COMPARATIVE THIN LAYER CHROMATOGRAPHY STUDY OF DIFFERENT BRANDS OF FIVE HERBAL REMEDIES

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I, Carla Urbani, declare that this research report is my own work. It is being submitted for the degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

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ABSTRACT

The belief that herbal remedies are less invasive on the human body than conventional medicine and the return of the consumer to a more natural lifestyle, has led to the development of a multitude of remedies, with many different uses. Because the use of these herbal remedies has increased drastically in the last decade, it is essential that the quality and efficacy of these products are well regulated. One of the objectives in this study includes the investigation of the presence of marker metabolites in five herbal remedies, namely *Serenoa repens*, *Silybum marianum*, *Hypericum perforatum*, *Echinacea purpurea* and *Gingko biloba*. Although most of the brands tested contained the active ingredients assayed for, a few exceptions were found. However, because this study used only thin layer chromatography for analysis of products, verification of these results should be obtained using other more modern methods for example high pressure liquid chromatography. Four brands of *Serenoa repens* were selected and assayed for the presence of β-sitosterol. All four brands tested indicated the presence of β-sitosterol. Five brands of *Hypericum perforatum* were selected and assayed for the presence of hypericin, rutin and chlorogenic acid. Four of the five products tested indicated the presence of hypericin, while three of five products indicated the presence of rutin and chlorogenic acid. Five brands of *Echinacea purpurea* were selected and assayed for the presence of β-sitosterol, chlorogenic and caffeic acid. Three of the five products indicated the presence of β-sitosterol, while only one of the five products contained chlorogenic acid. Caffeic acid was present in 3 of the 5 products. Seven brands of *Gingko biloba* were selected and assayed for the presence of rutin and bilobalide. Five of the seven products indicated the presence of rutin and bilobalide. Four brands of *Silybum marianum* were selected and assayed for the presence of both taxifolin and sylibin. Only two of the four products contained both taxifolin and silybin. The second objective of this study is to provide a literature review of the five herbal remedies mentioned above. Amongst the topics discussed were uses of these plants, evidence from studies conducted, chemistry and mechanism of action of the active molecules contained in the plants.

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INTRODUCTION

Since ancient times herbs have been renowned for their curative properties, and it is estimated that more than 52 000 plant species have been used for medicinal purposes. Many societies have developed their own traditions on plant use and some of these traditional medicines are now established commercial products eg. *Gingko biloba* was originally a Chinese medicine (Schippmann *et al*., 2002).

Various industries have capitalized on the drastic growth in the use of herbal remedies for health purposes over the past 10 years, which has developed due to the tendency of the consumer to return to a more 'natural lifestyle'. Additional factors which have led to this growth in the use of herbal remedies include the dissatisfaction of customers with modern healthcare due to the expense and unwanted side effects and the need of healthy people to prolong their healthy lifestyle into a healthy old age (O'Hara *et al*.,1998).

Both consumers and health professionals lack sufficient accurate information about the safety, efficacy and toxicity of these remedies. Although botanical herbal supplements have a more gentle effect on the body, and are believed to be less invasive than conventional therapies, they may not be as safe as patients perceive them to be. The undermining of the contraindications, side effect profile and toxicity of these remedies may be detrimental, and even fatal in some instances. The presence of many different products on the market renders it essential that both the patient and healthcare professional are aware that the quality standards, strength, and dosage differ not only from one product to another, but between different forms of the same product. The recent increased focus of manufacturers on the issues of quality, and standardisation in particular, has led to the differentiation between potent, good quality products and ineffective, unsafe and adulterated products (Ernst, 2000).

Marker metabolite analyses alone however, may not be sufficient to ensure the correct identification of raw materials used in consumer products. Numerous other physical characteristics (such as particle size, moisture content, homogeneity, pH, presence of

foreign matter etc.) which may affect the manufacturing process as well as product stability and bioavailability must be taken into consideration.

Because herbal medicines are often a mixture of more than one active ingredient, it may be uncertain as to which, or how many constituents are pharmacologically important. The use of modern scientific techniques ensures that products contain the specified amounts of marker metabolites, providing a guarantee of potency and hence product quality.

The number of molecules with unique chemical and physical properties contained in commercial preparations, unidentified constituents, and unknown therapeutic contribution of the active constituents, contribute to the difficulty of pharmacokinetics, making it one of the most difficult aspects of phytomedicines. This complexity impacts on quality control due to the standardisation of a variety of constituents, rather than one single constituent (USP conv, 1998). For this reason marker compounds (phytoconstituents) are used in standardisation to ensure product quality.

There is a great deal of confusion and misunderstanding as to what the purpose of standardisation is, and what it actually involves. Due to the phytochemical diversity botanicals may be highly variable in their chemical make up. Marker compounds may be chosen to ensure correct species identification or correct chemotypes required for a specific product, whereas universal constituents such as flavonoids may be indicators of product quality during manufacture or product stability during storage.

The growing evidence of efficacy is counterbalanced by inadequate regulation in some countries. Sales of herbal medicines are booming world wide. In the US, the market for herbal supplements is approaching 4 billion dollars a year (Ernst, 2000). The fastest growth record has been for St John's Wort, whose sales in 1998 increased by 2800% (Brevoort, 1998). It is due to the presence of figures like these that doctors are inclined to question the presence of evidence. Are there sufficient trials to illustrate their efficacy, and if so are these trials reliable and accurate? Does the efficacy of these herbal medicines outweigh the risks of administering them?

A great concern to both health professionals and patients is that most herbal medicines sold, especially in the US and the UK are sold as food supplements, therefore evading regulations regarding their safety and quality. In South Africa, it hasn't been until recently that the herbal remedies registered before 1965 were called up for re-registration. These previously unpopular remedies were registered without much ado, and it was only once the newer varieties began flooding the market, that it was felt insufficient information regarding the quality and safety of the registered products had been provided. The more stringent controls were introduced to ensure that all herbal remedies entering the market, and those already on the market are of good quality and safe, hereby protecting the consumer and preventing the sale of unregistered, potentially dangerous remedies.

This study intends to provide a thorough overview and identify marker metabolites of five herbal remedies, through extraction and thin layer chromatography methods. The herbal remedies to be used are saw palmetto (*Serenoa repens*), milk thistle (*Silybum marianum*), purple cone flower (*Echinacea purpurea*) St John's wort (*Hypericum perforatum*) and the Duck foot tree (*Gingko biloba*).

Objectives:

The main objectives of this study include:

- The investigation of the presence of the marker metabolites in the various selected brands of herbal remedies by thin layer chromatography.
- The determination of the best method in identifying these marker metabolites.
- The provision of a countercheck of the quality control protocol for each remedy.
- The determination of reasons for incongruence if inconsistencies are detected.

Consumer products of the selected five remedies were purchased from a number of pharmacies and health shops, in their commercialized form ie. They were already processed and packaged. A number of brands were represented: Bioharmony, Solgar, Bioforce, Herbology, Linkcare etc, as shown in the figures below.

Figure 1a: The different forms and brands of *Serenoa repens* used in the study.

Figure 1b: The different forms and brands of *Hypericum perforatum* used in the study.

Figure 1c: The different forms and brands of *Echinacea purpurea* used in the study.

Figure 1d: The different forms and brands of *Ginkgo biloba* used in the study.

Figure 1e: The different forms and brands of *Silybum marianum* used in the study

A standard method as outlined below was used in all five herbal remedies. Plant Drug Analysis was used to determine the assay method used for each species (Wagner and Bladt, 1996). The parameters which differed were:

-the weight of the samples between herbal remedies (These were kept within a constant range for each remedy ie. all the *Gingko* samples weighed between 0.35 and 0.36 grams, whereas all the *Hypericum* samples weighed 0.33grams). This was done to ensure that the same concentration of each product was tested.

-the solvent systems, which will be clearly stated in all instances.

-the length of time which the chromatogram was developed (this depends on the affinity of the solvent system for the constituents in the samples).

-the wavelength at which detection occurred (depends on constituents in sample).

-the spray reagent which was used.

The samples were weighed on an electronic scale, placed in polytubes, covered in 1ml of alcohol and capped. The reference standards, however, were not weighed as only very small amounts of these were available, due to their expense. Only a few grains of these were placed into a polytop, and just enough ethanol was added for dissolution. Samples were shaken up vigorously and left to stand for five minutes.

Once five minutes had passed, the solid herb particles of the specimens had settled on the bottom of the polytube, and the upper clear ethanol phase had separated out above the solid phase. Pipettes were used to transfer the liquid to clean polytops. Only one pipette per sample was used per product, so as to avoid contamination. The clean polytops were labelled clearly, to avoid mixing the products.

The silica TLC plates were cut according to the number of lanes needed ie. the number of products to be analyzed, and labelled accordingly. Products were spotted using calibrated pipettes. Twenty microlitres of sample were spotted in all cases, except in the reference standards, in which 10 microlitres were spotted. TLC plates were dried using a hairdryer, and placed in their various TLC tanks, in which the various solvent systems had saturated the chambers after being sealed with Vaseline^{R} and a glass lid for a minimum period of 5 minutes. The TLC plates were left to run until the solvent front had risen approximately 15 centimetres above the line of

application. After evaporation of the mobile phase, the plate was sprayed with various reagents depending on the active constituents (Wagner and Bladt, 1996). Each assay was repeated three times for each method, ie when one solvent system was used, but two different detection systems, a total of six plates were run (three for each detection system). However, if two separate solvent systems were run, with two different detection systems, a total of twelve plates were run (six for each system). The diagram below illustrates this more clearly.

The use of one solvent system(sys) and two detection systems produced six repeats in total:

Solvent system 1

detection sys 1	detection sys 2
three repeats	three repeats

The use of two solvent systems and two detection systems produced 12 repeats in total:

Solvent system 1		Solvent system 2	
detection sys 1	detection system 2	detection sys 1	detection system 2
three repeats	three repeats	three repeats	three repeats

Figure 1f. A diagram illustrating the number of repetitions performed in this study.

Table 1: Reference standards used in this study

Reference standard	**Brand name**	**Reference number**
Chlorogenic acid	Fluka	25700
Taxifolin	Sigma	T-4512
Rutin hydrate	Aldrich	R230-3
β-sitosterol	Sigma	S-1270
Hypericin	Sigma	H-9252
Caffeic acid	Sigma	C-0625

Spray reagents used:

Acetic anhydride reagent:

The TLC plate was sprayed with 10ml acetic anhydride and then heated at 150°C for approximately 30 minutes.

Natural products-polyethylene glycol reagent (NP/PEG)

The TLC plate is sprayed with 1% diphenylboric acid-β-ethylamino ester (diphenylboryloxyethylamine, NP), followed by 5% ethanolic polyethylene glycol-4000 (PEG), 10ml and 8ml respectively.

Fast Blue reagent

The TLC plate is sprayed with a solution of 0.5g Fast Blue B (tetraazotised di-o-anisidine) in acetone water (9:1 v/v). This solution should always be freshly prepared.

Iodine vapour

Some iodine crystals were placed in a closed chamber and left for a few minutes. The TLC plate was placed in the chamber for a few minutes.

Vanillin/sulphuric acid reagent

The TLC plate is sprayed with a 1% solution of vanillin in concentrated sulphuric acid, and heated at 100°C for 5 minutes.

10% Pyridine in ethanol

The TLC plate is sprayed with a 10% pyridine solution in absolute alcohol.
(Wagner and Bladt, 1996).

Serenoa repens (Saw palmetto)

Figure A

1. Saw palmetto (*Serenoa repens*)

i. Topography and physical appearance

Saw palmetto is endemic to North America. It is a member of the fan palm family, and is a low growing evergreen palm which grows to a height of approximately 4 metres. During Spring, 1 – 3 prominent clusters of white flowers are produced, which mature into fruit in the summer. The fruit are bluish-black berries, which are sized and shaped like olives. It bears multiple, persistent leaves protruding from the terminal end of long, horizontal stems which occur at or below ground level. Along the edges of the leaf are sharp, spine-like projections, which give the leaves a saw-like appearance, hence it's name, Saw palmetto (Tanner *et al*., 1996).

ii. Historical perspective

Saw palmetto berries have been consumed for many centuries to treat coughs from cold, urinary tract problems, menstrual cramps and ovarian enlargement, as an aid for increasing appetite, mammary gland stimulation and for impotency in both male and female. Moreover, saw palmetto has been used as a tonic for the male reproductive system and as a sexual rejuvenator. The medicinal value of the fruit has been described in scientific literature since 1800, however, it was not until 1898 that Hale proposed that tinctures of the fruit and crushed seeds can be used for the alleviation of prostate gland swelling and as an aphrodisiac. Today, more than a century later, the fruit is still used for benign prostate hyperplasia (BPH) (Tanner *et al*.,1996).

iii. Background

BPH is the enlargement of the prostate gland, which occurs in more than 50% of men aged between 40 and 59 (Wilt *et al*., 1998). It is a condition which is hormonal in origin and implicates and affects the reproductive system. BPH is directly related to an increase in testosterone and dihydrotestosterone (DHT) production, which in turn results in prostate gland enlargement. Figure 2 illustrates the 5α-reductase catalyzed conversion of testosterone to dihydrotestosterone. As the prostate enlarges, it surrounds the urethra progressively, compressing it and therefore not allowing the bladder to empty completely. Due to the impeded flow

of urine, the occurrence of bladder infections increases.

