997), anti-HIV (Bouic et al., 1999) and anti-inflammatory (Gupta et al., 1980) activity are ascribed to β -sitosterol and rooperol (aglucone of hypoxoside) (Van Wyk et al., 1997).

As regards, cytotoxicity, *In vitro* hypoxoside is non-toxic to cancer cells at concentrations up to 100 $\mu\text{g/ml}$. However, when it is hydrolysed to its aglucone, rooperol, by β -glucosidase, cytotoxicity is found at concentrations ranging from 2 to 10 $\mu\text{g/ml}$ (Albrecht et al., 1995a). Hypoxoside given orally to mice is deconjugated to form rooperol by bacterial β -glucosidase enzymes in the caecum and colon. The rooperol is then absorbed and converted to new conjugates (sulphates and glucuronides) by phase II biotransformation and then excreted into the bile and absorbed into mice portal blood. Even though no hypoxoside, rooperol, or rooperol metabolites entered the general circulation of the mice, the situation in humans was strikingly different and metabolites appeared in the serum of humans (Albrecht et al., 1995b). See Figure 1.5 for the biotransformation of hypoxoside in mice and primates. Albrecht et al. (1995b) demonstrates that these metabolites could be activated to become cytotoxic to melanoma cells, in culture, in the presence of β -glucuronidase as monosulphates of rooperol and dehydroxyrooperol (with glucuronic acid) are formed. They also found that extracts of human tumour (aqueous supernatant of human melanoma) could also deconjugate the metabolites and thus

activate them for their cytotoxic action. Hypoxoside could thus be a non-toxic pro-drug for cancer therapy (Albrecht et al., 1995b).

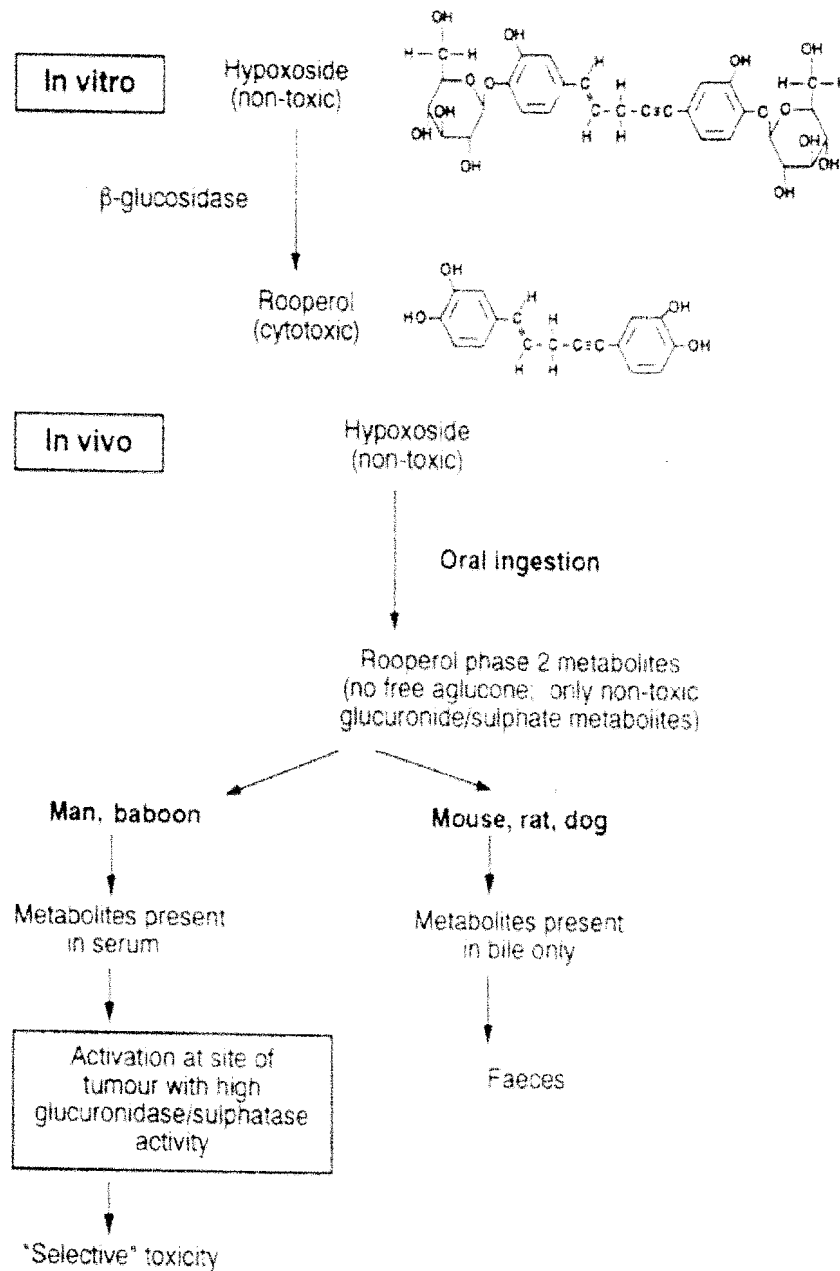


Figure 1.5: The biotransformation of hypoxoside to cytotoxic rooperol (Smit et al., 1995)

The cytotoxic effect of rooperol manifested as vacuolisation of the cytoplasm and formation of pores in the plasma membrane. The molecular basis of rooperol cytotoxicity still needs to be clarified, but

previous biochemical studies have shown that rooperol is a potent inhibitor of leukotriene synthesis in polymorphonuclear leukocytes at concentrations of 1 μM or less. The synthesis of cyclooxygenase products, TXB_2 (tromboxane B_2) and PGD_2 (prostaglandin D_2), are inhibited only at concentrations between 10 and 100 μM . Rooperol-induced growth inhibition occurs at concentrations ranging from about 0.6 to 8 $\mu\text{g/ml}$ which is equivalent to about 1 – 13 μM . It is, therefore, possible that the cytotoxicity is triggered by inhibition of leukotriene synthesis. Another possibility is that rooperol may be oxidised to form reactive semiquinone- and quinone radicals that could damage cell membranes directly (Albrecht et al., 1995b). This is known to occur with dicatechols such as the closely related cytotoxic agent, nor-dihydroguaiaretic acid (Albrecht et al., 1995b).

1.4.1.4 Extraction

Shredded *Hypoxis* corms are dehydrated in a convection oven at 70°C for 3.5 hours and then milled to a powder, which can be extracted with 25l of methanol at room temperature for 30 minutes (Kruger et al., 1994). The corms can also be freeze-dried with liquid nitrogen and extracted with 80% ethanol in the dark at 23 °C for 4 hours (McAllister & van Staden, 1994).

Another extraction method used locally, is that of Santjie Marx African potato product (promotional pamphlet). She proposes that c. 6.4 g of dried *Hypoxis* be simmered in 1200 ml water for 15 to 20 minutes and 600 ml should be taken daily.

1.4.1.5 Presentation and dosage

Santjie Marx Product's dosage is 200 ml {200 ml equals 416 mg dry extract as water only extracts 39% (Eloff, 1999)} three times daily (promotional pamphlet). Local preparations that are available in most pharmacies are Moducare® and Immunochoice®. These preparations contain only phytosterols and their glucosides (Immunochoice® contains absorption enzymes as well), without any hypoxoside or rooperol. Moducare® is available as capsules containing 20 mg phytosterols and 0.2 mg sterolins per capsule in an internationally patented formulation. The daily dosage is 60 mg daily (one capsule three times daily). Immunochoice® is available as capsules containing a 20 mg mixture of phytosterols (β -sitosterol, campesterol and stigmasterol) and sterol glucosides (sterolins), as well as 50 mg of enzymes (formula GR8CF-77) to promote the absorption of the phytosterols and sterolins per capsule. The absorption of β -sitosterol is only about 5% in comparison with the 50% absorption of cholesterol (Pegel, 1997). The dosage of Immunochoice® is also one capsule, three times daily.

Nutricare 2000® is also a South African phytosterol preparation (400 mg capsules, containing 58 mg fat, including sterols) but it differs from Moducare® and Immunochoice® in that hydroponically grown oilseed sprouts are harvested at their peak and freeze-dried in less than an hour, thus no chemical extractions are necessary (Anon, 2000). It is claimed that the absorption of the natural sterols in plants, especially in sprouts, is about 80% in comparison with the 5% absorption of sterol extracts (Personal communication with Dr. van Brummelen, BIOMOX PHARMACEUTICALS; Maria Ascencao, spokesperson of Bioharmony). Nutricare 2000® also contains digestive enzymes that are derived from wheat, barley, lupins and fenugreek sprouts and blended with the phytosterols derived from sprouted oilseeds. These enzymes are supposed to increase the bio-availability of the sterols (Anon, 2000). The dosage is one capsule three times a day, as well.

1.4.1.6 Efficacy

A study was performed by Berges et al. (1995) on 200 randomized patients who received either a placebo or β -sitosterol, at a dose of 20 mg, three times daily for 6 months. The results of the treatment group, in comparison with the placebo group, were promising. The IPSS decreased from 14.9 to 7.5 and the maximal urinary flow (Qmax) increased from 10.0 to 15.2 ml/s (Fitzpatrick, 1999).

1.4.1.7 Side effects

A study done to assess the toxicity of hypoxoside taken orally by 24 patients with lung cancer found no toxic effects in clinical examinations or biochemical or haematological measurements. Only one occasion of possible drug intolerance, at a dosage as high as 2400 mg standard *Hypoxis* plant extract (200 mg capsules) daily, with anxiety, nausea, vomiting and diarrhoea, was noted. The dosage of the particular patient was then reduced to 1200 mg per day, which was well tolerated without other incidents for another 36 days, after which the patient had cardio-respiratory failure (not attributable to the drug) (Smit et al., 1995).

1.4.2 *Prunus africana* (African plum)

1.4.2.1 General information

P. africana is the evergreen African plum tree (Lowe et al., 1998). This tall forest tree may reach a height of more than 30 metres. Buttress roots are often present and the bark is coarse, with a dark brown and black colour. It has dark green glossy leaves, small white flowers in elongated clusters and reddish-brown berries of about 10 mm in diameter (Van Wyk et al., 1997).

The bark is reported to be of value in treating chest pain (Van Wyk et al., 1997). Since the first registration by LABORATORIES DEBAT (France, 1969), an extract of the bark of *P. africana* has been used successfully in Europe and elsewhere for the treatment of BPH symptoms. LABORATORIES DEBAT marketed the *P. africana* extract as Tadenan[®] (Breza et al., 1998), but it is currently manufactured by LABORATORIES FOURNIER S.A., FONTAINE les Dijon, France. Other formulations (Prostata[®] etc.) are available throughout the United States, but almost all the research and clinical trials on *P. africana* bark extract have been done using the product Tadenan[®] (Lowe & Fagelman, 1999).

1.4.2.2 General composition

Lipophilic extracts of *P. africana* bark have been shown to contain at least three classes of active constituents that exert a beneficial effect on BPH. Phytosterols (present in both free and conjugated form); pentacyclic terpenes (including oleanolic, crataegolic and ursolic acids); and ferulic acid esters of fatty alcohols (Schulz et al., 1998).

1.4.2.3 Mechanism of action

P. africana bark extract (Tadenan[®]) has been used for the past 25 years in the treatment of micturition disorders associated with BPH at a non-surgical stage. Unfortunately the mechanism of action had not been clearly elucidated (Yablonsky et al., 1997).

Growth factors and their receptors' activation play an important role in the mechanisms of normal and pathological development of the prostate (Yablonsky et al., 1997). The effects of *P. africana* bark extract (Tadenan[®]) on the proliferation of rat prostatic stromal cells were studied by Yablonsky et al. (1997). The inhibitory activity of the extract was tested either on unstimulated or on basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), insulin-like growth factor-I (IGF-I) and keratinocyte growth factor (KGF), stimulated cells. The ability of Tadenan[®] to inhibit ³H-thymidine incorporation into stromal cells was also investigated. When increasing concentrations of the extract were added to either non-stimulated or growth factor stimulated cells, a dose-dependant inhibition of DNA synthesis was observed. However, Levin et al. (1996) found, in their experiments on rabbits with partially obstructed bladders, that the magnitude of increases in bladder mass and the level of DNA synthesis (³H-thymidine incorporation) were similar for the Tadenan[®] treated and untreated animals. Levin et al. (1996), therefore, concluded that Tadenan[®] had no effect on either the increase in bladder mass or the stimulation of DNA-synthesis induced by partial outlet obstruction. The

inhibition of DNA synthesis, according to Yablonsky et al. (1997), was significant at 1 µg/ml of the extract in bFGF; EGF and IGF-I treated cells and 5 µg/ml in non-treated cells. According to their results, stromal cells appeared to be more sensitive to bFGF and EGF than to IGF-I, while they did not show any sensitivity to KGF.

Using a rabbit model of partial outlet obstruction, Levin et al. (1997) identified three major cellular changes in the bladder as result of such obstruction: progressive denervation; mitochondrial dysfunction and disturbances of calcium storage, and release from the sarcoplasmic reticulum. Their hypothesis is that outlet obstruction results in bladder hypertrophy which induces ischaemia. This leads to a release of intracellular calcium, leading to activation of specific enzymes (calcium-activated protease and phospholipase A₂) and to generation of free radicals. These then attack the membranes of nerves, sarcoplasmic reticulum and mitochondria. By pretreating rabbits with *P. africana* extract (Tadenan[®]), Levin et al. (1997) found that the severity of both the contractile and metabolic dysfunctions, induced by partial outlet obstruction, were reduced. They, therefore, suggest that Tadenan[®] might either prevent the activation of degradative enzymes (or generation of free radicals), or protect the intracellular membranes against the destructive effects of free radicals or degradative enzymes.

