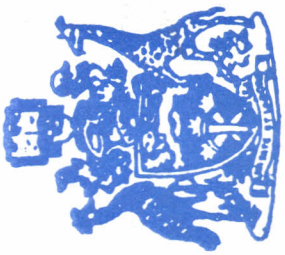

A COMPARISON OF MORPHOLOGICAL, PHYTOCHEMICAL AND PHARMACOLOGICAL PROPERTIES OF *MARSEDENIA SYLVESTRIS* R. BR FROM DIFFERENT REGIONS OF KENYA

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A thesis submitted in partial fulfillment of the requirements for the award of the degree of Masters of Science in Pharmacognosy and Complementary Medicine of the University of Nairobi.

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

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Date

This thesis has been submitted for examination with our approval as university supervisors.

Prof. Julius W. Mwangi. Date 25/08/2011

Dr. Faith A. Okalebo Date 25/08/2011

Dr. Beatrice Amugune Date 24/8/11

DEDICATION

To Judith Wambui my dear wife and my children Dr. Patrick Wacira, HDr. Lilian Nyaguthii, Beth Muthoni, Carolyne Njoki and Edwin Mutugi.

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LIST OF ABBREVIATIONS AND SYMBOLS

AIDS	Acquired Immune Deficiency Syndrome
ANOVA	Analysis Of Variance
b. w	Body weight.
Co	Company
DNA	Deoxynucleic Acid
Fig	Figure
Gm	Gramme
HIV	Human immune deficiency virus
H_2SO_4	Sulphuric Acid
HPLC	High Performance Liquid Chromatography
Kg	Kilogramme.
Ltd	Limited
ml	Millilitres
MSA	*Marsedenia sylvestris* Arabuko forest
MSG	*Marsedenia sylvestris* Gede
MSK	*Marsedenia sylvestris* Kisumu
Na^+	Sodium ion
Nacl	Sodium Chloride
NaOH	Sodium Hydroxide
Nm	Nanometre
Rf	Retention factor
STZ	Streptozotocin.
TB	Tuberculosis
TLC	Thin Layer Chromatography
µl	Microlitre

UV	Utraviolet
v/v	Volume over volume
WHO	World Health Organization
%	Percent

ABSTRACT

Mankind has over the centuries used plants to treat many diseases and many traditional herbal remedies are to be found. In fact many of the so-called conventional medicines like penicillins, artemesinin-based products and quinine are derived from plants. Natural medicines especially herbal medicine has of late gained great popularity and is undergoing renaissance the world over. The World Health Organization (WHO) has also recommended the evaluation of plants with folklore fame in treatment and managing of diseases. *Marsedenia sylvestris R.Br.* a woody climber is one of the promising plants and has been widely studied and researched. It has been traditionally used successfully particularly in India to manage and treat both type 1 and type 2 diabetes. *Marsedenia sylvestris R.Br.* species found in three different regions in Kenya were investigated. Plant materials were collected from Reru, West Seme Location in Nyanza Province, Arabuko Sokote Forest and Gede Ruins Forest in Coast Province.

The objective of this study was to carry out a comparison of phytochemical and hypoglycemic properties of *Marsedenia sylvestris R.Br.* from different geographical regions of Kenya. Their morphological characteristics, TLC, HPLC profiles, and hypoglycemic activities were also compared. Morphological studies showed that the three geotypes were similar save for minor differences in sizes of leaves which may be due to soil texture and fertility. Phytochemical studies showed that the leaves and twigs of *Marsedenia sylvestris R.Br.* contained saponins, tannins, cardiac glycosides and alkaloids. Anthracene glycosides were absent. Physical properties, TLC and the HPLC profiles were similar for the different geotypes. Methanolic Soxhlet extracts showed a significant hypoglycemic activity in oral glucose tolerance test. The extract from Nyanza province was slightly more potent than the others. This is suggestive that the plant grown in Kenya could be used for management and treatment of diabetes mellitus. The environmental conditions of Kisumu probably tend to favour the production of higher concentrations of the active ingredients.

The plant extracts were also found to increase the contractions of the rabbit heart, ileum and the rat uterus and cause relaxation of rat diaphragm in addition to increasing diuresis in rats. These

properties suggest that *Marsedenia sylvestris R.Br.* may also be useful in such clinical conditions like labour induction, prolonged labour, edema and cardiac failure and constipation. It is also a potential hypotensive agent. This is the first time this plant has been investigated in Kenya and further work need to be done particularly in identification and structural elucidation of the active principles. In addition more pharmacological studies need to be done particularly in the field of family planning and determine the optimum dose. Although the plant has been used in India for a long time toxicological and clinical studies should be done to assure the patients about its safety. It is after these studies are done should this plant be domesticated and farmed sustainably for its health and economic benefits.

CHAPTER I

INTRODUCTION

1.1 Plants as sources of pharmaceuticals

Mankind and diseases have co-existed since creation. During that time man has developed many types and systems to treat and heal himself. Such therapeutic systems include the conventional or orthodox medical system also known as the allopathic or chemical system. Others include herbal medicine, aromatherapy, ayurvedic medicine, unani and traditional Chinese medicine to mention but a few [1, 2].

The current conventional approach and treatment of diseases leave a lot to be desired. The disease-causing pathogens such as viruses, bacteria and parasites have developed resistance to most of these orthodox drugs. Many of these drugs are also very toxic to human beings and are very expensive as to make them out of reach of the common man. With the emerging challenges of new diseases like HIV/AIDS, old diseases like diabetes, asthma and TB are forming unholy alliances with HIV/AIDS and resisting conventional treatment [3]. There is therefore urgent need to develop safer and more efficacious drugs.

Mankind has over the centuries used plants to treat many diseases and many traditional herbal remedies are to be found. In fact many of the so-called conventional medicines like penicillins, artemesinin-based products and quinine are derived from plants [3]. Chloroquine and related 8- and 4- aminoquinoline synthetic compounds are based on the quinine pharmacophore.

Plants synthesize a wealth of unusual metabolites that have a secondary role in their ontogeny, such as self-defence, aggression or even communication as the need arises. These secondary metabolites exhibit remarkable diversity in terms of their structure and function. These natural products are known to posses a wealth of pharmacologically important activities including antimicrobial, antifungal, antiparasitic, antitumour, antihyperglycemic and pesticidal properties

[2]. Secondary metabolites are ubiquitous in natural distribution and have been obtained from organisms as diverse as bacteria, fungi, plants, insects, mollusks and sponges [4].
The plant kingdom holds many species with medicinal value. It is estimated that only 4% of the known plants have been screened for their pharmacological activity [5]. As a result of recent advances in chemical and physical techniques, pharmaceutical testing procedures, isolation and identification methods such as advanced chromatographic and spectroscopic methods, new plant drugs have been discovered. These new active compounds may be used as drugs for specific diseases or they may be used as lead compounds in the synthesis of synthetic or semi-synthetic compounds with increased potency, reduced toxicity and quicker onset of action with more durable activity. Therefore more time and finance is required for investigations of natural products since they present a potential source of drugs, some of which may become cures of presently difficult to treat or incurable diseases.

Plants have produced many important drugs in medical history such as herbal remedies or phytomedicines, natural products and nutraceuticals. Herbal remedies are derived from specific part of the plant or whole plant and include such remedies like St. Johns wort (*Hypericum perforatum*) used in the treatment of mild to moderate depression; the leaves and pods of senna (*Senna didymobotrya*) used for constipation and other stomach disorders; and *Prunus africana* bark used to treat benign prostatic hyperplasia [3]. Others include the leaves of *Ginkgo biloba* used for cognitive deficiencies and memory impairment and the flower heads of chamomile (*Chamomilla recutita*) which is used for mild gastrointestinal complaints and as an anti-inflammatory agent [3].

Natural products are pure chemical entities isolated from nature. They are sometimes produced synthetically and are referred to as "nature identical".These include quinine from *Cinchona* bark used to treat malaria; digoxin and other digitalis glycosides from *Digitalis species* used to treat heart failure; and morphine from opium poppy (*Papaver somniferum*) used as an analgesic. Others include taxol from the Pacific yew (*Taxus brevifolia*) used as an anticancer treatment and caffeine from the coffee shrub (*Coffea arabica*) used as a stimulant [2].

Nutraceuticals or functional foods are dietary natural products which have beneficial effects on health. These are exemplified by garlic, ginger, turmeric and black pepper. Others include carotenoid-containing plants like carrots, tomatoes and *Amaranthus species* and anthocynin- or flavonoid-containing plants like cocoa and red grapes [2].

Diabetes is one of the most common chronic diseases in modern times. In the past, it was regarded as the disease of the developed countries due to excessive indulgence in rich foods and beverages together with a sedentary lifestyle. Nowadays, it is to be found even in the developing world and more alarming, its incidence in children has increased.

The current conventional medical system uses drugs like insulin, sulphonyureas and biguanides to control blood sugar levels to some extent [3]. In spite of the advances in therapeutics, none of these drugs has been completely successful in maintaining euglycaemia and avoiding the late stage complications of diabetes. In fact synthetic (read conventional) hypoglycaemic medicines have been found wanting because of decreasing efficacy and potency, are too costly and not to mention their serious side effects. Non-pharmacological measures of diet, exercise and weight loss remain critical components of therapy but no significant progress has been achieved in diabetes as none of these measures corrects the basic disorder.

For a long time, natural medicines like African herbal remedies, Ayurvedic Indian medicines, Traditional Chinese medicines and other complementary medicines have been used successfully to treat and manage diabetes [1, 2]. The World Health Organization (WHO) has recommended the evaluation of plants with folkloric fame in treatment of and management of diabetes [1]. *Marsedenia sylvestris* is one such a plant.

1.2 Diabetes: Situational analysis

Diabetes is a metabolic disorder, specifically, an abnormality in the way the body utilizes glucose, due to an absolute or relative deficiency of the hormone insulin or resistance by the body tissues to the action of insulin. Diabetes mellitus was described more than three thousand years ago. The disease is aptly named as the word is derived from the Greek word "*diabetes*" meaning a siphon and the Latin word "*mellitus*" meaning honey-sweet. This is because the

passage of large amounts of sugar-laden urine is a key characteristic feature of poorly controlled diabetes [8].

Diabetes manifests itself in form of increased blood sugar, frequent urination and increased thirst, disturbances in vision, tiredness and itching in or around sex organs. Long term complications and consequences are kidney failure, blindness, amputations, neuropathy, ketoacidosis and impotence [6]. There are two main types of diabetes namely, insulin dependent diabetes mellitus (IDDM) also known as type one and non-insulin dependent diabetes mellitus (NIDDM) also known as type two. Type one diabetes occurs mainly in children and young adults below twenty years with a sudden onset while type two occurs mainly after the age of forty years.

Diabetes is a widespread disorder which has long been recognized in the history of medicine. The cause of much human suffering, diabetes places a considerable economic burden on individuals, families and healthcare systems. Before the advent of conventional medicine the major form of treatment involved the use of plants. More than 400 plants are known to have been recommended for diabetes management [7]

In 2004 more than 150 million people worldwide suffered from diabetes as reported in the World Health Organization report [1]. The incidence of diabetes is increasing rapidly and it is estimated that by the year 2025 this number will double [8]. Diabetes is in the top five of the most significant diseases of the developed countries and is fast gaining significance in developing countries. The number of people with obesity- related type 2 diabetes is rising sharply as the sedentary life style, behaviour and high-fat, high-sugar foods that characterize urban lifestyle replace the constant physical activity and vegetable-based diet of the rural lifestyle.

The Kenya Ministry of Health estimate that the prevalence of diabetes is around 10% of the population (4 million people) [9].This is mainly people in the urban areas but a significant increase has been noted in the rural areas and among young children and this is cause for worry. According to a survey conducted by Kenya Diabetes Management and Information Centre (DMI Centre) in partnership with Safaricom between May, 2005 and June, 2006, the national diabetes prevalence stood at 7.3% with a marginal deviation from the current Ministry of Health estimate

of 10% [10].This difference could be due to the fact that the DMI Centre study was a general population based survey as opposed to the Ministry of Health survey of hospital based population (clinically based estimation) which may introduce a bias. The study showed regional differential prevalence ranging from 2.3% in Kaiti of Makueni District to 12.8% in Mvita constituency in Mombasa. The sex distribution of diabetes compares well at a ratio of 1:1 and type one diabetes is about 10% while type two is about 90%.

1.3 Phytochemical and pharmacological studies on various anti-diabetic plants

Plant materials have played an important role in the traditional treatment of diabetes, particularly the type 2. In many regions of the world, herbal remedies continue to be more accessible and affordable than conventional drugs and represent the first line of treatment available to a diabetic patient [3]. Concurrently, within the societies with well-developed, modern health care systems, demand is growing for herbal remedies to complement prescribed modern therapies for many diseases including diabetes.

Diversity as well as similarity can be found in the use of plants across the world. Understandably, each region of the world has developed a *Materia Medica* of the antidiabetic remedies based on the local flora. Diversity is also seen in the range of plant families and types of phytochemicals associated with antidiabetic activity. At the same time, certain chemical groups such as alkaloids, saponins, xanthones and flavonoids and nonstarch polysaccharides appear to have effects of particular significance in diabetes treatment [11]

Certain plants such as *Momordica charantia* (Cucurbitaceae) and *Trigonella foenum-graecum* (Fabaceae) have been used in many regions across the world and this is evidence for their effectiveness. The extent to which various antidiabetic plants have been studied differs widely and some like *Momordica charantia, Trigonella foenum-graecum* and *Marsedenia sylvestris* (Apocynaceae) have had their active compounds isolated. Plants typically have more than one active component and are often associated with more than one mode of action [11]. Research suggests that using an antidiabetic plant in whole form or complex extracts may offer many benefits due to the presence of multiple active components where additive or synergistic effects undoubtedly occur. This is in conformity with the view of traditional herbalists that the activity

of a medicinal plant cannot be reproduced by the isolation of a single active component. However, identification of active ingredients, and their mode of action are important for drug development, and for validation, standardization and rational use of traditional herbal remedies.

Much research on antidiabetic plants has been undertaken in academia. This situation is likely to continue and hopefully increase in response to the growing prevalence of diabetes world wide. Many research studies have documented various plants with *in-vitro* anti-diabetic and hypoglycemic activities. Ethnobotanical surveys show that about 800 plants may possess anti-diabetic potential [11].

In a study to establish and document the hypoglycemic action of *Momordica charantia* in a validated animal model of diabetes, the alcoholic extract of the pulp was studied [12]. In the normal glucose-primed rat model, *Momordica charantia* fruit extract, 500 mg /kg, decreased the plasma glucose levels by 10-15% after 1 hour. Under similar conditions, tolbutamide 100 mg /kg, a standard conventional anti-diabetic drug caused approximately 40 % reductions in plasma glucose both at 1 and 2 hours. At 500 mg/ kg the efficacy of *Momordica charantia* was 25-30 % of tolbutamide. The reduction in plasma glucose in normal glucose primed rat was not accompanied by increased insulin secretion. These data suggested that the mechanism of action of *Momordica charantia* could be partially attributed to increased glucose utilization in the liver rather than an insulin secretagogue effect. The pharmacology of *Momordica charantia* extract has revealed that it has blood glucose lowering activity both in normal and streptozotocin-induced diabetic animals.

Kaleem *et al* conducted a study with the aim of analyzing the antidiabetic and antioxidant activity of oral administration of aqueous extract of *Annona squamosa* (Annonaceae) leaves on blood glucose, haemoglobin, glycosylated haemoglobin, plasma insulin, antioxidant enzymes and lipid peroxidation in the liver and kidney to streptozotocin-induced diabetic rats [13]. Oral administration of *Annona squamosa* aqueous extract to diabetic rats for 30 days significantly reduced the levels of blood glucose, lipids, and lipid peroxidation, but increased the activities of plasma insulin and antioxidant enzymes like catalase, superoxide dismutase, reduced glutathione and glutathione peroxidase. It has also been reported elsewhere that using a plant extract to treat

streptozotocin-induced diabetic rats resulted in activation of β- cells and insulinogenic effects [14]. Therefore this plant would be very useful for prevention or early treatment of diabetes mellitus.

In yet another study Kannabiran *et al* evaluated the hypoglyceamic activity of *Hemidesmus indicus* (Apocynaceae) on streptozotocin- induced diabetic rats [15]. The aim of the study was to evaluate the antidiabetic activity of an aqueous extract of the roots of *Hemidesmus indicus* on blood glucose, serum electrolytes, serum marker enzymes, liver microsomal Cytochrome P_{450} enzymes and lipid peroxidation in the kidneys and liver of streptozotocin-induced diabetic rats.

It was found that oral administration of *Hemidesmus indicus* aqueous extract to fed, fasted and glucose- loaded diabetic rats decreased blood glucose levels significantly at 5 hours and restored serum electrolytes, glycolytic enzymes and hepatic cylochrome P_{450} dependent enzyme systems by preventing the formation of liver and kidney lipid peroxides at the end of 12 weeks of the study period. On the basis of these findings, *Hemidesmus indicus* could be used as an antidiabetic and antioxidant agent for the prevention and treatment of diabetes mellitus.

Kumar *et al* studied *Phyllanthus reticulatus* (Euphorbiaceae) to validate the traditional use by the herbalists to treat diabetes mellitus [16]. *Phyllanthus reticulatus* is a large struggling or climbing shrub growing from 2.5 to 3 metres in height, *(Swahili-Mzizima).* The plant is used to treat a variety of ailments including diabetes, smallpox, syphillis, asthma, diarrhoea and bleeding gums. In the study, petroleum ether and ethanolic extracts of the leaves of the *Phyllanthus reticulatus* were tested at 500 mg and 1000 mg/kg for oral hypoglycemic effect in alloxan-induced diabetic mice. It showed antidiabetic activity at the dose of 1000 mg/kg. Phytochemical screening of the residues revealed the presence of terpenoids, glycosides, proteins, carbohydrates and absence of alkaloids and steroids.

Hypoglycaemic and other related actions of *Tinospora cordifolia* (Melianthaceae) roots were evaluated by Stanley *et al* [17]. *Tinospora cordifolia* is widely used in Indian Ayurvedic medicine for treating diabetes mellitus. Oral administration of an aqueous root extract to alloxan-induced diabetic rats caused an increase in body weight, total haemoglobin and hepatic

hexokinase. It also lowered hepatic glucose-6- phosphatase and serum acid phosphatase, alkaline phosphatase and lactate dehydrogenase.

A major review of plants that were pharmacologically tested and shown to have some value in diabetes mellitus was done [18]. These plants included such species like *Acacia nilotica, Allium cepa, Allium sativum, Aloe vera barbadensis, Azadirachta indica, Ipomea batatas, Momordica charantia, Trigonella foenum-graecum, Sclerocarya birrea, Catharanthus roseus* and *Gymnema sylvestre.*

1.4 Literature review of Marsedenia sylvestris

1.4.1 Taxonomic and botanical information on Marsedenia sylvestris

Originally, *Marsedenia sylvestris* R.Br was called *Gymnena sylvestre* R.Br and was classified in the Asclepidaceae family. However in the last two years it has been reclassified taxonomically and now belongs to the Apocynaceae family which belongs to the Contortae order. This order belongs to the class Dicotyledoneae which is a member of the angiospermae division [19].

The common Indian names for *Marsedenia sylvestris* are *Gudmar, Madhunashini, Gurmar* and *Meshashiringi*. It is also called "periploca of the woods" or "Rams horn" or simply Gymnema. The name *Gurmar* is Hindi which means "the destroyer of sugar" [19]. *Meshashiringi* is a Sanskrit name meaning "rams horn" due to the shape of its fruits.

