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**PHYTOCHEMICAL, ANTHELMINTIC AND ANTIMICROBIAL
INVESTIGATION OF *HAGENIA ABYSSINICA* (BRUCE)**

GMELIN

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U59/ 72061/08

**A thesis submitted in partial fulfilment of the requirements for the award of the degree of
Master of Science in Pharmacognosy and Complementary Medicine of University of
Nairobi**

Department of Pharmacology and Pharmacognosy

School of Pharmacy

University of Nairobi

2013

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
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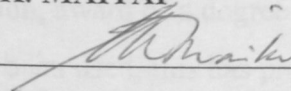
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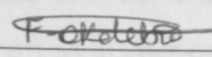
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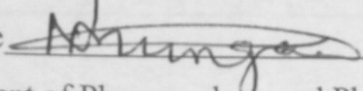
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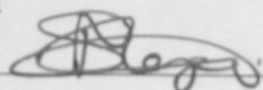
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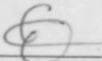
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DEDICATION

I thank the Almighty God for enabling me to carry out the research work.

My heartfelt gratitude goes to my research supervisors: Prof. C.E. Maudi, Dr. N.N. Mungu, Dr. P.A. Okalio and Dr. S. W. Ndung'u. Their advice, encouragement and thorough guidance throughout the research period has resulted in the successful completion of the research work.

I extend thanks to Prof. J. W. Mwangi who gave me insight into the study of the plant

This thesis is dedicated to my late parents Mr. and Mrs. J. Karumi; for raising me to be who I am today. Your memories remain in my heart forever.

May you rest in eternal peace.

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I am also grateful to my immediate family for their financial contribution towards the work. I cannot forget my father's name Mr. J. Karumi. My mother's name is Mrs. J. Karumi. I am also grateful to my friends who have been a source of inspiration.

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LIST OF ABBREVIATIONS AND SYMBOLS

%	Percent
CAM	Complementary and alternative medicine
CHCl ₃	Chloroform
cm	Centimeter
CNS	Central nervous system
°C	Degree Celsius
C.I	Confidence interval
¹³ C	Carbon 13
DARU	Drugs Analysis and Research Unit
DCM	Dichloromethane
DMSO	Dimethyl sulfoxide
DEPT	Distortionless Enhancement by Polarization Transfer
EtOH	Ethanol
H ₂ SO ₄	Sulphuric acid
hrs	Hours
HR	Hazard ratio
IC ₅₀	Half maximal inhibitory concentration
IR	Infra red
KBr	Potassium bromide
MeOH	Methanol
m	Multiplet
mg	Milligrams
min	Minutes
ml	Milliliter
mM	Millimolar
MHz	Megahertz
MS	Mass spectrometry
m/z	Mass to charge ratio
NMR	Nuclear magnetic resonance
NQCL	National Quality Control Laboratories

PACs	Proanthocyanidins
R _f	Retention factor
STH	Soil transmitted helminths
s	Singlet
t	Triplet
TLC	Thin layer chromatography
USA	United States of America
UV	Ultraviolet
WHO	World Health Organization
w/v	Weight by volume
v /v	Volume by volume

ABSTRACT

Hagenia abyssinica (Bruce) Gmelin is a flowering plant that is widespread in the afro-montane regions of Central and Eastern Africa. It is an important medicinal plant that societies relied on for generations for combating various ailments. Its inflorescence, for example, is used traditionally as an anthelmintic. Its roots are cooked in soup for treatment of malaria and the pounded bark is used for treatment of diarrhea. Besides being a source of medicine, *Hagenia* has been utilized for various other purposes such as construction, furniture, fuel and soil fertility management. As a result of its enormous significance, *H. abyssinica* is at risk of becoming an endangered tree species due to overexploitation. The objective of this study was to carry out phytochemical investigation of its chemical constituents and screen it for *in vitro* anthelmintic and antimicrobial activities.

The plant material was collected from South Kinangop, Nyandarua county in September, 2009. Phytochemical tests showed that the plant powders contained alkaloids, tannins, saponins and cardiac glycosides. The plant extraction was done by maceration and percolation using petroleum ether, chloroform, dichloromethane / methanol mixture and methanol. The petroleum ether and chloroform extracts were subjected to open column chromatography using solvents of varying polarity as the mobile phase. Thin layer chromatography was used to monitor the fractions. Two crystalline compounds were isolated from the chloroform extract and subjected to spectroscopic analysis. One of the compounds was identified as β -sitosterol. The second one was obtained in insufficient quantities and thus its structure could not be elucidated.

The *in vitro* anthelmintic activities of crude extracts of the stem bark and flowers were investigated. Screening for anthelmintic activity was carried out using *Panagrellus redivivus* model whereby the methanol and dichloromethane/ methanol extracts exhibited activity. Further tests were carried out on the stem bark methanol extract. These were the whole worm and the cut worm assays using *Caenorhabditis elegans* species. In these assays, the activity of the methanol extract was compared to levamisole to show the percentage of worms alive after incubation at room temperature for 24 hours for whole worm assay; and the time in minutes taken to paralysis in cut worm assay. The extract showed significant but weaker activity than levamisole.