Other possible mechanisms of action are competition with androgen precursors and inhibition of prostaglandin biosynthesis (anti-inflammatory) by the phytosterols in the extract. The pentacyclic terpenes exhibit anti-inflammatory activity by inhibiting glucosyl transferase and β-glucuronidase enzymes involved in the depolymerisation of proteoglycans in the connective tissues. Ferulic acid esters of fatty alcohols reduce the level of cholesterol in the prostate thereby limiting androgen synthesis (Schulz et al., 1998). When investigating the mechanism of action of selected plant extracts compared to finasteride, Rhodes et al. (1993), found that a concentration of as high as 63 ng/ml Tadenan[®] caused 50% inhibition (IC₅₀) of 5α-reductase. Only 1 ng/ml of finasteride was needed for 50% inhibition of the enzyme, thus *P. africana* bark extract, in the form of Tadenan[®], has weak 5α-reductase inhibitory activity.

1.4.2.4 Extraction

In Natal, the powered bark of the African plum tree was used in the form of a milk suspension for micturition problems (Schulz et al., 1998).

As Tadenan[®] is usually the product on which all the research and clinical trials have been conducted, no extraction is needed in these experiments, therefore, very little information regarding extraction of *P. africana* could be found. Tadenan[®] is manufactured by LABORATORIES DEBAT by solvent extraction of *P. africana* barks and the extract is dispersed in peanut oil. Tadenan[®] is administered orally in capsule form (Levin et al., 1996).

1.4.2.5 Dosage

The currently recommended daily dosage regimen is 50 mg of *P. africana* bark extract twice daily. However, Chatelain et al. (1999) compared the efficacy and safety of 50 mg Tadenan[®] twice daily and 100 mg Tadenan[®] once daily, and found the results to be similar.

1.4.2.6 Efficacy

Breza et al. (1998) did an open three-centre efficacy and safety study on Tadenan[®] in urology clinics in the Czech and Slovak Republics and in Poland. They used the IPSS and Quality of Life (QoL) as subjective assessments and urine flowmetry for objective evaluations. After a two-month treatment period with 50 mg Tadenan[®] twice daily, the mean IPSS was reduced by 40% and the QoL increased by 31%. The mean frequency and nocturia had decreased from 2.62 times per night before treatment to 1.66 times per night after two months of treatment with Tadenan[®]. Their evidence suggests that the beneficial effects of Tadenan[®] developed after one month of treatment, with a further improvement by the end of the second month. These rapid effects are advantageous in comparison with finasteride of which the onset of full activity appears to be slow (6-12 months) (Breza et al., 1998).

1.4.2.7 Side Effects

Acute and chronic toxicity tests in small animals showed *P. africana* to be devoid of severe side effects. Tests for mutagenesis and teratogenesis were negative. The extract appears to be well tolerated in humans following long-term administration (Schulz et al., 1998).

1.4.3 *Serenoa repens* (saw palmetto)

1.4.3.1 General information

Serenoa repens, also known as the American dwarf palm or saw palmetto, its lipoidal berry extract are the most widely used and most thoroughly tested BPH phytotherapeutic agent. The main source of *S. repens* is plantations in the south-eastern United States, especially in the state of Florida, where the plant was previously regarded as a weed (Marks & Tyler, 1999).

1.4.3.2 General composition

In general, *S. repens* extracts are composed of fatty acids [(90 wt %), 90% of which is lauric acid, oleic acid, myristic acid and palmitic acid] and a variety of alcohols e.g. n-alcohols; isoprenoidal alcohols; acyl glycerides; sterols (campesterol, dihydrobrassicasterol, stigmasterol, β -sitosterol, cycloartenol, 24-methylenecycloartenol), and a pentacyclic triterpenoid, lupeol (Quirke et al., 1998). Fractionation of *S. repens* extract IDS 89 (Strogen®) by Weisser et al. (1996), led to a non-saponifiable, a saponifiable and a hydrophilic subfraction. Weisser et al. (1996) found that IDS 89 inhibits 5 α -reductase (See Figure 1.3), but it was mainly ascribed to the saponifiable subfraction, whereas the non-saponifiable and the hydrophilic subfractions showed only a slight inhibition and no inhibition of 5 α -reductase respectively. As the fatty acids accumulate in the saponifiable subfraction, the inhibitory actions were attributed to the fatty acids in the extract. Only myristic and lauric acid, however, were capable of significantly inhibiting 5 α -reductase (Weisser et al., 1996).

1.4.3.3 Mechanism of action

The use of phytotherapy in the rational medical treatment of patients with LUTS has been limited by the lack of knowledge regarding their possible mechanism of action (Goepel et al., 1999). Because the extracts contain many different molecules, more than one mechanism may be involved (Marks & Tyler, 1999). *S. repens*, however, is one of the few plants on which substantial research has been done and some of the previously proposed mechanisms of action have been experimentally confirmed.

Goepel et al. (1999) demonstrate that *S. repens* extract inhibits radioligand binding to human α_1 -adrenergic receptors concentration dependently. Another well-researched mechanism of action is inhibition of the prostatic 5 α -reductase enzyme, thus reducing the conversion of testosterone to DHT. According to Bayne et al. (1999), Permixon® (*S. repens* extract) is an effective inhibitor of both

isomeric forms of 5 α -reductase expressed in the prostate. Di Silverio et al. (1998) find that, at the recommended dose (320 mg/day), Permixon® induces a 50% reduction of DHT-levels as result of this inhibition of 5 α -reductase. Di Silverio et al. (1998) state that Rhodes et al. (1993) questioned the capacity of this compound to inhibit 5 α -reductase because serum DHT levels did not decrease in their experiments, however, Rhodes et al. (1993) did a very short study (7-day) on healthy volunteers (age range 20-30 years), whose hormonal status differs from that of BPH patients. Furthermore, it has been clearly demonstrated by Toscano and Horton (1987) that circulating DHT does not reflect its peripheral formation. Di Silverio et al. (1998) also state that the levels of epidermal growth factor (EGF) are androgen-dependent and that a marked decrease of DHT causes EGF reduction, which is important, if one considers that DHT acts on cell proliferation mainly indirectly through growth factors.

Other possible mechanisms of action have been proposed, including inhibition of cyclo-oxygenase and lipoxygenase (leading to an anti-inflammatory and anti-oedemous effects), inhibition of sex hormone-binding globuline (Goepel et al., 1999), as well as competitive binding to cytosolic androgen receptors and antiestrogenic activity in prostatic tissue (Glenn et al., 1998). As far as I could ascertain not one of these mechanisms has yet been convincingly demonstrated.

1.4.3.4 Extraction

There are different extraction processes for *S. repens* e.g. supercritical fluid extraction with carbon dioxide as solvent, and lipophilization by 90% ethanol. The most extensively investigated preparation is an n-hexane liposterolic extract previously manufactured in France and sold as Permixon®, however, Permixon® is currently manufactured by GERMANIA PHARMAZEUTICA, Vienna, Austria. One result of the different extraction processes used, is that most plant extract preparations have different components, thereby complicating a direct comparison between various preparations from different companies (Lowe et al., 1998).

1.4.3.5 Dosage

The recommended therapeutic dosage of *S. repens* extract is 320 mg daily (160 mg twice per day) (Bayne et al., 1999 & Gerber et al., 1998).

1.4.3.6 Efficacy

Serenoa repens, taken over a period of six months by 50 patients, resulted in a significant improvement in their International Prostate Symptom Score (IPSS). However, there was no

significant change in peak urinary flow rate, serum PSA (prostate-specific antigen) levels or in any of the measured urodynamic parameters (Gerber et al., 1998). In a major European study comparing the effects of saw palmetto and finasteride, the two were found to have nearly equal effects, causing parallel and statistically significant symptom scores and increases in maximal flow rates (Carraro et al., 1999). When comparing the prices of Proscar[®] (5 mg finasteride) with an over-the-counter saw palmetto product (160 mg saw palmetto, as well as beta-carotene, vitamins B1, B2, B6, C and E, and zinc), one realises the cost-effective benefit of saw palmetto as phytotherapeutic agent against BPH. A packet of 28 Proscar[®] tablets (one tablet daily) costs c. R660 in comparison with a packet of 60 Formule Naturelle's Saw Palmetto[®] capsules (one capsule three times daily) for c. R75. Even though effective over-the-counter therapy is available for BPH, pharmacists and health shop personnel should advise their clients with LUTS to see a physician for a proper diagnosis and not to assume that it is BPH. It could be more serious e.g. carcinoma, sclerosis, fibrosis of the bladder neck, urethral stricture disease or urethral valves, smooth or striated sphincter dysynergia (Levin et al., 1996).

1.4.3.7 Side effects

The general safety profile of *S. repens* compares favourably with that of finasteride and sexual side-effects (erectile dysfunction, ejaculatory disturbance or altered libido) were less common with the plant product than with the drug. Aside from an occasional instance of gastro-intestinal upset, side-effects of *S. repens* extracts have not been reported (Marks & Tyler, 1999).

1.5 SIDE EFFECTS OF PHYTOSTEROLS

Adverse effects caused by β -sitosterol are usually mild and in most studies comparable to those of the placebo in frequency (Wilt et al., 1998). Gastro-intestinal side-effects (constipation and diarrhoea) are the most common and are more likely to occur higher doses as used for hypercholesterolaemia (3-30 g/d) (Pegel, 1997).

The fact that phytosterols and sterolins have a steroid structure, but no side-effects, is interesting, because steroids are generally associated with many adverse effects. However, critical chemical groups responsible for e.g. corticosteroidal activity (and its side-effects) are absent in phytosterols and their glucosides (See Figure 1.6), and, therefore, their long term use are not associated with the

side-effects of long-term corticosteroidal use e.g. metabolic side-effects (hyperglycemia; fat redistribution; skeletal muscle wasting; skin atrophy, and gastro-intestinal ulcers etc.), water retention, hypertension, immuno-suppression etc. (Hardman & Limbird, 1996).

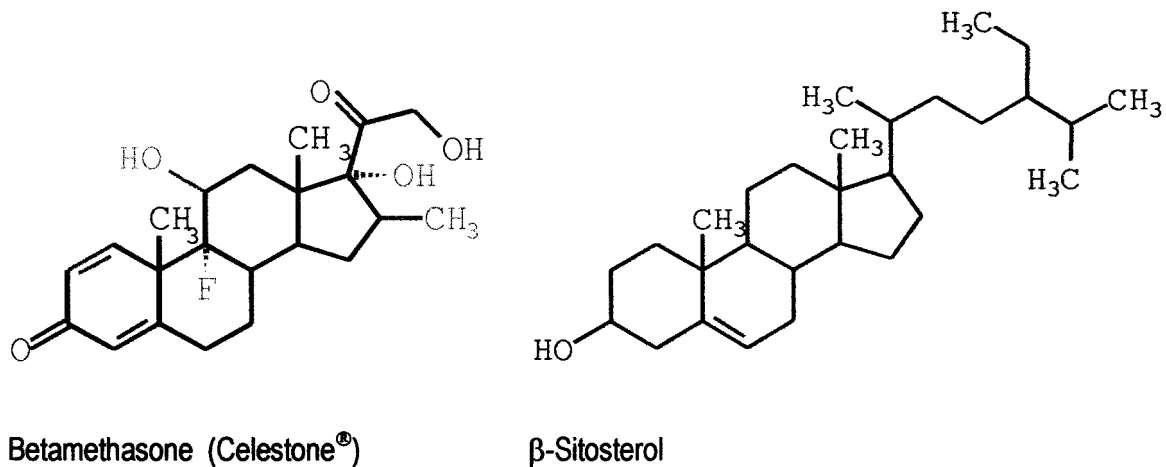


Figure 1.6: Structural differences between betamethasone and β-sitosterol. Red groups enable activity on the steroid receptor (intrinsic activity), and green groups increase activity on the steroid receptor (affinity). These critical functional groups are absent in β-sitosterol (composed from data from Hardman & Limbird, 1996).

1.6 CONCLUSION

Although the prejudice against phytotherapy still remains in many medical circles (probably because of a lack of knowledge regarding herbal therapy), treatment of BPH with certain scientifically-tested phytotherapeutic agents (e.g. *H. hemerocallidaceae*, *P. africana* and *S. repens*) is a definite option. Not only is the efficacy of *S. repens* extract comparable with finasteride, but the price is about a tenth of that of Proscar® for a month's supply. There are also very few side effects with the phytotherapeutics as opposed to allopathic medicines (dizziness, asthenia and hypotension with α -blockers; decreased libido and impotence with finasteride (Breza et al., 1998). Effective over-the-counter phytotherapeutics for BPH are available. However, pharmacists and health shop personnel should emphasise to their clients with lower urinary tract symptoms (LUTS), that they must consult their physicians first for proper diagnosis, and not assume that BPH is the cause of their micturition problems, as the cause could be more severe e.g. prostatic cancer.

As plant extracts are complex mixtures containing different active and inactive components, more than one mechanism is often involved in achieving the therapeutic effect. This is perhaps the main reason why nine years after the First International Consultation on BPH, in 1991, where the Scientific Committee proposed the standardisation of the evaluation of BPH pharmacological management, there still are uncertainties regarding the precise mechanism of action of many phytotherapeutics.

Despite the resistance by some conservative western physicians, the popularity of phytotherapy is increasing every year and, therefore, formal instruction in complementary and alternative medicine is now being offered in 64% of American medical schools (Marks & Tyler, 1999).

1.7 AIM OF THE STUDY

As BPH is a common medical condition in older men affecting as many as 40% of men aged 70 years and older (Wilt et al., 1998) and phytosterols and sterolins are regarded as the active compounds in many BPH phytotherapeutics, a study on the analysis of sterols and sterolins is undertaken.