Marsedenia sylvestis (Retz) is slow-growing perennial woody climber with soft and hairy pubescent leaves. It is a much- branched twinny shrub. The leaves are elliptic or ovate with an acute or acuminate apex (Fig 1). The leaves are opposite measuring about 3-5 cm by 1-2 cm and the lower surface is more pubescent with a rounded or cordate base. Non-glandular trichomes are present on both surfaces of the leaf [19, 20]. The green leaves which are tasteless have a pleasant and aromatic oduor but when chewed have a remarkable property of paralyzing the taste glands for a few hours against sweet and bitter taste [19]. The flowers are yellow in colour and form short- stalked umbellate cymes.

The petals are short tubes and hairy within, with the conspicuous corona appearing as fleshy lobes between the petals. The stamens are fused throughout and the pollinia are minute, erect and solitary in each anther cell. The stigma sticks out of the staminal column. The fruits are long pods about 5-10cm long with a characteristic rams-horn shape (Figure 1). The parts used are mainly the leaves and sometimes the roots.

FIGURE 1: *MARSEDENIA SYLVESTRIS* PLANT SHOWING THE AERIAL PARTS AND MATURE RAMS HORN-SHAPED FRUITS

1.4.2 Geographical distribution of *Marsedenia sylvestris*

Marsedenia sylvestris is widely distributed in central and western India, parts of Asia, Australia and tropical African countries like Kenya and Tanzania. [19,20,21]. In Kenya *M. sylvestris* is found in Ngai forest reserve Meru, Sakwa location in central Nyanza, West Seme location in Reru, Soklo Gulwe in Mfangano Islands, Arabuko Sokoke Forest, Gede Ruins Forest and Makodere in Shimba hills [20] (Fig 2).

Legend: Places *M. sylvestris* is found

FIGURE 2: MAP OF KENYA SHOWING DISTRIBUTION OF *MARSEDENIA SYLVESTRIS*

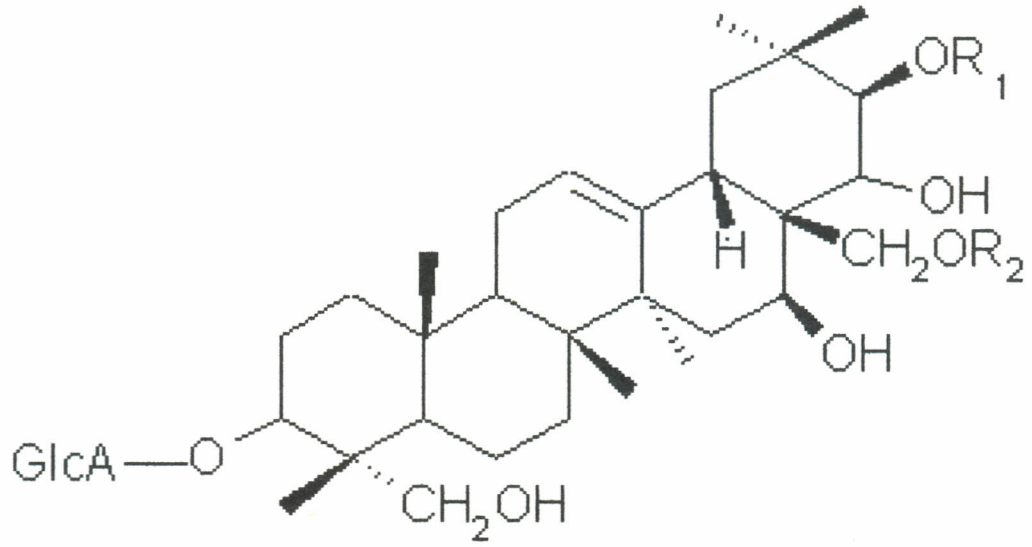
1.4.3 Ethnopharmacological and traditional uses

Marsedenia sylvestris, has a long history in Indian Ayurvedic medicine since 6th century BC [21]. It has been used all over India for controlling blood sugar. In ancient times, Ayurvedic physicians observed that chewing a few leaves of *Marsedenia sylvestris* suppressed the taste of sugar. The traditional primary application was for adult – onset diabetes, a condition for which it continues to be recommended for today in India and China. The leaves are also used for stomach ailments, constipation, water retention and liver diseases [21]. It has also been used for conditions such as malaria, snakebites, asthma, eye complains, inflammation and family planning. In addition it has been used in obesity, hypercholosteremia, edema,dental plaque and caries, arthritis and gout. In Kenya the plant has no known medicinal use for human beings but it is eaten by goats. A local name for the plant was not forthcoming from all the areas it was found growing.

1.4.4 Phytochemistry

Marsedenia sylvestris leaves contain triterpene saponins belonging to oleanane and dammarene classes. Oleanane saponins include gymnemic acids and gymnemasaponins, while dammarene saponin, include gymnemasides. Other plant constituents are flavones, anthraquinones, α and β-chlorophylls, two types of resins, (one soluble in alcohol), formic acid, tartaric acid, butyric acid and lupeol. It also contains β-amyrin related glycosides and stigmasterol [22-25]. In addition, quercitol and the amino acids derivatives betaine, choline and trimethylamine have been isolated.

The major biologically active constituents of *Marsedenia sylvestris* are the gymnemic acids. They are acylated derivatives of deacylgymnemic acid (DAGA) which is a 3-O glucuronide of gymnemagenin. Gymnemic acids have antidiabetic, antisweetener and anti-inflammatory activities [25, 36]. Gymnemic acid is a mixture of at least nine related acidic glycosides [Fig. 3].



	R_1	R_2
Gymnemic acid 1	**Tigloyl**	**Acetyl**
Gymnemic acid 11	**2-Methylbutyrl**	**Acetyl**
Gymnemic acid 111	**2-Methylbutyrl**	**Hydrogen**
Gymnemic acid 1V	**Tigloyl**	**Hydrogen**

FIGURE 3: STRUCTURES OF SELECTED GYMNEMIC ACIDS.

1.4.5 Pharmacological activity

Marsedenia sylvestris has many traditional uses. The main application however remains in lowering blood sugar and treatment of diabetes. The first scientific confirmation of this traditional use in human diabetics came more than 80 years ago when it was demonstrated that the leaves reduced urine glucose in diabetes [22].

Four years later it was shown that *Marsedenia sylvestris* had a blood glucose lowering effect when there was residual pancreatic function [26]. It was found to act on two sites, the taste buds in the oral cavity and the absorption surface in the intestines [27].

There are several mechanisms by which gymnemic acids may exert their hypoglycemic action. They may increase the secretion of insulin, promote regeneration of islet cells, and increase utilization of glucose by increasing the activity of enzymes responsible for utilization of glucose by insulin-dependent pathways. They cause a decrease in phosphorylase activity and decrease in gluconeogenic enzymes and sorbital dehydrogenase levels. They also cause inhibition of glucose absorption from the intestine [28].

The hypoglycemic effect of *Marsedenia sylvestris* brings about blood glucose homeostasis which in turn prevents increased glycosylation of proteins thus reversing the order of changes leading to micro-and macro-angiopathy. The glucose-like gymmenic acids fills the receptor locations in the absorptive layers of the intestines thereby preventing the intestines from absorbing the sugar molecules with the end results of lowering blood sugar level [25].

Since gymnemic acids have similar structure like glucose they therefore compete for taste buds receptors in the tongue. This interferes with the ability of the taste buds on the tongue to taste sweet and bitter compounds [27]. It is believed that by inhibiting the sweet taste sensation, people taking it will limit their intake of sweet foods. Therefore *Marsedenia sylvestris* is used to control sugar cravings. Gymnemic acid formulations have also been found useful against obesity, according to recent reports [29]. This is attributed to the ability of gymnemic acids to delay the glucose absorption in the blood.

1.4.6 Standardization of *Marsedenia sylvestris* products

According to the World Health Organization monograph [1], *Marsedenia sylvestris* for medicinal use should have more than 11.5 % (w/w) ash values, not less than 5.5 % (w/w) organic acids, the alcohol soluble extractives not more than 12 % w/w, a moisture content less than 10 % w/w, water soluble extractive value 25 % w/w, foreign matter less than 1 % and Gymnemic acids more than 10 % w/w by gravimetry.

1.5 Justification and rationale

Marsedenia sylvestris has a long therapeutic history. Its chemical, biological and pharmacological activities are well established. In Kenya, the *M. sylvestris* varieties have not been studied at all. There is no scientific report on their ethnomedical use or phytochemical studies. They are considered as troublesome woody climbers on the hedges with no known medical value. The Kenyan varieties may differ in gymnemic acid types and levels and hence their efficacy. There may also be some geographical and intra-species differences in term of chemical composition, levels and potency.

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In addition, since *M. sylvestris* market is established in many countries particularly in India and China it is imperative to carry out detailed studies of the local varieties in order to establish potential economic value and therapeutic uses. This will lead to development of useful pharmaceutical products and lead compounds in the fight against diabetes and other diseases. There is need to develop affordable and efficacious herbal medicines which can be integrated into the National Health delivery system. This is in line with United Nations Millennium Development Goals and the Kenyan Vision 2030. This study intends to test the null hypothesis that there is no statistically significance difference in the hypoglycaemic activity of various geotypes of *M. sylvestris* .

1.6 Hypothesis

1. Extracts of *Marsedenia sylvestris* in Kenya possess hypoglycemic activity responsible for the reported ethnomedicinal use.

2. There is no difference in hypoglycemic activity of extracts from *Marsedenia sylvestris* found in different geographical regions of Kenya.

1.7 Objectives of the study

1.7.1 General objectives

The general objectives of the study was to compare the botanical, phytochemical and hypoglycemic properties of *Marsedenia sylvestris* geotypes of Kenya

1.7.2 Specific objectives of the study

The specific objectives of the study were to:

1. Compare taxonomic characteristics of *Marsedenia sylvestris* from three different geographical regions in Kenya (Geotypes).
2. Screen for phytoconstituents of the extracts from the three different regions in Kenya
3. Compare the fingerprint TLC and HPLC profiles of the crude drugs and the extracts.
4. Compare the postprandial hypoglycemic activity of the extracts.
5. Study other pharmacological activities on selected isolated tissues.

EXPERIMENTAL

2.1 Materials

2.1.1 Equipment

Plant materials were ground using a hammer mill sourced from Ndume Ltd., Nakuru, Kenya. Microscopy was done using a light optical microscope, Olympus model, Japan.

Extraction was done using Soxhlet extractor (Quickfit, Birmingham, United Kingdom) mounted on an electrically-heated electrothermal isomantle (Fig. 4). The filtered crude extract was reduced to dryness under vacuum using Heidolph Laborata 4000 rotary vacuum evaporator connected to Heidoiph bath circulator (Heidolph Electro Gmmb and Co. G.Ketheim,Germany) and Knf Reyberger Laboport pump.

Thin layer chromatography was done using thin layer chromatography tank on aluminum plates coated with adsorbent silica gel $60F_{254}$ as the stationary phase (Merck ,West Germany). HPLC was done using Merck Hitachi Lachrom equipment with a C18 Zorbax Column XDB C 18Φ, 4.6 mm x150 mm with a particle size of 5 micron as the stationary phase. For hypoglycemic activity an Accu-check Active glucometer was used for testing for blood sugar.

Tissue movements were recorded using electronic recorder, oscillograph 2-channel recorder, Gemini 7070 2102, Comerio VA, Italy with a transducer, (Ugo, Basile S.R.L). Other apparatus used were an organ bath, Langendroff apparatus and a Havard recorder for recording the heart contractions.

2.1.2 Chemicals, reagents and solvents

All chemicals used were of analytical grade unless otherwise stated. For microscopy phloroglucinol, hydrochloric acid and chloral hydrate were purchased from Kobian (K) Ltd.

Extraction was done using GPR methanol sourced from Kobian (K) Ltd. Phytochemical testing was done using Dragendorff"s reagent, Mayer's reagent, Kedde's reagent, sulphuric acid, dilute ammonia, chloroform and alcohol. Acetic acid, vanillin, hydrochloric acid, ethanol, potassium hydroxide and glucose were obtained from Sigma Laboratories. Filtration was done using Whatman No 1,θ18 filter paper (Whatman International ltd, Maidstone, England). The reagents were freshly prepared.

Thin layer chromatography used chloroform, methanol, and acetic acid (5:1:1) as the mobile phase and vanillin in sulphuric acid for spray detection. For HPLC the following chemicals and reagents were required; 50 % ethanol, 12 % KOH, 4N- HCl, HPLC- quality acetonitrile (RFCL Ltd, New Delhi, India) and KH_2PO_4 (Loba Chemie Pvt Ltd, Mumbai, India). The hypoglycemic activity testing required 50 % glucose, glibenclamide 5mg tablets (Daonil®), normal saline, dimethyl sulfoxide (DMSO) and 50 % ethanol GPR (Kobian). For pharmacological studies physiological solutions Tyrode, Krebs-Henseleit, Locke and De Jalon were used.

2.1.3 Animals

White Sprague-Dawley rats of mixed sex were obtained from University of Nairobi, School of Pharmacy animal house and Kabete Campus where they had been housed and fed with rat pellets obtained from Unga Feeds Limited, Kenya and water *ad libitum*. They were housed in steel cages under a 12 hour dark light cycle at ambient temperatures and humidity. Ethical approval was obtained through the University of Nairobi.

2.2 Methodology

2.2.1 Determination of the botanical characteristics of *Marsedenia sylvestris*

2.2.1.1 Collection and determination of macroscopic and organoleptic characteristics of *Marsedenia sylvestris*

The leaves and twigs of *M. sylvestris* were collected from three geographical locations in Kenya, namely West Seme Location, Bondo in Nyanza Province, Arabuko Sokoke Forest in Coast Province and Gede Ruins in the north Coast. Collection was done in the morning and great care was taken to ensure that only the healthy leaves and young twigs were collected. Identification and collection was done by the writer being a herbalist himself. The morphological features were observed and compared in the field on fresh plant materials and close-up photographs taken in the three regions. Macroscopic evaluation and organoleptic characterization were carried out. Sufficient materials were collected and transported to Nairobi in sisal sacks that were well ventilated. Representative samples of each geotype were authenticated by the National Museums of Kenya, Nairobi where voucher specimens were submitted. Voucher specimens were also deposited at the School of Pharmacy, No. BBMM 1-9- 09.

The plant material was then air-dried in the shade at room temperature until completely dry. When the material was completely dry it was ground to coarse powder using a hammer mill. The coarse powder was properly labeled and stored in airtight containers until further use.

2.2.1.2 Determination of foreign matter, ash and alcohol extractive values

To determine the foreign matter, 100gm of the dried whole plant material of leaves was spread in a thin layer on a paper. It was examined at X6 magnification and the foreign matters such as other plant parts like twigs, stems and other organic matters were picked and weighed. This was similarly done for insect infestation and rodent faeces contamination and the results recorded.

Alcohol extractive values were determined using Soxhlet apparatus. About 100gm of powdered plant material was used for each geotype and the yields recorded. The ash values were determined by incinerating 2gm of the powdered plant material using the British pharmacopoeia protocol [46].The results were recorded.

2.2.1.3 Determination of presence of volatile oils

Clavenger-like apparatus was used .To a 100gm of the powdered material of each geotype was added 300ml of water in round-bottomed flask of the apparatus. This was connected to a volatile oil determination tube and then the latter connected to a reflux condenser. Water was added through the top of reflux condenser until the graduated tube of the volatile determination tube was filled and overflew to the round-bottomed flask. The flask was gently heated to boiling for about 5 hours until the volume of oil did not increase. Heating was stopped and after cooling the volatile oil determination tube was observed for the presence of volatile oil.

2.2.1.4. Determination of microscopic characteristics of *Marsedenia sylvestris*

2.2.1.4.1 Leaf surface examination

A fresh leaf section of the lamina with midrib was cut with a stainless steel surgical blade. Depending on whether it was the top epidermis or the lower epidermis to be examined, the cuticle of the opposite side was removed to allow light to pass through. The sample was mounted on the slide and treated with a few drops of chloral hydrate, the clearing reagent and warmed over a paraffin lamp flame until dry. Thereafter a few drops of water were added, the slide viewed and the observation recorded.

2.2.1.4.2. Test for lignification

A transverse section of the fresh leaf lamina was mounted on a slide after removing the upper layer. Lignification normally occurs in vascular bundles and associated fibres which are readily visible in a transverse section [3]. A few drops of alcoholic phloglucinol was added and allowed to stand for 2 minutes. Any excess alcohol was removed by filter paper. Concentrated hydrochloric acid was added and the slide was viewed microscopically.

2.2.1.4.3. Powdered drug examination and transverse section

A small amount of the powder of the three geotypes was mounted on a slide. A few drops of chloral hydrate, a clearing agent was added and then dried over a lamp flame. A few drops of

water were added, the slide viewed and the results recorded. A transverse section was cut. It was treated as above with chloral hydrate to clear and examined.

2.2.2 Phytochemical screening of M. sylvestris geotypes plant material

2.2.2.1 Test for alkaloids

About 1gm of *M. sylvestris* (M/S) leaf powder was mixed with 5 ml of 10 % sulphuric acid, warmed in a water bath for two minutes and filtered with Whatman No 1, θ18 filter paper. Then two drops of Mayer's reagent was added to 1ml of the filtrate. A white to buff precipitate would indicate the presence of alkaloids or nitrogen-containing compounds [3]. The rest of the filtrate was alkalinized using dilute ammonia and extracted with 2 ml chloroform. Then chloroform was evaporated off to leave a solid residue which was dissolved in 0.2 ml of 10% sulphuric acid. Dragendorff's reagent was added. The presence of an orange-red precipitate would confirm the presence of alkaloids or nitrogen-containing compounds [3].

2.2.2.2 Test for saponins

A little amount of *Marsedenia sylvestris* leaf powder was placed in a test tube and water was added. The mixture was vigorously shaken and left to stand. Foaming and frothing would indicate the presence of soap-like substances known as saponins [3]. Then 0.2gm of the powdered plant material were extracted in 10 ml warm water and filtered. Two test tubes were each put 2ml solution of 1.8% Nacl and to one of these was added 2ml distilled water and to the other 2ml of the plant extract.

Sprague-Dawley rat was sacrificed by cervical dislocation and blood was immediately put in coagulation vacutainers with sodium citrate. One drop of blood was added to each tube and the tubes inverted gently to mix the contents. Hemolysis in the tube would indicate the presence of steroidal and triterpenoid saponins [45]

2.2.2.3 Test for tannins

One gm of *M. sylvestris* was mixed with 10 ml of water and the mixture boiled in water bath. After filtering, to a 2 ml portion of the filtrate was added three drops of ferric chloride solution. If a brown-green precipitate formed this would indicate the presence of

hydrolysable tannins. A blue colour would indicate the presence of condensed tannins [44] . To another 2 ml portion was added 1ml of lead subacetate. A creamy-brown precipitate would indicate the presence of tannins [3].Then 4ml of the extract was shaken in 4ml of 10% ammonia solution. The formation of an emulsion would indicate the presence of hydrolysable tannins [44]

2.2.2.4 Test for glycosides

2.2.2.4.1 Test for cardiac glycosides

Two types of tests were used namely, the Kedde test and the Keller-Kilian test. One gm of *Marsedenia sylvestris* leaf powder was mixed with 10 ml of 70 % alcohol and heated for about two minutes in a water bath at 70°C. Filtration was done and after which, 10 ml of water and five drops of a strong solution of lead subacetate was added to the filtrate and filtered. To the filtrate, 10 % H_2SO_4 was added drop wise until no further precipitation occurred. The resultant solution was filtered and extracted with two successive 5 ml portions of chloroform. The two chloroform extracts were combined, washed with 1 ml of water and filtered. It was then divided into two equal portions in petri dishes and evaporated to dryness in an oven. The two extracts were then subjected to the following tests, namely Kedde test and Keller-Kilian test.

Kedde test

Two drops of Kedde's reagent were added to one of the dried extracts. A faint purple colour would indicate the presence of cardiac glycoside, whose aglycone has unsaturat lactone ring [3].

Keller-Kilian Test

About 0.4 ml of glacial acetic acid containing a trace amount of ferric chloride was added to the second extract. It was shaken gently to cause dissolution and then 0.5 ml of concentrated H_2SO_4 was added very carefully. A reddish-brown colour at the interface which gradually turned a distinctive blue-green wound indicate the presence of de- oxy sugars [3].