Some of the plant extracts were screened for both antibacterial and antifungal activities using agar diffusion method. Petroleum ether and dichloromethane/ methanol extracts showed activity against: *Staphylococcus aureus*, *Escherichia coli* and *Bacillus subtilis*. The respective diameters of zones of inhibition were 17, 16 and 16 mm for petroleum ether extract; and 19, 20 and 18 mm for dichloromethane/ methanol extract, while that of positive control was 20 mm for all the bacterial microorganisms. Chloroform extract at the same concentration only exhibited weak activity against *Bacillus subtilis* and the methanol extract did not exhibit any activity. All the *Hagenia* extracts tested lacked any antifungal activity at a concentration of 50 mg/ml.

The present study shows there may be a scientific basis for the traditional use of *Hagenia abyssinica* as an anthelmintic and also for treatment of diarrhea and livestock diseases caused by bacteria.

CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

1.1 General introduction

The limited availability and affordability of medicines means that the majority of the world's population depends on traditional medical remedies. The World Health Organization (WHO) estimates that approximately 80 % of world inhabitants rely mainly on traditional medicines for their primary health care. Plant products also play an important role for the remaining 20 % of the population who mainly reside in developed nations. About 20,000 species of higher plants are used medicinally throughout the world and about 74% of all plant derived pharmaceuticals are used in modern medicine in a way that correlates directly with their traditional use (Cragg and Newman, 2001; WHO, 2003). Plants have played a vital role in the prevention and treatment of disease since prehistoric times. This can be traced back over five millennia to written documents of early civilization in China, India and the Near East (Santos *et al.*, 2007). People in different parts of the world depend on plant resources for their basic needs and are aware of many useful species in their ecosystem. They have continuously developed their knowledge of traditional plant uses and plant resource management (Raedeke and Rikoon, 1997; Ellen and Harris, 2000; Warren *et al.*, 2000; Starzomski *et al.*, 2004).

Traditional medicine is widely practiced in Kenya with various uses of plants having been documented by various ethnobotanical surveys (Miaron *et al.*, 2004; Kareru *et al.*). The reasons for this range from the fact that in rural areas, the ratio of traditional medical practitioner to patients is much better at 1: 987 compared to the conventional doctor to patients' ratio which is 1:7,142. Secondly, there is limited accessibility and affordability

of conventional drugs to the rural folk because pharmacies and hospitals are mainly located in major towns and cities; and the imported conventional drugs are usually expensive. Traditional medicine is perceived as being a cheaper option and is also thought to be safer than conventional drugs because it is obtained from natural sources (Munguti, 1997; Tagboto and Townson, 2001; Miaron *et al.*, 2004).

Indigenous healers often claim to have learnt by observing that sick animals change their food preferences to nibble at bitter herbs they would normally reject (Huffman, 2003). Field biologists have provided corroborating evidence based on observation of diverse species. Lowland gorillas take 90% of their diet from fruits of *Aframomum melegueta*, a relative of the ginger plant that is a potent antimicrobial and apparently keeps shigellosis at bay. Sick animals tend to forage plants rich in secondary metabolites such as tannins and alkaloids (Hutchings *et al.*, 2003). Since these phytochemicals often have antiviral, antibacterial, antifungal and anthelmintic properties, a plausible case can be made for self-medication by animals in the wild. Researchers from Ohio Wesleyan University found that some birds select nesting material rich in antimicrobial agents which protect their young from harmful bacteria (Ichinda, 2004).

1.2 Plants as sources of drugs

Plants are the traditional source of many of the chemicals used as pharmaceuticals, biochemicals, fragrance, food colors and flavors. Many plants synthesize phytochemical substances that are useful in the maintenance of health in humans and other animals. These secondary metabolites possess chemical or structural complexity. They include aromatic substances, most of which are phenols or their derivatives such as tannins. In many cases, these substances serve as plant defense mechanisms against predation by

microorganisms, insects and herbivores. Many of the herbs and spices used by humans to season food yield useful medicinal compounds (Tapsell *et al.*, 2006).

Many well-known drugs listed in modern pharmacopoeia have their origin in nature. These include quinine from bark of the *Cinchona* species for the treatment of malaria. Quinine served as the lead for development of synthetic antimalarial drugs namely chloroquine, primaquine, amodiaquine and mefloquine. More recently the wider recognition of the anti-malarial activity of artemisinin from the herb *Artemisia annua* has led to development of synthetic and semisynthetic derivatives like artemether, artesunate and arteether which are more active against resistant strains of *Plasmodium* than artemisinin (Cragg and Newman, 2001; Tagboto and Townson, 2001; Houghton, 2002).

Ginseng from *Panax ginseng* and ginkgo from *Ginkgo biloba* have led to a herbal boom in Europe, USA, China and India (Cragg and Newman, 2001). Ginseng is one of the major botanical of US foreign trade. It is also used in Asia for treatment of anemia, diabetes, gastritis, sexual impotence, to decrease tiredness and invigorate patients. Ginkgo is used traditionally as an antiasthmatic, bronchodilator and for treatment of chilblains. Extracts of the leaf are used to improve peripheral and cerebral circulation in elderly patients with symptoms of loss of memory, hearing and concentration (Evans, 2002).