The aim of the study is to develop and apply two analysis techniques for the qualitative and quantitative analyses of sterols and sterolins: thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). A new TLC method will be developed and an existing HPLC method will be evaluated and adapted for plant extract analyses. The methods will be applied to investigate the sterols and sterolins in three BPH phytotherapeutics: *H. hemerocallidea*, *P. africana* and *S. repens*; as well as different sterol/sterolin containing products, qualitatively and quantitatively. As *H. hemerocallidea* is the main phytotherapeutic of interest, the analysis of hypoxoside (unique to Hypoxidaceae) will also be investigated. A study by Eloff (1999) revealed that water does not extract sterols or sterolins, and uncertainty remains regarding the actives in African potato tea. Water extraction of *H. hemerocallidea* as performed by Eloff (1999), will be repeated and if his finding is confirmed, other possible actives will be investigated.

Little data is available on the shelf-life of sterols and their stability against gamma irradiation, used to prevent microbial contamination of raw material and products. Therefore, the TLC and HPLC methods will also be applied to determine the stability of sterols. Information on the bioavailability of β -sitosterol from certain products is known, but no bio-equivalence study on the bioavailability of β -sitosterol from South African products has been published yet. Some companies claim that their products have very high bioavailability due to added enzymes or specialised growth and harvesting methods. Furthermore, there is a large discrepancy in bioavailability values found in literature. Therefore, a HPLC method used to determine the "bioavailability of β -sitosterol from *Pygeum africanum* extract in humans" (Emara et al., 1999), will be evaluated and applied to determine the bio-equivalence of four South African and three European sterol containing products. A clinical trial consisting of three parts will be performed: part 1 (pilot study), part 2 (South African products) and part 3 (European products).

It is important to develop analysis techniques, because up to now very few companies in natural medicine industry have quality control procedures in place and little information is available on the stability and/or bioavailability of phytotherapeutic products. This lack of knowledge is mainly due to the lack of proper methods to perform these analyses. By developing and improving methods to analyse phytosterols, the industry will be supplied with the necessary techniques that ensure the quality, safety and efficacy of their sterol/sterolin containing products.

CHAPTER 2

MATERIAL, STANDARDS AND METHODS

2.1. MATERIAL

2.1.1 Plant material

African potato 1 - Dried grated rhizomes of *Hypoxis hemerocallidea*, collected on the farm Knopfontein, in Nylstroom (Northern province) were obtained from the distributor, Mrs. Santjie Marx.

African potato 2 - Powder samples of African potato capsules (Batch 9908202).

African potato 3 – *Hypoxis* sp. powder (Batch 990910).

African potato 4 – SAVANNAH FOODS African potato powder (Batch 9910062).

Prunus africana bark was obtained from the botanical garden at Nelspruit.

Prunus africana leaves were obtained from the botanical garden at Nelspruit.

Prunus africana extract (5:1) (Batch 30240P429 & P11199011503) from WARREN CHEM.

Serenoa repens berry powder (Batch 9807506 & S02000020101).

Serenoa repens berry extract (4:1) (Batch S02199083017) from WARREN CHEM.

WARREN CHEM Betasitosterol (Batch 28757P94).

All of the above except African potato 1, *P. africana* bark and leaves were gifts from BIOMOX PHARMACEUTICALS, Pretoria.

2.1.2 Products

African potato 5 (627 mg of *H.hemerocallidea* powder per capsule) - DISCOVERIES OF NATURE, Pretoria.

African potato 6 (300mg *H.hemerocallidea* powder per capsule) - PLANTANICAL MEDICINE, Pretoria.

Harzol[®] (10 mg β -sitosterol and 0.1 β -sitosterolin per capsule) - HOYER GmbH & Co, Monheim, Germany.

Immunochoice[®] (20 mg plant sterols and sterolins and 50 mg absorption enzymes per capsule) - PHARMA CHOICE, Fourways.

Moducare[®] (20 mg plant sterols and 0.2 mg sterolins per capsule) - PHYTO LABS, Midrand.

Nutricare® (400 mg freeze-dried enzymes and sterol compound per capsule) - NUTRIGREEN Hydroponics, Umhlanga Rocks.

Permixon® (160 mg *S. repens* extract per capsule) - GERMANIA PHARMAZEUTICA, Vienna, Austria.

Phytogard® (10 mg glutathione, 75 mg food state selenium and 60 mg β -sitosterol per capsule) - SPORTRON INTERNATIONAL, Paulshof.

Prostol® (200 mg of *S. repens* berry powder, 200 mg of *P. africana* extract (5:1) and 100 mg pumpkin seed extract (4:1), per capsule) - BIOHARMONY, Wynberg.

Tadenan® (50 mg *P. africana* extract) - LABORATORIES FOURNIER S.A., FONTAINE les Dijon, France.

2.2 STANDARDS

Campesterol (c. 65%) was bought from ALDRICH.

Cholesterol (99+%) from SIGMA, was received as a gift from Magda Roseman, Dept. of Chemical Pathology, University of Pretoria.

β -Sitosterol (c. 40%, containing 20-30% campesterol and 10-30% dihydrobrassicasterol), derived from soybeans, was bought from ALDRICH.

Synthetic β -sitosterol (95.7%) was bought from SIGMA.

Stigmasterol (c. 95%) was bought from SIGMA.

Isolated hypoxoside was received as a gift from Prof. J. van Staden, Dept. of Botany, University of Natal.

Isolated β -sitosterolin was received as a gift from Prof. C.B. Rodgers, Dept. of Chemistry, University of Durban-Westville.

2.3 METHODS

The methods used in this study, are discussed in the individual chapters.

CHAPTER 3

TLC METHOD DEVELOPMENT AND APPLICATION

3.1 INTRODUCTION

3.1.1 History

Chromatography is a method of analysis in which a mobile phase passes over a stationary phase in such a way that a mixture of substances is separated into its components (Hanh-Deinstrop, 2000). Chromatography began in 1903 in Russia, when Michael Tsvett separated chlorophyll pigments in a tube filled with calcium carbonate. Long after Tsvett's adsorption chromatography, partition chromatography with silica gel columns was developed in 1941 by Martin and Synge to separate hydrophilic substances like amino acids. Three years later they experimented with filter paper and paper chromatography was born. The next separation technique, introduced in 1945, was gas chromatography developed by Hesse, Cremer, James and Martin. However, a milestone in the history of chromatography was reached in 1956 when a new concept, thin layer chromatography (TLC), was developed by Egon Stahl and his collaborators working on glandular hairs of plants. (Von Zahn & Rheinholz, 1980).

3.1.2 Aim of chapter

In this chapter a TLC method is developed and optimised to investigate the phytosterols and sterolins in *H. hemerocallidea*, *P. africana*, *S. repens*, and related herbal products. By evaluating different extractants, mobile phases and spray reagents, the best combination to investigate sterols and sterolins is selected. By comparison with standards, the sterol/sterolin contents of the three plant species and related products are qualitatively determined and compared. The information obtained from this technique, regarding the best extractant, method of extraction *etc.*, can then be applied in preparation of samples for analyses with high performance liquid chromatography (HPLC).

The results in this chapter will be discussed in two parts:

A – Method development

B – Application.

3.2 MATERIAL AND METHODS

3.2.1 Material

Plant material, products and standards as described in Chapter 2, 2.1 and 2.2 were used.

3.2.2 Methods

3.2.2.1 Extraction

3.2.2.1 (a) Selecting extractants according to polarity and solvent strength

The total interaction of a solvent molecule (mobile phase) with a sample molecule (solid phase) is the result of a combination of dispersion, dipole, hydrogen bonding and dielectric interactions. The larger this combined interaction is, the stronger is the attraction of solvent and solute molecules. The ability of a sample or solvent molecule to react in all four of these ways is referred to as the “polarity” of the compound. The “strength” of a solvent is directly related to its polarity. Solvent strength increases with solvent polarity in normal phase partition and adsorption liquid chromatography, but solvent strength decreases with increasing polarity in reverse phase liquid chromatography (Snyder & Kirkland, 1979). See Table 3.1.

Table 3.1: Solvent strengths, polarities and boiling points of the extractants used (from Snyder & Kirkland, 1979)

Solvent	Polarity (P')	Solvent strength (ϵ°)	Boiling Point ($^\circ\text{C}$)
Acetone	5.1	0.56	56
Methanol	5.1	0.95	65
Ethanol	4.3	0.88	78
Chloroform	4.1	0.40	61
Dichloromethane	3.1	0.42	40
Diethylether	2.8	0.38	35
Hexane	0.1	0.01	69

Table 3.1 indicates the polarities, solvent strengths and boiling points of the extractants used, and evaluated, in the study. The polarity parameter P' is based on the experimental solubility data reported by Rohrschneider and is an indicator of solvent strength in partition chromatography. The experimental adsorption solvent strength parameter ϵ° , measured on alumina, is a better index of solvent strength for adsorption chromatography (Snyder & Kirkland, 1979).

Note that acetone and methanol has the same polarity P' , according to Snyder and Kirkland (1979), whereas methanol is generally regarded as a solvent with much higher polarity than acetone. The table of Snyder and Kirkland (1979) was, however, used as an indication of polarity and solvent strength.

3.2.2.1 (b) Types of extraction used

3.2.2.1 (b) (i) Simple extraction

The plant material was usually extracted, with a ratio of 10 ml of extractant per gram plant powder e.g. 500 mg extracted with 5 ml extractant, by shaking the test tube vigorously on a horizontal shaking machine, for 5 minutes. The extract was then centrifuged in a Heraeus Labofuge 200 centrifuge at 3000 g for 5 minutes and decanted into a weighed amber vial (volume c. 8 ml). However, in the case of dense solvents e.g, chloroform, even high speed centrifugation did not result in sedimentation and the extracts had to be filtered. Qualitative filter paper (Whatman type 1 or 4) was wet before filtration and rinsed afterwards with the extractant, and the extract collected in a weighed amber vial. The extraction process was usually repeated three times with the originally weighed material and the supernatant was collected either in three different vials, for quantitative purposes or combined in the same vial. In the case of dense solvents, filtration was performed and the extraction process could only be repeated once, as the material was poured with the extract onto the filter paper. The extractant was usually removed by a stream of cold air in a fume cupboard, but in some cases (oily precipitates) to confirm the dryness after 24 hours, the extract was dried under vacuum in a dessicator. The dry extract was then redissolved, either in the extractant or in another solvent with particular polarity, to yield a known concentration of extract, usually 10 mg/ml.

All the extractants and solvents used for TLC purposes were analytical quality reagents: Acetone, methanol, ethanol, chloroform, dichloromethane, diethylether and hexane.

3.2.2.1 (b) (ii) Soxhlet extraction

In some cases of simple extraction, very little material was extracted per gram plant material exposed to the extractant, and a soxhlet extraction apparatus was used for continuous and complete extraction in a closed system. In these cases 20 g of plant material was placed in two porous paper thimbles (J Green's 603) and extracted, with excess extractant boiled on a heating mantle. The extraction period was two to three hours. The extract was dried and redissolved to yield a 10 mg/ml concentration, as with simple extraction.

3.2.2.1 (b) (iii) Water extraction of *H. hemerocallidea*

According to African potato 1's promotional pamphlet, one tablespoon (6.4 g) dried, grated corm of *H. hemerocallidea* must be simmered in six cups (1200 ml) water for 15 to 20 minutes and refrigerated until use.

Water extraction of the grated corm of African potato 1 was performed according to this method. The water extract was divided into four parts, one of which was dried to determine the concentration of the total volume; to the second ethylacetate was added as a water immiscible solvent with relatively high solvent strength ($\epsilon^{\circ}=0.58$), to remove the sterolins; to the third, chloroform was added as a water immiscible solvent with relative low solvent strength ($\epsilon^{\circ}=0.40$), to remove the sterols; and the fourth was kept unchanged as a control. See Table 3.1. Simple water extraction of powdered African potato 1 was also performed in duplicate. The extracts dried and redissolved in acetone and chloroform respectively.

To ascertain that ethylacetate does extract sterolins, the solubility of sterolins in ethylacetate was investigated. Simple extraction with methanol was performed (as methanol definitely extracts the sterolin as demonstrated in 3.3.1.2 (b) and Figure 3.3). The extract was redissolved in acetone as control (contains stigmasterolin after redissolving the methanol extract), methanol and ethylacetate. To determine whether methanol does not perhaps extract a low concentration of sterols, the methanol extract was also redissolved in chloroform.

3.2.2.2 Preparation of standards

The sterol standards were dissolved in chloroform to yield the required concentration, but β -sitosterolin was dissolved in acetone, and the hypoxoside in methanol.

3.2.2.3 Thin layer chromatography (TLC)

Pre-coated 0.25 mm silica 60 glass and 0.2 mm silica 60 aluminium plates were used. Glass plates (Macherey-Nagel DURASIL-25) were used to investigate the *red spot compound* (as will be discussed at a later stage) and cheaper aluminium plates (Macherey-Nagel ALUGRAM® SIL G) were used for sterols/sterolins and hypoxoside. Volumes of 5 to 10 µl were applied with a pipette to the plates. In all cases 100 µg of the extracts was applied (10 µg/µl x 10 µl). The mass of the standards applied, depended on the concentration, but in all cases 5 µl of the standards was applied. The plates were placed in a glass tank with porous paper on the sides and saturated with the particular mobile phase, for development. All mobile phase chemicals were analytical reagents:

BEA: Benzene:Ethanol:Ammonia in the ratios 18:2:0.2 or

CEF: Chloroform:Ethylacetate:Formic acid in the ratios 5:4:1 or

EMW: Ethylacetate:Methanol:Water in the ratios 10:1.35:1.

3.2.2.3 (a) Visualisation of separated components

After development, the plates were examined under ultraviolet (UV) light and usually sprayed with *p*-anisaldehyde spray (1 ml *p*-anisaldehyde, 18 ml ethanol and 1 ml sulphuric acid). Other spray reagents investigated were vanillin (0.1 g vanillin, 28 ml methanol and 1 ml sulphuric acid), phosphoric acid (15 ml 85% phosphoric acid diluted to 100ml with methanol), *p*-toluene sulphonic acid (20% *p*-toluene sulphonic acid in methanol), perchloric acid (20% aqueous perchloric acid), trichloroacetic acid (25% solution of trichloroacetic acid in chloroform) and a mixture of equal volumes of sulphuric acid and methanol (Stahl, 1969). The plates were heated in a Gallenkamp Hotbox oven at c. 110 °C until the colour bands were optimally visible and examined under UV light for a second time. With the phosphoric spray, it takes 15-30 minutes at 120 °C for development.