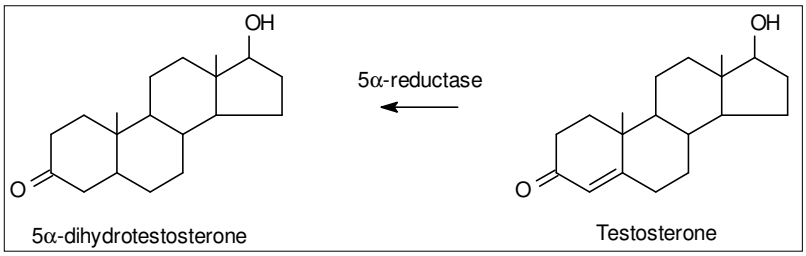

Figure 2. The conversion of testosterone to dihydrotestosterone

BPH and other prostate problems are often without pain. Pain may occur over the bladder during urination, or near the anus during ejaculation. Other symptoms include: difficulty in starting to urinate, the sensation of incomplete emptying, nocturia, frequent urination, urgency, dribbling after urinating and dysuria.

iv. Commercial source

Commercial saw palmetto products are derived from the ripe fresh or dried fruits of saw palmetto. These fruits are either sold whole, ground into a powder, and sold in the form of teas, capsules, tinctures, liquid and semi-solid extracts.

v. Uses

The Native Americans used saw palmetto as a sedative. The steam from cooking fruits was inhaled in bronchitis to relieve irritated mucous membranes and as an expectorant. The plant was first considered as a nutritive tonic and a useful remedy for relieving local irritations of the mucous membranes of the respiratory and digestive systems and the reproductive tract.

In females, saw palmetto has been used in dysmenorrhea and to increase the size of underdeveloped mammary glands. It has been used as a general tonic, for genitourinary problems, to increase sexual vigour, and as a diuretic.

Numerous studies have been published on the use of saw palmetto for treating lower urinary tract symptoms, secondary to benign prostatic hyperplasia. Saw palmetto was shown to be more effective than placebo in relieving the following symptoms: frequency, urgency, dysuria, nocturia and impaired urinary flow.

Advantages of using saw palmetto as compared to conventional drug therapies (eg. finasteride and terazosin) include the fact that it does not alter sexual function, or reduce prostate specific antigen (PSA) in the blood. An increased PSA level is indicative of prostate cancer. Some drugs reduce the level of PSA and therefore potentially mask the presence of prostate cancer (Bayne *et al*., 1999).

vi. Evidence from studies conducted with saw palmetto

In vitro studies

Several *in vitro* studies using cultured human foreskin fibroblasts have found extracts of saw palmetto to be specific inhibitors of 5α-reductase. Moreover, studies indicated that liposterolic extracts from saw palmetto inhibited both iso-enzymes of 5α-reductase, whereas finasteride only inhibits type 2.

In vivo animal trials

Significant inhibition of tissue growth was observed in mice treated with saw palmetto (Wilt *et al*., 1998) from a study conducted on hormonally treated castrated rats. It was found that administration of saw palmetto for 90 days inhibited hormone-induced total prostate weight, with maximum benefits observed 30 days after treatment had begun.

Distribution studies were performed in rats, which were given radioactive n-hexane liposterolic extract of saw palmetto. Radioactive labeled isolates of lauric and oleic acid and ß-sitosterol appeared in abdominal fat tissue, prostate tissue and in the skin. Small amounts also appeared in the liver and urinary bladder.

vii. Clinical trials

A number of clinical trials have been performed with saw palmetto, indicating favourable outcomes. A three month open trial of 305 patients with mild to moderate symptoms of BPH were treated with saw palmetto at 160 milligrams twice daily. The international prostate score, quality of life score, urinary flow rates, residual urinary volume, and prostate size were used to assess patients. Patients showed a significant improvement after 45 days of treatment. Therapeutic efficacy of saw palmetto was proven to be better than placebo and equal to finasteride in relieving symptoms of moderate BPH in a number of studies (Bach *et al*., 1997).

Recently, the Journal of the American Medical Association (2000) published a thorough review on saw palmetto. The selection criteria used in this review were very meticulous and only included studies which lasted more than 30 days and were both randomised and placebo controlled. The 2939 men involved in the studies ranged from 40 to 88 years of age, with a mean age of 65 years. A dose of saw palmetto 160mg twice daily was used for approximately 45 days. An overall 28% improvement in urinary symptom scale scores occurred, with nocturia improving by 25%, peak urinary flow by 24%, mean urine flow by 28%, and residual volume by 43%, when compared to placebo. Men taking saw palmetto were twice as satisfied with the results as were the placebo group and experienced fewer sexual complaints than those receiving finasteride (McCaleb *et al*., 2000).

viii. Chemistry

The principle chemical constituents consist of phytosterols, fatty acids, monoacylglycerides, ethyl esters and aromatic acids. Possible active constituents include ß-sitosterol and ß-sitosterol glucoside, free fatty acids such as capric acid, caprylic acid, lauric acid, myristic acid, linoleic acid, linolenic acid, palmitic acid, stearic acid, and glycerides. Two monoacylglycerides, specifically l-monolaurin and l-monomyristin have shown biological activity.

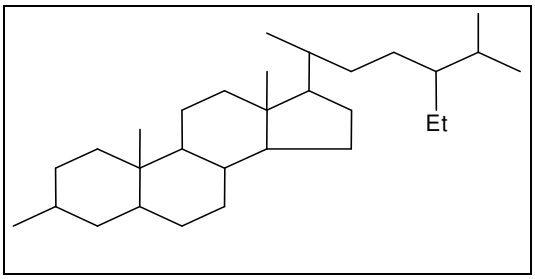

Figure 3. The chemical structure of β-sitosterol which was used as a reference standard for saw palmetto in this study.

ix. Mechanism of action of saw palmetto

The mechanism of action by which saw palmetto functions is not clearly understood. A number of studies have been conducted and from these the following explanations have been postulated:

Saw palmetto may block the formation of testosterone and DHT metabolites and it interferes with the binding of DHT to receptor sites on nuclei and cell membranes of prostate cells. ß–sitosterol, one of the active constituents of saw palmetto inhibits the binding of estradiol (which is formed by testosterone conversion) to receptor sites on the myothelium of the prostate gland. This group of researchers also think that saw palmetto helps the prostate to shrink in size (Van der Merwe, 2000).

The second theory claims that the functioning of saw palmetto is not hormonal at all, and it is due to this fact, that the negative adverse events associated with synthetic drugs (especially the loss of libido and sexual function) are absent with the use of saw palmetto. They tend to think that saw palmetto inhibits cycloxygenase and 5–lipoxygenase and 5 alpha-reductase pathways, in turn blocking the inflammatory response, hereby reducing the oedema in BPH and prostatitis. These researchers have observed changes at the cellular level, in that the epithelium of the prostate has shown to contract and the number of atrophied cells have increased. Although the total volume did not change, the epithelial contraction and increased number of atrophied cells indicates that saw palmetto slows the growth of prostate tissues and may be used as a potential deterrent of prostate cancer (Nieferprum *et al*, 1994).

x. USP – NF standards

The national formulary (NF) monograph for saw palmetto requires a lipophilic extract of at least 7% and the total fatty acids must be at least 9%. Moreover, microbial limits, heavy metals, volatile oil content and foreign organic matter are also specified. A number of methyl esters of fatty acids serve as marker substances for saw palmetto namely, methyl laurate, methyl oleate, methyl myristate, methyl palmitate, methyl linoleate, methyl caproate, methyl caprylate,

methyl caprate, methyl stearate and β-sitosterol.

xi. Dosage

Tea: 1-2 g/day

Liquid extract : 1:1 herb to extract ratio (HER) 0.6 – 1.5 ml three times daily

Tincture: 80% alcohol (fresh fruit 1:2 HER; dried fruit 1:5 HER) 1 – 2 ml up to four times daily. All preparations should be administered with food to minimize gastric disturbances (www.cami.usip.edu.com).

xii. Precautions

Studies performed on pregnant rats and rabbits showed no indication of teratogenic effects in doses of up to 600mg/kg. However, no clinical studies have been done on humans and due to this, saw palmetto use is not recommended.

xiii. Lactation

The use of saw palmetto in lactating females is not recommended because it is not known if saw palmetto constituents are distributed into breast milk.

xiv. Use in special populations

No age related adverse events have been reported with saw palmetto use in older adults. The use of saw palmetto in children has not been established, therefore its use is not recommended.

xv. Drug interactions

Endocrine or alpha adrenergic blocking effects may be possible when saw palmetto is used in combination with other drugs, but no studies have been conducted as yet (Berman-Fugh, 2000).

xvi. Contraindications

Although no contraindications have been reported with saw palmetto use, insufficient evidence exists in patients suffering from hormonal-dependent diseases (http:www.cami.usip.edu.com) and due to the anti-androgenic and anti-estrogenic effects of saw palmetto, its use is not recommended.

xvii. Toxicity

The lethal dose could not be determined through acute toxicity studies conducted on the rat and dog, in which oral doses of 10g/kg and 50g/kg were administered to these animals respectively. The animals displayed no adverse clinical signs at these doses. This lead to the researchers concluding that the lethal doses would have to be much higher than the above mentioned doses (Bombardelli *et al.*, 1997).

xviii. Adverse effects

Saw palmetto is not associated with any serious adverse events (Van der Merwe, 2000). Mild gastrointestinal complaints eg nausea and vomiting have been reported.

2. Materials and methods

The protocol adhered to for the TLC is outlined under the general materials and methods section. The information provided below is specific to the TLC performed on saw palmetto (Wagner and Bladt, 1996).

Reference compound:β-sitosterol

Solvent system:
I) toluene:ethyl acetate (70:30)

Adsorbent: Silica gel G/UV254 Alugram precoated TLC plates (Macherey-Nagel, Duren)

Spray reagents for detection:
I) vanillin-sulphuric acid reagent (vis 100°C for 5 minutes)

3. Results and discussion

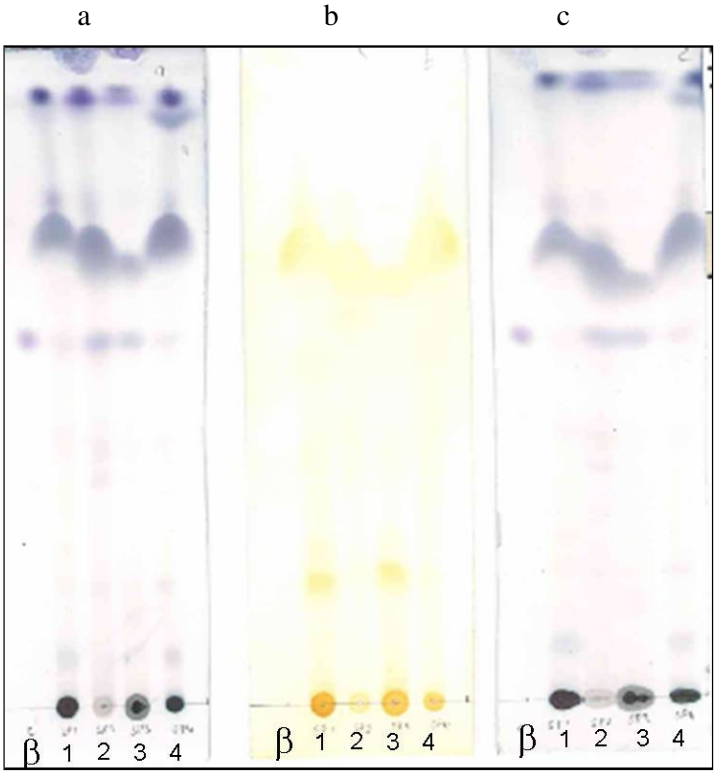

Figure 4: TLC plates of *Serenoa* samples using solvent system I, β-sitosterol as the reference standard (β) and a and c) vanillin-sulphuric acid b)iodine as detection reagent

Once the plates were sprayed with vanillin-sulphuric acid, the various substituents within the products were clearly distinguishable with product 2 (labelled as 2) in TLC plate 'a', above being the clearest and indicating the presence of a number of extra constituents, when compared to the other products. Products 2 and 3 showed the presence of β-sitosterol more clearly than the other 2 products at R_f 0.62. This could be interpreted as these products containing a higher concentration of this substance, when compared to products 1 and 4. If too much sample is spotted, or if the samples are too concentrated, streaking could occur, indicated by the arrow in TLC plate 'a' in figure 4 above. A series of bands occurred at a number of points. These were not all associated with being primary constituents of saw palmetto. When iodine replaced vanillin-sulphuric acid as the detection reagent (plate b), some of the substituents could not be seen as clearly. The same results as discussed above concerning the presence of β-sitosterol were evident. Only one method was used in the TLC of saw palmetto, as this is the most popular method used in a number of reference sources and the only method appearing in Wagner and Bladt for saw palmetto. However, changes were made to the protocol appearing in Wagner and Bladt (1996), and these included the baking of the TLC plates for 7 minutes, once vanillin-sulphuric acid had been used as the detection reagent. It was found that this brightened up the bands making them more conspicuous (Wagner and Bladt, 1996).

4. Conclusion

All products tested contained β-sitosterol, some more concentrated than others.

Hypericum perforatum (St John's Wort)

Figure B

2. St John's Wort (*Hypericum perforatum*)

i. Topography and physical appearance

Plants are glabrous and erect, with many woody stems, developing from a tap root. Leaves are elliptic to oblong, small, sessile, lineate, arranged opposite to one another. They are green or pale green in colour, and have an entire margin. Flowers are radiate with 5 sepals and 5 petals. Flower petals are yellow to yellow brown in colour, and may or may not have black dots. Both petals and leaves are characterized by many punctuate opaque, glands (Wagner and Bladt, 1996).

St John's Wort is native to all of Europe, Western Asia, North Africa, Portugal and has naturalized in North America and Australia. It does not grow in the Arctic region (Wagner and Bladt, 1996).

ii. Historical Perspective

Hippocrates, Pliny, Dioscorides and other ancient medical herbalists wrote about the medicinal properties of St John's Wort, describing its use as a balm for healing wounds and burns, in treating neuralgic conditions such as sciatica and as an anti-malarial. St John's Wort was also used in ancient times, in a more spiritual sense, to protect against demonic possession and evil spirits.

iii. Background

St John's Wort is a herbaceous perennial used traditionally in both herbal and homoeopathic remedies for the treatment of excitability, neuralgia, fibrositis, sciatica, menopausal neurosis, anxiety, depression and has been applied topically for the treatment of wounds (Barnes et al., 2001). Hypericum perforatum is believed to be derived from the Greek words hyper (over) and eikon (image). It is commonly known known as St John's Wort. There are a number of explanations as to how this name was derived. One refers to the fact that the flowers bloom around St John's day (June 24), another suggests that the red pigment exuded when buds and flowers are squeezed, is associated with the blood of St John the Baptist.