Several anti-neoplastics in clinical use have been isolated from plants or are semi synthetic derivatives of plant products. Examples include vincristine and vinblastine isolated from *Catharanthus roseus* (Madagascar periwinkle). Other antineoplastics are etoposide and teniposide which are semisynthetic derivatives of epipodophyllotoxin

which was isolated from roots of various species of genus *Podophyllum* (Cragg and Newman, 2001; Dewick, 2002).

Several alkaloids have been isolated from plants and are in clinical use for treatment of various ailments. Examples of these include tabersonine from seeds of *Voacanga africana* which is used as a central nervous depressant in geriatric patients. Reserpine, isolated from *Rauwolfia serpentine*, is an alkaloid used in psychiatric treatment and as an antihypertensive. Morphine is another alkaloid obtained from *Papaver somniferum* (Papaveraceae) and is used to alleviate severe pain (Cragg and Newman, 2001; Evans, 2002; Houghton, 2002; Mohammed, 2008).

Glycosides such as digoxin, gitoxin and digitoxin are obtained from the foxglove plant, *Digitalis purpurea* (Scrophulariaceae). They are clinically used for the management of congestive cardiac failure (Cragg and Newman, 2001).

Screening of compounds from plants for their pharmacological activity has been the vast source of innumerable therapeutic agents representing molecular diversity engineered by nature (Hammer *et al*, 1999).

1.3 Helminthiasis

Helminth is a general term for a parasitic worm. Worms causing infection in man fall either under the phylum, Platyhelminthes, which includes the cestodes and Trematodes, or under the phylum, Nematoda, which includes the nematodes or roundworms (Table 1.1). The most important of these worms are nematodes, which may inhabit soil and water habitats and are frequently encountered as parasites of humans or animals. Soil-transmitted helminths are the most common worldwide affecting the most deprived

communities. They include *Ascaris lumbricoides*, *Trichuris trichiura*, *Ancylostoma duodenale* and *Necator americanus*.