2.2.2.4.2 Test for anthracene glycosides

Borntragger test

About 0.5 gm of the *M. sylvestris* leaf powder was boiled in 5 ml dilute H_2SO_4 for 5 minutes. The extract was then filtered while hot, cooled and then an equal amount of carbon tetrachloride was added. After shaking in a separating funnel two layers formed. A few drops of dilute ammonia were added to the organic layer. Lack of no visible change would have indicated the absence of thracene aglycone and the presence of a characteristic red colour in the ammonia layer the presence of anthracene aglycones.

Modified Borntragger test

0.5 gm of the *Marsedenia sylvestris* leaf powder was treated in a similar manner as the borntragger test except that a few drops of 5 % ferric chloride were added when boiling the powder with water. A rose pink to red colour in the ammonia layer would have indicated the presence of the nthracene aglycones in the reduced state [3].

2.2.3 Extraction of the plant material

1kg of the plant material was packed in a fine cotton bag and inserted into the Soxhlet extraction chamber (Fig 4). Three litres of the methanol solvent were added and extraction was done until the materials were exhausted. This was indicated by the solvent in the extraction chamber turning colourless as chlorophyll was totally extracted. Total extraction took 44-48 hours. The extract was filtered first with cotton wool to remove large particulate insoluble matter such as cellulose and fibres then with Whatman filter paper No 1,150 mm θ.

The filtered crude extract was reduced to dryness under vacuum using rotary vacuum evaporator at temperatures below 80°c. The extract was dried in a Memmert incubator at 50°c and the yield

was determined. The extract was then stored in the cupboard at room temperatures. This was done for each geotype and the yields were computed.

FIGURE 4: EXTRACTION WITH SOXHLET APPARATUS

2.2.4 Determination of TLC and HPLC profiles of *Marsedenia sylvestris*

2.2.4.1 Thin layer chromatography of *Marsedenia sylvestris*

2.2.4.1.1 TLC of leaf powder

The method and protocol used was as reported by Natural Remedies Pvt. Ltd Research Centre Quality Control Department [37]. Fifty ml of mobile phase was made by mixing 35.5 ml chloroform, 7.25 ml methanol and 7.25 ml acetic acid. Pure chemicals and reagents were used to

avoid impurities. Fifty ml of absolute ethanol was added to 59 ml distilled water to make 50 % ethanol. Potassium hydroxide 11 % and 1N K0H were also made.

Five hundred mg of *Marsedenia sylvestris* leaf powder from each region was dissolved in 10 ml 50 % v/v ethanol. Two millilitres of 1N KOH were added and heated on a boiling water bath under reflux for an hour and then cooled. Then 1.8 ml concentrated HCl was added and heated on a water bath for 2 minutes. After cooling the pH was adjusted to 7.5-8.5 with 11% KOH .This solution was dissolved with equal amount of 50 % v/v ethanol and filtered. Ten µl of the extracts were applied on the TLC plates with Kieselgel 60 F_{254} as the stationary phase. TLC was run to 8 cms.

The plate was dried and viewed under UV light at wavelengths of 252 nm and 366 nm and spots marked. Thereafter the plate was sprayed evenly with vanillin in sulphuric acid. Then it was heated in a Memmert oven at 105°C for 5 minutes. A chromatogram was obtained.

2.2.4.1.2 TLC of *Marsedenia sylvestris* extract

2.2.4.1.2.1 Preparation of sample of the extract and running of TLC

All reagents were prepared as per TLC of the leaf powder.

Before hydrolysis.

Five hundred mg of each geotype *Marsedenia sylvestris* extract was dissolved in methanol.Ten µl of each were applied as a spot on the kieselgel coated TLC aluminium plates.

After hydrolysis.

One hundred mg of *Marsedenia sylvestris* extract from each region was dissolved in 10 ml of 50 % ethanol. Then 2 ml of 1N HCl was added and heated on a boiling water bath under reflux for an hour and then cooled. To this 1.8ml of concentrated HCl was added and heated on water bath for 2 minutes. After cooling the pH was adjusted to 7.5-8.5 with 11 % KOH. This was dissolved

with equal amounts of 50 % v/v ethanol and filtered. Ten µl of each solution was applied on the TLC plates.

All the samples were run together before (B) and after hydrolysis (H).This was repeated twice. Detection was done by observation under UV light at 254 nm and 366 nm. The plate was sprayed with vanillin in sulphuric acid. Then it was heated in a Memmert oven at 105°c for 5 minutes and chromatograms obtained.

2.2.4.2 HPLC of *Marsedenia sylvestris* leaf extract of the three geotypes

The method and protocol used is as reported by Natural Remedies Pvt Ltd Research Centre Quality Control Department [37].

2.2.4.2.1 Operating conditions and preparation of the sample

Gradient HPLC was done using two mobile phases namely acetonitrile:water (80:20)(eluent A) and potassium diphosphate : water (0.1:100) (eluent B). The eluation gradient was: % A=25-50 from 0 to 20 minutes at a flow rate of 1.5 ml per minute. Merck Hitachi Lachrom equipment was used with Zorbax Column XDB C 18Φ 4.6 mmx150 mm 5 micron as the stationary phase. The column temperature was maintained at 40° C and 20 µl of each geotype solution was injected. Detection was done at 210 nm using a UV detector.

About 0.75 gm of each geotype leaf extract was weighed and each was separately dissolved in 50 % ethanol to make 50 ml. To 10 ml of this solution was added 2 ml of 12 % KOH and heated on a boiling water bath for 1 hour. After cooling 5.5ml of 4N HCl was added and heated on a boiling water bath for 1 hour. This was then cooled and 12 % KOH was added to make a pH of between 7.5 and 8.5. Thereafter 50 % ethanol was added to make 100 ml. This solution was filtered by Whatman filter paper and subjected to HPLC.

2.2. 5 Determination of hypoglycemic effects of Marsedenia sylvestris

2.2.5.1 Testing for hypoglycemic activity in rats

2.2.5.1.1 Preparation of reagents and *M. sylvestris* extract stock solution

A stock solution of 50 % w/v was made by dissolving 50 gm of glucose in 100 ml distilled water with slight warming to increase solubility. Normal saline was prepared to act as the negative control. Glibenclamide 5 mg tablets (Doanil®) was dissolved in 3.25 ml normal saline to act as the positive control for each rat.

Stock solutions of 200 mg/ml were made by dissolving 4 gm of the extract for each respective geotype in 20 ml mixture of one drop DMSO, 1 ml 50 % ethanol and normal saline. In a similar manner stock solutions of the extracts of 50 mg/ml and 12.5 mg/ml were prepared by dissolving 1gm and 0.25 gm of the extracts respectively. From these stock solutions three concentrations were made to give the required doses of 2000 mg, 500 mg and 125 mg per kg of body weight.

2.2.5.1.2 Experimental

Thirty Sprague-Dawley rats were obtained from the University of Nairobi, School of Pharmacy animal house where they had been housed and fed with rat pellets and water *ad libitum*. They had been fasted overnight and their weight and blood glucose levels were taken. The doses of glucose and *Mersedenia sylvestris* extract stock solution were computed for each animal based on their body weight. The dose of glucose required for oral glucose tolerant test (OGTT) was 5 mg/ gm of body weight. A pilot study was initially carried out to optimize experimental conditions. The extract, the positive and negative control were administered by oral gavage to each respective rat. After one hour blood sugar levels were taken for each animal and then immediately given the respective glucose dose. Thereafter, the blood glucose levels were assayed at one hour intervals and the mean glucose levels were computed.

2.2.6 Determination of other pharmacological effects of *M. sylvestris*

2.2.6.1 Effect of *M. sylvestris* (Kisumu) plant extract on isolated rat diaphragm-nerve preparation

Marsedenia sylvestris extract from Kisumu (MSK) was used for all organs pharmacological studies.

2.2.6.1.1 Tissue and extract preparation

A 300 gm rat was sacrificed by cervical dislocation. It was left to bleed as much as possible to ensure the thorax was freed of blood. Dissection of the chest was done and the rat diaphragm muscle with the phrenic nerve was removed and transferred to a Petri dish containing Krebs Hensleit Physiological Solution at 37°C. The composition of the physiological solution was as follows in mg/ litre: NaCl 6.87; KCl 0.40; CaCl 0.28; $MgS0_4$ 0.14; H_2PO_4 0.16; $NaHCO_3$ 2.1 and Glucose 2.0. The organ was mounted in an organ bath filled with the physiological solution and coupled to an electric physiological recorder, the oscillograph 2-channel recorder, Gemini 7070. It was continuously bubbled with a mixture of 95 % O_2 and 5 % CO_2. Three concentrations of the MSK extracts of 100 mg in 1000 ml, 100 mg in 50 ml and 100 mg in 10 ml were prepared in normal saline.

2.2.6.1.2 Experimental

The organ was exposed to various concentrations of the extract in the following order: one exposure of 0.5 ml of 10 mg/ml, one exposure of 1 ml of 10 mg/ml, two exposures of 1 ml of 2 mg/ml and two exposures of 1 ml of 0.1 mg/ml. The results of the dose response relationship were recorded and the amplitude of the contractions computed before and after the administration of the extract.

2.2.6.2 Effects of *M. sylvestris* Kisumu (MSK) extract on isolated rat uterus

2.2.6.2.1 Tissue and the extract preparation

A young virgin female rat weighing 150 gm was injected intraperitoneally with 0.1mg stilboestrol 0.1 % 48 hours before it was sacrificed. This was to increase the sensitivity of the

uterus. The animal was sacrificed by a blow on the head and the uterine horns located and dissected out into a dish containing De Jalon ringer solution. The horns were separated from the animal just below the ovaries, cleaned and extraneous fat and connective tissue removed. The horns were separated at the bifurcation to yield two preparations.

A stock solution of MSK extract with strength of 100 mg in 10 ml was prepared. Normal saline was used as the solvent with one drop of DMSO and 1ml of 50% v/v ethanol to increase solubility.

2.2.6.2.2 Experimental

The organ was mounted on an organ bath and maintained at 37°C. It was first exposed to three doses of 0.1 ml acetylcholine 10 µg/ml equivalents to 1 µg and the tissue movements recorded. Thereafter, the organ was exposed to various concentrations of the MSK extract in the following order: 0.1 mg, 1 mg, 2 mg, 4 mg and 10 mg. The tissue movements were recorded.

2.2.6.3 Effects of *M. sylvestris* Kisumu (MSK) extract on the isolated rabbit heart

2.2.6.3.1 Preparation of the extract and the isolated tissue

A stock solution of MSK extract with strength of 100 mg in 10 ml was prepared. Normal saline was used as the solvent with one drop of DMSO and 1 ml of 50% v/v ethanol to increase solubility. Langendroff method was used to investigate the effects of the crude MSK extract on the cardiac muscle and on the coronary circulation without the modifying influences of the blood pressure. The Langendroff apparatus was set up and it was ensured that the perfusion fluid kept flowing to prevent the formation of trapped air bubbles which might cause air embolism. The temperature was maintained at 38°C.

A rabbit was sacrificed by a blow on the head and the chest wall and the rib cage quickly opened. The whole heart was removed and it was ensured that at least 1 cm of the aorta was left intact. The isolated heart was immediately placed in a dish of warm oxygenated Locke solution and

gently squeezed to wash out blood so that it did not clot inside the heart. The heart was freed of the extraneous tissue and immediately attached through the aorta onto the cannula at the base of the Langendroff apparatus, ensuring that no air bubbles were trapped in the heart.

The rate of perfusion and oxygenation was adjusted until the heart was beating satisfactorily. Then the ventricle end was hooked and attached by a thread through a pulley system to a starling heart lever. The normal heartbeat was recorded on a Harvard recorder.

2.2.6.3.2 Experimental

The organ was exposed to various concentrations of the extract by administering the drug through the rubber cap just above the cannula. The organ was exposed to various concentrations of the MSK extract in the following order:1mg, 2 mg and 4mg. The tissue movements were recorded.

2.2.6.4 Effects of *M. sylvestris* Kisumu (MSK) extract on isolated rabbit ileum

2.2.6.4.1 Preparation of the extract and the tissue

A stock solution of MSK extract with strength of 100 mg in 10 ml was prepared. Normal saline was used as the solvent with one drop of DMSO and 1 ml of 50 % v/v ethanol to increase solubility.

A rabbit was sacrificed by a blow at the back of the head to break the spinal cord. The abdomen was exposed and pieces of the jejunum were removed and put in an aerated Tyrode's solution. A piece of the jejunum was mounted on an organ bath containing aerated Tyrode's solution.

2.2.6.4.2 Experimental

The tissue was exposed to various concentrations of the extract in the following order: 2 mg, 4 mg, 8 mg, 10 mg and 20 mg. The ileum was washed three times and fresh solution introduced and given 3 minutes to recover after each drug administration. This was repeated with a time cycle of minutes. The tissue movements were recorded.

2.2.6.5 Diuretic effects of *M. sylvestris* Kisumu (MSK) extract in rats

2.2.6.5.1 Preparation of the materials and reagents

A stock solution of MSK extract with concentration of 100 mg in 10 ml was prepared. Normal saline was used as the solvent with one drop of DMSO and 1 ml of 50 % v/v ethanol to increase solubility. Sixteen Sprague-Dawley rats were starved for 24 hours before the experiment.

2.2.6.5.2 Experimental

The sixteen Sprague-Dawley rats were divided into four groups: A, B, C, D. Each animal was loaded by gavage with 5 ml warm water per 100 gm body weight. After 30 minutes the animals in group A received 333 mg / kg b.w. and Group B was given 66.6 mg / kg b.w. Group C was teated with frusemide 20 mg /kg b.w. and group D received normal saline to act as the negative control. Each group of the rats was placed in a metabolic cage. Then urine was collected from each group every ten minutes for ninety minutes and the results recorded. The pH of the urine was noted.

2.3 Data analysis

For the pharmacological studies descriptive and inferential data analysis was done using Microsoft excel software.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Macroscopic evaluation

3.1.1 Morphological evaluation

Morphological characteristics of the fresh plant materials for each geotype were generally the same. *Marsedenia sylvestris* plant was a much-branched woody shrubby climber with soft hairs. The leaves were green in colour, elliptic or ovate with an acute or acuminate apex (Figs.5).The leaves were opposite measuring about 3-5 cm by 1-2 cm. The dimensions were similar in the three regions sampled save for a few differences in size which may have been due to the fertility of the soil or other factors The leaves of young shoots were always larger. The lower surface of the lamina was more pubescent than the upper and the leaf had a rounded or cordate base.

The flowers were yellow in colour and formed short-stalked cymes. The petals were short tubes and hairy within, with the conspicuous corona appearing as fleshy lobes between the petals. The stamens were fused throughout and the pollinia were minute, erect and solitary in each anther cell. The stigma stuck out of the staminal column. The fruits were long pods about 5-10cm long with a characteristic rams-horn shape (Fig 5). The plant was many times found growing alongside *Harrisonia abyssinica* and *Lantana camara.*
These descriptive features and pictures were in agreement with the ones given earlier by Kakote and Agnew and confirmed the presence of this plant in Kenya [19, 20].

1= Fruit; 2 = Flowers; 3 and 4 = Leaves

FIGURE 5: *MARSEDENIA SYLVESTRIS* BUSH IN GEDE FOREST, MALINDI

3.1.2 Organoleptic characterization

The colour of the leaves were green, it was slightly aromatic or nearly odourless and slightly bitter with a remarkable property of paralyzing the taste glands for a few moments against sweet taste. These features were similar in the three geotypes examined. This is as reported by literature [19, 36]

3.1.3 Determination of foreign matter, ash and alcohol extractive values

The plant materials were examined to determine the presence or absence of foreign materials. It was observed that foreign matters were less than 1% and insect's infestation and rodent contamination were nil. These results were similar for the three geotypes.

The fine powders for each region were analyzed for the ash and alcohol extractive values which are presented in Table 1.

TABLE 1: ASH AND ALCOHOL EXTRACTIVE VALUES OF *M. SYLVESTRIS* LEAF POWDER

	MSK	MSA	MSG
Ash content %	14	13.95	14.2
Alcohol soluble extractives %	25	25	24.4

MSK= *M. sylvestris* Kisumu; MSA= *M. sylvestris* Arabuko; MSG= *M. sylvestris* Gede

This was consistent with the literature [1, 19] .The geotypes had similar values.

3.1.4 Determination of volatile oils

Using Clevenger-type, apparatus the presence of volatile oils was confirmed by observation of a few tiny oil droplets atop the aqueous layer at the top of the determination tube. This was observed for the three geotypes and explained the mild aromatic smell of the leaves.

3.2 Microscopic examination of plant materials

3.2.1 Leaf surface examination

The top epidermis was found to have polygonal cells with slightly wavy smooth walls (Fig.7). Covering or non-glandular trichomes and the cicatrices where the trichomes had been attached were visualized (Figs.6, 7). There were no stomata and the palisade cells were closely packed (Fig.6). The lower epidermis had more trichomes and cicatrices with numerous small paracytic stomata than the upper epidermis. Cluster crystals of calcium oxalate were seen (Fig.6). The three geotypes were found to be the same. The palisade cells were formed one layer on both the upper and lower surface of the leaf. This compares very well with the literature [19].

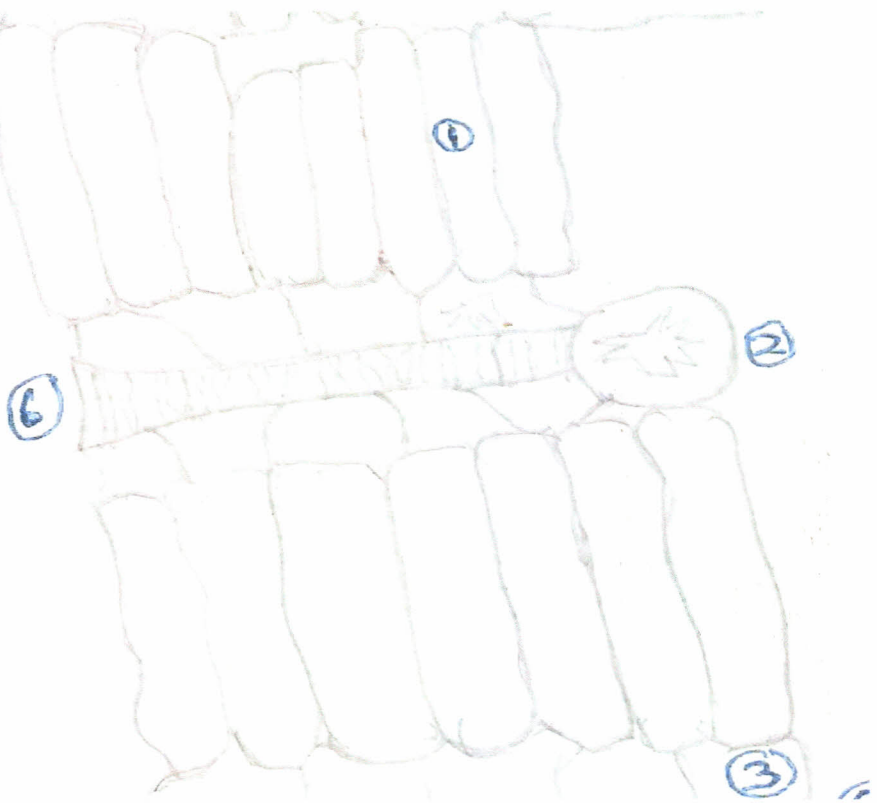


FIGURE 6: TRANSVERSE SECTION OF *MARSEDENIA SYLVESTRIS* TYPICAL LEAF

1; Lower and upper palisade cells. 2; Cluster of calcium oxalate crystals

3; Lower and upper epidermis. 4: Stomata on lower epidermis 5; Cuticle. 6; Fibres.