Another explanation is that which refers to an old English tradition of throwing the

flowers into a fire on the eve of St John's day (Hobbs, 1989).

iv. Commercial source

St John's Wort products are derived from fresh leaves, petals and seed capsules of the *Hypericum perforatum* plant. A variety of products including liquid tinctures, capsules, tablets, creams, gels and herbal infusions have been produced and are currently on the market.

v. Uses

St John's Wort is available in a number of different forms with various uses. The oil can be administered internally for gastric conditions like gastric ulcers, functional gastritis, inflammatory bowel syndrome and internal hemorrhoids. The infusion has been used as an astringent mouth wash and gargle to relieve pain in the mouth. The compress is used to treat swellings, bruises, bites and enlarged glands. The gels and salves are used as anti-inflammatories, vulneraries, analgesics and antimicrobials in treating bites, burns, external hemorrhoids, herpes lesions and sunburn (Van der Merwe, 2000).

The most common current uses of St John's Wort include its use as an anti-helminthic, as a tea for enuresis in children and as a diuretic, and in the treatment of neuralgic conditions eg. in the treatment of shooting nerve pain and neurological conditions including anxiety, insomnia, irritability, neuroses and migraine headaches (Melzer *et al*., 1991). It has proven to be a possible treatment of menopausal complaints, decreasing depression, relieving psychological (irritability, anxiety) physical (headaches, heart palpitations and sleep disturbances) and vasomotor (hot flushes and excessive sweating) menopausal symptoms. Its predominant use however, is for the treatment of mild to moderate depression and other psychological and neurological conditions. St John's Wort is also valued for its astringent properties.

St John's Wort could prove useful in treating patients with cardiac disease as it not only illustrated cardiotonic actions, but was also found to increase coronary flow in isolated guinea pig hearts (Goodman *et al*., 1993). Further studies would have to be conducted in humans in order to confirm these findings.

vi. Evidence from studies conducted with St John's Wort

In vitro studies

Bladt and Wagner (1996) reported that St John's Wort fractions with the greatest MAO (monoamine oxidase) inhibition contain the highest concentration of flavonoids. Computer modelling of St John's Wort constituents also suggest that flavonoids are most likely to inhibit MAO, due to their structural similarity to toloxotone and brofaromine, which are known inhibitors of MAO_A. The xanthone fraction was found to be a strong inhibitor of MAO_A *in vitro*. The MAO inhibition of St John's Wort has not yet been confirmed *in vivo*.

In vivo animal studies

A commercial standardized extract of St John's Wort illustrated increased activity in a water wheel test in mice, and reduced aggressiveness in isolated male mice. Moreover, when St John's Wort was compared to bupropin (synthetic antidepressant), it was found that both drugs resulted in similar effects including the tail suspension test in mice and the forced swim test in rats (Blumenthal *et al*., 1997).

In vivo human studies

Not only has St John's Wort proven to be significantly superior to placebo, and as effective as other standard antidepressants, such as maptroptiline, imipramine, and amitryptiline, through a meta analysis of 23 randomised St John's Wort trials, but another trial investigating the antidepressant effects of St John's Wort in comparison to fluoxetine, indicated that the 2 treatments are equipotent in their antidepressant effects when using the HAM-D scale and that patients exhibited less adverse effects when using St John's Wort as compared to fluoxetine. Moreover, a clear advantage of St John's Wort over fluoxetine is its safety (McCaleb *et al*., 2000).

A study done by Demisch *et al.* (1991), showed that *Hypericum* resulted in an increased production of nocturnal melatonin, hereby functioning as a sedative and allowing a person to relax. Melatonin is also useful in counteracting jetlag (Demisch *et al*., 1991).

vii. Clinical trials

The use of St John's Wort in individuals presenting with fatigue in an uncontrolled pilot study showed a significant improvement after six weeks. Individuals with an obsessive compulsive disorder of at least 12 months duration who received a fixed daily dose of an extended release formulation of St John's Wort for 12 weeks, illustrated significant improvements in Yale Brown Obsessive Compulsive Scale scores (Barnes *et al*., 2001).

St John's Wort is undergoing clinical trials in the USA as an antiviral in an open pilot study. Recent interest has focussed on its potential as an antiviral agent can be attributed mainly to the rapid spread of AIDS (Acquired immune deficiency syndrome). Eighteen patients with AIDS (at various stages) were treated with intravenous and tablet forms of St John's Wort. Eighty nine percent of patient's with good compliance showed stable or increased CD_4 counts over a 40 month period. CD_4:CD_8 ratios in most patients also improved. Eighty seven percent of patients remained clinically stable and only thirteen percent developed an opportunistic infection during the 40 month period. It is not yet evident, however, if these results can still be obtained using oral doses only (Bauer *et al*., 1998).

viii. Chemistry

The genus St John's Wort has an extremely complex and diverse chemical composition. Several classes of compounds are present in St John's Wort, namely the naphtodianthrone derivatives eg. hypericin, flavonoids eg catechin, xanthones, coumarins eg. scopoletin, carboxylic acids eg. caffeic acid, phloroglucinol derivatives, essential oil components eg. monoterpenes, n-alkanes, n-alkanols, carotenoids and phytosterols. The conditions in which the plant is grown affects its final composition, for instance, hyperoside and rutin content is higher in plants which grow in dry conditions. The hyperoside and tannin content of St John's Wort is higher when grown above 14°C. The harvesting time and way in which plants are grown affects the concentration of active constituents. Plants of northern slopes have a higher concentration of flavonoids, with this concentration being highest in the leaves at full bloom, but highest in the flowers at the start of flowering (Bauer *et al*.,1998). The essential oil component is greatest in the mature seed capsule, with the highest

concentration being recorded before flowering. The phloroglucinols, hyperforin and psuedohyperforin occur exclusively in flowers and capsules, with the levels increasing considerably during capsule formation. The flowers yield carotenoids and contain the highest concentration of xanthones. The highest concentration of napthodianthrones and flavonoids occur in the middle to top part of the plant, therefore harvesting of the lower leaves will result in a significant lower concentration of constituents. The naphthodianthrones and phloroglucinols are photosensitive and require protection from the sun (Bauer *et al*., 1998). St John's Wort can be differentiated from adulterants by the presence of hyperforin and rutin, which are absent or negligible in other species.

As previously mentioned, St John's Wort's biological activity is due to a number of constituents, rather than a single constituent. Table 2 below illustrates the activity of the various constituents (Blumenthal *et al*., 1997)

Table 2: Activity of various constituents in St John's Wort.

Constituent	Activity
Amentoflavone	Antiinflammatory, antiulcerogenic
GABA	Sedative
Hyperforin	Antibacterial against Gram-positive bacteria, wound healing, neurotransmitter inhibitor, potential anticarcinogenic
Hypericin	Antiviral
2 methyl-butenol	Sedative
Proanthocyanidins	Antioxidant, antimicrobial, antiviral, vasorelaxant
Pseudohypericins	Antiviral
Quercitin	MAO inhibition illustrated in vitro
Xanthones	Antidepressant, antimicrobial, antiviral, diuretic, cardiotonic, MAO_A inhibitor

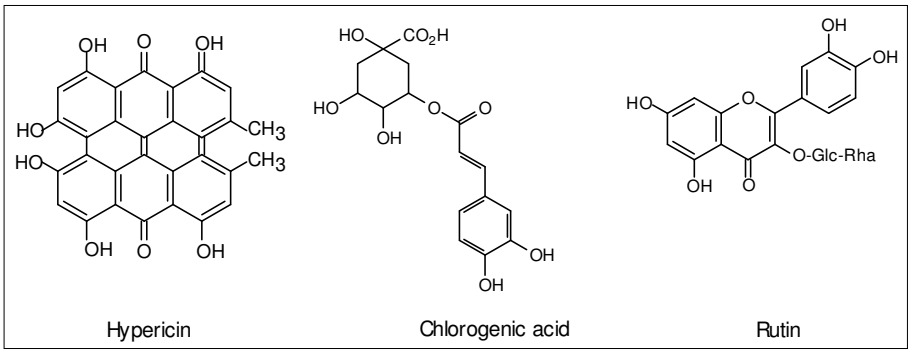

Figure 5. The chemical structures of hypericin, chlorogenic acid and rutin which were used as reference standards for St John's Wort in this study.

ix. Mechanism of action

It is probably due to the several classes of compounds present in St John's Wort that various effects including wound healing activity, blood vessel protection, bactericidal actions, antiviral effects, antidepressive, psychotropic, diuretic, antihelmintic and uterotonic effects have been observed through its use.

St John's Worts' antimicrobial effects are due to the essential oil, phloroglucinol and flavonoid content. While the essential oil and water soluble part of an alcoholic extract show minor antifungal and significant antibacterial activity, the resin part of the extract has also shown to be active against Gram-positive organisms. The tannins and flavonoids were reported to inactivate *E. coli* at certain concentrations (Upton, 1997).

Proposed mechanisms of action for antidepressant activity, include effects on serotonin and cytokines. It has been reported that St John's Wort extracts inhibit serotonin receptor expression at 25 micrograms/millilitre. The inhibition of serotonin uptake was reported at 6.2 micrograms/millilitre (Cott *et al*., 1976). It seems unlikely, however, that either of these concentrations could be achieved in the totality of any animal (Perovic and Mueller, 1995). A novel proposal is that St John's Wort extract reduces cytokine expression (Interleukin-6), which induces depression in susceptible individuals (Cott, 1977). St John's Wort inhibits the sodium dependent uptake of catecholamines and amino acids into synaptic nerve endings by interfering with mechanisms which control the synaptic sodium concentration. The infusion of hyperforin resulted in a delayed reduction of acetylcholine release and a concomitant choline level increase (Buchholzer *et al*., 2002).

The crude extract of St John's Wort showed significant receptor affinity for adenosine, $GABA_A$ and $GABA_B$, serotonin, benzodiazepines, inositol triphosphate and MAO_B. The significance of hypericin binding to GABA is presently unknown, but sufficient literature indicates that GABA plays a role in affective disorders. It has been reported that GABA levels are low in unipolar and bipolar depression and that benzodiazepines which enhance GABA activity are effective antidepressants and anxiolytics. Moreover, GABA neuronal systems also regulate dopamine-induced behaviour (Cott *et al*., 1976).

The form in which the hypericin appears is also very important in determining its activity. Although hypericin from crude extracts of St John's Wort inhibits MAO, 95% synthetic hypericin lacked significant MAO_A or MAO_B inhibition. A concentration of up to 10 micromoles of synthetic hypericin had affinity for NMDA (N-methyl-D-aspartic acid) receptors only. This could explain its antiviral activity, since NMDA antagonism prevents neurotoxicity (Cott, 1997).

Due to the inhibition of protein kinase C, hypericin was reported to inhibit gliomal cell growth. This activity is said to be equal to, or greater than the activity of Tamoxifen^R, which is used to treat cancer. The inhibition of protein kinase C may also contribute to the anti-inflammatory effects of hypericin, which in turn inhibits arachidonic acid and leukotriene B_4 release.

Although both hypericin and pseudohypericin have shown to inhibit a wide variety of encapsulated viruses, including Herpes simplex I and II and the HIV I virus, it is not known how the antiviral activity is achieved. It has been suggested that the antiviral action is due to the non specific association of hypericin with cellular and viral membranes. Others tend to think that the antiviral activity is due to a photoactivation process which inactivates viral fusion and syncytia formation (Diop *et al*., 1994).

Quercetin is a flavonoid which mainly occurs in plants as glycosides, such as rutin. Quercetin and rutin are both used for blood vessel protection, strengthening and modulating permeability of blood vessel walls, by inhibiting platelet aggregation and improving blood circulation (Sheu *et al*., 2004). Rutin also functions as an antioxidant and has good superoxide anion scavenging activity (www.phytochemicals.info.)

x. Dosage

It is difficult to draw valid conclusions from heterogenic studies, because not only are the herbal preparations available in a variety of forms, but the dosages differ between the same forms, manufactured by a different supplier. The possibility that the constituent being tested for is not the only active constituent, can not be ignored. Dose standardization and adequate trial length is required in order to draw valid conclusions. However, the usual doses are 300mg of dry extract one to three times daily and half a

teaspoon of the liquid extract of fresh plant (1:1 HER) three times per day (Burch, 2003).