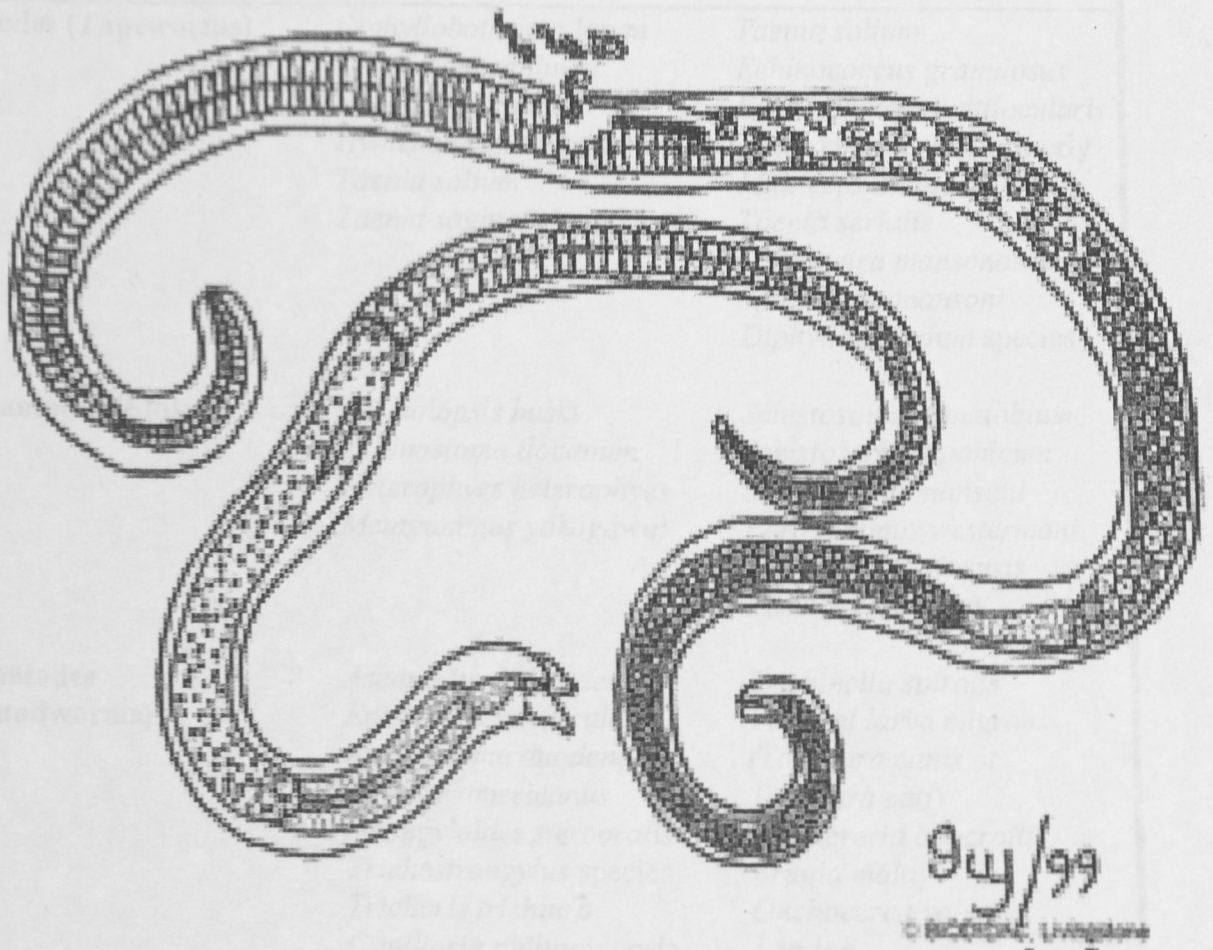


Figure 1.1. An illustration of male and female *Trichinella* roundworms

Image courtesy of BIODIDAC available at:
<http://biodidac.bio.uottawa.ca/thumbnails/catquery.htm?kingdom=Animalia&phylum=neematoda>

Table 1.1. Classification of helminthes (Reference: Baron, 1996)

Class	Intestinal	Tissue (Larval Forms)
Cestodes (Tapeworms)	<i>Diphyllobothrium latum</i> <i>Dipylidium caninum</i> <i>Hymenolepis nana</i> <i>Hymenolepis diminuta</i> <i>Taenia solium</i> <i>Taenia saginata</i>	<i>Taenia solium</i> <i>Echinococcus granulosus</i> <i>Echinococcus multilocularis</i> <i>Taenia multiceps</i> (formerly <i>Multiceps multiceps</i>) <i>Taenia serialis</i> <i>Spirometra mansonoides</i> <i>Spirometra mansoni</i> <i>Diphyllobothrium</i> species
Trematodes (Flukes)	<i>Fasciolopsis buski</i> <i>Echinostoma ilocanum</i> <i>Heterophyes heterophyes</i> <i>Metagonimus yokogawai</i>	<i>Schistosoma hematobium</i> <i>Schistosoma japonicum</i> <i>Schistosoma mansoni</i> <i>Paragonimus westermani</i> <i>Opisthorchis sinensis</i> <i>Fasciola hepatica</i>
Nematodes (Roundworms)	<i>Ascaris lumbricoides</i> <i>Enterobius vermicularis</i> <i>Ancylostoma duodenale</i> <i>Necator americanus</i> <i>Strongyloides stercoralis</i> <i>Trichostrongylus</i> species <i>Trichuris trichiura</i> <i>Capillaria philippinensis</i>	<i>Trichinella spiralis</i> <i>Visceral larva migrans</i> (<i>Toxocara canis</i> or <i>Toxocara cati</i>) <i>Wuchereria bancrofti</i> <i>Brugia malayi</i> <i>Onchocerca volvulus</i> <i>Loa loa</i> <i>Dracunculus medinensis</i>

1.3.1 Microbiology of helminths

Compared to other eukaryotic parasites, most helminthes are relatively large with some exceeding one meter in length. Their bodies have well-developed organ systems, especially reproductive organs and most are active feeders. The bodies of flatworms are flattened and covered by a plasma membrane, whereas roundworms are cylindrical and covered by a tough cuticle. Flatworms are usually hermaphroditic whereas roundworms have separate sexes; both have an immense reproductive capacity. Frequently helminthes

possess elaborate attachment structures such as hooks, suckers, teeth or plates. These structures are usually located anteriorly (Nelson and William, 2007; Pommerville, 2009).

Nematodes are dioecius and in many species clear sexual dimorphism exists. Males are usually smaller than females and both have copulatory organs. They are round non-segmented worms, usually tapering at both ends and are bilaterally symmetrical (Nelson and William, 2007).

1.3.2 Transmission of infection

Helminths are transmitted to humans in many different ways. The simplest is by accidental ingestion of infective eggs of such species as *Ascaris*, *Echinococcus*, *Enterobius*, *Trichuris* or larvae of some hookworms. Other worms produce larvae that actively penetrate the skin (hookworms, schistosomes, *Strongyloides*). In several cases, infection requires an intermediate host vector. In some cases the intermediate vector transmits infective stages when it bites the host to take a blood meal as is the case with arthropod vectors of filarial worms. In other cases, the larvae are contained in the tissues of the intermediate host and are taken in when a human eats that host (*Clonorchis* in fish, tapeworms in meat and fish, *Trichinella* in pork). The levels of infection in humans therefore depend on: standards of hygiene, the climate, ways in which food is prepared and on the degree of exposure to insect vectors (Pommerville, 2009).

1.3.3 Epidemiology

Helminthic infections are among the most common infections in man; infection is most common and most serious in poor countries. The distribution of these diseases is

determined by climate, hygiene, diet and exposure to vectors (Wakelin, 1996). Parasitic helminthes infect more than 2 billion people in the world today (Table 1.2) and cause marked morbidity and disability. Over 400 million of infected individuals are children of school age and 300 million suffer from a heavy worm burden (WHO, 2000; Hotez *et al.*, 2003).