3.2.2.3 (b) The retardation factor (R_f)

The position of a substance zone in TLC can be described with the aid of the retardation factor R_f . This is defined as the quotient obtained by dividing the distance between the substance zone and the starting line, by the distance between the solvent front and the starting line (Hahn-Deinstrop, 2000).

By comparing the fractional movement of a solute band, relative to the distance moved by the solvent front of the separated components with that of the standards, sterols and sterolins could be identified. R_f values are usually reproducible under a given set of circumstances. Differences in

mobile phase composition, room temperature, and saturation of the development tank with the solvent system, could however cause R_f -values to vary.

3.2.2.4 Fluorescence

To detect fluorescent compounds, the TLC plates were observed with a CAMAG TL-900/U UV lamp, with wavelengths of 254 nm and 350 nm before and/or after they were sprayed with the reagent. Nothing could be seen before spraying with the reagent. With *p*-anisaldehyde spray, which was used most often, the sterols and sterolins were not found to be fluorescent and UV examination was usually not performed. However, with other spray reagents e.g. phosphoric and especially *p*-toluene sulphonic acid, the sterols and sterolins were clearly visible at the longer UV wavelength.

3.2.2.5 Hydrolysis of “stigmasterolin”

To determine qualitatively whether an extra low intensity band seen with TLC in the 95% stigmasterol standard was really the stigmasterolin, it was attempted to hydrolyse it to the sterol. Acid hydrolysis with 5% hydrochloric acid were performed. A mixture of methanol:chloroform:water (MCW) in the ratios 12:5:3, was used to dissolve stigmasterol standard (50 mg stigmasterol dissolved in 5 ml mixture) and 5% hydrochloric acid was added. At this ratio the methanol make the chloroform and water miscible, with water being necessary for hydrolysis.

The experiment was repeated with African potato 1, as a component with similar R_f and colour as the “stigmasterolin”, was noticed in its acetone and methanol extracts with TLC. In the rest of the study this unknown substance is referred to as the *red spot compound*. African potato 1 was extracted with the same MCW mixture and acidified. The acidified stigmasterol solution and African potato 1 MCW extract was refluxed on a heating mantle for an hour and then dried under vacuum (Büchi Rotavapor R-114 and KNF Neuberger Laboport). The dried stigmasterol residue was then washed with distilled water to remove the acid, dried again and redissolved in chloroform to yield a concentration of 10 mg/ml. An unhydrolysed control of stigmasterol in MCW was prepared in the same way. The African potato 1 extract however, was not dried but a $\frac{1}{6}$ of the volume methanol and a $\frac{1}{4}$ of the volume chloroform were added, to form two immiscible phases in which the sterols and sterolins could separate. To determine the concentration of the African potato 1 extract, a small volume was dried and the concentration of the total extract was calculated. This was done because it is often difficult to redissolve the material after it has been dried completely, even if it is redissolved in the same solvent as was used for extraction. Again, an unhydrolysed control was prepared using the same method.

3.2.2.6 Isolation of the *red spot compound* with preparative thin layer chromatography (PTLC)

A pre-coated 2 mm silica 60 glass plate (Merck precoated PLC silica gel 60 F-254) was used. The *H. hemerocallidea* acetone extract was concentrated and c. 200 μ l was applied over the length of the PTLC plate and developed in CEF. After development, a 2 cm strip on the right of the plate was sprayed with *p*-anisaldehyde spray and overheated for maximum visibility of *red spot compound*. The identified zone was scraped off the plate using a spatula, and collected. The silica scrapes were ground to a fine powder and then placed into a glass tube. Acetone was passed through the silica to dissolve the *red spot compound*. The solution was dried (to remove the TLC mobile phase components) and redissolved in 0.5 ml deuterated acetone.

3.2.2.7 Structure elucidation of the *red spot compound* with Nuclear Magnetic Resonance Spectroscopy (NMR)

The samples were analysed by Dr. J.J. Retief, SASOL Technology, Research and Development. A 400 MHz Varian Unity Inova NMR spectrometer was used for proton (^1H) analysis of the *red spot compound*. For the small volume with low concentration, an indirect detection (ID) probe was used and the sample was moved upwards so that the centre of the sample and the centre of the NMR coils coincided. To increase the concentration, only 0.5 ml of deuterated acetone was used, instead of the usual 0.7 ml.

3.3 RESULTS AND DISCUSSION

A: METHOD DEVELOPMENT

3.3.1 Extractant evaluation

3.3.1.1 The solubility of phytosterols in acetone, methanol and chloroform

The solubility of the 95% stigmasterol standard and Warrenchem Betasitosterol were determined in acetone, methanol and chloroform. It was found that acetone dissolved 17.4% stigmasterol and 66.2% Warrenchem Betasitosterol. Methanol dissolved only 3.2% stigmasterol and 8.4% Warrenchem Betasitosterol, while chloroform dissolved 90.6% of the stigmasterol and 98.8% Warrenchem Betasitosterol. This is consistent with Merck index (Stecher et al., 1968), which states that stigmasterol is insoluble in water, but soluble in usual organic solvents. The stereochemical angle of rotation $[\alpha]_D^{25}$ of β -sitosterol, campesterol and stigmasterol is measured in chloroform in the Merck Index (Stecher et al., 1968). It seems that stigmasterol is less soluble than β -sitosterol in all three solvents.

It is important to note that the term β -sitosterol frequently represents a defined extract of phytosterols with β -sitosterol as the main component (Berges et al., 1995). According to the certificate of analysis, Warrenchem Betasitosterol contains 46.6% β -sitosterol, 28.8% campesterol and 17.6% stigmasterol.

3.3.1.2 Extraction

The extractants are discussed here in order of polarity as indicated in Table 3.1 in 3.2.2.1 (a).

Extraction will be discussed under the following headings:

- (a) (i) Acetone extraction
- (a) (ii) Acetone extract redissolved in methanol, diethylether and water
- (b) Methanol, ethanol and chloroform extraction
- (c) Dichloromethane and diethylether extraction
- (d) Hexane extraction
- (e) Best extractant evaluation

3.3.1.2 (a) (i) Acetone extraction

Acetone is a solvent with an intermediate polarity. It dissolves many polar and non-polar substances and gives a good indication of the polarity of the components in a plant, as well as which other solvents to consider (with regards to their polarities). Furthermore, acetone is miscible with water, volatile and evaporates quickly when drying the extract and when applying it on a TLC plate, to give a neat thin line.

The African potato 1 and 2; *S. repens* berry powder; *P. africana* bark extract (5:1), and Warrenchem Betasitosterol (composed of mainly β -sitosterol, but also campesterol and stigmasterol) were extracted with acetone and chromatographed on TLC, developed in BEA and sprayed with *p*-anisaldehyde and vanillin respectively. See Figure 3.1.

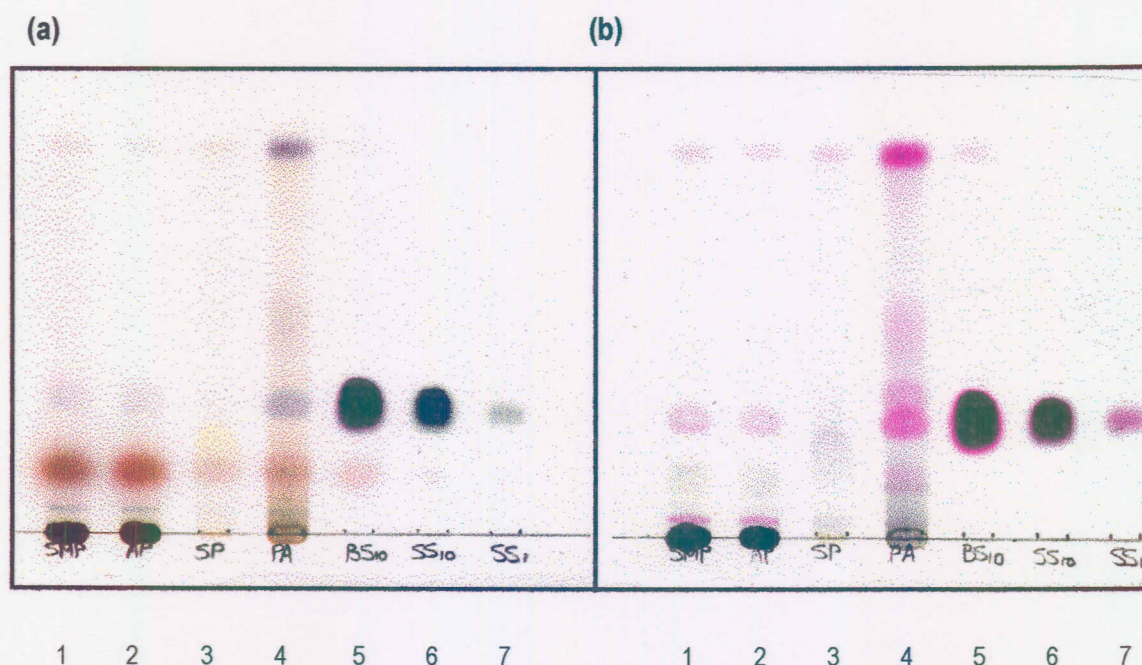


Figure 3.1: TLC on aluminium plates of acetone extracts of African potato 1(1) and 2 (2), *S. repens* berry powder (3), *P. africana* bark extract (4) and Warrenchem Betasitosterol (5). The plates were developed in BEA and sprayed with (a) *p*-anisaldehyde and (b) vanillin. On the right are 10 mg/ml 95% stigmasterol standard (6) and 1 mg/ml stigmasterol standard (7).

In all cases 100 μ g of the extracts was applied.

With BEA the majority of the compound bands were on the lower half of the plate, which is not ideal. Warrenchem Betasitosterol and the stigmasterol standards had the same R_f -value and colour

reaction with the spray reagents. Warrenchem Betasitosterol (composed of three phytosterols) and 95% stigmasterol standard had the same retardation factor ($R_f = 0.26$). TLC with BEA could not separate the different phytosterols in Warrenchem Betasitosterol – a blue “phytosterol-complex” was visible. When the chemical structures of these sterols are considered, it is understandable why their R_f -values were the same (See Chapter 1, 1.2). A similar blue spot with $R_f = 0.26$ was visible in all the samples in Figure 3.1, except *S. repens* berry powder, and it was postulated to be a “phytosterol-complex” as well.

On the moderately heated aluminium plates, a pink-red spot with $R_f = 0.12$ in BEA was noticed below the “phytosterol-complex” in Warrenchem Betasitosterol and 10 mg/ml stigmasterol standard (not 1 mg/ml). This was, however, only seen on the plates sprayed with *p*-anisaldehyde, but not on the plates sprayed with vanillin. The compound responsible for the second spot in stigmasterol standard was thought to be stigmasterolin. A similar pink-red spot was visible in African potato 1 and 2; *S. repens* berry powder, and *P. africana* bark extract (5:1). At the time β -sitosterolin standard was not available, but it was identified retrospectively as the blue band with $R_f = 0.053$ (BEA) in African potato 1 and 2 and in *P. africana* bark extract (5:1). See Figure 3.2 where the isolated β -sitosterolin is included. If the pink-red spot was a complex of sterolins (similar to the “phytosterol-complex”), it did not include β -sitosterolin. In the rest of the study, the “pink-red spot” mentioned, is referred to as the *red spot compound*. The pink-red spot in stigmasterol standard could be stigmasterolin, as the standard was 95% pure and sterols never exist on their own in nature, they are always found in combination with their glucosides (Vanderhaeghe & Bouic, 1999). Despite the same R_f -value, the *red spot compound* however, is not necessarily stigmasterolin, but it will be discussed as a possible sterolin in the rest of the study.

Vanillin did not reveal the “stigmasterolin” band in stigmasterol standard, but the type of TLC plate (aluminium vs. glass) also determines the visibility of “stigmasterolin” and the *red spot compound*. The *red spot compound* was substantially more visible when the TLC plates were overheated, but in the case of aluminium plates sprayed with *p*-anisaldehyde, the background turned pink when overheated and this reduced the visibility of the *red spot compound*.

Conclusion: Glass TLC plates (overheated) are best for investigation of the red spot compound.

Assuming that the *red spot compound* is a sterolin (glucoside), alkaline conditions would cause the hydroxyl groups to be in undissociated form (the sugar hydroxyl in the –OH instead of –O⁻), which would reduce the polarity and increase the solubility in non-polar extractants. To determine the effect

of increased pH on extraction of African potato 1, 20% ammonium hydroxide was added to the acetone extractant. The result was an increase in the intensity of the *red spot compound* on TLC. It is also would appear that as if the removal of the extractant and redissolution (in the same or another solvent), does not cause degradation (e.g. hydrolysis) of the *red spot compound*. See Figure 3.2.