Swiss researchers attempting to determine the optimum dosage of hypericin required to produce antidepressant activity found that an improvement in mood was measured using the Hamilton psychiatric scale for depression (HAM-D) at the end of the 6th week. A 50% decrease in depression was noted, with small differences between dose levels, at this time period.

xi. Precautions

Although ultraviolet treatment must be avoided during St John's Wort treatment, due to its photosensitizing action, use with tyramine containing foods and MAOIs is not contraindicated as it is with other selective serotonin reuptake inhibitors (http:www.mskcc.org/html).

xii. Lactation

The use of St John's Wort in lactation is a controversial subject. Some sources claim that it stimulates milk stimulation and alleviates breast engorgement, while others claim it to be unsafe for use during this period (www.holisticonline.com).

xiii. Use in special populations

Because liver and kidney function is known to be compromised in the elderly, it is essential that precautions are taken in this population and that St John's Wort is only administered under the supervision of a physician. The use of St John's Wort is not recommended in children.

xiv. Drug interactions

St John's Wort was antagonized by drugs which are known to be dopamine reducing agents for eg. haloperidol and sulpiride. It was this fact that lead to the conclusion that St John's Wort exerts its anti-depressive effects via dopaminergic activation. St John's Wort significantly prolongs narcotic induced sleeping times (except with barbiturates - time is reduced) and antagonizes reserpine effects. Although the mechanism for these interactions is unknown, it may be due to pharmacokinetic alterations of the co-

administered drug (Upton, 1997).

xv. Contraindications

St John's Wort should not be used in pregnancy due to uterotonic effects reported in animals (Van der Merwe, 2000).

xvi. Toxicity

Toxicity in humans using crude St John's Wort preparations is rare and its safety, when consumed within normal therapeutic dosages is well established. The toxicity in grazing animals is predominant in white-skinned cattle, horses and sheep, exposed to strong sunlight after consuming large amounts of fresh St John's Wort material. Pigmented animals and those not exposed to bright sunlight show no symptoms of intoxication. Signs of intoxication in grazing animals include an elevated temperature, rapid pulse and respiration, diarrhoea and dermatitis. St John's wort is a photosensitizing plant which can cause illness and seldom death in grazing animals, if consumed in large amounts. However, because the first symptoms of St John's Wort intoxication include appetite loss, hypericin absorption becomes self limiting.

Photosensitivity associated with St John's Wort occurs because hypericin is absorbed from the intestine and concentrates near the skin. When the human or animal skin is exposed to sunlight, an allergic reaction occurs, which can damage the liver.

xvii. Adverse effects

Photosensitization and sun burn is common in fair skinned individuals. Caution should be taken in fair-skinned individuals consuming large amounts of hypericin. Gastrointestinal symptoms, allergic reactions and fatigue are amongst the most common adverse effects reported with St John's Wort use (Burch, 2003).

2. Materials and methods

The protocol adhered to for the TLC is outlined under the general materials and methods section. The information provided below is specific to the TLC performed on St John's Wort (Bladt and Wagner, 1996).

Reference compound: hypericin, chlorogenic acid, rutin

Solvent system:

I) ethyl acetate-formic acid-glacial acetic acid-water 100:11:11:26

Adsorbent: Silica gel G/UV254 Alugram precoated TLC plates (Macherey-Nagel, Duren)

Spray reagents for detection:

I) NP/Peg No 28 (The plate is sprayed with 1% methanolic diphenylboric acid-β-ethylamino ester (10ml), followed by 5% ethanolic polyethylene glycol-4000 (PEG) (8ml) respectively.

II) 10% pyridine in ethanol

Solvent system 1

ethyl acetate-formic acid-glacial acetic acid- water (100:11:11:26)

ds1	ds2	ds3	ds4
PEG 10% in ethanol	pyridine	iodine	vanillin-sulphuric acid

Solvent system 2

Toluene-ethyl-formate-formic acid (50:40:10)

ds1	ds2	ds3	ds4
PEG	pyridine	iodine	vanillin

Figure 6. An illustration of the two solvent systems and four detection systems (ds) used for St John's Wort.

3. Results and discussion

These samples did not produce very clear results, even after several repetitions. The best method was found to be using solvent system 1 (ethyl acetate-formic acid-glacial acetic acid- water (100:11:11:26) which was sprayed with NP PEG and viewed under uv-365nm and with the visible spectrum. Products 1, 2 and 4 showed distinct bands at R_f 0.73, R_f 0.65, R_f 0.54, R_f 0.50 and R_f 0.34 in the visible spectrum, therefore indicating the presence of hypericin at R_f 0.54 as a greenish-brown band and rutin at R_f 0.34 as a orange-yellow band. Product 3 illustrated the presence of hypericin as a very feint band. This may be due to the instability of hypericin in a liquid form. The product may have been standing for a long time (although not expired), or it may have been produced from the root of the plant and not the leaf. One of the products was omitted in this final round of testing as it was devoid of any active constituents. It illustrated this consistently with the use of all the various methods and in all previous repetitions.

At the UV 365nm spectrum, the red fluorescent zones observed at R_f 0.73 and R_f 0.77 indicated the presence of hypericins. The greenish band at R_f 0.34 can be identified as rutin, the yellowish band at R_f 0.54 as hyperoside, and the bright blue band at R_f 0.4 as chlorogenic acid.

Figure 7: TLC plate of *Hypericum* samples run using solvent system I, hypericin (H) and chlorogenic acid (Cl) as reference standards and NP PEG as detection reagent, when viewed in the visible spectrum.

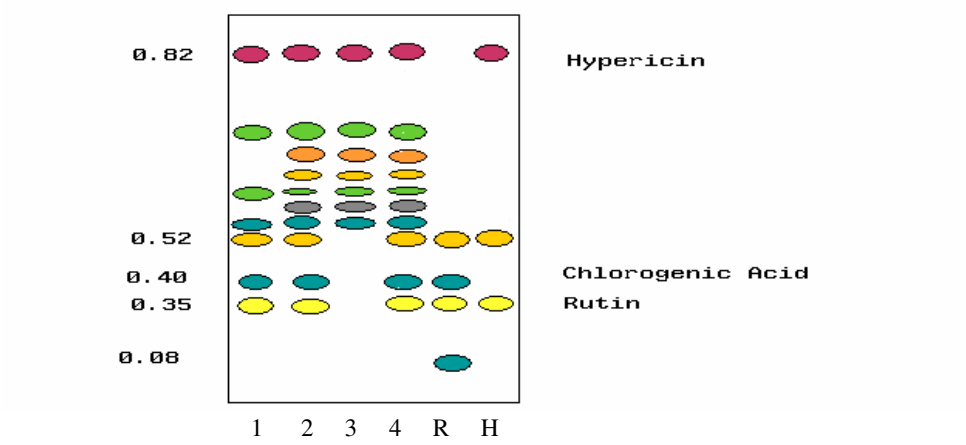

Figure 8: TLC plate of *Hypericum* samples run using solvent system solvent system I, hypericin (H) and rutin (R) as reference standards and NP PEG as detection reagent, when viewed under UV light.

When the products were run using solvent system 2 (toluene-ethyl formate-formic acid 50:40:10) hypericin was seen in all, and all products illustrated the presence of hyperoside at R_f 0.5. Although this method produced results which could be easily interpreted, the constituents were not as distinct as when solvent system 1 was used.

The presence of rutin was more evident when using 10% pyridine in ethanol as the detection system. The rutin appeared as bright orange bands. When iodine was used as the detection system, clear results were achieved, but the TLC plates faded quickly. The use of vanillin-sulphuric acid reagent resulted in the production of TLC plates devoid of any substituents at all. This can be interpreted as the absence of essential oil components such as terpenoids or phenylpropanoids in St John's Wort products.

4. Conclusion

Products 1, 2 and 4 contained all three marker metabolites tested for ie. hypericin, chlorogenic acid and rutin with product 2 containing the highest concentration followed by product 4 then product 1. Product 3 indicated the presence of hypericin at R_f 0.54. The best method was that in which solvent system 1 was used and the chromatograms were viewed after the detection of NP PEG as detection system. One of the products not shown in Figure 8 was devoid of all active ingredients assayed for.

Echinaceae purpurea (Purple cone flower)

Figure C

3. Purple coneflower (*Echinacea purpurea*)

i. Topography and physical appearance

Echinacea purpurea is one of nine coneflowers from the daisy family (Asteraceae), characterized by spiny flowering heads with an elevated receptacle forming the core. The other eight species include *E. angustifolia, E. pallida, E. tennesseensis, E. laevigata, E. paradoxa, E. simulata, E. atrobes* and *E. sanguinea* (Foster, 2000). Of the above mentioned, the three most commonly used in herbal preparations include *E. purpurea, E. angustifolia* and *E. pallida*. Although *E. purpurea* is the most widespread and easiest to cultivate, it is less abundant than *E. angustifolia* and *E. pallida* (Awang *et al*., 1991). It is essential that the constituents of consumer products are distinguished due to the fact that significant quantitative and qualitative differences occur in the chemical composition of extracts, for instance isobutylamides, which result in a local anaesthetic effect, are major constituents of *E angustifolia* roots and are present in high quantities in the roots of *E. purpurea*, but constitute very low concentrations of *E. pallida* roots (Awang *et al*., 1991).

The purple coneflower is a class of herbal perennials whose stems are simple or branched, upright and may ascend from fibrous roots. The leaves towards the roots have stalks, but they become stalkless and smaller as they approach the top of the plant. A few common names by which *Echinacea* is known include purple coneflower, purple Kansas coneflower, black sampson, red sunflower, comb flower, cock up hat, indian head (Van der Merwe, 2000).

ii. Historical perspective

Plants of this genus were first described approximately 300 years ago and records exist of its medicinal use for more than 200 years (Awang *et al*., 1991). The first historical mention were by Clayton in the Flora Virginica in 1762 (Clayton *et al.*, 1762). Samples of the purple coneflower date back to the archaelogical sites of the Lakota Sioux village in the 1600's, but only became popular among European based settlers from the turn of the 19th century (Van der Merwe, 2000). Its popularity in the last few years has risen tremendously, due to the increased interest in the

functioning of the immune system, and due to the fact that it is one of the most promising immune strengtheners and modulators.

Purple coneflower may have been collected initially in Virginia and sent to European botanists in 1680. The purple coneflowers were classified into the genus *Echinacea* by Moench in 1794. Native American tribes used herbs and other internal medicines extensively, and it was these tribes which introduced it to the German lay physician, Meyer, in Nebraska in the early 1870's (Lloyd, 1921).

iii. Background

The name *Echinacea* is derived form the Greek *echinos* meaning 'hedgehog' or 'sea urchin', referring to the sharp pointed bracts of the receptacles (www.viable-herbal.com). The purple coneflower is indigenous to the United States of America and was widely used for centuries by the Indians of the central plains of America. Native American indians used the purple coneflower internally for headaches, stomach aches, an anti-tussive, as an anti-dote for snake bites and internal sores (Awang *et al.*, 1991). It has regained its importance in the 1980's due to the emergence of HIV and its ability to strengthen the immune system. The leaves and roots are used due to their anti-bacterial, anti-viral and anti-fungal actions. Purple coneflower is used to boost the body's defences against foreign organisms, through macrophage activation, which has been demonstrated very convincingly in numerous studies (Bauer *et al*., 1988).

The most widely used herbal products in the world today include preparations made from purple coneflower. This can be attributed to the wide variety of uses this plant possesses, its immunostimulatory action, and to its use in both the prevention and treatment of a wide variety of infectious and immunological diseases, which are becoming increasingly predominant for example HIV and tuberculosis (Barrett, 2003). It is highly unlikely, however, that the activity of this plant can be attributed to a single active constituent. There are currently numerous purple coneflower products on the market and because of the heterogeneity between those commercial products, it is essential that the various active ingredients are correctly identified, accurately quantified and taken into consideration in any comparative study. The interpretation of research literature on the purple coneflower is difficult, not only

because of the above mentioned points, but also because commercial products are not typically standardised to one particular component (Kligler, 2003).

Purple coneflower is a plant remedy which is often adulterated in the market. *Parthenium integrifolium* is the most common adulterant sold as the purple coneflower, because the roots of *Parthenium* are larger and easier to harvest. Other plants which are also substituted for purple coneflower in preparations include *Lespedeza capitata* and *Eryngium aquaticum*.

Fortunately, most commercial manufacturers have converted to using certified organic herbs in the last few years (Nussein, 2000). Adulteration may not only result in the product being inefficient, and therefore in customer dissatisfaction, but may more seriously result in dangerous adverse reactions, such as anaphylaxis. It is therefore essential that measures are implemented which can distinguish between authentic and adulterated products. A measure used in the laboratories at present, is testing for the presence of certain reference standards depending on the various species in the product. This does, however, have certain downfalls, for example, although echinoside has been used as a reference standard in some products containing purple coneflower, a study done indicated that this compound is neither particularly active, nor present in *Echinacea purpurea*. The best candidates for standardization are most likely to be active compounds such as the alkylamides (Clifford, 2002).

iv. Commercial source

Purple coneflower dietary supplements typically consist of fresh, aerial parts of the plant, harvested at flowering time. The radix may also be used in some preparations. The products on the market include liquid tinctures, tablets, capsules, creams and herbal infusions. The quality of the starting herb and the care taken in the manufacturing process is more important than the dosage form eg powder or liquid. Extracts are of best quality when they are made from fresh plants.

v. Uses

Depending on the various tribes, the purple coneflower had many different names

and as many different uses, ranging from healing a sore mouth and gums to coughs, dyspepsia and chronic infections of the respiratory tract. Purple coneflower preparations have also been used from as early as 1887 for a wide variety of more serious ailments including insect, spider and snake bites, diptheria, typhoid, gonorrhea, eczema, cystitis,
burns leucopenia, blood and food poisoning and syphilis (Melchart, 1994).

Purple coneflower is indicated in the treatment of mild to moderate infections of all body parts and systems. It has been tested in clinical trials for the treatment and prevention of the common cold and upper respiratory tract infections, due to its immunostimulatory activity (Cott *et al*., 1976). Purple coneflower is used in combination with antibiotics and chemotherapy. In serious ailments or emergency conditions, it is only to be used as an adjuvant therapy to support other treatments. Purple coneflower plays an important role in supporting and enhancing the immune system in all age groups (Jurcic *et al*., 1995)

Purple coneflower reduces both the growth and the reproductive rates of *Trichomonis vaginalis* and prevents the recurrence of *Candida albicans*. It has been used as supportive therapy in the treatment of urinary tract infections, when administered internally, and for the treatment of superficial wounds when administered externally. Parenteral preparations given to animals and humans produced immunostimulating effects such as an increase in the number of white blood cells, the activation of phagocytosis by human granulocytes and elevation of body temperatures. Antiviral, anti inflammatory and antibacterial properties have often been reported in *in vitro* studies. Bronchitis, bacterial and viral infections of the respiratory tract have all been treated using *Echinacea*. *In vivo* studies indicate that the therapeutic effects of purple coneflower are due to the stimulation of the cellular immune response (Jurcic *et al*., 1995).