Table 1.2. Global Prevalence and Distribution of Helminth Infections

Helminth infections	Total cases	Major geographical areas
STH infections	≥ 2 billion	
Ascariasis	1.221 billion	Sub-Saharan Africa, India, China and East Asia
Trichuriasis	795 million	Sub-Saharan Africa, India, China and East Asia
Hookworm	740 million	Sub-Saharan Africa, Americas, China and East Asia
Schistosomiasis	187 million	
<i>S. haematobium</i>	119 million	Sub-Saharan Africa
<i>S. mansoni</i>	67 million	Sub-Saharan Africa, Americas
<i>S. japonicum</i>	1 million	China and East Asia

Source: De Silva *et al.*, 2003

Parasitic nematodes are also among the most common and economically important infectious diseases of grazing livestock, especially in small ruminants in the tropics and subtropics.

1.3.4 Pathogenesis

Helminths pose a threat to public health and contribute to a high prevalence of malnutrition, anemia, eosinophilia and pneumonia. Infections contribute to morbidity, especially in children, through malnutrition, vitamin deficiencies, diarrhoea and anemia (Montresor *et al.*, 2002; WHO, 2008). In addition to their health effects, helminthic

infections also impair physical and mental growth in childhood, thwart educational advancement and hinder economic development.

Many of the pathogenic consequences of worm infestations are related to the size, movement and longevity of the parasites. This is because the host is exposed to long-term damage and immune stimulation, as well as to the sheer physical consequences of being inhabited by large foreign bodies (Pommerville, 2009).

The most obvious forms of direct damage are those resulting from the blockage of internal organs or from the effects of pressure exerted by growing parasites. Large *Ascaris* or tapeworms can physically block the intestine, and this may occur after some forms of chemotherapy; migrating *Ascaris* may also block the bile duct. *Capillaria philippensis* and *Strongyloides stercoralis* causes diarrhoea and malabsorption whereas *Enterobius vermicularis* cause perianal pruritis. *Trichostrongylus* species cause anemia. Granulomas that form around schistosome eggs may block the flow of blood through the liver and this may lead to pathological changes in that organ and elsewhere. Blockage of lymph flow, leading to elephantiasis, is associated with the presence of adult *Wuchereria* in lymphatics. Pressure atrophy is characteristic of larval tapeworm infections (hydatid cyst, the larva of *Echinococcus granulosus*) where the parasite grows as a large fluid-filled cyst in the liver, brain, lungs or body cavity. The multilocular hydatid cysts caused by *Echinococcus multilocularis* have a different growth form, metastasizing within organs and causing necrosis. The larvae of *Taenia solium*, the pork tapeworm, frequently develop in the central nervous system (CNS) and eyes. Some of the neurological

symptoms of the resulting condition, called *cysticercosis*, are caused by the pressure exerted by the cysts (Pommerville, 2009).

Intestinal worms cause a variety of pathologic changes in the mucosa, some reflecting physical and chemical damage to the tissues, others resulting from immunopathologic responses. Hookworms actively suck blood from mucosal capillaries. The anticoagulants secreted by the worms cause the wounds to bleed for prolonged periods, resulting in considerable blood loss. Heavy infections in malnourished hosts are associated with anemia and protein loss. Protein-losing enteropathies may also result from the inflammatory changes induced by other intestinal worms. Diversion of host nutrients by competition from worms is probably unimportant, but interference with normal digestion and absorption may well aggravate undernutrition (Pommerville, 2009).

Many helminths undertake extensive migration through body tissues, which both damage tissues directly and initiate hypersensitivity reactions. The skin, lungs, liver and intestines are the organs most affected. Petechial hemorrhages, pneumonitis, eosinophilia, urticaria and pruritus, organomegaly and granulomatous lesions are among the signs and symptoms produced during these migratory phases (Martindale, 2000; Pommerville, 2009).

Feeding by worms upon host tissues is an important cause of pathology, particularly when it induces hyperplastic and metaplastic changes in epithelia. For example, liver fluke infections lead to hyperplasia of the bile duct epithelium. Chronic inflammatory changes around parasites (for example, the granulomas around schistosome eggs in the bladder wall) have been linked with neoplasia, but the nature of the link is not known.

The continuous release by living worms of excretory-secretory materials, many of which are known to have direct effects upon host cells and tissues, may also contribute to pathology (Pommerville, 2009).

All helminths are “foreign bodies” thus they are antigenic and therefore stimulate immunity. Immune-mediated inflammatory changes occur in the skin, lungs, liver, intestine, CNS and eyes as worms migrate through these structures. Systemic changes such as eosinophilia, edema and joint pain reflect local allergic responses to parasites. The permeability of the mucosa changes, fluid accumulates in the gut lumen and intestinal transit time is reduced. Prolonged changes of this type may lead to a protein-losing enteropathy. Heavy infections with the whipworm *Trichuris* in the large bowel can lead to inflammatory changes, resulting in blood loss and rectal prolapse (Martindale, 2000; Pommerville, 2009).