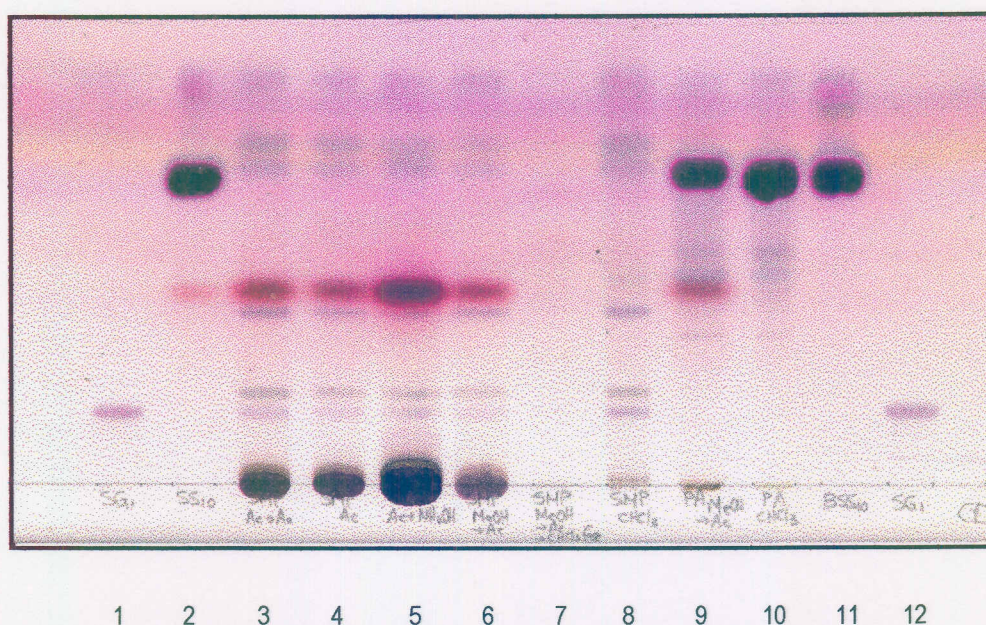


Figure 3.2: Overheated TLC on a glass plate, of different extracts of *H. hemerocallidea* powder and *P. africana* extract (5:1), developed in CEF and sprayed with *p*-anisaldehyde. On the left are 1 mg/ml isolated β -sitosterolin, 10 mg/ml 95% stigmaterol standard and on the right are 10 mg/ml 40% β -sitosterol standard and 1 mg/ml isolated β -sitosterolin. In all cases 100 μ g of the extracts was applied.

In Figure 3.2 from the left are 1 mg/ml isolated β -sitosterolin (1) and 10 mg/ml 95% stigmaterol standard (2). The order of the African potato 1 extracts are as follows: Acetone, dried and redissolved in acetone again (3), acetone, not dried (4), alkaline acetone (5), methanol, dried and redissolved in acetone (6), acetone insolubles from 6 redissolved in 50/50 $\text{CHCl}_3/\text{H}_2\text{O}$ – chloroform phase (7) and chloroform, dried and redissolved in chloroform (8). The extracts of *P. africana* extract (5:1) are methanol, dried and redissolved in acetone (9) and chloroform, dried and redissolved in

chloroform (10). On the right are 10 mg/ml 40% β -sitosterol standard (11) and 1 mg/ml isolated β -sitosterolin (12).

Conclusion: The red spot compound is more soluble and better extracted at an alkaline pH.

3.3.1.2 (a) (ii) Acetone extract redissolved in methanol, diethylether and water

Acetone extracted a substantial amount of African potato 1, African potato 2, *P. africana* extract (5:1) and *S. repens* berry powder, but when the dried acetone extract was redissolved in acetone, a large quantity of material did not dissolve. This was true of all the samples, except *S. repens* berry powder where 96.5% of the dried extracted material dissolved in acetone. The reason for the insoluble residue could be complex formation during drying.

The acetone-insoluble residue of the other three samples, was then redissolved in methanol. Methanol dissolved 72.9% and 75.9% respectively of the acetone-extracted material of African potato 1 and African potato 2 (filtered), but only 10.3% of *P. africana* extract (5:1). See Table 3.2. Subsequently the *P. africana* extract (5:1)'s acetone and methanol insoluble material was redissolved in a non-polar solvent, diethylether. None of the material dissolved. It was decided to try using a very polar solvent, water, to redissolve the acetone-, methanol- and diethylether-insoluble material of *P. africana* extract (5:1) – 63.8% material dissolved.

TLC of the extracts in Table 3.2 (results not shown), sprayed with *p*-anisaldehyde, indicated that acetone removed certain polar compounds from both African potato samples, which were not present in the methanol solution. The acetone-insoluble compounds that dissolved in methanol, were so polar, that the methanol solution did not separate in CEF (although it separated in EMW).

The acetone solution of *P. africana* extract (5:1) separated in CEF, but EMW was too polar. On the other hand the water solution of *P. africana* extract (5:1) did not separate in either CEF or EMW. Even EMW was too non-polar in this case.

Table 3.2: Percentage of material extracted with acetone from *H. hemerocallidea*, *P. africana* bark extract (5:1) and *S. repens* berry powder and residue redissolved in different solvents

Plant material	% (w/w) Extracted with Acetone	% (w/w) Residue redissolved in Acetone	% (w/w) Residue redissolved in MeOH	% (w/w) Residue redissolved in Ether	% (w/w) Residue redissolved in Water	Best Solvent
African potato 1 powdered	2.4%	23.7%	72.9%	-	-	MeOH
African potato 2 capsules filtered	2.3%	16.6%	75.9%	-	-	MeOH
<i>P. africana</i> ext.	2.3%	31.0%	10.3%	0	63.8%	Water
<i>S. repens</i> pwd.	5.8%	96.5%	-	-	-	Acetone

Conclusion: Methanol is the best solvent for redissolution to yield a final concentration of H. hemerocallidea acetone extracts, water for P. africana (5:1) acetone extracts and acetone for S. repens acetone extracts.

3.3.1.2 (b) Methanol, ethanol and chloroform extraction

As mentioned above, in many cases acetone extracted a substantial amount of material, but when the dried extract was redissolved in acetone, a significant quantity was acetone-insoluble. This resulted in a low concentration of the extract. Due to this and because alcohols, especially ethanol, are often used in the extraction of *H. hemerocallidea* (McAllister & van Staden, 1994), *S. repens* (Lowe et al., 1998) and *P. africana* (Yablonsky et al., 1997), methanol and ethanol were also applied for extraction. As it was shown with stigmasterol standard and Warrenchem Betasitosterol that sterols are very soluble in chloroform, this non-polar solvent was applied as well. Three different samples of *H. hemerocallidea* powder were extracted with methanol, ethanol and chloroform respectively and evaluated.

Methanol extracted the highest quantity of material from all three *H. hemerocallidea* samples, ethanol c. 50% and chloroform c. 1% of the quantity extracted by methanol. See Table 3.3. It was noted that very little material was extracted by chloroform. Although absorption onto the filter paper during filtration of these extracts, could have been a reason. It was postulated that perhaps chloroform selectively extracts sterols. This was confirmed with TLC.

Table 3.3: Percentage of material extracted with methanol, ethanol and chloroform from three sources of *H. hemerocallidea* powder and residue redissolved in different solvents

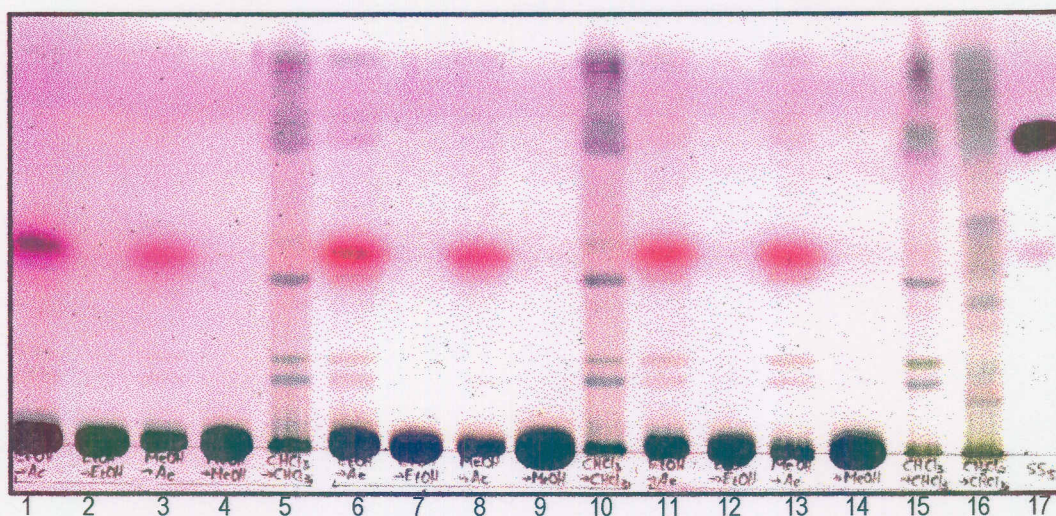
Plant material	Extractant	% Extracted (w/w)	% (w/w) Residue redissolved in Acetone	% (w/w) Residue redissolved in MeOH	% (w/w) Residue redissolved in EtOH	% (w/w) Residue redissolved in CHCl ₃
African potato 1 powdered	Methanol	4.2%	6.7%	77.1%	–	–
“	Ethanol	1.8%	10.9%	–	54.3%	–
“	Chloroform	0.06%	–	–	–	100%
African potato 2 capsules	Methanol	6.0%	6.6%	64.2%	–	–
“	Ethanol	3.6%	3.3%	–	59.3%	–
“	Chloroform	0.06%	–	–	–	100%
African potato 3 powder	Methanol	6.8%	5.3%	65.5%	–	–
“	Ethanol	4.2%	0	–	77.4%	–
“	Chloroform	0.08%	–	–	–	100%

Instead of dissolving the extract in the extractant to give a particular concentration, the ethanol and methanol extracts were dissolved in sufficient acetone to yield 10 mg/ml concentrations, but afterwards the remaining acetone insoluble precipitates were redissolved in ethanol and methanol respectively, to yield again 10 mg/ml concentrations.

The chloroform extract was dried and redissolved in chloroform for a 10 mg/ml concentration.

TLC of the 10 mg/ml extracts in Table 3.3 and 5 mg/ml stigmasterol standard was performed, using CEF and EMW solvent systems and *p*-anisaldehyde as spray reagent. It showed, that although the percentage of material extracted by methanol, ethanol and chloroform differed for the three African potato samples, the components extracted are similar and all three samples can, therefore, be considered as representing *H. hemerocallidea*. See Figure 3.3.

(a)



(b)

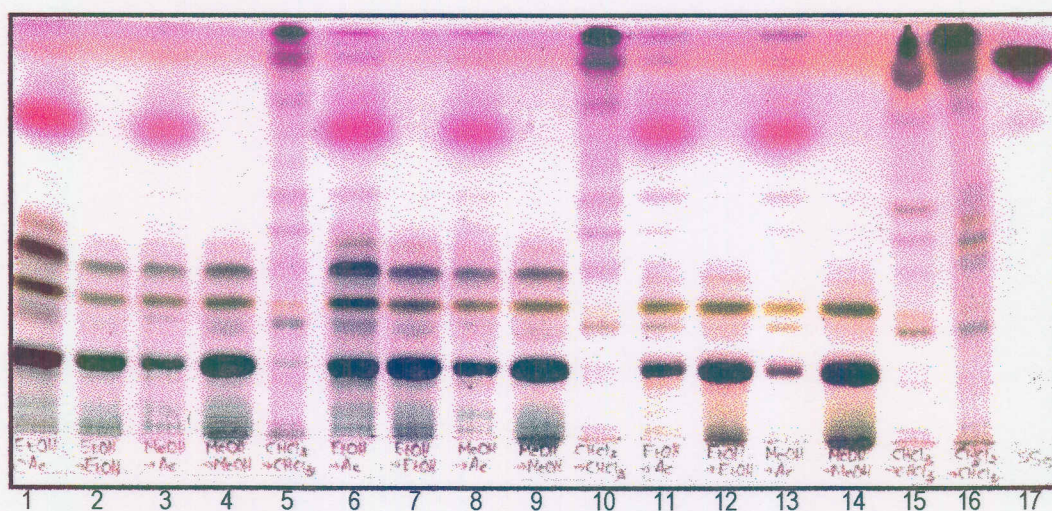


Figure 3.3: Overheated TLC on glass plates of ethanol, methanol and chloroform extracts of *H. hemerocallidea* powder from three sources (African potato 1, 2 and 3). The plates were developed in CEF (a) and EMW (b), using *p*-anisaldehyde as spray reagent.

In all cases 100 μg of the extracts was applied.

In Figure 3.3 the identity of the different lanes are as follows: African potato 1 (lanes 1-5), African potato 2 (lanes 6-10) and African potato 3 (lanes 11-15). From the left: ethanol, dried and redissolved in acetone (1, 6 & 11), ethanol, dried and redissolved in ethanol (2, 7 & 12), methanol, dried and redissolved in acetone (3, 8 & 13), methanol, dried and redissolved in methanol (4, 9 & 14), chloroform, dried and redissolved in chloroform (5, 10 & 15). On the right a soxhlet dichloromethane extract of African potato 1, which was redissolved in chloroform (16) and 5 mg/ml 95% stigmasterol standard (17).

At the time, hypoxoside standard was not available, but retrospectively hypoxoside was identified as the black compound ($R_f = 0.17$) in all the methanol and ethanol extracts (dissolved in acetone or the particular alcohol) on the overheated plate, developed in EMW. On moderately heated plates sprayed with *p*-anisaldehyde, the compound was purple. Hypoxoside is a norlignan diglucoside unique to the Hypoxidaceae. Due to the two glucose molecules in its structure, the molecule is polar and therefore only separates in the highly polar EMW. On the TLC plates, it seemed that methanol and ethanol extracted the same components: the *red spot compound*; a low concentration β -sitosterolin (visible in the alcohol extracts dissolved in acetone in CEF) and also hypoxoside. Methanol, however, extracted substantially more material. See Table 3.3. The isolated hypoxoside dissolved extremely well in methanol, and a concentration of 10 mg/ml was achieved without shaking. Koch and Brosch-Starzengruber (1991), and Marini-Bettolo and Galeffi (Nicoletti et al., 1992) also used methanol extracts for hypoxoside analyses.

Conclusion: Methanol was identified as the best extractant for extracting hypoxoside.