Clinical reports from various commercial purple coneflower products indicate that the injectable preparations are used in polyarthritis, general infections and wounds, pertussis in children, colds and flu, urinary tract infections, tuberculosis, bronchitis, eczema, warts, chronic prostatitis, allergies, pelvic inflammatory disease and chronic

skin ulcers. The topical use of purple coneflower has been mentioned in a number of trials treating inflammatory skin conditions eg burns, eczema and ulcerative gingivitis. Oral dosage forms of purple coneflower proved to be useful in treating psoriasis, candidiasis, upper respiratory tract infections, and in stimulating the immune system (Perry, 2000).

vi. Evidence from studies conducted with purple coneflower

The efficacy of purple coneflower as an immuno modulator has been reviewed by a number of controlled clinical trials. Although, its administration at the first signs of an URTI inhibited progression and resulted in quicker relief of symptoms than with placebo, the use of it in the prevention of URTI's could not be considered as valid due to deficiencies in study designs, such as insufficient or flawed statistical analysis, undefined URTI diagnostic criteria, inadequate sample sizes, absence of randomisation, lack of patient and evaluator blinding, the absence of dose standardisation across brands, the number of and reasons for subject withdrawl, defiencies in methods and insignificant evidence for prophylactic efficacy (Melchart *et al*., 1995).

vii. Clinical trials

Jurcic *et al* (1995) reported the results of two randomized placebo-controlled human trials. They reported a 20% increase in phagocytic activity at day 4, in a single-blind study of 27 volunteers, which were injected intravenously with an *Echinacea* preparation. In the second, randomized and double-blind study, 24 male subjects who ingested 30 *Echinacea* drops three times daily for five days, illustrated an increase in polymorphonuclear phagocytic activity. No changes in white cell counts were noted.

viii. Chemistry

Although there is a lot more to learn about the active constituents of purple coneflower, the alkylamides, cichoric acid derivatives, polysaccharides, glycoproteins and caffeic acid derivatives have proven to be essential components in the pharmacological action of the purple coneflower. Flavonoids, monoterpenes, hydrocarbons such as N-alkanes, high molecular weight polysaccharides, essential

oils and pyrrolidizidine alkaloids also play a role in the activity of the purple coneflower.

A number of compounds have been isolated from *Echinacea*, including:

- Essential oils
- Pyrrolidizine alkaloids
- Phenolic derivatives derived from caffeic acids, including chlorogenic and dicaffeoylquinic acids
- Monocaffeate, dicaffeate and ferulate of tartaric acid (The dicaffeate form is abundant in *E. purpurea*)
- Sugar esters of caffeic acid (echinoside)
- Numerous unsaturated aliphatic compounds including aliphatic amides, isobutylamides of polyene and polyenyne acids
- Polysaccharides including arabinogalactin and fucogalactoxyloglucoside

It is important to realize that active compounds of the purple coneflower (like those of all other herbal remedies) degrade during manufacture and storage, due to a number of processes. It is therefore essential that these remedies are manufactured, packaged and stored in conditions conducive to their optimal activity and existence (Blumenthal *et al*., 1997).

Cichoric acid is highly susceptible to enzymatic degradation during the preparation of purple coneflower products. The degradation of caffeic acid derivatives can be inhibited by the addition of antioxidants to the extraction solvents. Studies indicated that polyphenols are responsible for oxidative degradation of caffeic acid derivatives, and not peroxidases as previously thought. The addition of 40% ethanol and 50mM ascorbic acid to an aqueous extract of *E. purpurea* herba, resulted in a constant amount of cichoric acid over four weeks (Nussein, 2000).

The level of alkylamides diminished by more than 80% during storage at 24° C for 64 weeks. A significant drop also occurred at storage of 18° C (Clifford, 2002).

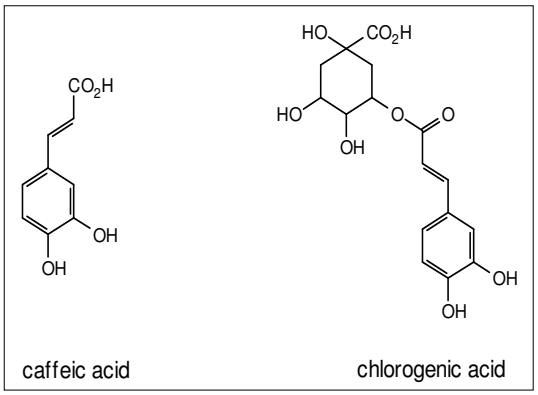

Figure 9. The chemical structure of caffeic and chlorogenic acids which were used as a reference standard for the purple coneflower in this study.

ix. Mechanism of action

Claims that immune reactions work by the law of all or nothing have been made ie when immune modulators reach a critical dose, an immune response which can not be increased further is achieved. An increased concentration in some cases may even lead to immune suppression (Jurcic *et al*., 1995).

Both cichoric and Dodeca 2E, 4E, 8Z, 10E/Z tetraenoic acid isobutylamide are described as active components of the purple coneflower with immunostimulatory action. The quantity of the two substances vary greatly amongst the variety of products on the market, depending on the type of remedy eg homoeopathic mother tincture or herbal tablets, on the species of plant used eg *E. purpurea* as compared to *E. pallida*, and on the part of the plant used eg root or leaf. The age of the plant, and when it is harvested also has an impact on determining the content of active phytochemicals (Nussein, 2000).

Alkylamides from purple coneflower root illustrated anti-inflammatory activity in *in vitro* models through the inhibition of cycloxygenase I and II enzyme, 5-lipoxygenase, and hyaluronidase activity. Moreover, mosquitocidal evidence was illustrated against *Aedes aegyptii* larvae (Clifford, 2002).

x. Dosage

Recommendations for oral dosage and dosage forms are difficult due to the wide variety of preparations on the market.

2-3ml *E. purpurea* juice/ 1-2ml of an extract 3-4 times a day in juice, water, or sublingually. A solid dosage form containing 150 - 300mg of a dry powder administered 3 times a day if juice can not be tolerated with maximum of 1g three times daily. A cream/ ointment containing 15% expressed juice is recommended for external use (Mahady *et al*., 1999).

xi. Precautions

Caution should be taken in autoimmune conditions such as Lupus syndrome. The stimulation of an overactive immune system is not advised. Although the use of purple coneflower has been recommended in the German Commission E Monographs for parenteral and oral use, the preparation containing the root is not recommended due to lack of data (Nussein, 2000).

xii. Lactation

The safety of the purple coneflower in lactation has not been established.

xiii. Use in special populations

No age related adverse effects have been observed with the use of purple coneflower in the aged.

xiv. Drug interactions

Because the purple coneflower enhances immune system function, it interferes with drugs used to suppress the immune system after organ transplants and in other conditions. It should not be taken in conjunction with azathioprine, cyclosporine, daclizumab, mycophenalate, sirolimus and tarcolimus. Because the purple coneflower undergoes hepatic metabolism, it might also interfere with drugs which are processed by the same enzymes in the liver for example fexofenadine, itraconazole, vincristine, vinblastine, paclitaxel, lovastatin, etoposide and oral contraceptives (www.drugdigest.org).

xv. Contraindications

The purple coneflower is well tolerated with a low frequency of adverse events as mentioned previously. Due to its minimum toxicity and favourable side

effect profile, patients are recommended to administer purple coneflower, except in pregnant and lactating women as safety has not been established in these groups. Purple coneflower is contraindicated in those with autoimmune and progressive systemic diseases such as tuberculosis, leukemia, multiple sclerosis, collagen disorders and diabetes mellitus, because it may exacerbate immune system dysfunction (Barrett, 2003).

xvi. Toxicity

Experimental studies indicate that there should be no concern regarding the safety of the purple coneflower.

xvii. Adverse effects

Purple coneflower has shown a lack of side effects in long term studies, however its immunostimulating properties may decline with continued use, therefore preparations should not be administered for longer than eight consecutive weeks (O'Hara *et al*., 1998). No adverse effects other than aversion to taste have been reported. It is well tolerated and safe.

2. Materials and methods

The protocol adhered to for the TLC is outlined under the general materials and methods section. The information provided below is specific to the TLC performed on the purple coneflower (Wagner and Bladt, 1996).

Reference compounds: chlorogenic acid, caffeic acid, β-sitosterol.

Solvent system:

I) toluene:ethyl formiate-formic acid:water (5:100:10:10)

II) toluene:ethyl acetate (70:30)

Adsorbent: Silica gel G/UV254 Alugram precoated TLC plates (Macherey-Nagel, Duren)

Spray reagents for detection:

I) NP/Peg No 28 (UV-365nm)

II) vanillin-sulphuric acid reagent (Vis 100° C/5min)

3. Results and discussion

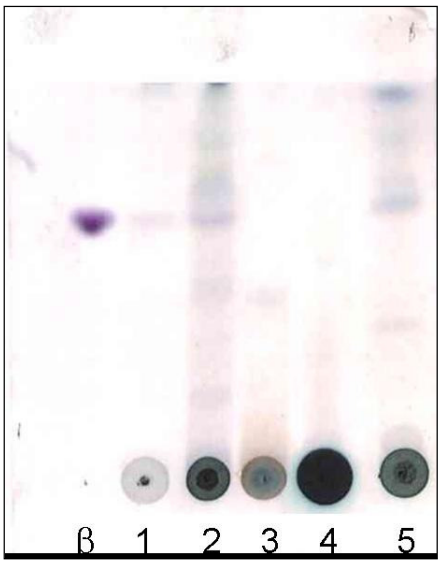

Figure 10: TLC plates of *Echinacea* samples run using solvent system II, β-sitosterol as reference standard, and vanillin-sulphuric acid as detection reagent

It was established that the best results were achieved using solvent system II (toluene:acetone 70 : 30) and vanillin-sulphuric acid as detection reagent, as the bands were more pronounced and less streaking was seen.

All products except samples 3 and 4 showed the presence of β-sitosterol at R_f 0.56. Both the above mentioned products were liquid forms. Perhaps ß-sitosterol is another of those constituents which is unstable in solution, or disintegrates during storage.

Product 2 contained a number of constituents, more than it should have, by virtue of its extraction and staining methods. It contained 10 bands, as compared to a five or six maximum of the other samples. Although, it is difficult to tell at this stage, and further tests would be required to confirm it, this may be due to one of three reasons:
-the presence of an adulterant
-a mixture of root, stem and leaf constituents, rather than a product manufactured from a single source (part of plant)
-the presence of a contaminant
Parthenium integrifolium is well known as a substituent of the purple coneflower presenting as 10-12 weaker bands of caffeic acid derivatives in the 0.2-0.8 range. Although further confirmation would be necessary to prove it, a speculation that product 2 is the above mentioned adulterant would not be inconceivable.

Because product 5 showed the darkest band at R_f 0.56, it can be assumed that it contained the most β-sitosterol when compared to the other samples, followed by product 2 , then product 1. Quantification of β-sitosterol, however, would have to be verified by HPLC.

When *Echinacea* samples were run with chlorogenic acid as the reference standard (plates not shown), only product 2 showed that it contained this organic acid. Product 4 was blank and product 5 was devoid of any chlorogenic acid.

Products 1 and 2 showed an additional band at R_f 0.55. When the same products were run with a caffeic acid standard, products 1, 2 and 3 were shown to contain caffeic acid at R_f 0.80, product 5 showed the presence of an unidentified band at R_f 0.60. Samples 4 and 5 were devoid of any caffeic acid.

When the purple coneflower products were run in solvent system 1 (toluene: ethyl formiate : formic acid: water; 5:100:10:10) and sprayed with NP PEG, the results appeared clearer when the TLC plates were baked in the oven for between three to eight minutes, after spraying with the detection reagent. The same results were achieved as discussed above, but clarity was not as precise.

4. Conclusion

It can be concluded that products 1, 2 and 5 contained β-sitosterol. Only 1 product contained chlorogenic acid (product 2), and 3 products contained caffeic acid (products 1, 2 and 3). More importantly only 1 of the 5 products (product 2) contained all three of the above active ingredients, but it also contained a lot of other unidentified constituents. Product 4 did not contain any of the marker metabolites assayed for.

Gingko biloba (Gingko)

Figure D

4. Duck foot tree (*Gingko biloba*)

i. Topography and Physical appearance

The duck foot tree is a plant indigenous to China which has been used by the Chinese people as a medicinal plant for thousands of years. It was initially referred to as 'The fountain of youth' herb. The tree reaches an average height of eight metres and can live for up to 1000 years. The duck foot tree can adapt to almost any environment and lives very long. It is also been referred to as the 'Maidenhair tree' because the foliage resembles some of the Maidenhair ferns. Presently the duck foot tree planted in cities with a subtropical climate, although it is also able to adapt to colder climates (Huh and Staba, 1992).

The seeds and fan-shaped leaves of the duck foot tree are dried and produce standardised extracts in the form of capsules, tinctures and tablets have been used for food and in medicine preparations for thousands of years. This tree has evolved over a period of two million years. '*Gingko'* means silver apricot after the trees fruit and '*biloba'* describes the lobe like appearance of the leaves (Huh and Staba, 1992).

ii. Historical perspective

The duck foot tree is the only living representative of a group of gymnosprems composed of the Gingkoacea family, consisting of approximately 18 members, with its earliest fossils dating back 270 million years ago to the Permian period. The duck foot tree was commonly found in North America and Europe but became extinct seven million and two and a half million years ago respectively, during the ice age. It survived in China and was mainly found in monestaries in the mountains, and in palace and temple gardens, where Buddhist monks cultivated the tree for its many good qualities. The Chinese duck foot tree survived essentially unchanged. Fossilized leaf material from the Permian period is remarkably similar to the present day duck foot tree. From China it spread to Japan and Korea around 1192 AD (Kwant, 2003).

iii. Background

The duck foot tree is so resilient to insects, pollution, toxins, and parasitic invasions that seeds were found in Japan after the Hiroshima attack. Damaged duck foot trees

began to bud again after the attack without any major deformities.