Helminth infections are associated with down modulated responsiveness. In some cases there results in measurable attenuation of responses to bystander antigens, routine vaccination or allogenic tissue transplants. Helminthiasis is characterized by a strong type 2 immune profile which can down-regulate an anti-viral type 1 reaction. The immunological responses to frequent challenge from helminth parasites and other pathogens may influence rates of infection by HIV and/or progression to AIDS (Fincham *et al.*, 2003; Maizels *et al.*, 2003, 2004; Borkow *et al.*, 2004). Studies have shown that a balanced type 1/type 2 response is significantly related to long-term non-progression of HIV infection. When the maternal immune profile is imbalanced by helminthic infection,

the risk of transmitting HIV to babies appears to increase (Imami *et al.*, 2002; Gallagher *et al.*, 2005).

1.3.5 Treatment of helminthiasis

Most of the commonly used anthelmintics belong to one of 3 chemical classes: benzimidazoles, imidazothiazoles or macrocyclic lactones. However, there are other classes of anthelmintics as shown in Table 1.3.

The benzimidazoles are a large chemical family used to treat nematode and trematode infections in humans and domestic animals. They are characterized by a broad spectrum of activity against roundworms (nematodes), an ovicidal effect and a wide safety margin. They act by inhibiting microtubule synthesis that irreversibly impairs glucose uptake; thereby intestinal parasites are immobilized and die slowly. The adverse effects associated with this class of drugs include abdominal pain, nausea, vomiting, diarrhea and sometimes drowsiness and dizziness. However, thiabendazole may cause severe adverse effects such as erythema multiforme and fatal Stevens-Johnson syndrome. These drugs are contraindicated in pregnancy and in children below 2 years of age.

Pyrantel pamoate is also a broad-spectrum anthelmintic and it acts as a depolarizing neuromuscular blocking agent leading to worm paralysis. Its side effects are gastrointestinal disturbance, drowsiness, headache, insomnia, rash and fever.

Within the various classes of anthelmintics, all individual compounds act in a similar fashion. Thus, resistance to one particular compound may be accompanied by resistance to other members of the group. This phenomenon is known as cross-resistance. Levamisole resistance appears to be associated with alteration of cholinergic receptors

whereas that of benzimidazoles is associated with an alteration in beta- tubulin genes which interferes with the binding of benzimidazoles to tubulin. Resistance to an anthelmintic is expressed by passage of increased numbers of parasite eggs, higher survival rates of adult worms in the host and greater numbers of larvae on the pasture after treatment than would be seen if the parasites were susceptible to the drug. Reports of drug resistance have been made in every livestock host and to every anthelmintic class. This may have been due to heavy use of drugs to control livestock parasites over several decades. Even resistance to anthelmintic drugs in humans has been reported (Prichard, 1994; Geerts and Gryseels, 2001; Kaplan *et al.*, 2004; Sanyal, 2005; Sanyal and Dubey, 2010). To minimise the problem of drug resistance, the realistic strategy would be to develop novel approaches that decrease the need for treatment and to use the anthelmintics that remain effective in a manner that minimizes the risk.

Table 1.3. Classes of anthelmintics

Anthelmintic class	Examples
Benzimidazoles	mebendazole, flubendazole, fenbendazole, oxfendazole, oxibendazole, albendazole, thiabendazole, thiophanate, febantel, netobimin, and triclabendazole
Imidazothiazoles	Tetramisole, levamisole
Macrocyclic lactones	Avermectins: ivermectin, abamectin, doramectin, selamectin Milbemycins: milbemycin oxime, moxidectin
Tetrahydropyrimidines	Pyrantel pamoate, pyrantel tartrate and oxantel
Organophosphates	Metrifonate, trichlorfon, haloxon, naftalofos, coumaphos

1.3.6 Role of traditional medicine in treatment of helminthiasis

The traditional medical practitioner extensively uses plants in treatment of helminthiasis. A number of medicinal plants, that may provide possible alternatives, and are used by pastoralists and smallholder farmers in Kenya as deworming agents for their livestock and equines include: *Aframomum sanguineum*, *Albizia anthelmintica*, *Hagenia abyssinica*, *Ananas comosus*, *Annona squamosa*, *Dodonea angustifolia*, *Hildebrandtia sepalosa*, *Olea europaea* var. *africana*, *Azadirachta indica* *Myrsine africana* and *Rapanea melanophloeos* (Githiori, 2004).

A number of plants, either those used in African traditional medicine as anthelmintics or species closely related to them, have been tested elsewhere and some have been found to be useful. Raw garlic (*Allium sativum*), used in Nigeria as a vermifuge (Dalziel, 1937), was shown to have some activity against *Ascaridia galli* in chicken (Das and Thakuria, 1977) and to inhibit the embryonation of the eggs of *Necator americanus* and *Ancylostoma caninum* (Bastidas, 1970). The juice of *Citrus aurantifolia* is used as a vermifuge in Senegal and Sierra Leone and for treatment of dysentery in West Africa (Dalziel, 1937). A number of *Citrus* species such as *C. decumara*, *C. acida*, *C. aromaticum* and *C. medica* have been found to be active against *Ascaris lumbricoides* (Kaleysa, 1975).