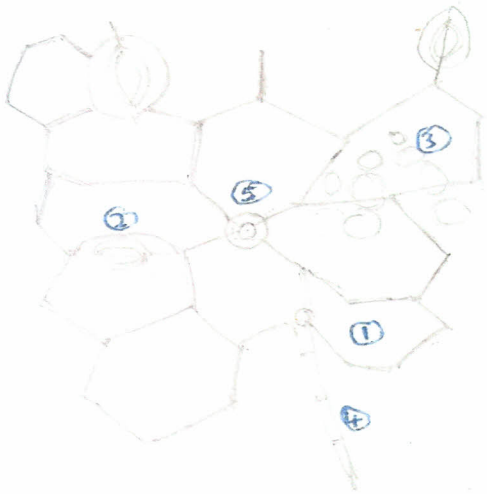


FIGURE 7: LOWER EPIDERMAL SURFACE OF *MARSEDENIA SYLVESTRIS* TYPICAL LEAF

1: Polygonal cells. 2: Paracytic stomata. 3: Underlying palisade cells.

4: Non-glandular trichome. 5; Cicatrix

3.2.2 Test for lignification

Transverse section of a leaf lamina was used.

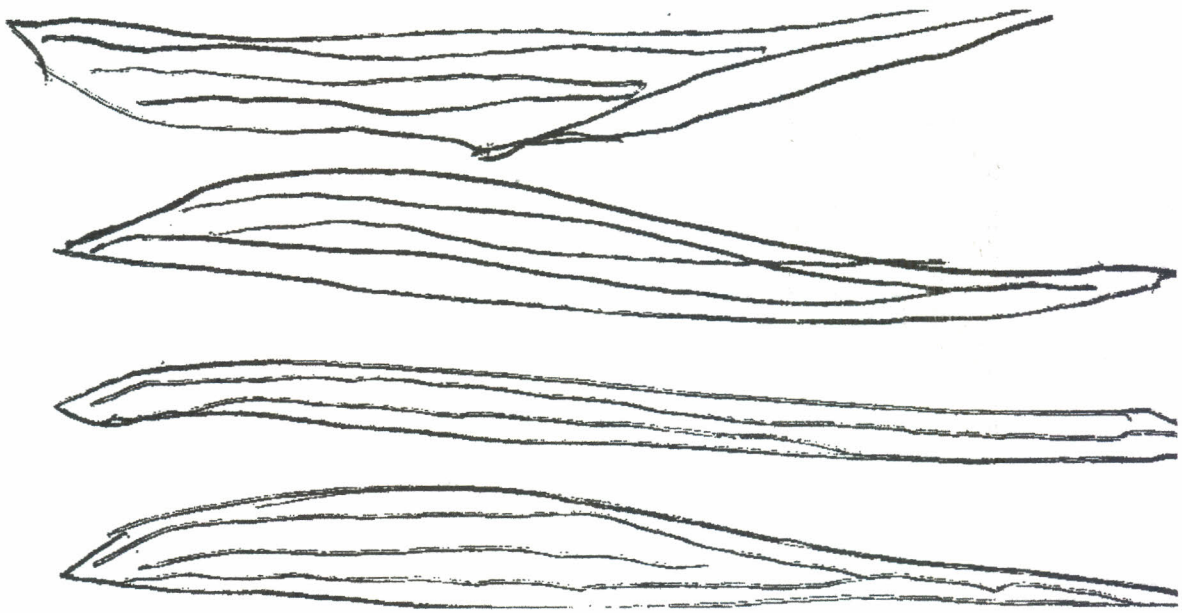


FIGURE 8: TRANSVERSE SECTION--- BUNDLES OF PINK/RED FIBRES OF LIGNIFIED TISSUE

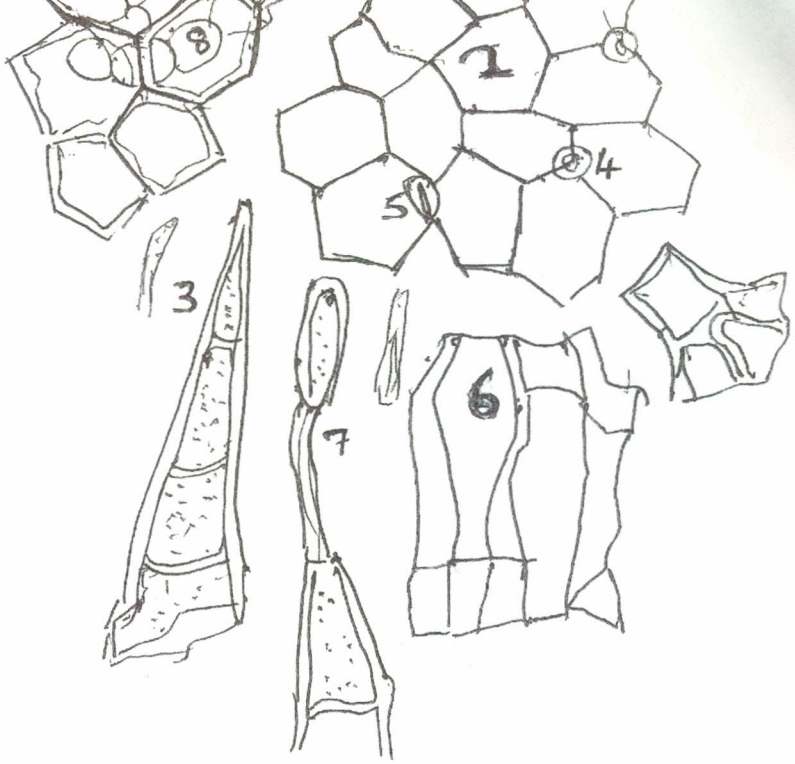


FIGURE 9: WHOLE POWDERED DRUG OF *MARSEDENIA SYLVESTRIS* LEAF

1: Upper epidermis view and underlying palisade cells.

2: Lower epidermis showing polygonal cells and paracytic stomata.

3: Non-glandular trichomes. 4: Cicatrix. 5: Paracytic stomata. 6: Cortical parenchyma.

7: Trichome showing collapsed cell. 8: palisade cells.

3.2.3 Examination of powdered leaf

Many multicellular non-glandular trichomes were seen (Fig.9). They were found attached to fragments of the epidermis and many others scattered singly or in dense felted masses. They were conical and almost straight and tapering gradually towards the apex. The walls were moderately thickened and the lumen was visible. Paracytic stomata were also seen.

3.2.4 Leaf transverse section

The leaf was treated with chloral hydrate and examined. It was observed that the plant had a lot of multicellular non-glandular trichomes which were more abundant on the lower side (underneath) of the lamina. Trichomes which are also called the plant hairs were tubular elongated outgrowths of the epidermal cells and consisted of root in the epidermis and body outside the epidermis. These trichomes were not lignified and had 3-6 cells each. The palisade cells were in one layer but on both sides of the leaf. The fibres and calcium oxalate crystals were visualized in the midrid (Fig. 10).

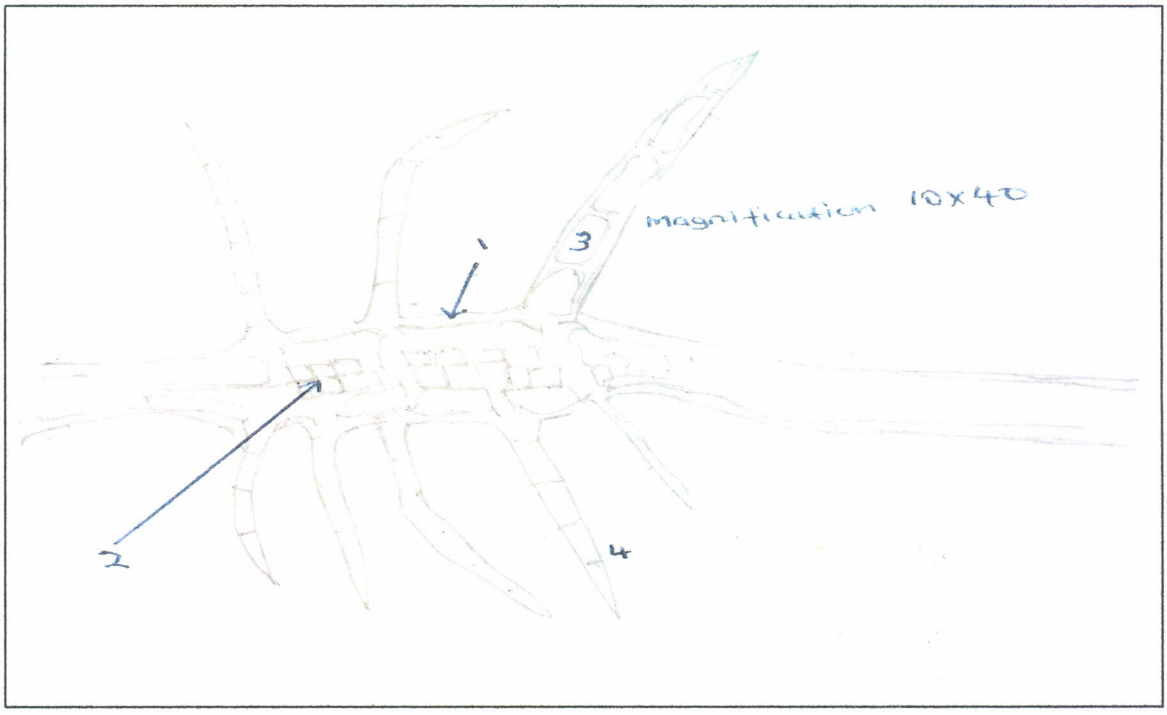


FIGURE 10: A SKETCH TRANSVERSE SECTION THROUGH THE MIDRIB

1 Epidermis. 2; Vascular bundles; 3-4; Multicellular non-glandular trichomes

3.3 Phytochemical test results

The three geotypes gave the same results on phytochemical screening indicating that their phytochemical composition is similar. Alkaloids, saponins, tannins and cardiac glycosides were detected (Table 2).The presence of alkaloids or nitrogen-containing compounds was demonstrated by the formation of a white to buff precipitate with Mayer's reagent and an orange-red precipitate with dragendorff's reagent. Alkaloids are organic products which are basic in nature and contain one or more nitrogen atoms, normally of heterocyclic nature and possess significant physiological actions on the human body [19]

Saponins are glycosides characterized by their property of producing a frothing solution. They have haemolytic properties and are used as fish poisons. The presence of saponins was demonstrated first by persistent frothing on vigorous shaking of the plant materials in aqueous solution. Secondly the hemolytic effect of the plant extract on rat fresh blood demonstrated the presence of either or both steroidal and triterpenoid saponins. The presence of hydrolysable tannins was indicated by the formation of a brown-green precipitate with ferric chloride and the formation of an emulsion with 10% ammonia solution. [3].Tannins are secondary metabolites containing complex organic substances in which polyphenols are present and thus have the capacity to combine with tissue proteins and precipitate them [19]. They are therefore used in medicines as mild antiseptics, in treatment of poisonings and diarrhoea and stopping small haemorrhages.

Cardiac glycosides presence was indicated by formation of a faint purple colour with Kedde's reagent and a reddish-brown colour at the interface in the Keller-Kilian Test. These results are in conformity with earlier ones reported in literature [22-25]. No anthracene glycosides were however

demonstrated by both the ordinary and modified Borntragger tests. This is a significant observation because literature review shows that the Indian species has anthraquinones. The Kenyan geotypes seem to be different in this respect. Anthracene glycosides are associated with numerous adverse effects of plants.

TABLE 2: PHYTOCHEMICAL COMPONENTS OF *M. SYLVESTRIS* GEOTYPES

Alkaloids	+
Saponins	+
Tannins	+
Cardiac glycosides.	+
Anthracene glycosides	–

Glycosides exert therapeutically significant effects on humans and animals and many plants containing them are used in traditional and conventional medicines due to their cardiotonic, purgative, analgesic, anti-rheumatic and other useful actions [19]

This very diverse phytochemical composition of *M. sylvestris* explains and validates the wide ethnopharmacological use of the plant. Such traditional uses are for stomach ailments, constipation, water retention and liver diseases [21]. It has also been used for conditions such as malaria, snakebites, asthma, eye complaints, inflammation and family planning.

3.4 Yields on extraction

The yields on methanolic extraction showed minimal variations with the Kisumu geotype giving a slightly higher yield. The yields were very high and ranged from 24 to 30 % as shown in table 3 below;

TABLE 3: YIELDS OF METHANOL EXTRACT OF *M. SYLVESTRIS* PLANT MATERIAL

Plant material 1Kg	**Extract yield Gm**	**Yield %**
MSK1	250.50	25.05
MSK2	296.82	29.68
MSK3	259.46	25.95
MSG	248.96	24.90
MSA	259.00	25.90

MSK = *M. sylvestris* Kisumu. MSA= *M. sylvestris* Arabuko. MSG= *M. sylvestris* Gede

3.5 TLC of *Marsedenia sylvestris* extract

TLC is used in the detection and monitoring of compounds through a process that occurs due to the differences of the two underlying principles of adsorption and partition equilibrium [33, 35].The Rf values, being the distance traveled by the component divided by the solvent front is then very characteristic of the particular compound. Other factors such as the stationary phase, the mobile phase, nature, composition and purity of the solvent together with environmental factors being the same, the Rf values depend on the polarity of the substance being resolved [34]. Silica gel 60 F_{254}

was used as the stationary phase and polar compounds showed a higher affinity for the stationary phase and therefore moved slowly up the plate as the solution migrated (Figs.11-13). They therefore showed relatively small Rf values. The non-polar compounds had less affinity for the stationary phase and moved comparatively quickly up the plates and had a relatively larger Rf values.

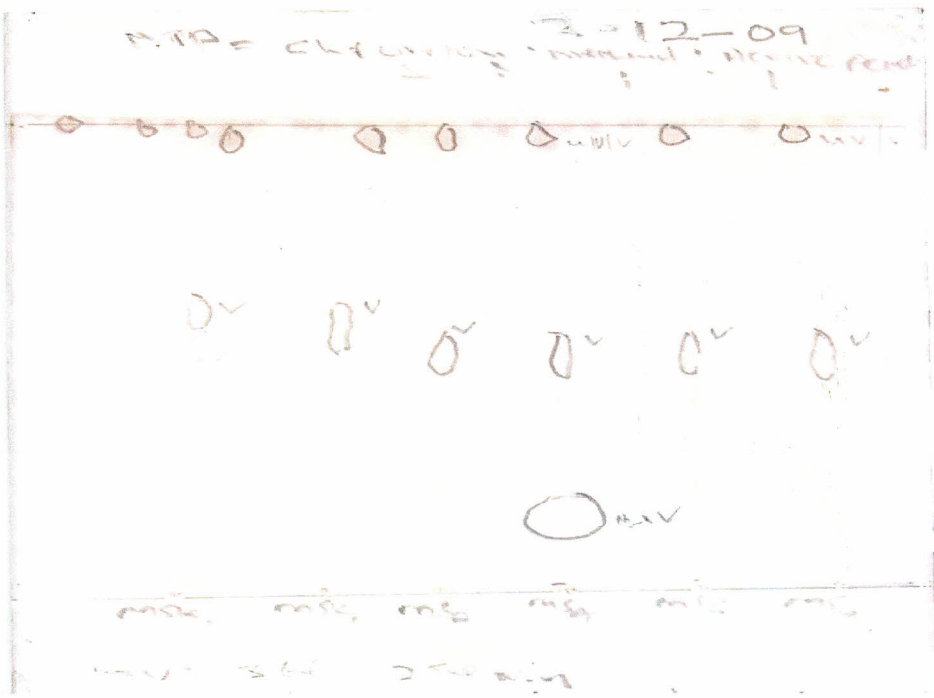


FIGURE 11: TLC OF *THE MARSEDENIA SYLVESTRIS* LEAF POWDER

V=detection after vanillin spray. UV=under UV light

All spots appeared under both wavelengths 254 nm and 366 nm.

Mobile phase: Chloroform, Methanol and Acetic acid (3: 1: 1).

Stationary phase: Silica gel $60F_{254}$. Samples: 2 MSK, 2MSA, 2MS

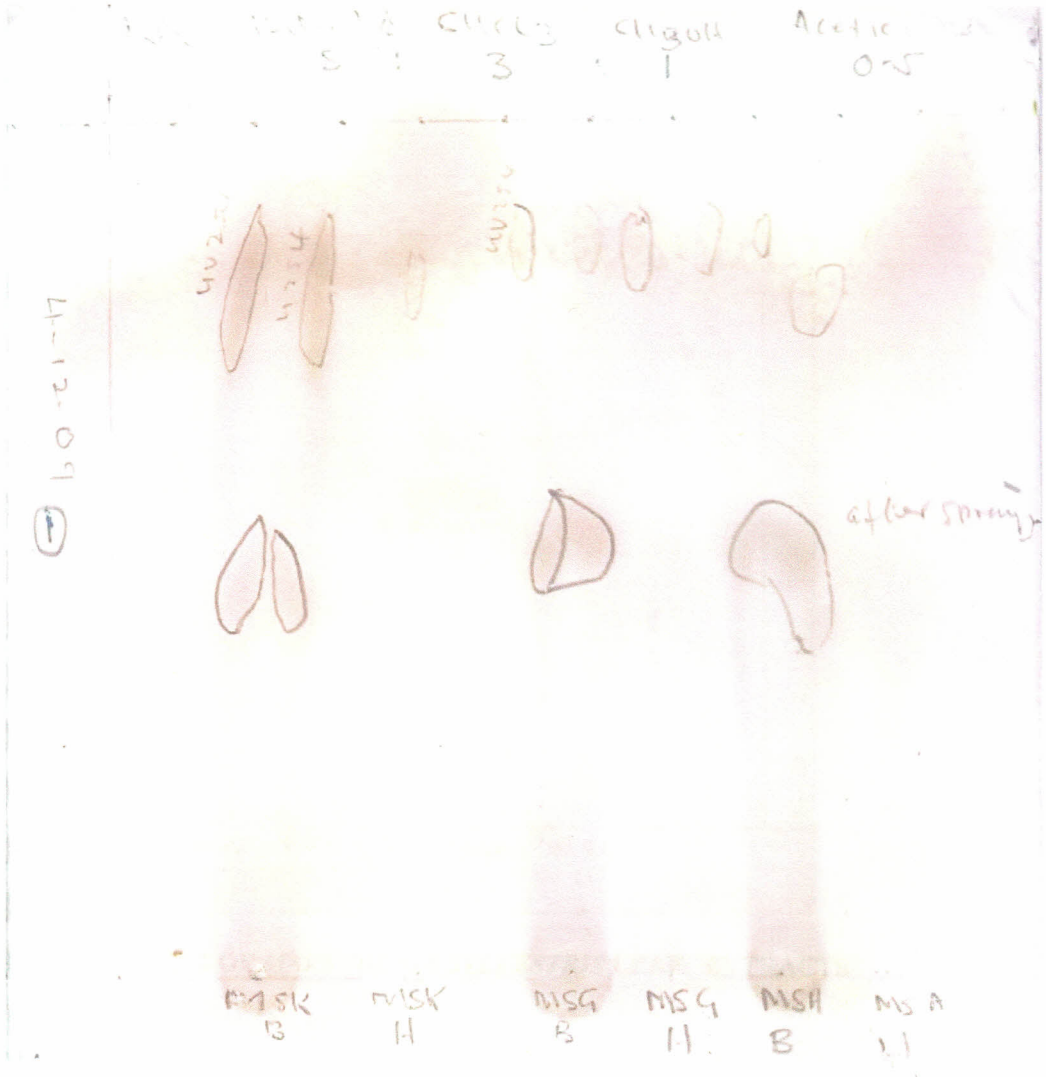


FIGURE 12: TLC 1 OF *MARSEDENIA SYLVESTRIS* LEAF EXTRACTS

TLC chromatogram 1: B = before hydrolysis. H = after hydrolysis

Mobile phase: Isopropyl alcohol, Chloroform, Methanol and Acetic Acid (5:3: 1: 0.5)

Stationary phase: Silica gel $60F_{254}$.