The tree was initially described as, and named "Gingko" by Engelbrecht Kaempfer approximately 300 years ago, who introduced the tree to Europe from Japan in the early 1700s and to America later that century. The tree was introduced to North America in 1784 at Woodlands near Philadelphia. Thereafter, in 1771 Linnaues gave it the binomial name *Gingko biloba* (Clifford, 2002).

iv. Commercial source

The leaves, stems and seeds of the duck foot tree are used to manufacture liquid tinctures, tablets, capsules and herbal infusions. Preparations currently available on the world market include tinctures, dried leaves, homoeopathic preparations and various extracts.

v. Uses

The earliest recorded use of the duck foot tree included the use of its thorns to drain abcesses. The thorns were boiled in water and drunk to decrease swelling (Clifford, 2002). The leaves were used as early as 3000BC to improve memory and the seeds were used to treat lung disorders, to improve circulation and digestion. The nuts from the duck foot tree are mentioned in Japanese textbooks from 1492 they were used at tea ceremonies as sweets and dessert. In the Edo period (1600-1897) people ate them as vegetables and ingredients for pickles. In the 18th century the nuts were used as a side dish when drinking sake. Today they are used in a steamed egg dish or in Japanese fondues. The grilled nuts are still often eaten in Japan when drinking sake (Michael *et al*., 1988).

In Chinese medicine, the leaves from the duck foot tree were used in improving brain function, as an astringent for lungs, for cough relief, relief of asthma symptoms and in filiarisis treatment. The leaf was initially mentioned in 1436 for its external use to treat skin, head sores and freckles. The internal use of the leaf was initially recorded in 1505 to treat diarrhoea (Van der Merwe, 2000). It is also used in the symptomatic treatment of disturbed performance in organic brain syndromes within the regimen of a therapeutic concept in cases of demential

symptoms showing the following:
memory deficits, disturbances in concentration, depression, dizziness, tinnitus and headache. The primary target groups are dementia syndromes such as primary degenerate dementia and vascular dementia (Warburton, 1988).

The duck foot tree alleviates mild to moderate forms of depression. A study including 40 elderly patients using 80mg three times daily, showed a marked decrease in the total score measured by the Hamilton depression scale. It counteracts one of the major changes in brain chemistry associated with aging, for example, reduction in the quantity of serotonin receptor binding sites by improving receptor synthesis. Patients using the duck foot tree to treat cerebrovascular insufficiency also showed improvement in their mood (Chatterjee, 1988).

The duck foot tree is a circulatory herb, with one of its main actions being its ability to increase blood flow through aging vessels, especially in the brain. It also decreases red blood cell aggregation and blood viscosity in humans. It increases oxygen supply to all cells of the body, and improves blood circulation to the brain, resulting in enhanced memory, mental agility, alertness and concentration. It is due to this that the duck foot tree might be a therapeutic option for Alzheimer's disease and multiinfarct dementia.

Furthermore, it improves energy levels and general health by slowing down aging, limiting free radical damage and acts as a potent anti-oxidant. It improves hearing and eyesight, and is used for a wide range of connected disease and disorders eg. diabetic retinopathy or cataracts (Warburton, 1988).

vi. Evidence from studies conducted with the duck foot tree.
The following effects have been established from experimental studies conducted with the duck foot tree:

- Improvement of hypoxic tolerance, especially cerebral tissue through the inhibition of the development of traumatically or toxically induced cerebral oedema and acceleration of its regression.
- Reduction of retinal oedema and of cellular lesions in the retina.

- Inhibition of age related reduction of muscarinergic cholinoreceptors and adrenoreceptors as well as the stimulation of choline uptake in the hippocampus.
- Increased memory performance and learning capacity.
- Improvement in the compensation of a disturbed equilibrium.
- Improvement of blood flow especially with regards to microcirculation.
- Improvement of rheological properties of the blood.
- Inactivation of toxic oxygen radicals by flavonoids.
- Antagonism of the platelet activating factor (PAF by gingkolides).
- Neuroprotective effect (gingkolides A and B, bilobalide) (Hindmach, 1988).

In vivo studies

A study performed on patients with organic and neurological angiopathy illustrated beneficial physiological changes resulting from exercise after having ingested the duck foot tree. This indicates that the duck foot tree would be useful in treating central and peripheral vascular disease, including diabetic angiopathy, because:

-it lowered blood pressure and dilated peripheral blood vessels in patients recovering from thrombosis.

-it increases peripheral blood flow without decreasing cerebral circulation. Chemical vasodilators accumulate in the expanded vessels rather than circulate to the veins that feed the central nervous system. The duck foot tree however, increases blood flow to both the periphery and the brain.

-Increases blood supply to the brain in Parkinson's disease secondary to cerebral arteriosclerosis (Hoffman).

It was found that one hour after a single 60mg dose of the duck foot tree in patients between the ages of 25 and 40, a significant improvement in the short term memory parameters was demonstrated, as measured by a battery of tests, indicating specific activity on central cognitive processes (Chatterjee, 1985).

viii. Chemistry

The chemistry of the leaves and seeds are similar, but the quantities of the constituents vary slightly. The duck foot tree contains flavoglycosides, bioflavonoids (including ginkgetin, quercetin, kaempherol, bilobetin), terpene lactones, proanthocyanidins and organic acids. The wide variety of healing abilities of the duck foot tree can be attributed to the combination of all of its ingredients and not to a single ingredient (Pietta *et al*., 1997).

The duck foot tree contains 22 - 27% flavonone glycosides determined as quercetin and kaempferol. Between 2.8 and 3.4% consists of gingkolides A, B, C and approximately 2.6 - 3.2% bilobalide and gingkolic acids. It contains a number of pharmacologically active flavonoids eg kaempferol, quercetin, and terpenes eg gingkolide and bilobalide (Blumenthal *et al*., 1997).

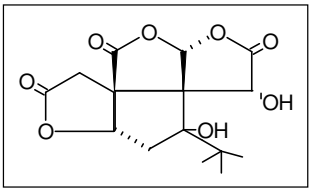

Figure 11: Chemical structure of bilobalide which was used as reference standards for the duck foot tree in this study.

ix. Mechanism of action

The standardized extract of the leaves is used in a wide variety of physiological dysfunctions and pathological conditions, including those involved with blood conditions and circulation. The extract can scavenge free radicals and inactivate the formation of radicals not affected by uric acid. It inhibits lipid peroxidation of membranes, hereby maintaining integrity and permeability of cell membranes (Foster, 1996). Reviewed studies show that extracts from the duck foot tree have vascular tone regulating properties, anti-hypoxic effects and aids in the modulation of cerebral energy metabolism.

The bioflavonoids in the duck foot tree, namely quercetin, proanthocyanidin, isohamnetin and kaempferol are responsible for its potent anti-oxidant activity (Pietta *et al*., 1997). Gingketin has been reported to function as a phospholipase A_2 inhibitor (Chong *et al*., 1999). They are also involved in reducing platelet aggregation in the arteries, which lead to deep vein thrombosis, stroke, lung embolisms and heart attacks. Because the duck foot tree can penetrate the blood brain barrier, the antioxidants it contains are then able to neutralise the free radicals inside the brain to prevent lipid peroxidation of the nerve cell membrane. Other constituents with antioxidant effects include gingkolide B, which inhibits PAF (which plays a role in cerebral hypoxia-mediated damage). These extracts affects a number of neurotransmittor receptors and levels, including muscarinic alpha-2, acetylcholine, norepinephrine, serotonin, and gamma amino butyric acid (GABA), and are therefore related to changes in neurotransmission. Moreover, it inhibits β-amyloid mediated cell toxicity and death *in vitro*. A study done by Le Bars *et al*. (1997), illustrated that treatment with the duck foot tree may delay Alzheimer's disease symptom progression.

The terpene lactones, namely the gingkolides and bilobalides give the duck foot tree its unique abilities to improve mental function and peripheral blood flow to the brain and all parts of the body so that tissues get more oxygen and nutrients. They also protect the nerve cells through out the body and result in improved memory and concentration. Novel diterpene lactones unique to the duck foot tree include the gingkolides A, B, C and M and a sesquiterpene, bilobalide. These various gingkolides have different degrees of potency. Gingkolide B is considered to be the most active of the three. They are induced by platelet activating factor (PAF) and are therefore very selective antagonists of platelet aggregation. Because of the PAF receptor antagonist activity, the hope of the use of the duck foot tree in a wide variety of diseases has increased (Warburton, 1988).

Gingkolides inhibit platelet activating factor which is implicated in asthma and other allergic conditions. The duck foot tree has a direct relaxing effect on vascular smooth muscle through the stimulation of endothelium derived relaxing factor (EDRF) and prostacyclin. The duck foot tree has been used for the treatment of premenstrual stress in females and for the treatment of urinary and prostate problems in males. It is well

known for its positive effect in the treatment of male impotence due to its increased blood flow to the penis.

The extract improves blood flow and decreases red blood cell aggregation and blood viscosity in humans. The mechanisms behind these vasomodulatory and rheologic activities may involve attenuated nitric oxide and prostacyclin effects (Warbuton, 1988).

It is thought to treat cerebrovascular insufficiency by improving the function of various neurotransmitters including serotonin, dopamine and noradrenaline in the brain which are responsible for the transfer of messages along the neurons. In essence, the duck foot tree improves message transfer and in so doing leads to improved memory, concentration and mental alertness. It has illustrated the ability to increase acetylcholine binding to its receptors. This is important because reduced receptor binding of acetylcholine has been reported in Alzheimer's disease (Warburton, 1988).

x. Dosage

120 - 240mg dry extract in 2-3 divided doses (Van der Merwe, 2000)

xi. Precautions

Use with caution in patients administering other medications acting on GABA, $5HT_2$ and $alpha_2$ receptors.

xii. Lactation

Chinese herbalists claim that the duck foot tree is safe in lactation and it encourages milk flow. It will also help in mastitis and congestion of the breasts. The safety of its usage in lactation has not been established.

xiii. Use in special populations

The use of well defined standardised extract in the treatment of common conditions in elderly populations include dizziness, depression, tinnitus, vasoconstriction, short term memory loss, prevention and treatment of various stages of dementia,

improvement in peripheral and cerebral blood flow, anti-radical and protective effect on the blood brain barrier (Chatterjee, 1985).

xiv. Drug interactions

An Alzheimer's disease patient presented with a coma after three days of starting trazodone (which works on the benzodiazepine receptors). This is because flavonoids contained in the duck foot tree increase the production of 1-(m-chlorophenyl) piperazine, an active metabolite of trazodone which releases GABA hereby inducing an agonistic action on presynaptic $5HT_2$ and $alpha_2$ adrenergic receptors. The duck foot tree has no effect on hormonal plasma levels nor on the hepatic microsomal oxidation system (Berman-Fugh, 2000).

xv. Contraindications

The use of the duck foot tree alone or with aspirin/warfarin has been associated with cases of serious bleeding (Goodman *et al*., 1993). It is also contraindicated in individuals with hypersensitivity to duck foot tree preparations (www.thorne.com)

xvi. Toxicity

Consumption of large amounts of the nuts from the duck foot tree can cause vomiting, stomach ache, diarrhoea, spasm, anxiety and breathing difficulties (www.alternativehealing.org)

xvii. Adverse effects

Stomach or intestinal upset occur seldomly, headaches and allergic skin reactions (www.thorne.com)

2. Materials and methods

The protocol adhered to for the TLC is outlined under the general materials and methods section. The information provided below is specific to the TLC performed on the duck foot tree (Wagner and Bladt, 1996).

Reference solutions: rutin, chlorogenic acid, bilobalide

Solvent system:

I) ethyl acetate-glacial acetic acid-formic acid-water (100:11:11:26)

II) chloroform-acetone-formic acid (75:16.5:8.5)

II) toluene-acetone (70:30)

Adsorbent: Silica gel G/UV254 Alugram precoated TLC plates (Macherey-Nagel, Duren)

Spray reagents for detection:

I and II) Natural products – polyethylene glycol reagent (NP/Peg No 28)

III) Acetic anhydride-TLC plate was sprayed and heated for 5-10 Minutes at 120° C

3. Results and discussion

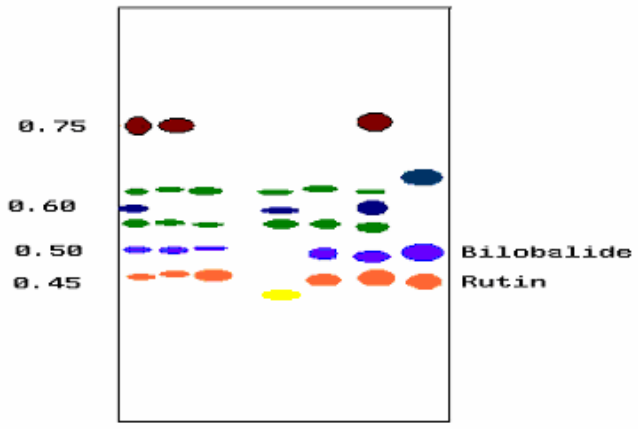

Figure 12. TLC plates of the duck foot tree using solvent system I, rutin as the reference standard (R) and NP PEG as detection reagent.

The literature states that duck foot tree leaves are characterized at UV 365nm using NP PEG reagent, by 8 - 10 green-yellow or orange-yellow fluorescent flavonol glycoside zones in the R_f 0.2 - 0.75 range. However, once the products had run and were examined, it was noted that blue-purple bands also appeared. The presence of bands in the 0.8 - 0.9 region were recorded. These bands could be indicating the presence of sciadopitysin, but we could only be certain if we ran these samples against a sciadopitysin reference standard. Another reason for the presence of these bands could be due to the fact that the products were not only composed of aerial parts of the duck foot tree, but may have contained other parts of the duck foot tree plant eg roots, or that the products tested contained contaminants.