Diospyros mespiliformis is used in Nigeria as a veterinary vermifuge and as a remedy for dysentery in humans (Dalziel, 1937). Diospyrol from the plant *Diospyros mollis* was shown to be superior to bephenium against *Necator americanus* in hamsters and against *Hymenolepis nana* in mice (Sen et al, 1975). Decoctions of the berries of *D. mollis* have

been used in antihookworm campaigns in Thailand (Sadavongvivad, 1980). Similarly several *Cucurbita* species, such as *C. maxima*, *C. moschata* and *C. aromatica*, have been shown to be taeniocidal in mice, rats and man (Srivastava and Singh, 1967; Albert *et al.*, 1972; Plotnikov *et al.*, 1973; Lozoya, 1978).

Red algae, *Digenia simplex*, have been used as a vermifuge for hundreds of years. Its active ingredient is called α -kainic acid and is marketed as a broad-spectrum anthelmintic against whipworm, roundworm and tapeworm. A chemically related compound, domoic acid isolated from the red algae *Chondria armata* and *Alsidium corallinum*, also has anthelmintic properties (Evans, 2002).

Evaluation of the effect of these plants contributes to the development of the scientific basis for their therapeutic application; and also considerably enriches the therapeutic arsenal for the treatment of a number of diseases (Castello *et al.*, 2002).

1.4 Infectious diseases and challenges in their management

Infectious diseases are the leading cause of premature deaths, killing almost 50,000 people every day. These diseases are also a major cause of disability, social and economic disruption for millions of people (WHO, 2003; Breman *et al.*, 2004). It is estimated that infectious diseases account for approximately one-half of all deaths in tropical countries and that they are the underlying cause of death in 8 % of the deaths occurring in the U.S.A (Pinner *et al.*, 1996).

In recent years, drug resistance to human pathogenic bacteria has been reported all over the world. With the continuous use of antibiotics, microorganisms have become resistant.

In addition to this problem, antibiotics are sometimes associated with adverse effects such as hypersensitivity and depletion of beneficial mucosal microorganisms. These negative health trends call for a renewed interest in infectious disease in the medical and public health communities and renewed strategies for treatment and prevention. The development of new antimicrobials is encouraged as one of the solutions to the problem (Fauci, 1998; Mukesh *et al.*, 2012).

Despite the existence of safe and effective interventions for the management of infectious diseases, many people lack access to needed preventive and treatment care. The lost productivity due to morbidity and high health care costs caused by infectious diseases directly impact negatively on families and communities. The adverse impact is most severe amongst the people with limited or no access to integrated health care, prevention tools and medication. The emergence of new infectious diseases such as Human Immunodeficiency Virus (HIV) has further complicated the situation. Between 14 and 17 million people still die each year due to infectious diseases- majority of whom live in resource limited countries (UNAIDS, WHO 2007). The emergence of the Human Immunodeficiency Virus (HIV) has spurred intensive investigation into the plant derivatives which may be effective, especially for use in resource limited nations with little access to expensive Western medicines.

The public is becoming increasingly aware of problems with the over prescription and misuse of traditional antibiotics. A multitude of plant compounds, often of unreliable purity, is readily available over-the-counter from herbal suppliers and natural-food stores, and self-medication with these substances is common place. The use of plant extracts, as well as other alternative forms of medical treatments, is enjoying great acceptance.

Mainstream medicine is increasingly receptive to the use of antimicrobial and other drugs derived from plants. This is because traditional antibiotics, which are products of microorganisms or their synthesized derivatives, have become ineffective and also new diseases, particularly viral, remain intractable to these types of drugs.

1.5 Use of plants as antimicrobials

Though most clinically used antibiotics are produced by soil microorganisms or fungi, higher plants have also been a source of antibiotics. Examples of plants with antimicrobial activity are *Allium sativum* (garlic) which contains allicin and *Hydrastis canadensis* (goldenseal) which contains berberine (Evans, 2002).

Cranberry juice has antimicrobial effects and has been used traditionally to prevent and even cure urinary tract infections. Cranberry's ability to maintain urinary tract health is not due to the acids in the juice as commonly thought, but rather to distinct flavonoids in cranberries known as proanthocyanidins (PACs). Proanthocyanidins help prevent urinary tract infections by blocking uropathogenic bacteria from adhering to the uroepithelium and proliferating to cause infection. These proanthocyanidins have an uncommon A-type intermolecular double linkage that appears to impart its unique antiadherence effect (Sobota, 1984; Zafriri *et al.*, 1989; Kyu and MacArthur, 2004). By preventing urinary tract infections, cranberry can reduce the need for antibiotics, which decreases the tendency for bacteria to develop antibiotic resistance.

Plants thus represent a vast untapped source of antimicrobial agents. Evaluating plants from the traditional African system of medicine can provide clues as to how these plants can be used in the treatment of disease.