Samples: MSK, MSG, and MSA before and after hydrolysis.

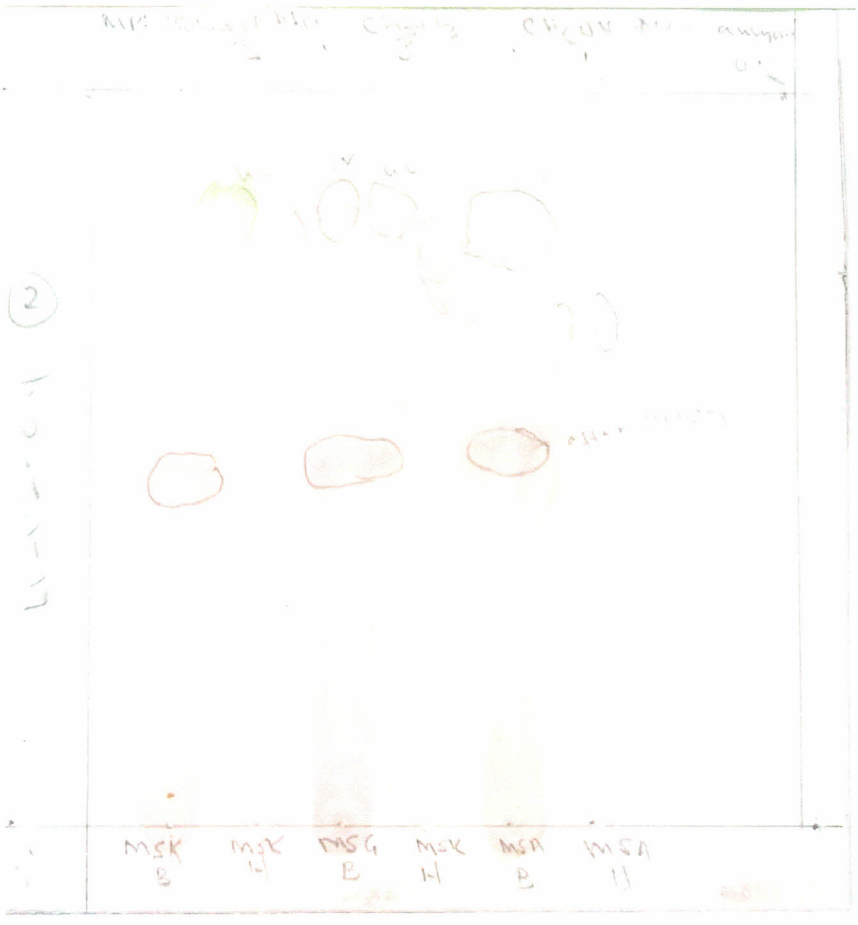


FIGURE 13: TLC 2 OF *MARSEDENIA SYLVESTRIS* LEAF EXTRACTS

TLC Chromatogram 2:

B = before hydrolysis. H = after hydrolysis.

Mobile phase: Isopropyl alcohol, Chloroform, Methanol and Acetic acid (5:3: 1: 0.5).

Samples: MSK, MSA, MSG before and after hydrolysis.

The polar compound showed Rf values of approximately 0.52 and 0.48 for the crude drug and the extract respectively. The non-polar compounds showed Rf values of 0.96 and 0.83 for the crude drug and the extract respectively (Figs. 11-13). The retention characteristics of the three extracts of *Marsedenia sylvestris* geotypes indicate that they have similar chemical compounds. They were all detected with UV light at 254 nm and 366 nm. After hydrolysis no spots appeared in the middle zone. The chromatograms obtained both for the crude drug extract and the leaf extract were similar for the three geotypes.

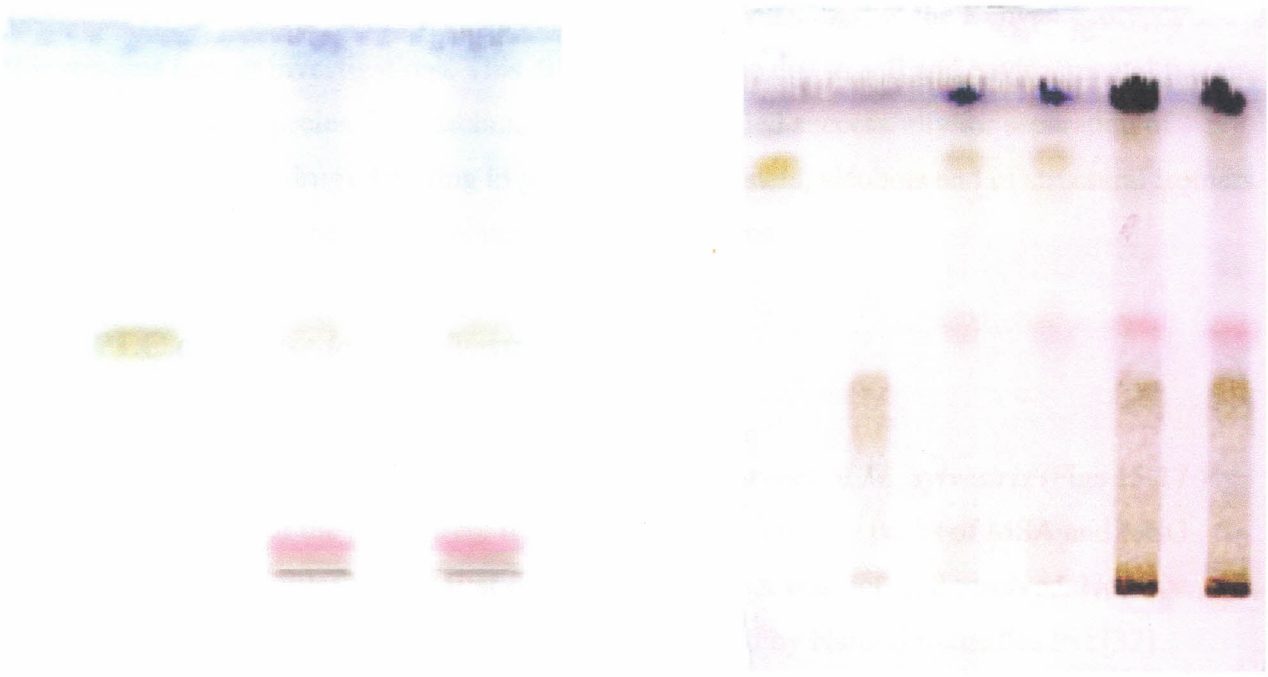


FIGURE 14 TLC *OF M. SYLVESTRIS* BY NATURAL REMEDIES RESEARCH CENTRE PVT LTD

S – Gymnemagenin (Reference Standard)
T1 - *Gymnema sylvestris* **reference sample**
T2 - *Gymnema sylvestris* **test sample**

S1 - Gymnemagenin (Reference Standar
S2-Gymnemic acid (Reference Standard)
T1 & T2 - *Gymnema sylvestris* extract **(after hydrolysis)**
T3 & T4 - *Gymnema sylvestris* extract **(before hydrolysis)**

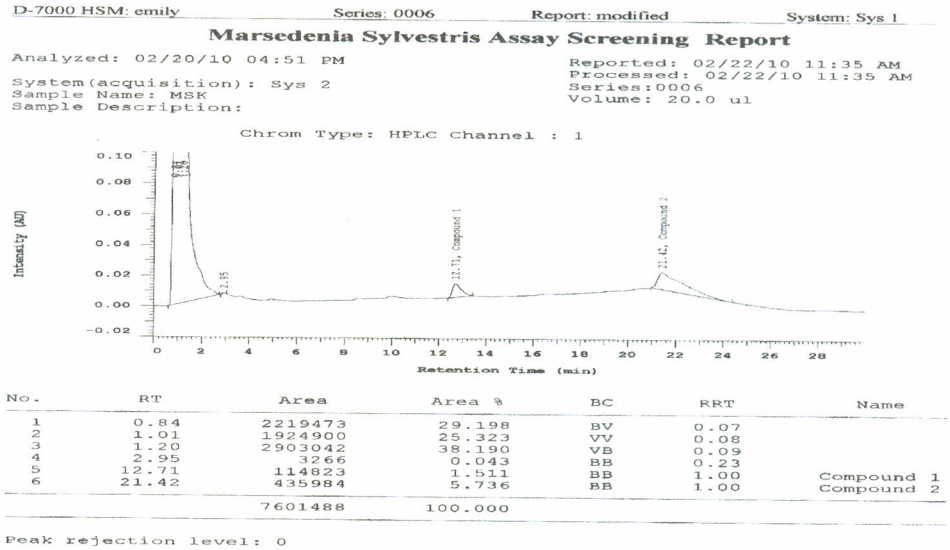
A comparison of our chromatograms with the ones obtained by Natural Remedies Pvt shows that our polar compounds are the gymnemic acids, reference standard S_2 and our non-polar compounds are the aglycone gymnemagenin, reference standard S_1 (Figure 14). Hydrolysis broke down the gymnemic acids to the glycone and the non-polar aglycone gymnemagenin hence the disappearance of the polar spot. Reliable identification and confirmation should be done using spectroscopic investigations such as infrared, nuclear magnetic resonance and mass

spectrometry or coupling thereof with TLC [7]. However, the TLC chromatogram from Natural Remedies Pvt also shows some pink spots which are not visualized in the Kenyan geotypes and this requires further investigations. This difference may be due to soil and environmental factors or variations in the species. TLC technique has been applied successfully for separation of compounds in herbal drugs differing in polarity such as esters, alcohols and of structural isomers exhibiting different energies of interaction with the adsorbent [19].

3.6 HPLC of *Marsedenia sylvestris* extract

Gradient HPLC profiles were carried out on the three geotypes of *M. sylvestris* (Figs 15-17) The retention times of the peaks were recorded (Table 4). The key peaks of MSA and MSG extracts were very similar but the first peak of MSK extract was not well resolved. However, the HPLC profiles obtained were similar to the ones presented by Natural Remedies Pvt [37] (Figures 18- 19). Retention times were different as a column of different size and type was used. Natural Remedies used Wakosil 11 5C 18Φ 46 mm x 250 mm column and in this study Zorbax XDB C 18Φ 4.6 mm x 150 mm 5 micron as the stationary phase. The profiles for the MSK and MSA geotypes were very similar but the MSG profile was slightly different.

No direct HPLC was done on the Indian plant and the HPLC conditions of Natural Remedies Pvt were not identical to ours hence the differences. However this being a comparative study of Kenyan geotypes was adequate.



No.	RT	Area	Area %	BC	RRT	Name
1	0.84	2219473	29.198	BV	0.07	
2	1.01	1924900	25.323	VV	0.08	
3	1.20	2903042	38.190	VB	0.09	
4	2.95	3266	0.043	BB	0.23	
5	12.71	114823	1.511	BB	1.00	Compound 1
6	21.42	435984	5.736	BB	1.00	Compound 2
		7601488	100.000			

Peak rejection level: 0

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Column: Zorbax XDB C 18Φ 4.6 mm x 150 mm 5 micron
Mobile phase A: acetonitrile:water 80:20. B: potassium diphosphate: water 0.1:100
Flow rate: 1.5 ml per minute. Elution gradient: % A=25-50 from 0 to 20 minutes.

FIGURE 15: HPLC OF *MARSEDENIA* SYLVESTRIS (KISUMU) LEAF EXTRACT (MSK)

TABLE 4: RETENTION TIMES OF *MARSEDENIA SYLVESTRIS* LEAF EXTRACT

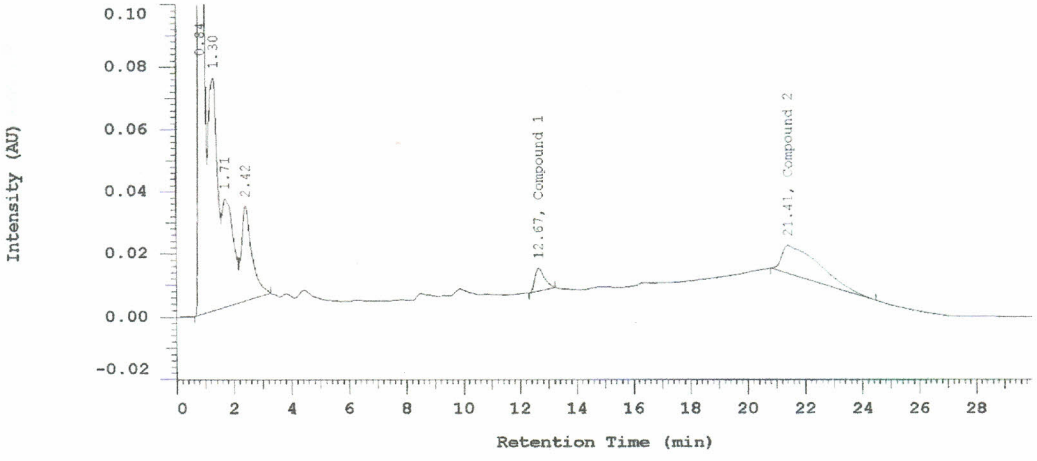
Extract	MSK	MSA	MSG
Peak No			
1	0.84	0.84	0.84
2	1.01	1.30	1.31
3	1.20	1.71	1.72
4	2.95	2.42	2.45
5	12.71	12.67	12.68
6	2242	21.41	16.36
7			21.40

D-7000 HSM: emily Series: 0006 Report: modified System: Sys 1

Marsedenia Sylvestris Assay Screening Report

Analyzed: 02/20/10 05:22 PM
Reported: 02/22/10 11:35 AM
Processed: 02/22/10 11:35 AM
System(acquisition): Sys 2
Series:0006
Sample Name: MSA
Volume: 20.0 ul
Sample Description:

Chrom Type: HPLC Channel : 1



No.	RT	Area	Area %	BC	RRT	Name
1	0.84	1855970	45.795	BV	0.07	
2	1.30	810456	19.997	VV	0.10	
3	1.71	486273	11.998	VV	0.13	
4	2.42	378250	9.333	VB	0.19	
5	12.67	83795	2.068	BB	1.00	Compound 1
6	21.41	438072	10.809	BB	1.00	Compound 2
		4052816	100.000			

Peak rejection level: 0

FIGURE 16: HPLC OF *MARSEDENIA SYLVESTRIS* (ARABUKO) LEAF EXTRACT (MSA)

The compound peak was separated by changing the buffer ratio to give better resolution of the polar compounds. The HPLC profiles produced were similar for the three geotypes (Appendix C, D, and E)

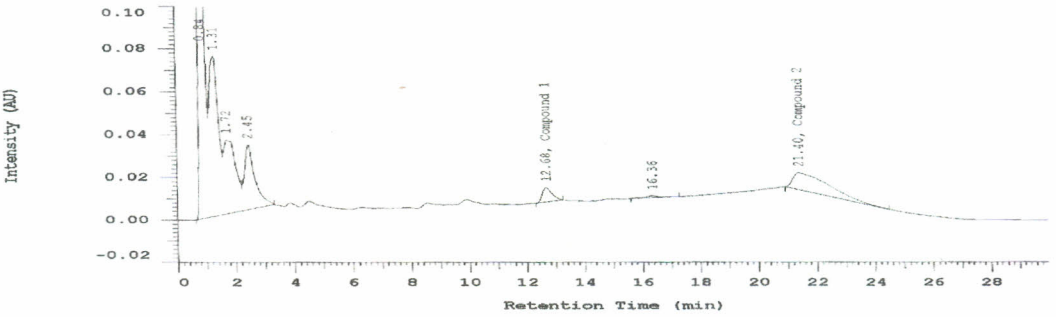
D-7000 HSM: emily Series: 0006 Report: modified System: Sys 1

Marsedenia Sylvestris Assay Screening Report

Analyzed: 02/20/10 05:53 PM
System(acquisition): Sys 2
Sample Name: MSG
Sample Description:

Reported: 02/22/10 11:35 AM
Processed: 02/22/10 11:35 AM
Series:0006
Volume: 20.0 ul

Chrom Type: HPLC Channel : 1



No.	RT	Area	Area %	BC	RRT	Name
1	0.84	1794280	45.326	BV	0.07	
2	1.31	813423	20.548	VV	0.10	
3	1.72	517292	13.068	VV	0.14	
4	2.45	378013	9.549	VB	0.19	
5	12.68	78136	1.974	BB	1.00	Compound 1
6	16.36	14597	0.369	BB	0.76	
7	21.40	362837	9.166	BB	1.00	Compound 2
		3958578	100.000			

Peak rejection level: 0

FIGURE 17: HPLC OF *MARSEDENIA SYLVESTRIS* (GEDE) LEAF EXTRACT (MSG)

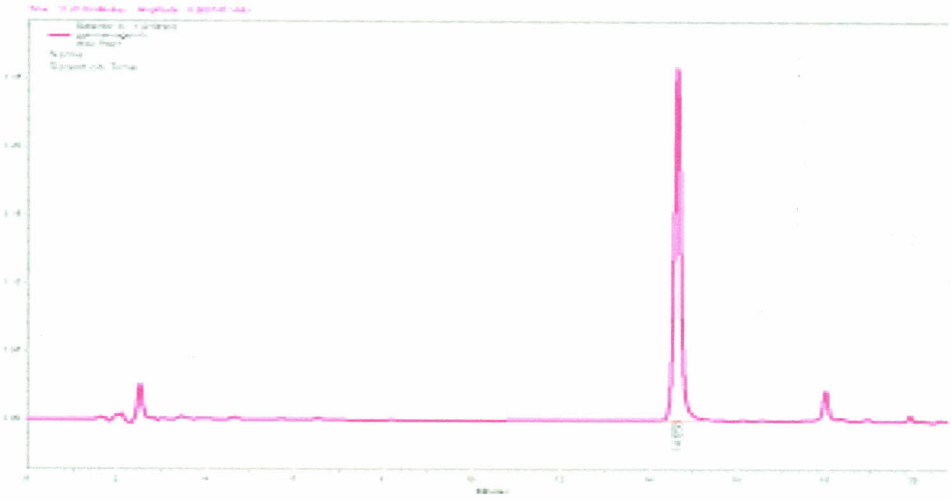


FIGURE 18: HPLC OF GYMNEMAGENIN BY NATURAL REMEDIES RESEARCH CENTRE PVT LTD

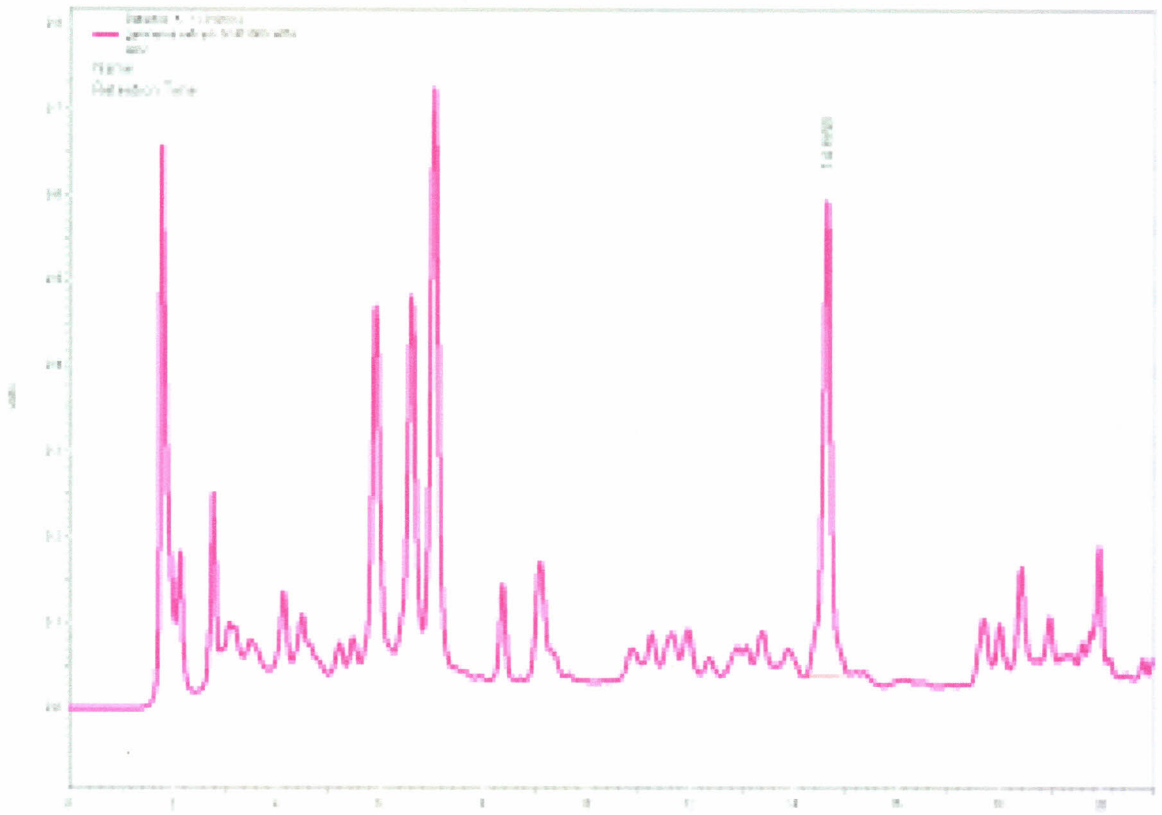


FIGURE 19: HPLC *OF M. SYLVESTRIS* BYNATURAL REMEDIES RESEARCH CENTRE PVT LTD

3.7 Hypoglycemic activity of *Marsedenia sylvestris* extract

M. sylvestris extracts and the crude drug were tested for hypoglycemic activity using glucose loaded Sprague-Dawley rats. Tables 5, 6 and 7 reported the effects of *M. sylvestris* extracts given by oral route at doses ranging from 125 to 2000 mg /kg b.w. on the blood glucose levels. The extracts induced the lowering of blood sugar in a dose –dependent manner (Fig. 20).This is in line with what is reported in literature by other workers [25, 36]. The effects of the three geotypes namely Kisumu (MSK), Arabuko Sokoke (MSA) and Gede (MSG) are shown by Figures 20-23 and Tables 6-9. *M. sylvestris* extracts, particularly the 2000 mg/ kg b.w. produced results comparable to the standard hypoglycemic drug glibenclamide. The 500 mg /kg b.w. dosage was also fairly effective and the MSK extract seemed to be slightly more potent. They seemed euglycemic as sugar levels never fell below baseline values.