The best method is that using solvent system 1 (ethyl acetate: glacial acetic acid: formic acid: water 100:11:11:26). The products were run against the rutin reference standard, products 1, 2, 3, 6 and 7 showed the presence of rutin as a bright orange band at R_f 0.45. Product 7 showed the darkest and brightest band, followed by product 6, then products 3, 2 and 1 respectively. Rutin was absent in product 5 and product 4 was devoid of all constituents. This was said to be the best method, because the bands were clear and the greatest number of bands were distinguishable.

Flavonoid glycosides fluoresce brown or dark green at UV-365nm. In a repeat run using this solvent system, and spraying with NP PEG reagent, all products except product 5, showed that they contained the following flavonoids: isoquercetin, astragalin, dihydro-kaempferol-7-O-glucose at R_f 0.72 and narcissin, isoharmnetin-rutinoside at R_f 0.45. Products indicated the presence of 6 hydroxykynurenic acid kaempferol-quercetin-3-O-6''' (*trans*-p-coumaroyl-4" glucosyl)–rhamnoside at R_f 0.5.

It is also interesting to note that the enriched and standardized extracts of commercial duck foot tree preparations are free of bioflavonoids and because of this, do not show yellow zones at the solvent front.

When solvent system 2 (chloroform: acetone: formic acid; 75:16.5: 8.5) was used and the products were run against the rutin reference standard, the rutin appeared in products 2, 3, 6 and 7, as a green band, but not in products 1 and 4. This could be due to its

absence in these products, or due to the fact that it is present in extremely low concentrations, at which it can not be seen. But this would probably infer that its effects at this concentration would be negligible. This system is used to identify bioflavonoids.

When solvent system 3 (toluene: acetone; 70:30) was used with acetic anhydride spray reagent, the bands were detected as fluorescent zones at UV 365nm. The products were run against a bilobalide standard. This system is used in the identification of gingkolides (Wagner and Bladt, 1996). Products 1, 2, 3, 5, 6 and 7 showed a band at R_f 0.48 identifiable as bilobalide. Products 1, 5 and 7 showed bands at R_f 0.60. These bands could be ginkgetin. The only way to clarify this would be to run these samples against a gingketin reference standard. Product 4 was devoid of any actives. Products 1, 2 and 7 showed feint bands at R_f 0.75, which could be flavonoglycosides. Product 5 showed very feint bands at R_f 0.45. These were not in the same position as the bilobalide reference standard but appeared in the same shade of orange, only much lighter. It is not clear what these constituents are, or if they were added to the products, perhaps intending to deceive the consumer.

Detection systems: When solvent system I (ethyl acetate-glacial acetic acid-formic acid-water; 100:11:11:26) is sprayed with NP PEG, flavonoids and bioflavonoids fluoresce yellow-orange and green at 365nm. When solvent system III is sprayed with acetic anhydride, gingkolides and bilobalides fluoresce on heating at 120° C for 5-10 minutes. Unfortunately, due to the fact that the duck foot tree TLC plates required viewing under UV light, the photographs produced are unclear and of no use.

4. Conclusion

Products 1, 2, 3, 6 and 7 showed the presence of rutin using solvent system 1. Products 1, 3, 6 and 7 showed the presence of rutin using solvent system 2. Products 1, 5 and 7 showed the presence of gingketin. Products 1, 2, 3, 5, 6 and 7 indicated the presence of bilobalide. Products 4 and 5 were devoid of rutin when assayed by all methods. Product 4 was devoid of all active substituents assayed for. Product 5 contained an unidentified ingredient. Only products 1 and 7 showed the presence of rutin, bilobalide and gingketin.

Silybum marianum (Milk thistle)

Figure E

5. Milk thistle (*Silybum marianum*)

i. Topography and physical appearance

Milk thistle is a member of the Asteracea family, which includes daisies and thistles. It is also known as wild artichoke, holy thistle, marian thistle, Our lady's thistle and is widely distributed throughout Europe and has since been established in the eastern US, California, South America and Southern Australia. The name 'Milk thistle' is derived from its characteristic spiked leaves with white veins, which were thought to carry the milk of the Virgin Mary. The full grown plant has large light purple flowers and many stout spines (Van der Merwe, 2000).

ii. Historical perspective

Its use as a liver protecting herb dates back to approximately AD 23-79, when Pliny, the first century Roman physician/naturalist wrote of its use as a vegetable and noted that a mixture of plant juice and honey was excellent for carrying off bile. Discorides, a Greek herbalist in ancient times used a tea of milk thistle seeds to cure a bite of a poisonous snake. Milk thistle was quoted to be "the best remedy against melancholy". Milk thistle was used for the treatment of liver diseases initially by Otto Brunfels in 1534. In 1787 Culpepper re-discovered it to be an excellent remedy for hepatic and splenic obstructions. Infusions from fresh roots and seeds were curative for jaundice, and for breaking and expelling stones. At the turn of the 20^{th} century, a school of herbalists, the Eclectics were using milk thistle extracts for liver congestion, varicose veins, menstrual disorders and abnormalities of the spleen and kidneys (Van der Merwe, 2000).

iii. Background

Chronic hepatitis is a global problem affecting approximately five percent of the world population. It is due to the high prevalence of liver diseases including the above mentioned and cirrhosis, that there is a great need for efficient and cost effective treatments (Blumenthal, 1999). The potential benefit of milk thistle in the treatment of liver diseases remains a controversial issue. Besides having a good safety record with only rare case reports of gastro-intestinal disturbances and allergic skin reactions, it is one of the few herbal therapies that have no real equivalent in the

conventional medicine world.

As the interest in alternative therapies has increased over the last decade, throughout the world, so has the number of patients taking milk thistle, with little understanding of its purported properties.

iv. Commercial sources

The fruit, leaves and root and hull of this plant are used to produce liquid tinctures, capsules, tablets and herbal infusions.

v. Uses

Silymarin is a derivative of milk thistle and has been used for centuries as a natural remedy for upper GI disturbances, diseases of the liver and biliary tract, menstrual disorders, varicose veins and other conditions.

Milk thistle is said to work as an anti-oxidant, scavenging free radicals and inhibiting lipid peroxidation. It also protects against genomic injury, increases protein synthesis in hepatocytes, decreases the activity of tumour promoters, stabilizes mast cells, chelates iron and decreases calcium metabolism.

vi. Evidence from studies conducted with milk thistle

Milk thistle has been used for centuries to treat liver diseases. It is said to be effective in improving acute or chronic viral, drug or toxin-induced and alcoholic hepatitis. It was found to protect against histologic changes found in livers of pregnant females and females on birth control pills. Furthermore, it reduces hepatic injury caused by some drugs, including halothane, acetaminophen, carbon tetrachloride, benzopyrene and intoxication from the *Amanita phalloides* mushroom.

In vitro incubation with milk thistle in a concentration equivalent to the usual therapeutic dosage, increases the superoxide dismutase of lymphocytes in patients with alcoholic cirrhosis. Superoxide dismutase is the enzyme which catalyzes the dismutation reaction of toxic superoxide radicals to molecular oxygen and hydrogen peroxide, thus forming an important part of the cellular antioxidant reaction. The antitoxic effects of milk thistle

are due to its antioxidant and free radical scavenging properties. Silymarin has been found to suppress nuclear factor kappa β (NF-kβ) DNA binding activity and its dependent gene expression in the hepatoma cell line. NF-kβ controls many genes encoding the proteins of the hepatic acute phase response and in turn is an important regulator of inflammatory and immune reactions. Milk thistle blocks TNF-alpha induced activation of NF-kβ in a dose dependent manner through the inhibition of phosphorylation and degradation of an inhibitor of NF-kβ (Ferenci *et al*., 1989).

In vivo studies

The inhibition of the 5-lipoxygenase pathway, especially leukotriene B_4 at low silibinin concentrations is achieved *in vivo.* This could be an important pharmacological property even though high concentrations of silybinin are necessary to diminish free radical formation by activated Kupffer cells (Saller *et al*, 2001)

The antioxidant properties of silibinin have been evaluated using relevant biological reactive oxygen species (ROS) such as superoxide anion radical ($O_{2\text{-}}$), hydrogen peroxide ($H_2 O_2$), hydroxyl radical (HO^-) and hypochlorous acid (HOCl). The effect on lipid peroxidation was also investigated. It was found that silibinin is not a good scavenger of $O_{2\text{-}}$ and no reaction with H_2O_2 was observed. It reacts rapidly with HO^- radicals in free solution, prevents the effects of tissue necrotic factor alpha, induced with alpha amanitin, hereby indicating an involvement of reactive oxygen species in the amanitin potentiating effect of TNF-α (Ferenci *et al*., 1989).

vii. Clinical trials

Most of the clinical trials designed to assess milk thistle efficiency are flawed in the number of subjects, variance in etiologies, differences in severity of the liver diseases, inconsistencies in alcohol usage by the patients, and therefore the interpretation and correlation of the results must be carefully undertaken.

Double blind studies on humans with acute viral hepatitis suggest that milk thistle therapy not only decreases complications, but it hastens recovery, and hereby shortens hospital stays. A study in which patients were administered 140mg of milk thistle three times daily for three weeks, indicated that after five days, aspartamine transaminase

(AST), alanine
transaminase (ALT) and bilirubin (BRB) levels were significantly lower than in the control group. Moreover, a greater number of patients showed normalisation of BRB, AST and ALT levels within three weeks, when compared to the control group (Bach *et al*., 1996).

Significant changes were seen in a group of 36 patients with chronic alcoholic liver disease in a six month double-blind clinical trial. Their liver function tests illustrated the normalisation of serum bilirubin, AST, alanine aminotransferase and decreased the activity of gamma glutamyl transferase (Feher *et al*., 1989).
Confounding factors in clinical trials of liver cirrhosis are complicated by various factors including:
-persistence of harmful substances eg. alcohol
-concomitant therapies such as supportive treatments or corticostreroids
-patient compliance
-abstinence is a major factor in the pathogenic process of cirrhosis in the alcoholic patient.
The pharmacological rationale for using silymarin in alcoholic patients is due to the following reasons:
-the inhibition of 5 lipoxygenase pathway, especially LTB_4
-ROS scavenging properties of the compound
-Inhibition of the activation of NF-kβ, kinases and capsases may play a role in cytoprotection
-Decrease in collagen and procollagen III content after biliary obstruction in the rat
-Decreased mortality rate as compared to placebo patients
-Liver related mortality decreased with the administration of milk thistle. The pooled liver mortality per year was 4.9% in patients treated with silymarin and 9.3% in placebo patients. However, one of the drawbacks of clinical studies using silymarin in patients with liver cirrhosis, is that all the studies considered death as the primary end point (Bach *et al*.,1996).

viii. Chemistry

Milk thistle is a process-standardised special extract product consisting of four flavonolignone isomers, namely, silibinin 50 - 60%, isobilinin 5%, silidianin 10% and silychristine 20% and other components eg taxifolin 5%. Silibinin is the most active biologically. Silibinin, silidianin and silychristin are collectively known as silymarin. The flavonoids function as scavengers of free radicals and stabilizers of plasma membranes. Other chemical constituents of milk thistle include tyramine, histamine and gamma linoleic acid (http:www.viable-herbal.com). Once Flora *et al*. (1998), had reviewed the history and pharmacology of milk thistle, as well as its properties and the clinical trials in patients with acute and chronic liver disease, they concluded that milk thistle may be effective in improving the clinical courses of acute and chronic, viral, drug and alcohol induced hepatitis (Ferenci *et al*., 1989). Trials must be interpreted with care, however due to the small numbers of patients enrolled, heterogenecity of diagnoses within the same study, the lack of standardization of milk thistle preparations and the dose administered.

The standardised extract obtained from the dried seeds of milk thistle contains approximately 60% silymarin (as measured by HPLC). Pharmacokinetic parameters of silymarin and the active constituents of milk thistle containing products are standardized as silymarin. The bioavailability of silibinin from the extract is low and depends on a number of factors such as

-the content of accompanying substances with solubilising characters eg flavonoids, phenol derivatives, amino acids, proteins, tocopherol and cholesterol.

-the concentration of the extract itself

-the complexation of silibinin with phosphatidylcholin or β-cyclodextrin

-choice of capsule material

The variation in content, dissolution and oral bioavailability of silibin between different commercially available silymarin products, despite the same declaration of content are significant.

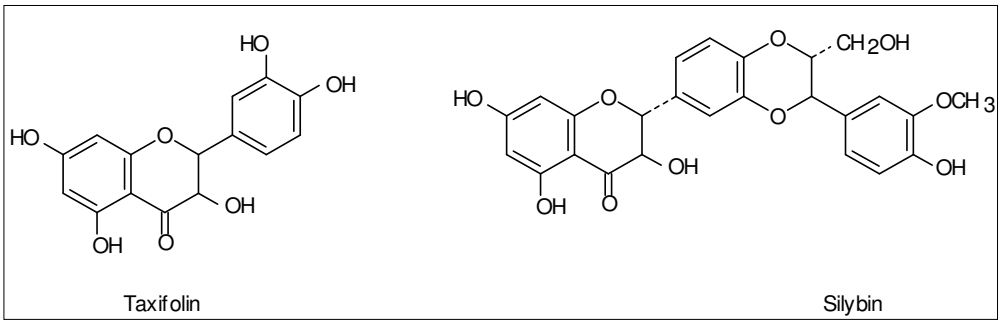

Figure 13. Chemical structures of taxifolin and silybin which were used as reference standards for milk thistle in this study.

ix. Mechanism of action

The exact mechanism of action of milk thistle and its constituents are unknown, but a number of theories have been proposed, such as:

- Milk thistle provides hepatocellular protection through the stabilisation of hepatic cell membranes. It alters the structure of the outer hepatocellular membrane so as to prevent liver toxin penetration into the cell (http:www.mskcc.org).
- It stimulates nucleolar polymerase A resulting in increased ribosomal protein synthesis, hereby increasing the regenerative ability of the liver and the formation of new hepatocytes (http:www.mskcc.org).
- It interrupts the entero-hepatic recirculation of toxins and promotes the regeneration of damaged hepatocytes (http:www.mskcc.org).
- The flavonoids in milk thistle are said to induce anticancer effects by resulting in G1 and S phase arrest in rapidly dividing cancer cells (http:www.mskcc.org).
- Milk thistle inhibits leukotriene and prostaglandin synthesis, mast cell stabilisation, and Kupfer cell functioning (Saller *et al*., 2001).
- It functions by the modification or occupance of cell membrane receptor sites, thus inhibiting the binding of drugs or toxins to these sites and prevents the inhibition of RNA polymerase and RNA synthesis by the toxin (Saller *et al*., 2001).
- Milk thistle decreases HMG CoA reductase leading to a decrease in cholesterol synthesis (Saller *et al*., 2001).
- Silybin functions as an anti-oxidant by reacting rapidly with oxygen free

radicals (Saller *et al*., 2001).