TLC in Figure 3.3 shows that the acetone removed all the *red spot compound* and β -sitosterolin (not all the hypoxoside) from the methanol and ethanol extracts (lanes 1, 3, 6, 8, 11 & 13) and, therefore, the alcohols used to rinse the vials after acetone (lanes 2, 4, 7, 9, 12 & 14), did not contain the *red spot compound*. The *red spot compound* was yellow-brown on the normally heated glass plate sprayed with *p*-anisaldehyde, but when overheated it turned a beautiful, pink-red. When aluminium plates were used, the *red spot compound* was quickly visible, but with overheating the background turned pink so that the pink-red compound could no longer be distinguished.

Chloroform extracted a radically different spectrum of components, among others, the “phytosterol-complex” ($R_f = 0.72$ in CEF and 0.91 in EMW), which was not visible in the alcohol extracts, and even β -sitosterolin ($R_f = 0.17$ in CEF, but not clear in EMW). As β -sitosterolin is a glucoside (polar), it was not expected to be extracted by chloroform (non-polar).

In both CEF and EMW solvent systems the sterols and sterolins were visible. Stigmasterol (standard) and the “phytosterol-complex” had a $R_f = 0.72$ in CEF and 0.91 in EMW, and stigmasterolin and the *red spot compound* had R_f -values = 0.44 in CEF and 0.73 in EMW. However the sterolin of the 5 mg/ml stigmasterol standard (as opposed to the 10 mg/ml concentration discussed previously) could not be seen when heating the glass plates as usual at 105 °C. Overheating the plates until the background turned pink-red (*p*-anisaldehyde), made even the lower concentrations of sterolins (e.g. in 5 mg/ml stigmasterol standard) visible. This led to a theory that overheating the glass plate caused hydrolysis of the sterolin to the sterol. Assuming that the sterol reacts more readily with the spray reagent (*p*-anisaldehyde), the hydrolysed sterolin is more clearly visible. This formed the basis of the experiments as described in 3.2.2.5

3.3.1.2 (c) Dichloromethane and diethylether extraction

As simple chloroform extraction of African potato 1, 2 and 3 [described in 3.3.1.2 (b)] only extracted $0.06 - 0.08\%$ plant material (See Table 3.3), it was decided to apply soxhlet extraction for continuous and complete extraction in a closed system. The plant material of African potato 1 was placed in two porous paper thimbles and extracted with excess extractant. Solvent strength (ϵ°), instead of solvent polarity (P') was used as polarity parameter: Dichloromethane's solvent strength (ϵ°) is 0.42 , which is very close to that of chloroform ($\epsilon^\circ = 0.40$), was used as extractant because it boils at 40 °C whereas chloroform boils at 61 °C. It was felt that the active extract constituents could possibly be degraded at the higher temperature. The solvent polarity (P') of dichloromethane and chloroform, however, differ by one unit (3.1: 4.1). See Table 3.1.

TLC of the soxhlet dichloromethane African potato 1 extract. compared to the simple chloroform extract, indicated that the dichloromethane extracted a greater variety of compounds (See Figure 3.3). Soxhlet dichloromethane extraction of African potato 1, extracted 1.705% compared to only 0.04% with simple extraction.

Soxhlet extraction of African potato 1 was repeated with diethylether. Again solvent strength was used as polarity parameter: As previously explained with dichloromethane, diethylether's solvent strength ($\epsilon^\circ = 0.38$) is close to that of chloroform ($\epsilon^\circ = 0.40$) as well, although the boiling point is much lower (35 °C: 61 °C). Yet again the solvent polarity P' (as opposed to the solvent strength, ϵ°) differs substantially, with $P' = 4.1$ for chloroform and $P' = 2.8$ for diethylether. See Table 3.1.

TLC of the soxhlet diethylether extract compared to the simple chloroform extract, indicated that diethylether extracted the same components as chloroform. Soxhlet diethylether extraction however, extracted a substantially higher concentration of each component than simple chloroform extraction. Soxhlet diethylether extraction of African potato 1, extracted 0.125% compared to the 0.06% with simple extraction.

3.3.1.2 (d) Hexane extraction

Hexane is a very non-polar solvent and its extraction capability of sterols was tested and compared with chloroform. Hexane extracted 0.04% material from African potato 1, compared to the 0.06% extracted by chloroform [See 3.3.1.2 (b)]. TLC of the chloroform and hexane extracts revealed that chloroform is in fact a better extractant than hexane (results not shown).

Conclusion: Chloroform was identified as the best extractant for sterols.

3.3.1.3 Best extractant evaluation

When the acetone extracts of African potato 1 are compared with the methanol extract (redissolved in acetone) in Figure 3.2, it would seem that as if the composition and contents of the same concentration of methanol (lane 6) and acetone extracts (lane 3 & 4) of African potato 1, are very similar. However, methanol extracts substantially more material from African potato 1 than acetone (4.2%:2.2%) See Table 3.2 and 3.3. Furthermore, the methanol extract (redissolved in methanol) in Figure 3.3 does not separate in CEF, as it contains very polar compounds (it separates in EMW) thus, the extracting properties of acetone and methanol are not the same, despite the fact that their solvent polarity ($P' = 5.1$) is the same, according to the data of Snyder and Kirkland (1979). See Table 3.1.

However, it is important to realise that other factors e.g. large dipole and proton donating-and-accepting properties influence solvent selectivity. The data of Rohrschneider, used to define solvent polarity values P' , was plotted on a triangular diagram by Snyder & Kirkland (1979). This diagram

grouped various solvents into clusters of similar selectivity and, according to these groups, methanol is a good proton acceptor with a large dipole (group II), and acetone is a better proton donor with a smaller dipole (group VIa). This partly explains the different extracting properties. On the other hand, acetone and methanol's solvent strength (ϵ°) differs significantly, with $\epsilon^\circ = 0.56$ for acetone and $\epsilon^\circ = 0.95$ for methanol (Snyder & Kirkland).

Thus acetone extracts less material from African potato 1 but it does extract the *red spot compound* and β -sitosterolin. Therefore, acetone is seen as a more selective extractant (w/w) for sterolins (including the *red spot compound*) than methanol. Furthermore, acetone is easier to work with, as the extracts dry quickly for concentration determination and, the volatility also results in neat thin lines on TLC plates.

Conclusion: Acetone is the best extractant for sterolins (including the red spot compound).

Chloroform was shown to be the best extractant for sterols and methanol for hypoxoside. If required that all three compounds, that is phytosterols, sterolins and hypoxoside be indicated with one extract, methanol should be used as extractant.

3.3.2 Mobile phase evaluation

Three different solvent systems were used as mobile phases for the development of TLC plates. In order of polarity:

*BEA: Benzene:Ethanol:Ammonia in the ratios 18:2:0.2 (separates non-polar compounds).

*CEF: Chloroform:Ethylacetate:Formic acid in the ratios 5:4:1 (separates compounds of intermediate polarity).

*EMW: Ethylacetate:Methanol:Water in the ratios 10:1.35:1 (separates polar compounds).

All three the systems showed the phytosterols clearly, although none of the systems could separate the individual sterols (β -sitosterol, campesterol and stigmasterol) and a "phytosterol-complex" was visible. In BEA (non-polar system), the sterols had low R_f -values and the compounds of interest were, therefore, on the lower half of the chromatogram. The *red spot compound* and β -sitosterolin were visible as well. In CEF (intermediate polarity) the sterols and sterolins of interest had intermediate R_f -values and were spread over the entire chromatogram. Both the *red spot compound* and β -sitosterolin were visible. In EMW (polar system), the sterols and sterolins had high R_f -values and the compounds of interest dominated the upper half of the chromatogram. Therefore, with

balance in distribution on the chromatogram and its general appearance, as the main reason, CEF was chosen as the best mobile phase for separation of the sterols and sterolins. However, only EMW was able to separate hypoxoside from the polar methanol extracts and was used for hypoxoside determinations.

Conclusion: CEF is the best mobile phase to separate sterols and sterolins and EMW for hypoxoside determination.

3.3.3 Spray reagent evaluation

3.3.3.1 *P*-Anisaldehyde spray reagent

P-Anisaldehyde reacts with the sterols, the *red spot compound* and β -sitosterolin to reveal the coloured substances in daylight. When glass plates are moderately heated, the *red spot compound* is brown-yellow, but becomes a pink-red when the plate is overheated. β -Sitosterolin is a light purple line and the phytosterols form a dark, blue-purple mass – “phytosterol complex”.

Sterols and sterolins do not fluoresce at 254 nm or 350 nm when sprayed with *p*-anisaldehyde.

3.3.3.2 Vanillin spray reagent

Eloff (1999) found in his experiments that vanillin does not reveal the sterolins. However, when these experiments were repeated, it was found that vanillin does show the sterolins present in high concentrations e.g. isolated β -sitosterolin and “stigmasterolin” in 10 mg/ml stigmasterol standard. Overheating glass plates made the “stigmasterolin” in the 10 mg/ml standard more visible. The *red spot compound* and β -sitosterolin in African potato 1 and *P. africana* extract (5:1) were also visible when sprayed with vanillin. See Figure 3.4 and compare with Figure 3.2 (sprayed with *p*-anisaldehyde).

Sterols and sterolins fluoresce slightly at 350 nm when sprayed with vanillin. Five compounds were visible under 350 nm UV light that were not visible in daylight or with *p*-anisaldehyde. Three of them had R_f -values higher than the *red spot compound* and lower than the “phytosterol-complex” and were present in the alkaline acetone (lane 5) and in the methanol extracts (lane 6 and 7) respectively. Two compounds were present in the *P. africana* extract (5:1) methanol and chloroform extracts respectively, with R_f -values lower than the *red spot compound*.

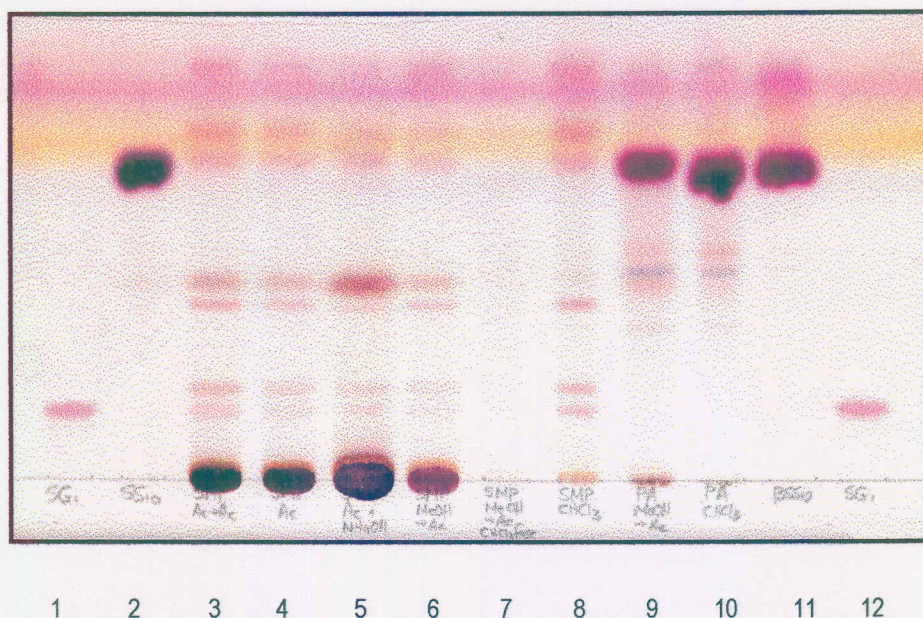


Figure 3.4: Overheated TLC on a glass plate of different extracts of *H. hemerocallidea* powder and *P. africana* extract (5:1), developed in CEF and sprayed with vanillin. On the left are 1 mg/ml isolated β -sitosterolin, 10 mg/ml 95% stigmasterol standard and on the right are 10 mg/ml 40% β -sitosterol standard and 1 mg/ml isolated β -sitosterolin. In all cases 100 μ g of the extracts was applied.

In Figure 3.4 from the left are 1 mg/ml isolated β -sitosterolin (1) and 10 mg/ml 95% stigmasterol standard (2). The order of the African potato 1 extracts are as follows: Acetone, dried and redissolved in acetone again (3), acetone, not dried (4), alkaline acetone (5), methanol, dried and redissolved in acetone (6), acetone insolubles from 6 redissolved in 50/50 $\text{CHCl}_3/\text{H}_2\text{O}$ – chloroform phase (7) and chloroform, dried and redissolved in chloroform (8). The extracts of *P. africana* extract (5:1) are methanol, dried and redissolved in acetone (9) and chloroform, dried and redissolved in chloroform (10). On the right are 10 mg/ml 40% β -sitosterol standard (11) and 1 mg/ml isolated β -sitosterolin (12).

3.3.3.3 Perchloric acid spray reagent

Perchloric acid chars the organic compounds on the TLC plate and they turn brown or black depending on the quantity present. It only charred the compounds that were revealed by *p*-anisaldehyde, which indicates that there are not other organic compounds present in high

concentrations. The sterols and sterolins (*red spot compound* and β -sitosterolin) fluoresce at 350 nm, when sprayed with perchloric acid. The sterols fluoresced orange and β -sitosterolin light-pink

3.3.3.4 50% Sulphuric acid in methanol spray reagent

50/50 H₂SO₄/MeOH also chars the organic compounds. Again no other compounds than those revealed by *p*-anisaldehyde, were shown, therefore, it can be assumed that *p*-anisaldehyde reveals all the organic compounds present in high concentrations.

The sterols and sterolins do not fluoresce when sprayed with 50/50 H₂SO₄/MeOH at either 254 nm or 350 nm.