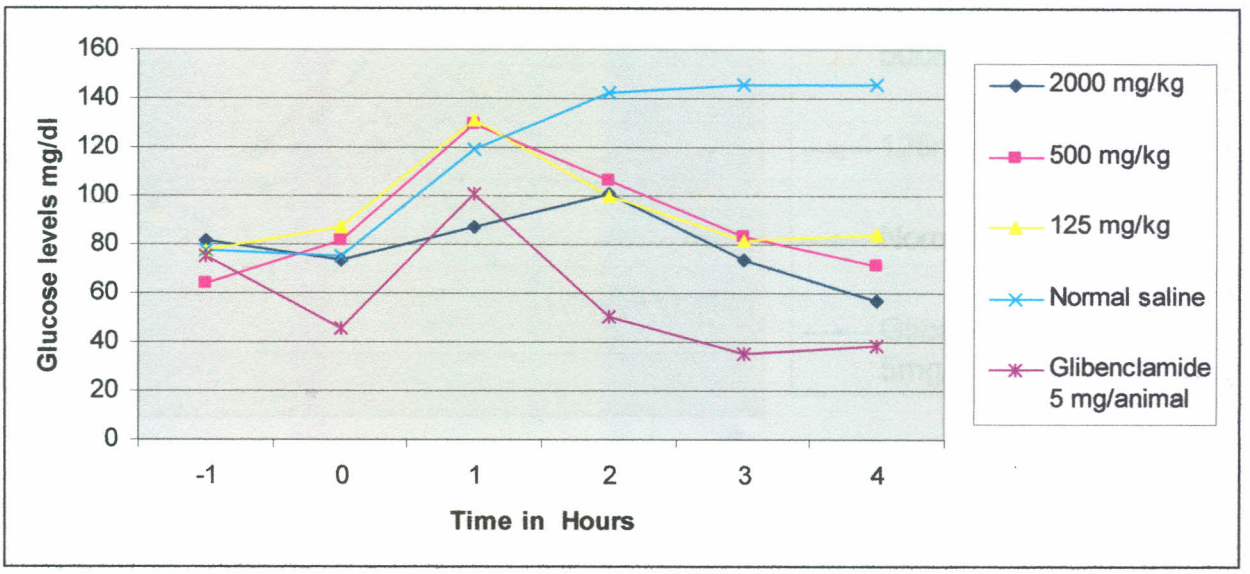


FIGURE 20; HYPOGLYCEMIC EFFECTS OF GRADED DOSES OF MSA PLANT EXTRACT

TABLE 5: MEAN BLOOD GLUCOSE LEVELS OF ANIMALS TREATED WITH MSA AND GLIBENCLAMIDE (n = 3)

Time (hours)	Mean b.w gms	-1	0	1	2	3	4
2000 mg/kg	261±5.00	82 ± 4.00 (n=2)	73.5±17.50	87.5±36.50	100.5±36.50	73.5±18.50	56.5±6.50
500 mg/kg	253.20±13.25	64 ±11.60	81.67±1.41	129.67±6.68	106.33±11.64	83.33±4.32	71.33±12.83
125 mg/kg	206.80±43.17	77.25±5.31	87.5±2.81	131±7.84	100±9.80	81.5±3.90	84.25±7.90
Normal saline 3.25 ml	366.90±44.70	78±8.29	75.33±6.65	119±5.71	142.33±19.22	146±18.06	146±18.02
Glibenclamide/Animal	309.10±35.96	75±0.82	46±6.68	101±1.63	50.33±10.50	35.33±4.19	38.33±2.87

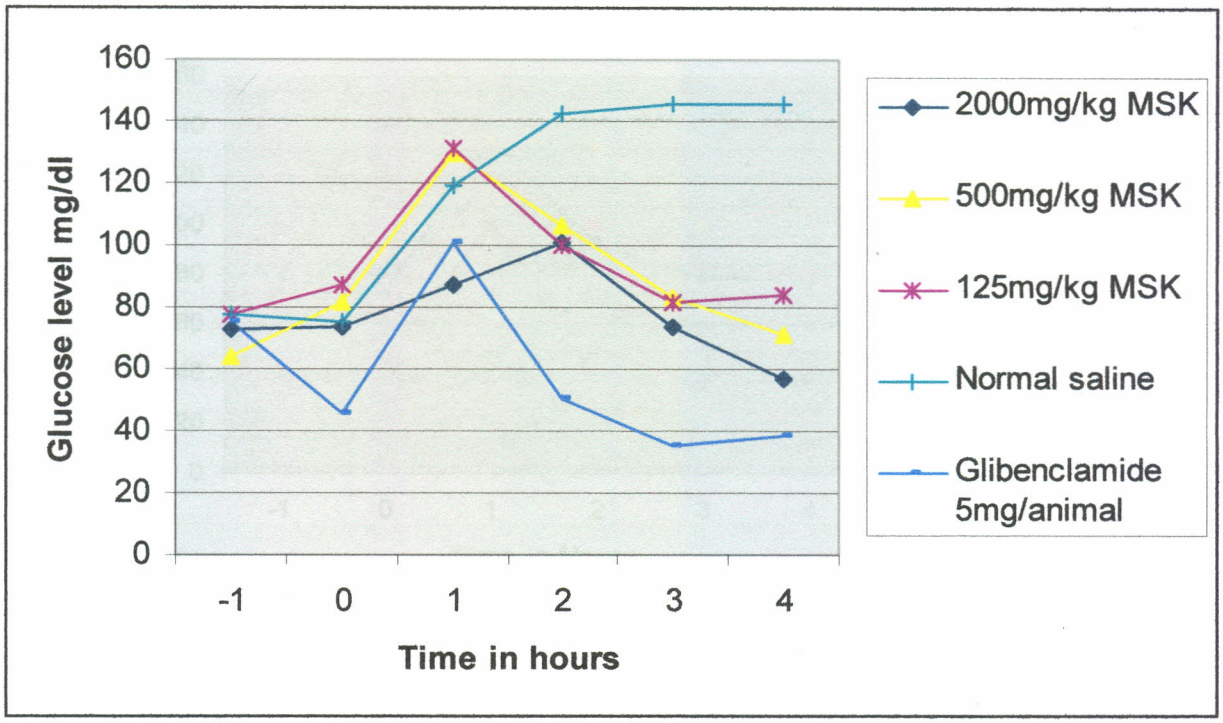


FIGURE 21: HYPOGLYCEMIC EFFECTS OF GRADED DOSES OF MSK PLANT EXTRACT

TABLE 6: MEAN BLOOD GLUCOSE LEVELS OF ANIMALS TREATED WITH MSK AND GLIBENCLAMIDE

Time(hours)	Mean b.w gms	-1	0	1	2	3	4
2000mg/kg MSK	247.50±43.70 N=4	72.75±15.58	83.75±17.68	87.50±13.37	100.50±21.57	73.50±12.55	56.50±16.16
500mg/kg MSK	260.80±29.54	64.00±17.91	81.65±13.42	129.65±24.09	106.30±11.26	83.30±16.68	71.30±28.11
125mg/kg MSK	246.90±19.65 N=4	77.25±3.49	87.50±11.01	131.00±38.20	100.00±34.6	81.50±24.91	84.25±27.77
Normal saline 3.25 ml	366.90±44.70	78.00±8.29	75.30±6.65	119.00±5.71	142.30±19.22	146.00±18.6	146.00±18.02
Glibenclamide/ Animal	309.10±35.96	75.00±0.82	46.00±6.68	101.00±1.63	50.30±10.50	35.30±4.19	38.30±2.87

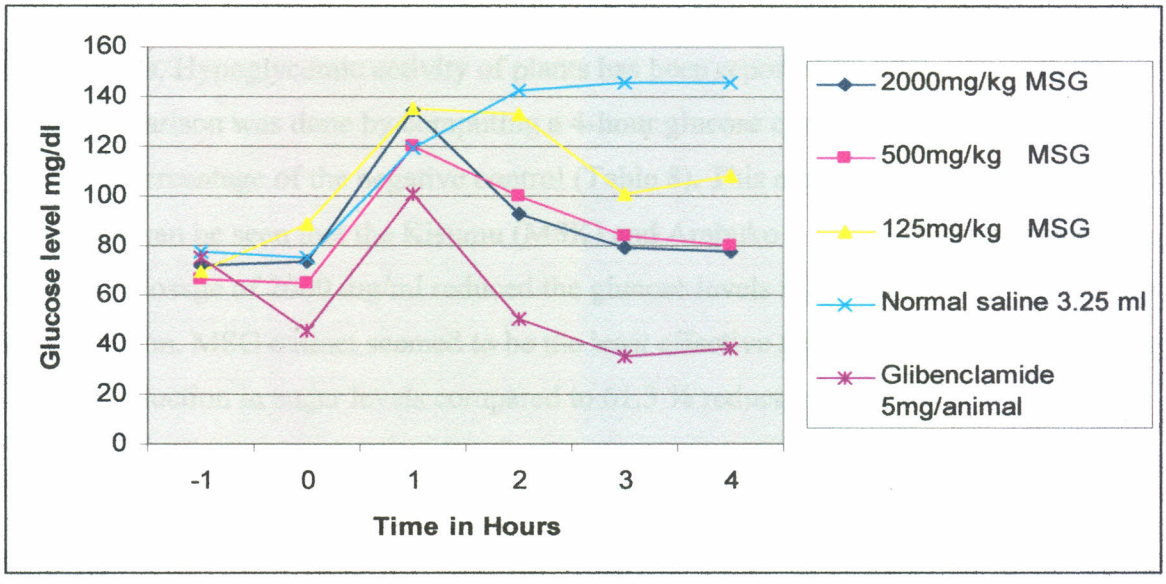


FIGURE 22: HYPOGLYCEMIC EFFECTS OF GRADED DOSES OF MSG PLANT EXTRACT

TABLE 7: MEAN GLUCOSE LEVELS FOR ANIMALS TREATED WITH MSG AND GLIBENCLAMIDE (n = 3)

Time(hours)	Mean b.w gms	-1	0	1	2	3	4
2000mg/kg MSG	22.60±41.67	69.30±4.5	88.65±17.17	135.00±4.32	132.65±7.13	100.65±4.03	100.33±23.
500mg/kg MSG	244.90± 6..28	66.65±1.25	65.00±5.73	120.30±29.78	99.65±38.59	84.30±22.10	80.00±18.0
125mg/kg MSG	230.50± 12.42	72.00± .24	73.65±9.18	134.65±2.86	92.65±7.59	79.30±12.25	77.65 ±12.
Normal saline	366..90±44.70	78.00±8.29	75.30±6.65	119.00±5.71	142.30±19.22	146.00±18.08	146.00±18.
Glibenclamide 5mg / animal	309.10± 25.96	75.00±0.82	46.00±6.68	101.00±1.63	50.30±10.50	35.30±4.19	38.30±2.87

A dose response relationship was established by this study. This is in line with what is reported in the literature that *Marsedenia sylvestris* has hypoglycemic activity [22, 26-29]. The geotypes compared favourably with the standard drug glibenclamide. The mechanism by which these extracts lowers the blood sugar need to be studied. It may be due to increase in circulating insulin level (hyperinsulinemia) or by enhancing peripheral utilization of glucose. Hyperinsulinemia may be due to either an increase in pancreatic secretion of insulin from the β-cells or its release from the bound form [12, 15]. Further *M. sylvestris* contain a number of minerals such as potassium, calcium, magnesium [25]. Minerals sometimes play a contributing role in enhancing medicinal properties including hypoglycemic activity of plants [39, 40]. The mineral element may be associated with the mechanism of insulin release and its activity or glucose tolerance factor as described in different laboratory animals and in human beings [41]. Further studies are

necessary to elucidate in details the mechanism of action of the plant at the cellular and molecular levels. Hypoglycemic activity of plants has been reported by other workers [42]. A further comparison was done by computing a 4-hour glucose concentration for all three geotypes as a percentage of the negative control (Table 8). This enabled us to do a better comparison. It can be seen that the Kisumu (MSK) and Arabuko Sokote (MSA) extracts particularly at dosage of 2000 mg/ml reduced the glucose levels significantly. This shows a long duration of action. MSG extract seemed to be the least effective since at 2000 mg / kg it caused only a 32 % reduction in sugar levels compared to 61.3 % reduction of MSK.

TABLE 8: 4-HOUR GLUCOSE CONCENTRATION AS % OF NEGATIVE CONTROL

Dose mg /kg	**MSA**	**MSK**	**MSG**
125	51.70	57.70	53.20
500	48.90	48.80	54.80
2000	36.70	38.70	68.70

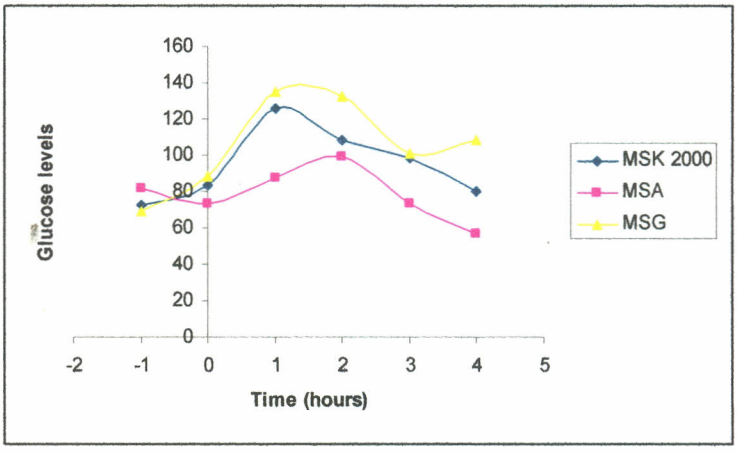
However, on univariate analysis, at most points there were no statistically significant difference across the doses of each extract (Table 9). A comparison of the effects of the extracts with the negative control at various time points and at various doses showed that there was not

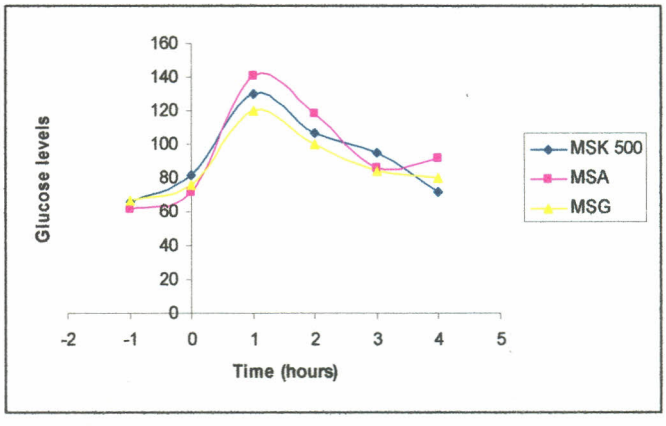
TABLE 9: GLUCOSE LEVEL UNIVARIATE ANALYSIS ACROSS THE DOSES AT 3HR AND 4HR

Extract vs normal saline	At time 3HR	At time 4HR
MSA	0.78(p=0.4894), 2 df	0.18 (P=0.8405) 2 df
MSK	1.13 (p=0.3942)	2.42 (p=0.1842)
MSG	1.14 (p=0.3796)	1.69 (p=0.2613)

statistically significant difference in the glucose levels of the extracts and the negative control at baseline and at time 0 (Fig 23, Appendix-D).Glibenclamide had the fastest onset of action as the glucose levels at 1 hour post-prandial were statistically significantly different from that of the

negative control. Activity was significant at 3 and 4 hours and as early as 1 hour it was observed that glucose levels were less than for the control group.





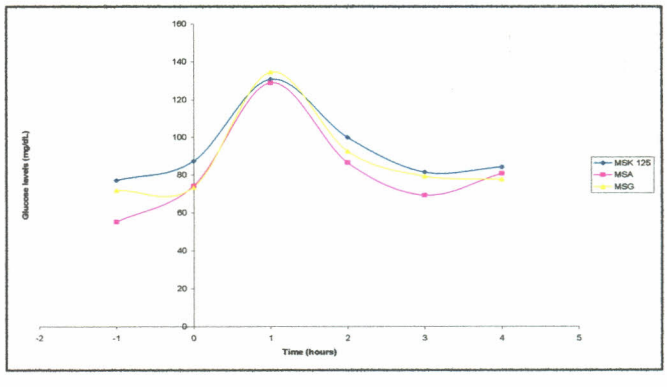


FIGURE 23: GLUCOSE LEVELS AT A DOSE OF 125, 500 AND 2000MG/KG OF BODY WEIGHT

From the graphs there seemed to be no significant difference in the biological activity of the three geotypes. A comparison of the efficacy of the three geotypes at various dose levels was done. An inferential data analysis showed that there was no statistically significant difference in biological activity at all three doses (Table 10).

TABLE 10: UNIVARIATE ANOVA FOR DIFFERENCE ACROSS THE 3 GEOTYPES AT VAROIUS DOSES

	F	P	Degrees of freedom
125 mg	0.18	0.8425	2
500 mg	0.26	0.7798	2
2000 mg	2.91	0.1309	2

However, the sample size may have been too small to detect a difference in the biological activity. It is worth noting that from the graphs MSA gave the lowest glucose levels at dose levels 125mg and 2000mg.