- Milk thistle is said to increase hepatocyte protein synthesis by stimulating the activity of ribosomal RNA, decrease hepatic and mitochondrial glutathion oxidation, decrease the activity of tumour promoters, stabilize mast cells, protect against radiation-induced suppression of hepatic and splenic DNA and RNA synthesis, and may slow calcium metabolism (Bach *et al*., 1996).

Comparisons between studies should be carried out with caution considering the differences between the analytical methods used eg TLC and whether free, conjugated or total silibinin is the object of measurement (Flora *et al*., 1998).

A plasma silibinin maximum concentration of between 200 and 1400 micrograms per litre was reached after approximately two hours after a single oral administration of a standardized dose of silibinin of between 100mg and 360mg in male volunteers. Approximately 75% of the silibinin was present in the conjugated form. An elimination half life of approximately six hours is estimated. No accumulation is observed after the administration of multiple doses. Also the administration of a single dose of silibinin does not modify the kinetics of alcohol, but in patients with cirrhosis, the maximum plasma concentrations of silibinin was lower and the maximum concentration delayed (Ferenci *et al*., 1989).

There is very little evidence of a therapeutic effect of milk thistle in toxic liver diseases, other than mushroom poisoning, eventhough a considerable number of pharmacological studies have been reported in animals. Milk thistle therapy has been evaluated for its role in acute poisoning with the *Amanita phalloides* mushroom. Isolated livers from a variety of mammals were exposed to high doses of phalloidin. Histologic injury was minimised by the administration of milk thistle before sacrifice. A study was done in dogs showing that if the dogs did not receive milk thistle before administering lyophilized *Amanita phalloides*, they would die. Milk thistle has a regulatory action on cellular and mitochondrial membrane permeability, with an increase in membrane stability against xenobiotic injury. It decreases the cellular absorption of noxious xenobiotics, hereby providing cell

protection. Milk thistle is not known to affect viral replication from a pharmacological view, but may inhibit the inflammatory and cytotoxic cascade of events triggered by viral replication (Saller, 2000).

The *Amanita phalloides* mushroom contains amanitin, which is a cyclic octapeptide inhibiting RNA polymerase II and therefore interferes with protein synthesis. This process causes damage to cell membranes, organelles and nuclei. Phalloidin, a cyclic heptapeptide that accompanies amanitin may interfere with actin polymerisation. The use of silybinin as opposed to silymarin is due to the fact that it inhibits phalloidin and amanitin transporters and has cytoprotective effects on tissues which are not yet injured (Carducci *et al*., 1996).

x. Dosage

For use as a protectant : 175mg/day of a seed extract containing 80% silymarin.

For restorative and therapeutic effects: up to 600mg/day of an 80% silymarin extract (www.naturalproducts.org)

xi. Precautions

This herb might have a mild laxative effect (www.usadrug.com).

xii. Lactation

No information is available for the use of milk thistle in pregnancy or lactation (www.naturalproducts.org).

xiii. Use in special populations

Because liver function is not optimal in the elderly, it is wise to use this product with caution in this population, especially since milk thistle functions directly on the liver.

The recommended adult dose must be adjusted according to the child's weight before it is used (www.usadrug.com).

xiv. Drug interactions

Because milk thistle may reduce the lipoperoxidation of cell membranes and insulin resistance, it decreases endogenous insulin level production, increasing the need for

exogenous insulin administration (Hakova *et al*., 1993). It inhibits cytochrome P450 therefore increased levels of medications metabolised via this enzyme will occur and doses must be adjusted accordingly in some drugs eg ketoconazole, erythromycin, triazolam (http:www.mskcc.org.com).

xv. Contraindications

Sensitive persons may develop an allergic reaction as with all other natural products (Murphy *et al.*, 2000).

xvi. Toxicity

A mild laxative effect may occur in the first few days of use, because of its stimulating effects on the liver and gallbladder. No long term risks are associated with milk thistle (Murphy *et al.*, 2000).

xvii. Adverse effects

Although adverse reactions to milk thistle are rare, patients may experience a mild laxative effect. Safety data on milk thistle has been reviewed from data from clinical trials which report sufficiently on adverse effects of patients and adverse effects registered in a central database of the main producers of silymarin (Flora *et al*., 1998). These studies included, headaches, pruritus, upper gastrointestinal symptoms, bloating, dyspepsia, nausea, irregular stools, diarrhoea, malaise asthenia and vertigo. Uterine and menstrual stimulation has been reported in females (http:www.mskcc.org.com).

2. Materials and methods

The protocol adhered to for the TLC is outlined under the general material and methods section. The information provided below is specific to the TLC performed on milk thistle (Wagner and Bladt, 1996).

Reference compound: taxifolin, silybin

Solvent system:
I) chloroform-acteone-formic acid (75:16.5:8.5)

Adsorbent: Silica gel G/UV254 Alugram precoated TLC plates (Macherey-Nagel, Duren)

Detection:
I) NP/Peg No 28 (UV-365nm)
II) Fast blue salt reagent (FBS No 15-vis)

3. Results and discussion

Although the bands at R_f 0.6 were not green-blue as specified in the literature, but rather purple-blue in colour, they were distinct and indicated the presence of silybin in products 3 and 4. Products labelled 1 and 2, however, did not show the presence of either taxifolin or silybin. Product 2 showed an unidentified band at R_f 0.48. Product 1 was devoid of all bands. Products 3 and 4 showed the presence of taxifolin at R_f 0.4.

Both the methods (ie the one sprayed with NP PEG and the method using the FBS reagent) produce clear and reproducible results. The third method, the exposure of the plate to iodine, also illustrated some of the bands clearly, but when the bands were in close proximity, they appeared as smudges, making it difficult to determine the R_f values of the individual bands. Iodine is useful in illustrating the presence of conjugated double bonds, but doesn't provide clear separation of the bands.

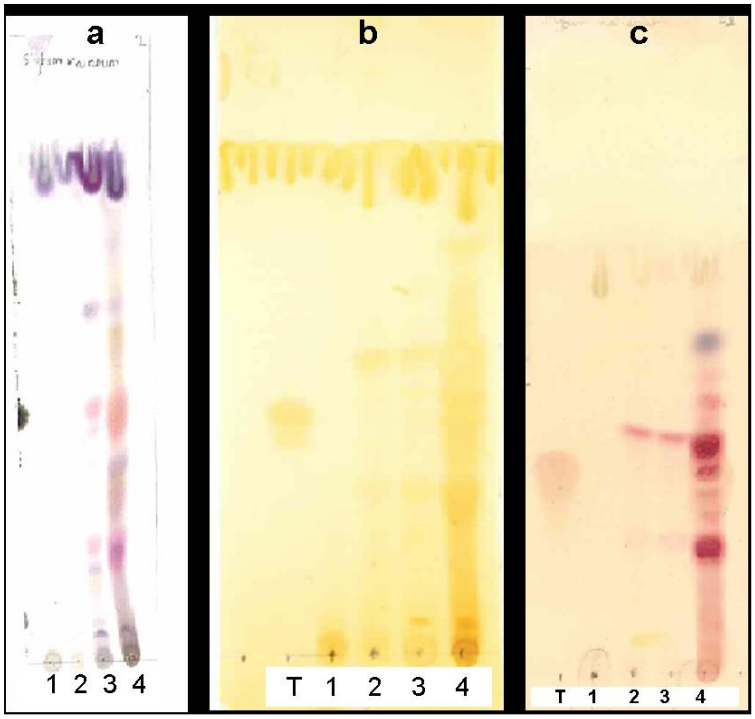

Figure 14: TLC plates of *Silymarin* samples run using solvent system I, taxifolin as reference standard(t) and a)vanillin-sulphuric acid reagent b)iodine and c)fast blue.

4. Conclusion

Products 3 and 4 indicated the presence of silybin and taxifolin, while products 1 and 2 did not show the presence of either. Product 4 indicated the presence of unidentified constituents, while product 1 was devoid of all substituents being assayed for.

GENERAL CONCLUSIONS

From this study, it can be concluded that there are a vast variety of herbal and homoeopathic remedies on the market, some of which don't accurately reflect the constituents and their concentrations, on the product label, and some of which may even contain adulterants. It is essential that both the health professionals and patients are made aware of this, and that more stringent regulatory controls are implemented to avoid it. This may have serious implications in that a patient may use one of these remedies containing an ingredient to which he/she is allergic, because it does not appear on the label. A doctor assessing the allergic reaction would not be able to identify the allergen, and might treat the patient incorrectly. Moreover, it is essential that healthcare professionals change their attitudes towards the use of complementary and herbal medicines, so that the patients are not reluctant to tell them what products they are using. This would enable the healthcare professional to predict herb-drug interactions and to advise the patient accordingly.

The determination of the best method in identifying the marker metabolites is not always a simple task. It involves a lot of experimentation and modification of methods. As discussed in the materials and methods, the definition of 'best' in the phrase 'the best method' is the simplest method resulting in the clearest results on repetition. However, what I've found to be the best method, may not work as well for someone else. The best TLC methods for the various products were as follows:

Saw palmetto: Toluene: ethyl acetate (70:30) solvent system and vanillin-sulphuric acid reagent

St John’s Wort: Ethyl acetate-formic acid-glacial acetic acid-water (100:11:11:26) as solvent system and NP PEG reagent .

Purple coneflower: Toluene:acetone (70:30) solvent system and vanillin-sulphuric acid reagent.

Duck foot tree: Ethyl acetate-formic acid-glacial acetic acid-water (100:11:11:26) solvent system and NP PEG detection reagent.

Milk thistle: Chloroform-acetone-formic acid (75:16.5:8.6) and NP PEG or FBS reagent.

The tables below illustrate which product brands contain the various ingredients assayed for.

Table 3: Different product brands of Saw palmetto illustrating β-sitosterol content.

Product brand	Presence of β-sitosterol
Product 1	+ve
Product 2	+ve
Product 3	+ve
Product 4	+ve

Table 4: Different product brands of St John's Wort illustrating Hypericn, Chlorogenic and Rutin content.

Product brand	Hypericin	Chlorogenic acid	Rutin
Product 1	+ve	+ve	+ve
Product 2	+ve	+ve	+ve
Product 3	+ve	-ve	-ve
Product 4	+ve	+ve	+ve
Product 5	-ve	-ve	-ve

Table 5: Different product brands of the purple coneflower illustrating β-sitosterol, caffeic and chlorogenic acid content.

Product brand	B-sitosterol	Caffeic acid	Chlorogenic acid
Product 1	+ve	+ve	-ve
Product 2	+ve	+ve	+ve
Product 3	-ve	+ve	-ve
Product 4	-ve	-ve	-ve
Product 5	+ve	-ve	-ve

Table 6: Different product brands of the duck foot tree illustrating Rutin and Bilobalide content.

Product brand	Rutin	Bilobalide
Product 1	+ve	+ve
Product 2	+ve	+ve
Product 3	+ve	+ve
Product 4	-ve	-ve
Product 5	-ve	+ve
Product 6	+ve	+ve
Product 7	+ve	+ve

Table 7: Different product brands of milk thistle illustrating Taxifolin and Silybin content.

Product brand	Taxifolin	Silybinin
Product 1	-ve	-ve
Product 2	-ve	-ve
Product 3	+ve	+ve
Product 4	+ve	+ve

From the above tables it is evident that discrepancies across products brands occur.

Some reasons for these inconsistencies may include:

- The usage of adulterants in products.
- The usage of different parts of the plants eg the roots of a plant will not have the same chemical constituents as the leaves, or stems.
- The site/origin of plant growth.
- The age of the plant when it is picked.
- The time of day at which the plant was picked.
- Environmental conditions in which the plant grew.
- Whether the herb is manufactured into a liquid or solid form. Liquids tend to be more unstable then solids.
- The length of time which the products stands before being consumed. The longer it stands after manufacture, the more unstable the ingredients become.

This study illustrates that although all the products on the market are said to go through a quality control protocol, some clearly don't and some of those which do, are still of a poor quality, in that they contain contaminants, or the active ingredients may be present in extremely low concentrations, different to that stated on the product label, or even totally absent.

It can be concluded that:

-some brands are of better quality than others across all herbal variants.

-some brands produce forms of different quality for eg. although the tablet form may contain all the active ingredients, as stipulated on the package, the liquid form may not be of the same high quality, either containing insignificant amounts of the active ingredients, or none at all.

-some products are devoid of any active ingredients eventhough these are stated on the packaging as being present.

It is essential that minimum standards exist and are constantly monitored in the manufacture, packaging and storage of herbal and homoeopathic remedies in order to ensure the production and maintenance of optimal products for consumer use.

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