3.3.3.5 Phosphoric acid spray reagent

The colour reaction, that takes place when spraying the plates with phosphoric acid, was not satisfactory. However, under 350 nm UV light, apart from the usual compounds revealed by *p*-anisaldehyde, seven bands could be seen in the African potato 1 methanol extracted, acetone-insoluble, chloroform phase (lane 7 in Figure 3.2 and 3.4, but now sprayed with phosphoric acid). *P*-Anisaldehyde did not reveal anything in this extract, but vanillin showed one compound at 350 nm UV. the sterols fluoresced orange and β -sitosterolin light-pink, as with perchloric acid.

3.3.3.6 *P*-Toluene sulphonic acid spray reagent

Similar to phosphoric acid, the colour reaction, that reveals compounds in daylight, was not satisfactory. However, two blue bands with R_F-values higher than the *red spot compound*, were visible in the *P. africana* extract (5:1)'s methanol and chloroform extracts. As with perchloric and phosphoric acid, at 350 nm the sterols fluoresced orange and β -sitosterolin was light pink. The *red spot compound* was not clearly visible.

3.3.3.7 Trichloroacetic acid spray reagent

After spraying the plates with trichloroacetic acid, nothing could be seen in daylight. However, at 350 nm UV the sterols looked light blue, but β -sitosterolin was not clear.

3.3.3.8 Best spray reagent evaluation

Apart from *p*-anisaldehyde and vanillin, the other spray reagents made the sterols/sterolins visible under UV light, but not in daylight. Two other important spray reagents widely used in herbal

medicinal TLC, fast blue and natural product reagent, also gave no colour reaction in daylight, when used by Eloff (1999) for sterol/sterolin analyses, and were, therefore, not applied.

P-Anisaldehyde was most often used as spray reagent. Although not all compounds (that were visible under UV light) were revealed by it, they were not the major compounds of interest. The important compounds, e.g. the sterols/sterolins (including the *red spot compound*) and even hypoxoside, were revealed by *p*-anisaldehyde and were clearly visible in daylight.

Conclusion: P-Anisaldehyde was regarded as the best reagent tested to indicate sterols and sterolins and hypoxoside.

B. APPLICATION

3.3.4 TLC Application

3.3.4.1 Analyses of different plants used for benign prostatic hyperplasia (BPH)

3.3.4.1 (a) *H. hemerocallidea* from three different sources – extracts compared

Comparison of methanol, ethanol and chloroform extracts of three sources of African potato revealed no differences on a qualitative basis with TLC. Although the percentage (w/w) of material extracted by methanol, ethanol and chloroform differed for the three African potato samples, the components extracted were similar, and all three samples could be considered as pure *H. hemerocallidea*. TLC can, therefore, be used to identify qualitatively unknown corns as *H. hemerocallidea* by comparing and matching with the TLC template of a known corn. Furthermore, TLC can indicate batches that do not comply to set standards e.g. minimum sterol/hypoxoside levels, again by comparison with a satisfactory TLC template of *H. hemerocallidea*. The compound levels in plants can be influenced by e.g. time of harvest, absence/availability of essential ground nutrients, climate *etc.* See 3.3.1.2 (b) and Figure 3.3.

3.3.4.1 (b) Comparison of sterols/sterolins in BPH phytotherapeutics: *H. hemerocallidea* powder, *P. africana* extract and *S. repens* powder/extract

The aim was to compare the sterol/sterolin contents of different plants, specifically African potato 1, *P. africana* extract (5:1), *S. repens* powder and extract (4:1). Furthermore the composition and concentration differences of the actives in the powder and extract were also compared in the case of *S. repens*.

Methanol extracts were used to compare sterolins (including the *red spot compound*) and chloroform extracts for the sterols. The methanol extracts were redissolved in acetone and the chloroform extracts in chloroform. The TLC plate was developed in CEF with *p*-anisaldehyde as spray reagent. See Figure 3.5.

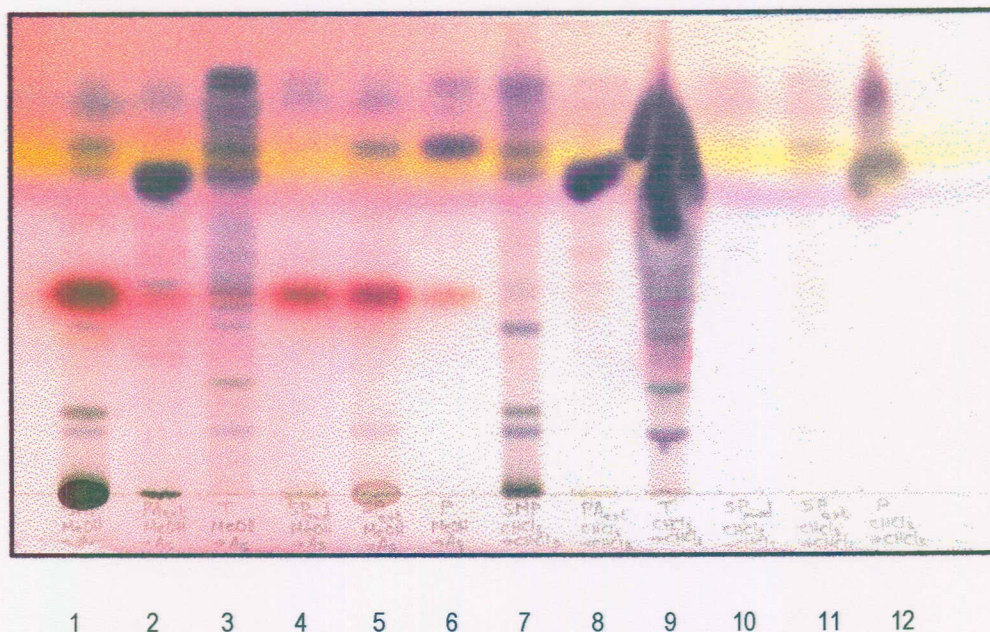


Figure 3.5: Overheated TLC on a glass plate of methanol (lanes 1-6) and chloroform (lanes 7-12) extracts of African potato 1 (1 & 7) *P. africana* extract (2 & 8), Tadenan[®] (*P. africana* extract) (3 & 9), *S. repens* powder (4 & 10) and extract (5 & 11) and Permixon[®] (*S. repens* extract) (6 & 12). The plate was developed in CEF and sprayed with *p*-anisaldehyde. In all cases 100 µg of the extracts was applied.

Methanol extracted a high concentration of phytosterols and a low concentration of the *red spot compound* and β -sitosterolin from *P. africana* extract (5:1). See that the methanol extract of *P. africana*, in the form of Tadenan[®] (lane 3), contained β -sitosterolin ($R_f = 0.14$). Methanol, however, extracted a low concentration of phytosterols, a substantial amount of the *red spot compound*, as well as hypoxoside (not visible in CEF, but visible in EMW – not shown) and β -sitosterolin from African potato 1. Methanol extracted the *red spot compound* from *S. repens* powder and extract (4:1), and a low concentration β -sitosterolin and sterols from the extract (4:1). Apparently chloroform extracted the same quantity of sterols from *P. africana* extract (5:1) than methanol, which was surprising. On the other hand chloroform extracted almost nothing from either *S. repens* powder or the extract (4:1). According to literature *S. repens* extracts are composed of 90% fatty acids and a variety of alcohols (including sterols) and a pentacyclic triterpenoid, lupeol (Quirke et al., 1998). Therefore, sterols were not expected, but it was strange that the fatty acids were not noticeable in the chloroform extracts of *S. repens*.

Table 3.4: Relative amounts of sterols, sterolins and hypoxoside (indicated by 1+ to 4+) extracted by methanol and chloroform respectively from *H. hemerocallidea* powder, *P. africana* extract (5:1), *S. repens* powder and extract (4:1), deduced from Figure 3.5

Methanol extraction				
	Phytosterols	β -Sitosterolin	Red spot compound	Hypoxoside
<i>H. hemerocallidea</i> pwd.	+	++	++++	+++
<i>P. africana</i> ext	++++	+	+	-
<i>S. repens</i> pwd.	-	-	++	-
<i>S. repens</i> ext.	+	+	+++	-
Chloroform extraction				
	Phytosterols	β -Sitosterolin	Red spot compound	Hypoxoside
<i>H. hemerocallidea</i> pwd.	++	+++	-	+
<i>P. africana</i> ext	++++	-	-	-
<i>S. repens</i> pwd.	+	-	-	-
<i>S. repens</i> ext.	+	-	-	-

Conclusion: From the results one can conclude that *H. hemerocallidea* contains sterols, sterolins (including the red spot compound) and also hypoxoside. *P. africana* extract (5:1) contains a very high concentration of sterols and a low concentration of β -sitosterolin and the red spot compound. *S. repens* extract (4:1) contains hardly any sterols, low concentrations β -sitosterolin and relatively high concentrations of the red spot compound (lower than *H. hemerocallidea*).

****Note** that the extractability of compounds and their sensitivity to the spray reagent may differ. For example, in Table 3.4 the concentration of phytosterols in *H. hemerocallidea* may be higher than the concentration of the red spot compound. However, if the red spot compound is more soluble in the extractant used and it reacts more readily with *p*-anisaldehyde than the phytosterols, the red spot compound band will have a higher intensity on TLC and wrongly give the impression of a higher

concentration in the plant. Therefore only comparisons of the same compound using the same extractant should be made.

3.3.4.2 Analysis of different phytosterol products

The aim was to compare the phytosterol levels in four different phytosterol containing products (Moducare[®], Immunochoice[®], Phytogard[®] and Nutricare[®]), four *H. hemerocallidea* products (African potato 1, 4, 5, 6), a BPH product [Prostol[®] contains *S. repens* berry powder, *Prunus africana* extract (5:1) and pumpkin seed extract (4:1)] and *S. repens* extract (4:1) and *P. africana* extract (5:1). For phytosterol comparison, chloroform extraction was performed and aluminium TLC plates were suitable. See Figure 3.6.

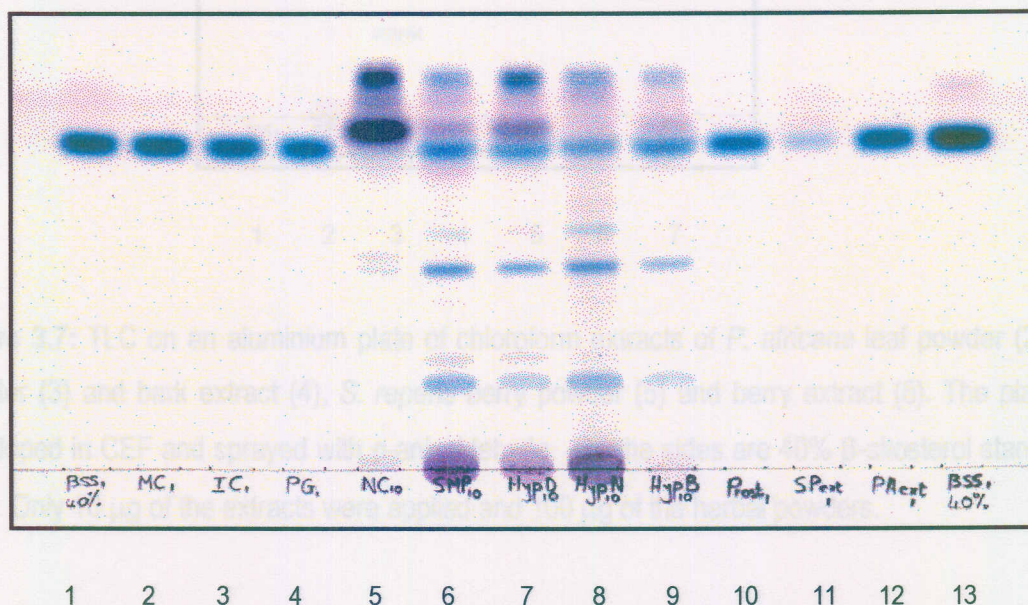


Figure 3.6: TLC on an aluminium plate of chloroform extracts of different phytosterol products. From the left are 40% β -sitosterol standard (1), Moducare[®] (2), Immunochoice[®] (3), Phytogard[®] (4) and Nutricare[®] (5), African potato 1 (6), African potato 4 (7), African potato 5 (8), African potato 6 (9), Prostol[®] (10), *S. repens* extract (11), *P. africana* extract (12) and 40% β -sitosterol standard (13). The plate was developed in CEF and sprayed with *p*-anisaldehyde.

In the case of Nutricare[®] and the *H. hemerocallidea* samples, 100 μ g was applied, but in all other cases 10 μ g was applied.

TLC of the chloroform extracts of Moducare[®], Immunochoice[®], Phytogard[®], Prostol[®] and *P. africana* extract (5:1) were similar - only the “phytosterol-complex”, as seen in the 40% β -sitosterol standard, was visible. Nutricare[®] appeared different from the other phytosterol products and the usual “phytosterol-complex” was not visible. The four *H. hemerocallidea* products appeared similar although the band intensities differed. *S. repens* extract (4:1) contained a very low concentration phytosterols, compared to *P. africana* extract (5:1). This confirmed the results of 3.3.4.1 (b). The phytosterol complex of Prostol[®] could probably also be attributed to *P. africana* extract (5:1) – the phytosterol contents of pumpkin seed was not investigated.

3.3.4.3 Analysis of herb powder versus the herbal extract of *P. africana* and *S. repens*

In the natural medicine industry herbal extracts are claimed to contain substantially higher concentrations of the particular active components when compared to the herb powders from which they have been extracted. These extracts are often also very expensive. This was investigated by comparing the phytosterol contents of chloroform extracts of *P. africana* bark powder extract (5:1) and *S. repens* berry powder and extract (4:1). The phytosterol contents of *P. africana* leaves were investigated as well. See Figure 3.7.

The *P. africana* leaf powder contained phytosterols (“phytosterol-complex”) in similar concentrations as the bark powder, but both contained less than *P. africana* bark extract (5:1). The chloroform extract of *P. africana* bark powder is complex and more than fourteen different bands were visible on TLC, whereas there was only one band (“phytosterol-complex”) visible in the bark extract (5:1). The “phytosterol-complex” in *P. africana* bark extract (5:1) is at least five times as intense as that of the bark powder.