3.8 Effects of the *Marsedenia sylvestris* (MSK) plant extract on isolated rat diaphragm nerve preparation

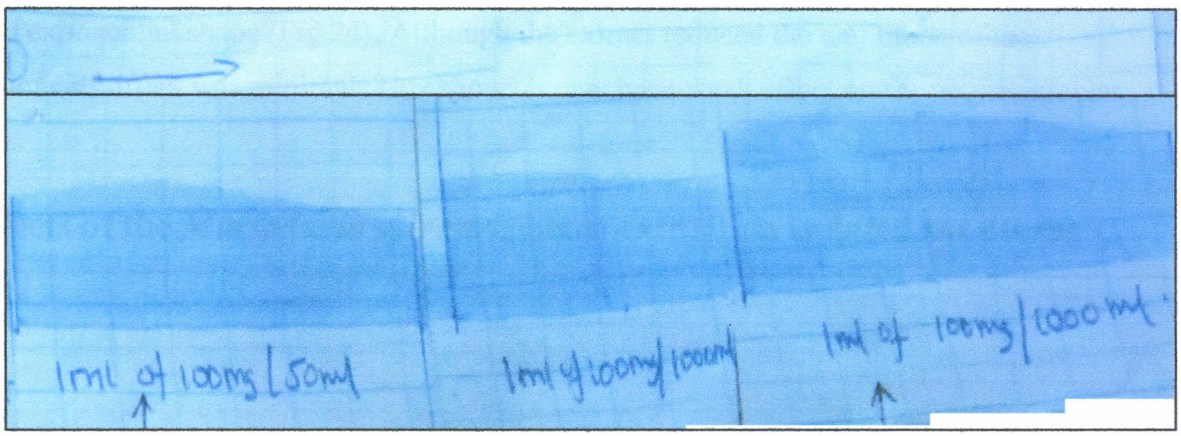


FIGURE 24: EFFECTS OF DIVERSE STRENGTHS OF MSK ON ISOLATED RAT DIAPHRAGM

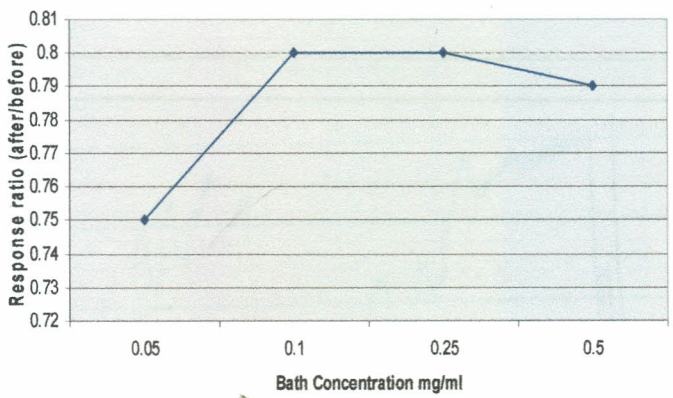


FIGURE 25: DOSE-- RESPONSE RATIO OF *M. SYLVESTRIS* (AFTER/BEFORE).

Further pharmacological study was done on the effect of the plant extract on isolated rat diaphragm nerve preparation (Figs 24). The amplitude of the contraction was measured before and after administration of each dose of the extract. The results show a mild relaxation dose-

response relationship as demonstrated by the response ratio. As the dose increased the relaxation increased. The extract may acts as an antagonist of acetylcholine and hence blocks its effect leading to a decrease in contractility of the diaphragm. The muscle contraction reduces to a maximum as the concentration of the drug increases. The dose-response curve showed the expected exponential shape (Fig 24). Although the extract reduced the amplitude of the contractions its effect was minimal. This shows it may have weak effect on the neuromuscular junction.

3.9 Effects of the *Marsedenia sylvestris* plant extract on isolated rat uterus

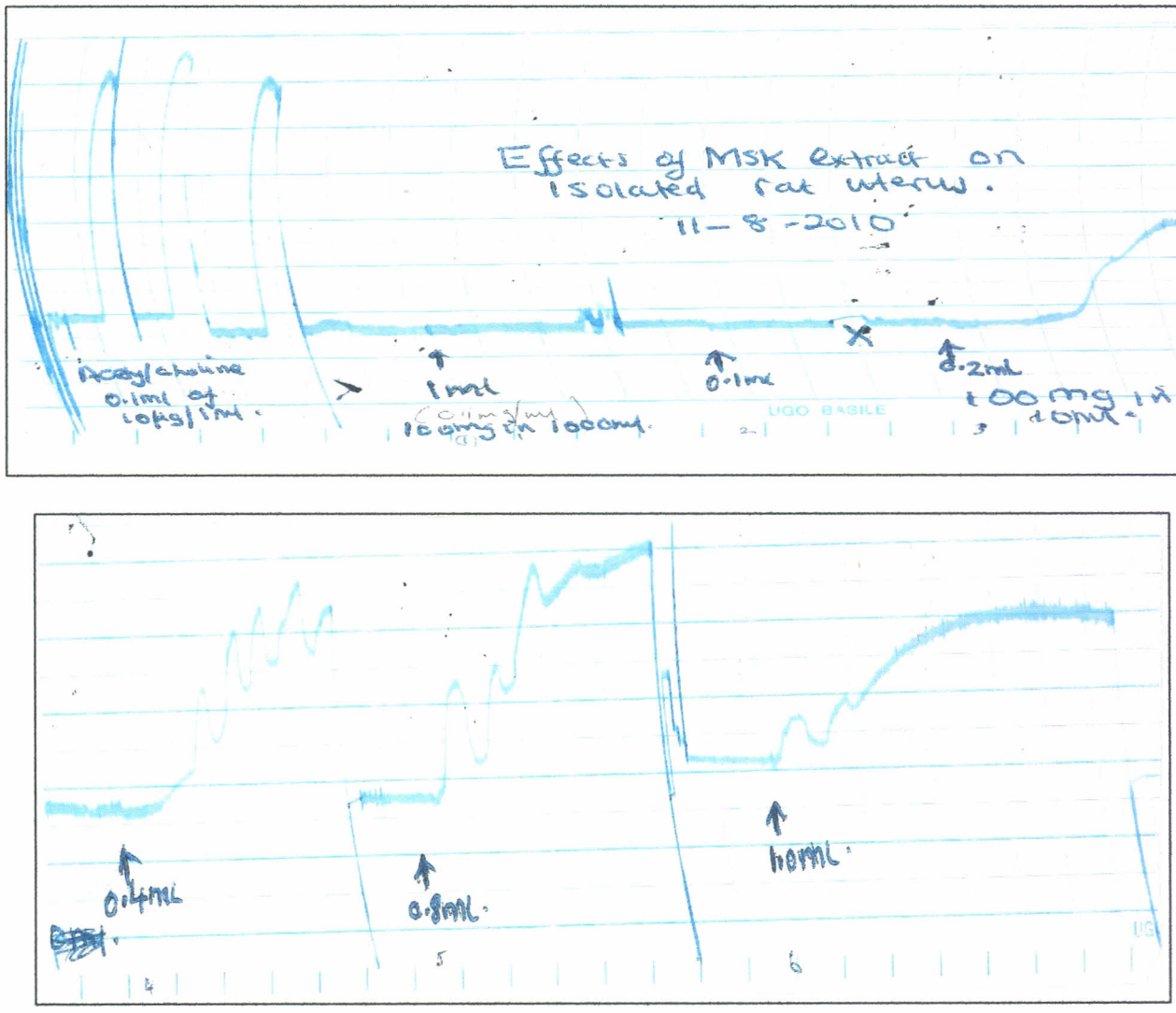


FIGURE 26; EFFECTS OF THE MSK PLANT EXTRACT ON ISOLATED RAT UTERUS

The effect of the plant extract on the isolated rat uterus is demonstrated in figure 26 above. The uterus has a thick muscular wall made of three layers of smooth muscles namely the perimetrium, myometrium and endometrium. It is innervated chiefly by utero-vaginal plexus, which is a mainly sympathetic and parasympathetic fibres from the pelvic nerve. Stimulation of the parasympathetic nerves leads to increased uterine contraction while sympathetic stimulation or by drugs which act as these leads to relaxation. Stimulation of the uterus is affected by the stage of the menstrual cycle, pregnancy, temperature and the amount of circulating hormones in the blood such as estrogens and progesterone [43]. Estrogens like oxytocin and stilboestrol increase the excitability of the myometrium while progesterone reduces.

The uterus was pretreated with stilboestrol to prime it. Acetylcholine acted by binding with muscarinic receptors setting in motion a sequence of events leading to contraction of the uterus [43]. Low doses of the extract produced no response. Doses from 0.2 ml of 100 mg in 100 ml solution upwards produced progressive increase in the frequency and force of uterine contraction until a maximum was reached. At higher doses above 0.4 ml, application of the drug resulted in multiple contractions. Drugs with this oxytocic activity find clinical application in induction of labour, abortion, reduction of prolonged labour and management of post-partum haemorrhage and retained placenta. Natural examples are ergot alkaloids such as ergometrine and their derivatives [3]. This action validates the traditional use of *M. sylvestris* in India for family planning [21].

3.10 Effects of the MSK plant extract on isolated rabbit heart

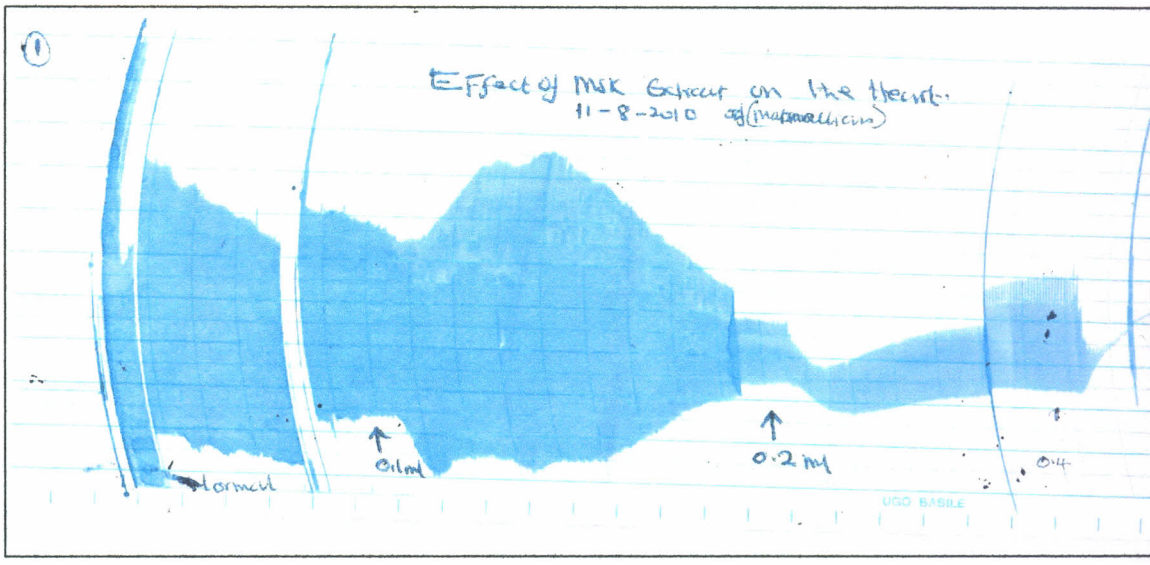


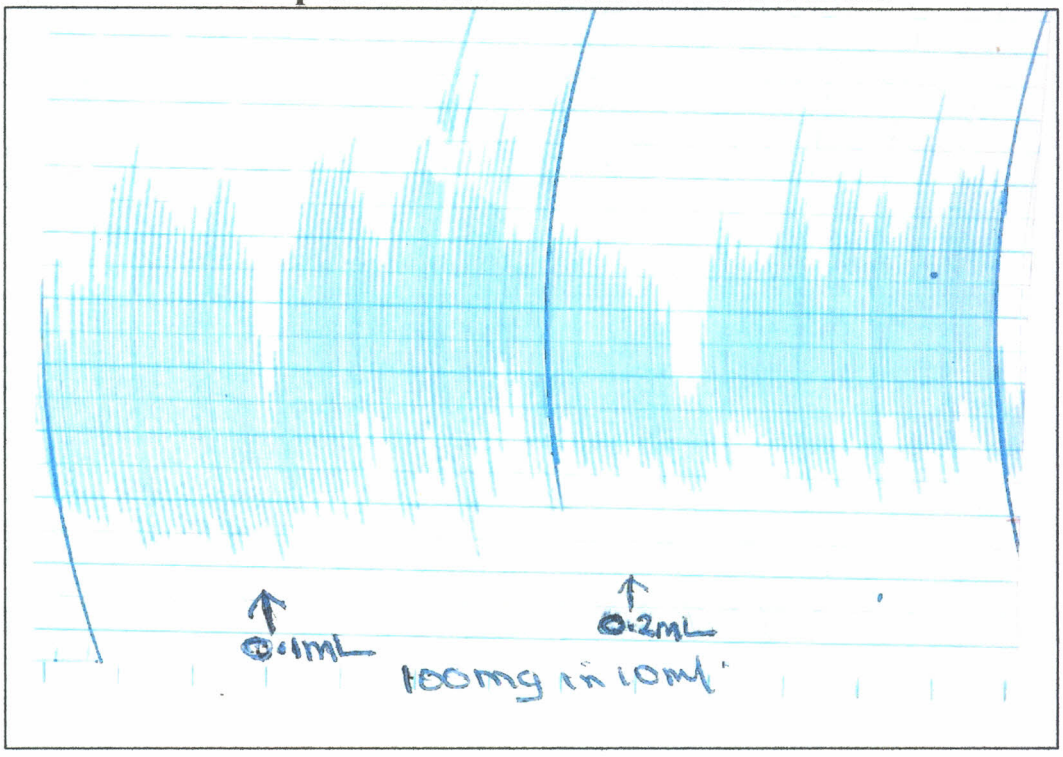
FIGURE 27: EFFECTS OF THE MSK PLANT EXTRACT ON ISOLATED RABBIT HEART

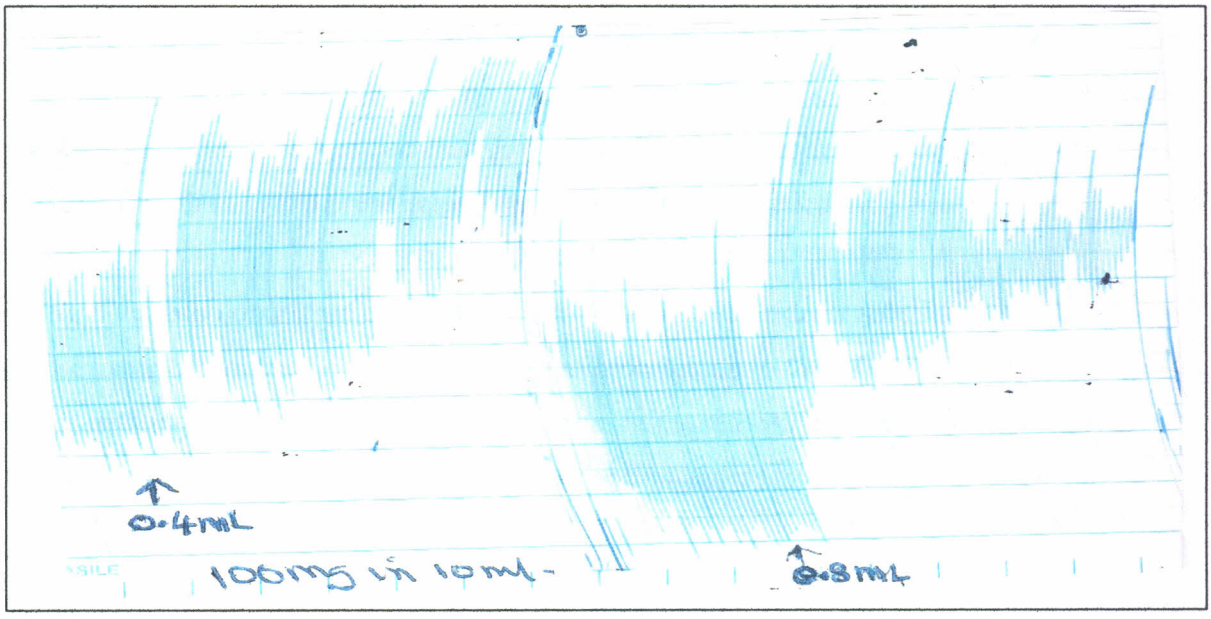
The effect of the plant extract on the isolated rabbit heart is shown in figure 27 above. The effect of the drug on the heart may be on the rate and rhythm, myocardial contraction or metabolism and blood flow [43]. These effects are not independent of each other and therefore a drug which affects the electrical properties of the myocardial cell membranes is likely to affect both the rate and rhythm of the heart and its contraction. Similarly a drug which affects contraction will inevitably alter metabolism and blood flow.

An isolated rabbit heart was used to prevent the interference of the physiological regulatory factors such as parasympathetic and sympathetic stimulation, and influence by hypothalamus, medulla oblongata, baroreceptors and carotid bodies. These tissues control the heart in an intact animal [43]. Therefore only the effects of the drug acting directly on the heart were shown. *M. sylvestris* extract 0.1 ml of 100 mg /10 ml (1mg) solution first caused a mild slowing of the heart followed by positive chronotropic and ionotropic effect. Administration of 0.4 ml of the extract caused a negative ionotropic effect and the tissue never recovered but died slowly. The extract therefore showed positive ionotropic effects at low doses and negative ionotropic effects at high doses. This is comparable to what happens in the uterus. The effects on the heart could be attributed to cardiac glycosides which were detected during phytochemical screening. An

increase in the force and rate of heart contraction leads to increased cardiac output. Therefore low doses of this extract may be useful in clinical conditions like arterial fibrillation, paroxysmal supraventricullar tachycardia and cardiac failure.