The chloroform extract of *S. repens* berry powder is much simpler than that of *P. africana* bark powder and approximately four bands are visible on TLC. Only two bands of *S. repens* berry extract (4:1) are visible on TLC. The “phytosterol-complex” in *S. repens* berry extract (4:1) appears lighter than that of *S. repens* berry powder, but the concentration was ten times less than that of the powder. However, the total quantity extracted should also be taken into consideration with these comparisons.

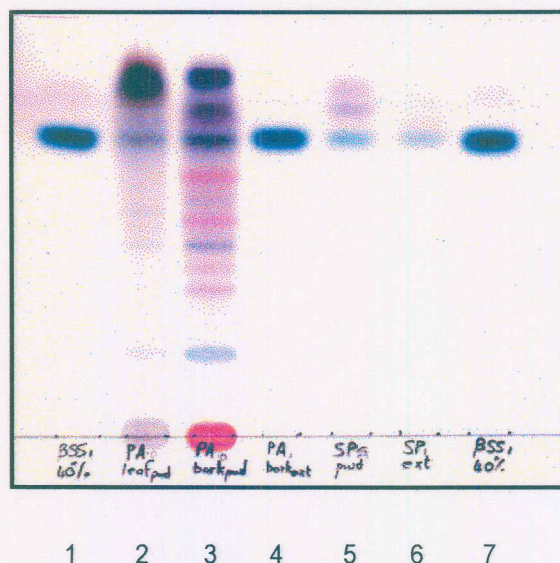


Figure 3.7: TLC on an aluminium plate of chloroform extracts of *P. africana* leaf powder (2), bark powder (3) and bark extract (4), *S. repens* berry powder (5) and berry extract (6). The plate was developed in CEF and sprayed with *p*-anisaldehyde. On the sides are 40% β -sitosterol standard (1 & 7). Only 10 μ g of the extracts were applied and 100 μ g of the herbal powders.

3.3.4.4 Hydrolysis of “stigmasterolin” and the red spot compound

See the method of hydrolysis in section 3.2.2.5.

“Stigmasterolin” was visible in the unacidified 10 and 5 mg/ml stigmasterol standards (lanes 2 & 4), but it disappeared in the acidified stigmasterol solutions (lanes 1 & 3). Thus, as expected the hydrochloric acid hydrolysed the “stigmasterol glucoside” and cleaved the sugar bond to form stigmasterol. See Figure 3.8

However, hydrolysis of the *red spot compound* in the MCW extract of African potato 1 (water-methanol phase) was not observed (results not shown). It is possible that the *red spot compound*'s concentration was too high in proportion to the HCl added and that the reaction was not complete. In the MCW-chloroform phase of African potato 1, new bands appeared after hydrolysis, while others disappeared.



Figure 3.8: Overheated TLC on a glass plate of unhydrolysed (1) and hydrolysed 10 mg/ml 95% stigmasterol standard (2) and unhydrolysed (3) and hydrolysed 5 mg/ml 95% stigmasterol standard (4).

3.3.4.5 Isolation of the *red spot compound* with preparative thin layer chromatography (PTLC)

See the PTLC method in section 3.2.2.6.

After dissolving the *red spot compound* on the silica powder in acetone, the acetone was removed and c. 500 mg precipitate remained. Usually at least 1 mg, but preferably 10 mg pure sample is needed for NMR structure elucidation. The sample was sent for structure elucidation to SASOL.

3.3.4.6 Structure elucidation of the *red spot compound* with Nuclear Magnetic Resonance Spectroscopy (NMR)

See the method in section 3.2.2.7.

From SASOL's ACD/HNMR Dictionary the ^1H spectra of β -sitosterol, campesterol and stigmasterol could be obtained. See Appendix A.

As there was very little sample, special measures were taken to improve the sensitivity of NMR (indirect detection probe *etc.*) as discussed in 3.2.2.7. However, NMR ^1H spectrum revealed that the sample was impure and there was too little to identify the major compound present.

TLC of the NMR analysed samples (CEF with *p*-anisaldehyde) revealed that they contained at least three different components. Apparently, the separation of the extract on the thicker preparative plate (2 mm), as discussed in 3.2.2.7, was not as good as on the ordinary glass TLC plates (0.25 mm). The bad separation on the preparative plate is consistent with Cannell (1998), who states that when attempting to separate a mixture with PTLC (preparative TLC) it should not contain more than four main components. The acetone extract of *H. hemerocallidea* (African potato 1) contained at least nine different compounds, therefore, NMR analysis of the *red spot compound*, isolated with PTLC, was unsuccessful.

3.3.4.7 Water extraction of African potato 1

African potato 1 is marketed for various medicinal indications, e.g. immunity stimulation, anti-cancer effects, arthritis, *etc.* (promotional pamphlet). These effects are attributed to the sterols and sterolins in the plant (Van Wyk et al., 1997 & Bouic et al., 1999). The extraction method suggested by the distributor [See this chapter, 3.2.2.1 (b) (iii)] hardly extracts any phytosterols or sterolins (Eloff, 1999). To determine whether sterols/sterolins are extracted by water, the work of Eloff (1999) was repeated as it is possible that the particular sample analysed by Eloff (1999) had a very low concentration of sterols/sterolins. For the complete method see this chapter, 3.2.2.1 (b) (iii). The prepared extracts were chromatographed and developed in EMW (*p*-anisaldehyde as spray reagent) as the water extract is polar and does not separate well in CEF. See Figure 3.9.

From TLC, it seemed that water extracted the *red spot compound* and a little hypoxoside from African potato 1, but not β -sitosterolin – as retrospectively identified when isolated β -sitosterolin was obtained (lane 4). It does not appear as if water extracted the sterols, as to be expected (lanes 8 & 9), as the sterols are too non-polar. The methanol extract, dried and redissolved in methanol (lanes 3 & 11), contained hypoxoside but not the *red spot compound*. The methanol extract, dried and redissolved in acetone (lane 12), on the other hand, contained hypoxoside and the *red spot compound*. Possible reasons were that the *red spot compound* did not dissolve in the methanol again after drying the methanol extract, or that the methanol extract did not separate completely and that the *red spot compound* was present, but was not visible. Note that all that is extracted does not necessarily dissolve again, due to possible complex formation *etc.*

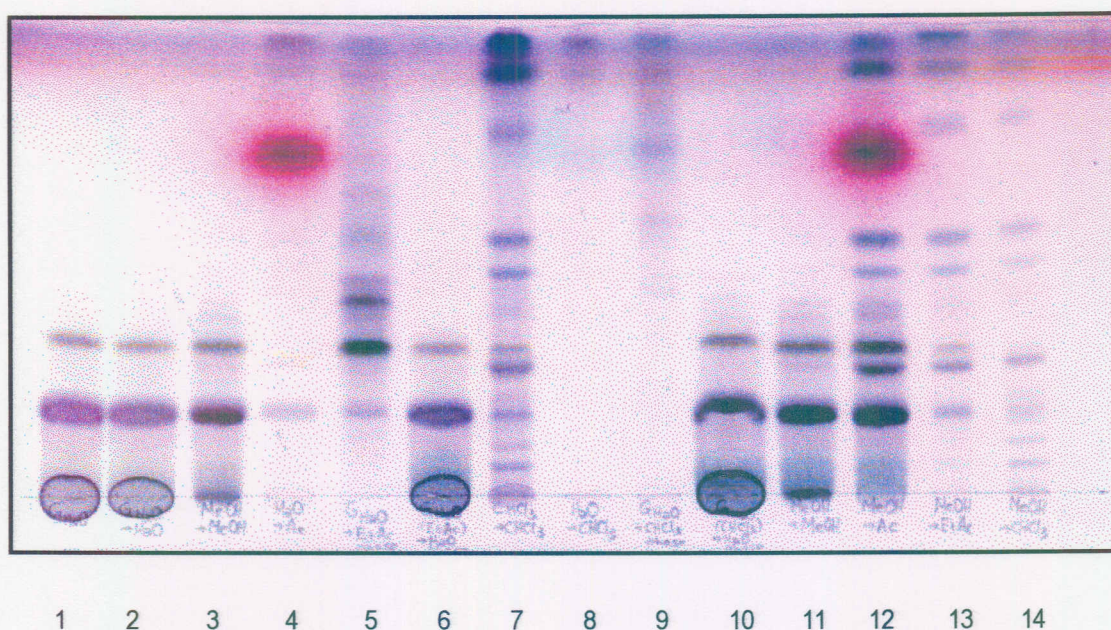


Figure 3.9: Overheated TLC on a glass plate of water and other extracts of grated African potato 1 corms (G) and powdered African potato 1, developed in EMW and sprayed with *p*-anisaldehyde.

In Figure 3.9 the identity of the different lanes are as follows: Grated (G) corms water extract (1); G water extract dried and redissolved in water (2); powder methanol extract (redissolved in methanol) (3); powder water extract (redissolved in acetone) (4); G water extract (ethylacetate's) organic phase (5); G water extract (ethylacetate's) watery phase (6); powder chloroform extract (redissolved in chloroform) (7); powder water extract (redissolved in chloroform) (8); G water extract (chloroform's) organic phase (9); G water extract (chloroform's) watery phase (10); powder methanol extract (redissolved in methanol) (11); powder methanol extract (redissolved in acetone) (12); powder methanol extract (redissolved in ethylacetate) (13), and powder methanol extract (redissolved in chloroform) (14).

The therapeutic effect of African potato 1, extracted with water, may be ascribed to hypoxoside, or perhaps even the *red spot compound* or other novel compounds present, but apparently not to β -sitosterol.

3.4 CONCLUSION

In order to develop an optimum TLC method for phytosterols and sterolins, the best extractant, mobile phase and spray reagent were determined. The developed TLC method could be applied to qualitatively compare *H. hemerocallidea*, *P. africana* and *S. repens*, as well as different phytosterol containing products, in terms of their phytosterol and sterolin contents.

Acetone was the best extractant for sterolins (β -sitosterolin and the *red spot compound*), chloroform for phytosterols and methanol for hypoxoside. The best separation of the sterols/sterolins was found with chloroform:ethylacetate:formic acid (5:4:1) as mobile phase and the best spray reagent was *p*-anisaldehyde.

Apart from the extractant, mobile phase and spray reagent, it seems that even the type of TLC plate (aluminium vs. glass) can influence the visibility of compounds on TLC. Overheated plates are not always negative, as the *red spot compound* was more prominent and therefore noticed for the first time, after the glass plates (sprayed with *p*-anisaldehyde) were overheated by accident.

In our results, *H. hemerocallidea* powder contained sterols, sterolins (including the *red spot compound*) and hypoxoside; *P. africana* extract (5:1) contained a very high concentration of sterols and a low concentration of β -sitosterolin and the *red spot compound*, but *S. repens* extract (4:1) contained hardly any sterols, low concentrations β -sitosterolin and relatively high concentrations of the *red spot compound* (lower than *H. hemerocallidea*).

The developed TLC method thus provides the natural medicine industry with a simple, cost-effective and reproducible method to impose the necessary quality control procedures.

As African potato tea was used traditionally for centuries and is still marketed for medicinal application, the water extract of *H. hemerocallidea* was also investigated. From the TLC results on water extraction of *H. hemerocallidea* powder, it appeared that water does not extract phytosterols or β -sitosterolin. This was concerning, as currently the phytosterols (especially β -sitosterol) and sterolins, are regarded as the main active components of *H. hemerocallidea* and also of African potato tea. These results should however be confirmed with further research, possibly using more sensitive techniques. The water extract did contain the *red spot compound* and hypoxoside.

Hypoxoside is metabolised *in vivo* to rooperol and has anticancer activity and could thus possibly be considered as one of the main actives. See Chapter 1, section 1.4.1.3.

Whether the *red spot compound* has therapeutic effect is unknown, as the structure could not be identified. Apparently it is a common compound in phytosterol-containing material, as it could be detected in acetone, methanol and water extracts of *H. hemerocallidea* and in acetone extracts of *P. africana*, *S. repens*, Moducare®, Harzol®, Immunochoice® and Nutricare®. Although the R_f -value was the same as that of, or what was considered to be, “stigmasterolin” in the 95% stigmasterol standard, the *red spot compound* is not necessarily stigmasterolin and its identity has to be determined. In this study, however, it was regarded as a sterolin and reported as such. An attempt to hydrolyse the *red spot compound* did not cause the band intensity to decrease, as could be shown with “stigmasterolin” in the 95% stigmasterol standard. PTLC isolation of the *red spot compound* was unsuccessful as the separation on the PTLC plate was not as effective as on TLC. Column chromatography could perhaps provide a clean sample, but a substantial quantity of *H. hemerocallidea* will have to be extracted with acetone.

It is important to note that the compared *P. africana* and *S. repens* extracts (and other products containing extracts) were produced and/or extracted by different companies, probably using different extraction procedures. As noticed in the different compounds extracted e.g. with acetone versus chloroform from *H. hemerocallidea*, the extraction procedure largely determines the composition of the product and the compounds that it contains. Therefore, the botanical product of one company may differ dramatically from that of another regarding specific components present and, thus also effectiveness, as we often do not know what the active components of these products are. This holds true, even if they use material that originates from the same plant. Then there is also natural variability among the plants themselves (Lowe & Fagelman, 1999).

The implication is that the results of basic or general research and clinical trials cannot be automatically accepted and/or transferred from one manufacturer's product to another. The different preparations from each individual manufacturer need to be evaluated individually. The necessity of this principle was emphasised by the Committee on Other Medical Therapies of the Fourth International Consultation on Benign Prostatic Hyperplasia (Lowe & Fagelman, 1999). If this is not possible, the products should at least be qualitatively compared. This is where a well-developed and reliable technique, such as the TLC method developed in this study, could play an important role.