3.11 Effects of the MSK plant extract on isolated rabbit ileum





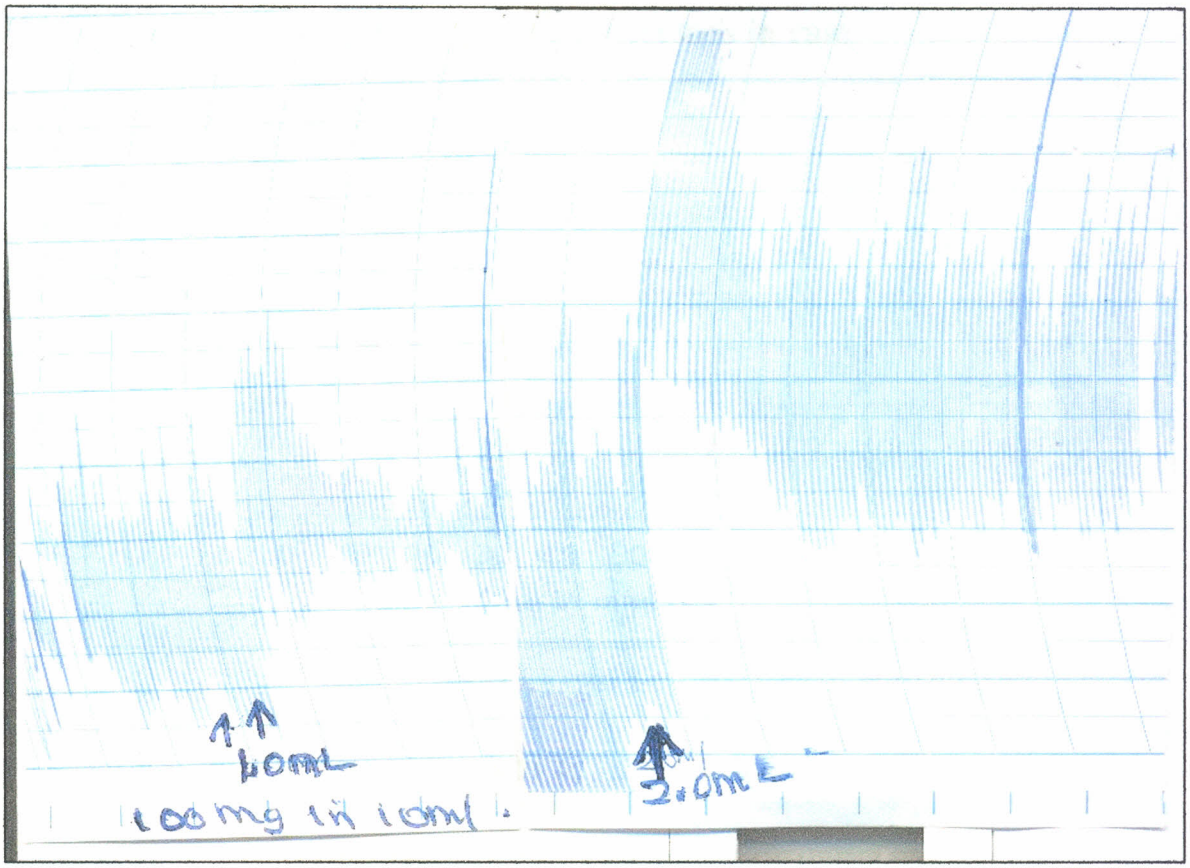
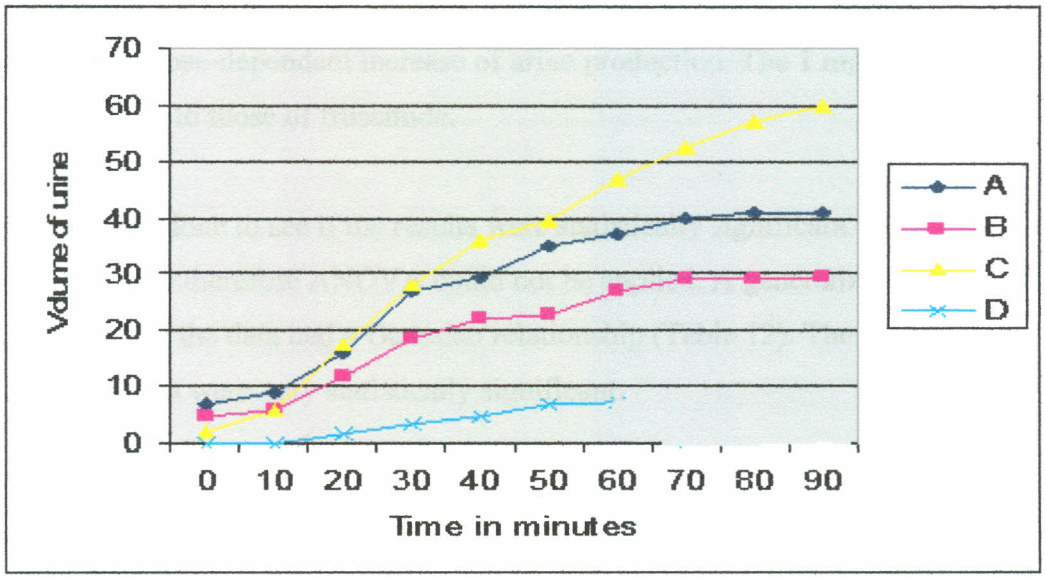


FIGURE 28: EFFECTS OF THE MSK PLANT EXTRACT ON ISOLATED RABBIT ILEUM

The effect of the plant extract on the isolated rabbit ileum is shown in figure 28 above. The ileum receives intrinsic and extrinsic innervation from the parasympathetic and sympathetic nervous system. The sympathetic nerves decrease motility and the tone while the parasympathetic nerves increase them. In the *in vitro* preparation we have only the intrinsic innervation.

In this study various concentrations of *M. sylvestris* 10 mg /ml extract solution first relaxed the muscle followed by contraction. This pattern was repeated up to the 2.0 ml dose of 100 ml in 10 ml extract with increased force and amplitude. This increased contraction leads to increased gastric motility. This drug may be useful in constipation and dyspepsia.

3.12 Diuretic effects of *Marsedenia sylvestris* in rats



A = MSK 1, B = MSK 2, C = FRUSEMIDE, D = NORMAL SALINE

FIGURE 29: DIURETIC EFFECTS OF *M. SYLVESTRIS* IN RATS

TABLE 11; CUMULATIVE VOLUME OF URINE WITH TIME

Time (minutes)	0	10	20	30	40	50	60	70	80	90
A (msk 1) Vol.mls	7	9	16	27	29.5	35	37	40	41	41
B (msk 2) Vol.mls	5	6	12	18.5	22	22.8	27	29	29	29.5
C (frusemide) Vol.mls	2.1	5.8	17.5	28	36	39.5	47	52.5	57	60
D (saline) Vol.mls	0	0	1.6	3.6	5.00	7.00	7.20	0	0	0

MSK 1: 2 mg /ml. MSK 2: 1 mg / ml. . Frusemide: 10 mg / ml

Note: Volume of urine was cumulative.

The pH of urine was 7 for all groups.

The diuretic effect of *M. sylvestris* was investigated in this study (Fig. 29). Table 11 shows the volume of cumulative urine collected every ten minutes. Frusemide is a loop diuretic acting in the loop of Henle [43]. It was used as the positive control because it is a very potent diuretic and considerably increased the urine output. Normal saline, a 0.85 % NaCl solution was the least

efficacious with a mild diuretic effect. It increases the Na^+ levels in the plasma leading to water retention. This leads to sequence of events precipitating decreased output of urine. *M. sylvestris* extract showed a dose-dependent increase of urine production. The 1 mg /ml dose produced results comparable to those of frusemide.

Data analysis was done to see if the results were statistically significant. All the data (Table 11) was parametric and therefore ANOVA could not be applied. A generalized linear model was used assuming that the data had a Gaussian relationship (Table 12). The P- values were zero and therefore the results were very statistically significant.

TABLE 12: T-TEST RESULTS OF THE DIURETIC EFFECT

	z	P value
All extracts	-3.37	0.001
MSA vs normal saline	-9.11	0.000
MSK vs normal saline	-9.07	0.000
MSG vs normal saline	-7.11	0.000

Cardiac glycosides are also known to cause increased diuresis and therefore they could have been responsible for the increased urine output noted. This diuretic effect is useful therapeutically since it could lead to reduced blood pressure which frequently occurs together with diabetes mellitus.

CHAPTER 4

CONCLUSION AND RECOMMENDATIONS

In this study *Marsedenia sylvestris* species found in three geographical locations in Kenya were studied. Macroscopic and microscopic investigations were carried out, Phytochemical tests, pharmacological tests, TLC and HPLC finger printing and profiles were done. A comparison was done among the three geotypes involving the taxonomic characteristics, crude extracts yields, phytoconstituents and pharmacological activity.

This study has confirmed that the three geotypes had similar macroscopic and microscopic characteristics and TLC fingerprints. The HPLC profiles had little differences in Retention Times and particularly the Gede geotype. The pharmacological activity was found to be similar except the Kisumu geotype which seemed to have more potency. This also confirms and validates ethnopharmacological uses by the herbalists for the control and treatment of diabetes mellitus. It is also known that diabetes mellitus and obesity are closely associated to hypertension and stroke. The hypoglycemic and the diuretic effects demonstrated by *Marsedenia sylvestris* suggest that this plant is a potential hypotensive and a powerful anti-diabetic agent. Further work and more research should be done to validate this.

The effects demonstrated on rat diaphragm nerve preparation, isolated rabbit heart and ileum together with isolated rat uterus all support and validate the herbalists' claims. The leaves have been used traditionally for stomach ailments, constipation and water retention. They have also been used for conditions such as family planning.

This is the first time this plant has been studied and investigated in Kenya. Further work therefore needs to be done to confirm whether the active principles in Kenyan plants are the same as those found elsewhere. The study also demonstrates the need to investigate other plants found and studied elsewhere but the Kenyan species has not been worked on. Such plants include *Achranthes aspera, Hypoxis hemerocallidea* and *Solanum nigrum*. The effect on geographical

variation and agronomical studies should also be investigated. Though this plant has been used by herbalists in India for many years, toxicological studies for the Kenyan plants need to be done to verify their safety. In this study it has been demonstrated that the local species have very high yield of extract and it is hereby recommended that they should be domesticated as a cash crop. This will benefit the local communities economically and in health care.

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LIST OF APPENDICES

D-7000 HSM: emily Series: 0013 Report: modified System: Sys 2

Marsedenia Sylvestris Assay Screening Report

Analyzed: 09/04/10 03:48 PM

System(acquisition): Sys 2
Sample Name: MSA (1)
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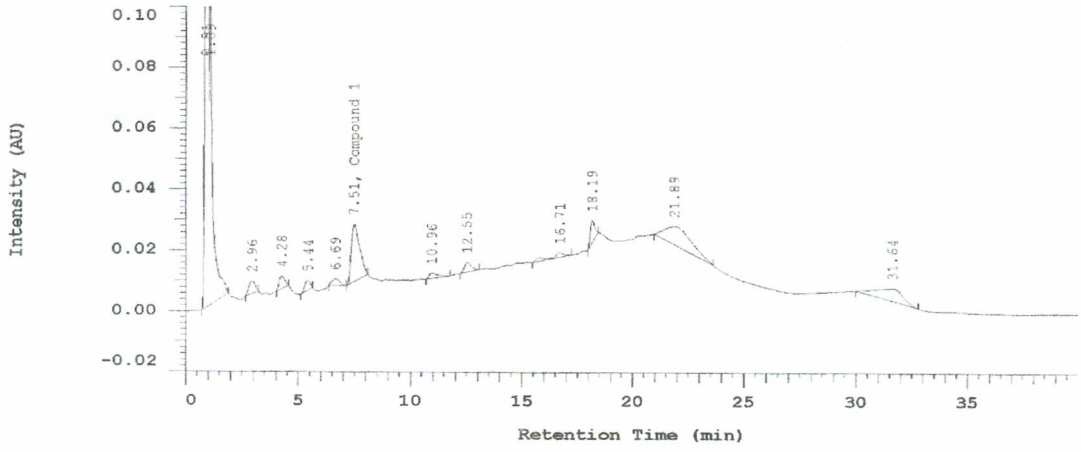
Processed: 09/06/10 09:40 AM
Series:0013
Volume: 20.0 ul

Chrom Type: HPLC Channel : 1



No.	RT	Area	Area %	BC	RRT	Name
1	0.91	1029987	41.979	BV	0.12	
2	1.09	492301	20.064	VB	0.15	
3	2.96	39465	1.608	BB	0.39	
4	4.28	34906	1.423	BB	0.57	
5	5.44	26265	1.070	BB	0.72	
6	6.69	24803	1.011	BB	0.89	
7	7.51	235648	9.604	BB	1.00	Compound 1
8	10.96	23515	0.958	BB	1.46	
9	12.55	34248	1.396	BB	1.67	
10	16.71	31564	1.286	BB	2.23	
11	18.19	48891	1.993	BB	2.42	
12	21.89	259506	10.577	BB	2.92	
13	31.64	172503	7.031	BB	4.21	
		2453602	100.000			

Peak rejection level: 0

APPENDIX A: HPLC OF *M. SYLVESTRIS* EXTRACT (MSA)—RESOLVED PEAK 1

Marsedenia Sylvestris Assay Screening Report

Analyzed: 09/04/10 04:29 PM

System(acquisition): Sys 2
Sample Name: MSK (1)
Sample Description:

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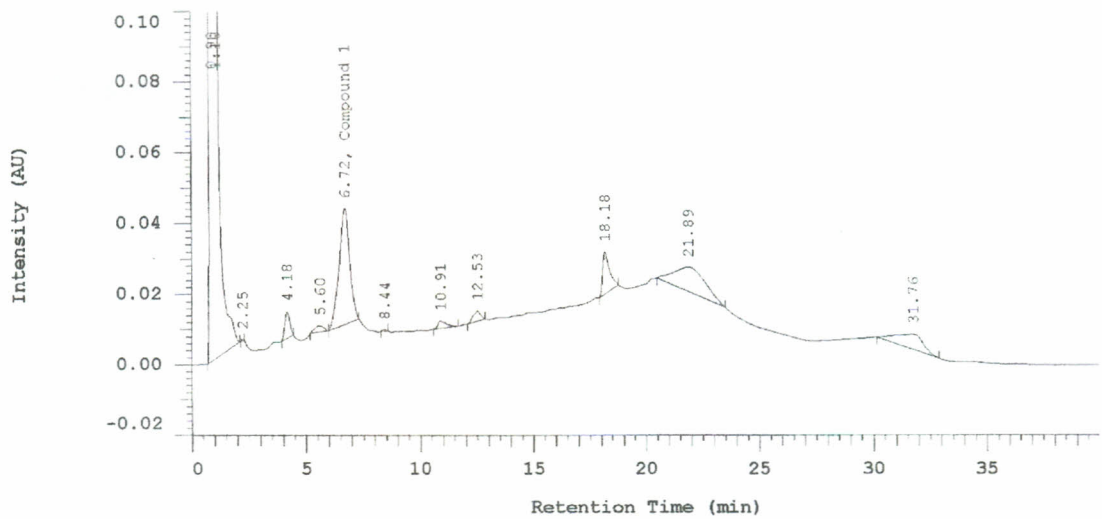
Processed: 09/06/10 09:40 AM
Series:0013
Volume: 20.0 ul

Chrom Type: HPLC Channel : 1



No.	RT	Area	Area %	BC	RRT	Name
1	0.90	2547858	52.120	BV	0.13	
2	1.10	1046614	21.410	VB	0.16	
3	2.25	1301	0.027	BB	0.34	
4	4.18	56089	1.147	BB	0.62	
5	5.60	24862	0.509	BB	0.83	
6	6.72	520173	10.641	BB	1.00	Compound
7	8.44	2563	0.052	BB	1.26	
8	10.91	24869	0.509	BB	1.62	
9	12.53	32171	0.658	BB	1.87	
10	18.18	122546	2.507	BB	2.71	
11	21.89	335502	6.863	BB	3.26	
12	31.76	173940	3.558	BB	4.73	
		4888488	100.000			

Peak rejection level: 0

APPENDIX B: HPLC OF *M. SYLVESTRIS* EXTRACT (MSK)—RESOLVED PEAK 1

Marsedenia Sylvestris Assay Screening Report

Analyzed: 09/04/10 05:10 PM

System(acquisition): Sys 2
Sample Name: MSG (1)
Sample Description:

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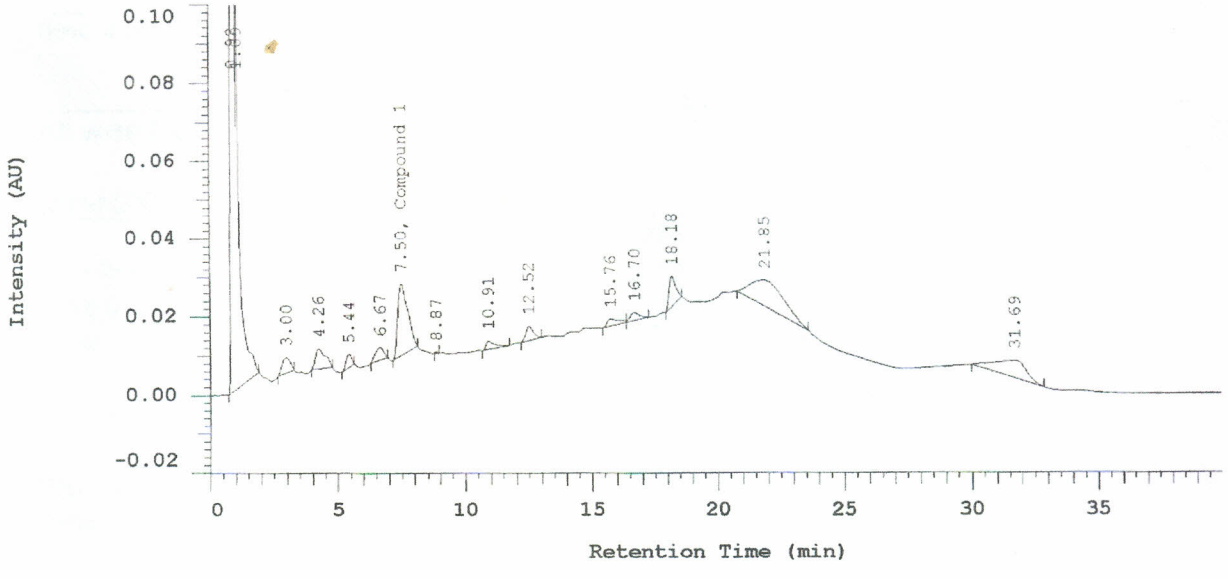
Processed: 09/06/10 09:40 AM
Series:0013
Volume: 20.0 ul

Chrom Type: HPLC Channel : 1



No.	RT	Area	Area %	BC	RRT	Name
1	0.92	1020946	39.217	BV	0.12	
2	1.09	489844	18.816	VB	0.15	
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4	4.26	68282	2.623	BB	0.57	
5	5.44	25293	0.972	BB	0.73	
6	6.67	34949	1.343	BB	0.89	
7	7.50	243003	9.334	BB	1.00	Compound
8	8.87	979	0.038	BB	1.18	
9	10.91	26232	1.008	BB	1.46	
10	12.52	35765	1.374	BB	1.67	
11	15.76	27605	1.060	BB	2.10	
12	16.70	25450	0.978	BB	2.23	
13	18.18	66890	2.569	BB	2.42	
14	21.85	307344	11.806	BB	2.91	
15	31.69	187599	7.206	BB	4.23	
		2603330	100.000			

APPENDIX C: HPLC OF *M. SYLVESTRIS* EXTRACT (MSG)—RESOLVED PEAK 1

Dose 125

	MSA	MSK	MSG	Glibenclamide
Baseline	p>0.05	p>0.05	p>0.05	
Time 0	p>0.05	p>0.05	p>0.05	
Time 1	0.31 (p=0.5875)	**12.52 (p=0.0241)**	**9.97(p=0.0340.0342), 1 df**	**18.34 (p= 0.0128)**
Time 2	4.79(p=0.491) 1 df	2.26 (p= 0.2072)	0.44 (p= 0.5414)	**35.28 (p= 0.0040)**
Time 3	**17.50 (p= 0.0013) 1 df**	**20.41 (p= 0.0107)**	**11.65 (p= 0.0269)**	**70.39 (p=0.0011)**
Time 4 hours	**16.31 (p=0.0016) 1 df**	**11.92 (p= 0.0260)**	3.27 (p=0.1448)	**69.65 (p=0.0011)**

All was 1 df.

Dose500

	MSA	MSK	MSG
Baseline	p>0.05	p>0.05	p>0.05
Time 0	p>0.05	p>0.05	p>0.05
Time 1	0.37 (p=0.5752)	**12.52(p=0.0241)**	**0 (p= 0.9534))**
Time 2	5.22 (p= 0.0843)	2.26 (p= 0.2072)	1.96 (p=0.234)
Time 3	**15.04 (p= 0.0179)**	**20.41 (p= 0.0107)**	**9.15 (p= 0.0390)**
Time 4 hours	**10.00 (p= 0.0341)**	**11.92 (p= 0.0260)**	**13.39 (p= 0.0216)**

Dose2000

	MSA	MSK	MSG
Baseline	p>0.05	p>0.05	p>0.05
Time 0	p>0.05	p>0.05	p>0.05
Time 1	0.45 (p= 0.5341)	1.29 (p= 0.3381)	**9.97 (p= 0.0342)**
Time 2	3.30 (p= 0.1288)	1.90 (p= 0.2619)	0.44 (p= 0.5414)
Time 3	**11.94 (p= 0.0181)**	**11.17 (p= 0.0443)**	**11.65 (p= 0.0269)**
Time 4 hours	**18.64 (p= 0.0076)**	**27.24 (p= 0.0137)**	3.27 (p= 0.1448)

APPENDIX D: A COMPARISON OF EXTRACTS WITH CONTROL AT VARIOUS DOSES AND POINTS