1.6 Literature review of *Hagenia abyssinica*

Hagenia is one of the genera in Rosaceae family which is discussed below in 1.6.1.

1.6.1 Rosaceae family

The rose family (Rosaceae), in the order Rosales, is a large plant family containing more than 100 genera and about 3,000 species of trees, shrubs and herbs. The rose family is probably one of the six most economically important crop plant families. Some of them are cultivated as edible fruit trees such as apples, pears, quinces, medlars, loquats, almonds, peaches, apricots, plums, cherries, strawberries and raspberries. Others are highly valued ornamental shrubs such as *Crataegus*, *Potentilla*, *Prunus*, *Pyracantha*, *Rhodotypos*, *Rosa*, *Sorbus* and *Spiraea* (Watson and Dallwitz, 1992).

All plants in this family have powerful therapeutic, esthetic and aromatherapy properties. This is due to their high content of volatile oils essential for use in perfume industry and also the high quantity of vitamins contained in the fruits. Rosaceae wood is used in carpentry whereas stems and roots are used for making tannin extract and young leaves are used as a substitute for tea (Beentje, 1994; Aslam, 2002).

1.6.2 *Hagenia abyssinica* (Bruce) Gmel

It is a monotypic genus, *Hagenia abyssinica* which is a species of flowering plant native to the Afromontane regions of Central and Eastern Africa (Beentje, 1994). A photograph of the plant is presented in figure 1.3.



Figure 1.2. A photograph of a mature *Hagenia abyssinica* tree

(Photograph taken by H. C. D. de Wit, Biosystematics group; available at <http://www.prota4u.org>)

Hagenia abyssinica (Bruce) Gmel is known in English as African redwood and by various local names. These local names are presented in Table 1.4 (Kokwaro, 1976; Gachathi, 1989; Beentje, 1994). It is commonly known as *Kosso* in Ethiopia. It was formerly known as *Brayera anthelmintica* Kunth, *Banksia abyssinica* Bruce, *Hagenia anthelmintica* (Kunth) Eggeling, and *Hagenia abyssinica* (Bruce) Gmelin var. *viridifolia* Hauman (Jansen, 1981).

Table 1.4. Various local names of *Hagenia abyssinica* in Kenya

Community	Local name(s)
Kiswahili	<i>Omukunakuna</i>
Kikuyu	<i>Kamonde, Muinyeri, Mumondo</i> or <i>Muthithiku</i>
Maasai	<i>Olboldo</i>
Kipsigis	<i>Bondet</i>
Meru	<i>Mujogajoga</i>
Marakwet	<i>Seweruya</i>
Nandi	<i>Mjororuet</i>
Pokot	<i>Sorwa</i>

1.6.2.1 Ecology and distribution of *Hagenia abyssinica*

Hagenia abyssinica is found in the mountainous regions of Ethiopia, Kenya, Tanzania, Malawi, Zimbabwe, Zambia, Democratic Republic of Congo, Burundi, Rwanda, Uganda and Sudan (Jansen, 1981). This plant was once abundant in the semi-humid mountain woodlands with the altitudinal range of between 2,450 and 3,250 m. In forest depressions, it can descend to 2,000 m and gives a wider altitudinal range of 1,850 to 3,700 m. The species occurs naturally within the undifferentiated Afromontane (mixed *Podocarpus* forest) and dry, single-dominant Afromontane forest (*Juniperus* forest) or forest dominated by *Hagenia*. It is often found in association with tree species such as *Schefflera abyssinica* Harms, *S. volkensii* (Harms) as well as with the mountain bamboo,

Arundinaria alpine (Graham, 1960). In Kenya it is mainly dominant in the woodland zone just above the bamboo and also in moist forest below bamboo (Beentje, 1994).

1.6.2.2 Botanical description of *Hagenia abyssinica*

Hagenia abyssinica is a slender tree 5 to 25 m tall, with a short trunk and has thick branches; with branchlets covered in silky brown hairs and ringed with leaf scars (Gachathi, 1989). It often has a crooked bole and the bark is thick, brown or reddish-brown and readily peels as shown in figure 1.3.

The plant has compound leaves with 3-6 pairs of leaflets plus a terminal leaflet (Fig 1.4), each about 10 cm long. The leaflets are pale or bright green on the upper side with silvery hairs on the lower side but are normally reddish and sticky when young. The leaf margins are serrated and fringed with long hairs and the leaf stalks are 12 cm long, with expanded wings formed from the stipules, and are densely hairy on the underside.

The flowers form handsome multibranched, terminal, drooping panicles up to 60 cm long and 30 cm wide (Fig. 1.5). They are polygamo-dioecious with pinkish-red female heads while the male heads are feathery orange-buff to white. The fruits are usually small, dry, winged, asymmetric, single seeded, brown syncarp with a single ovoid carpel and fragile pericarp (Beentje, 1994).



Figure 1.3: A photograph showing a readily peeling *Hagenia abyssinica* bark



Figure 1.4: A photograph showing *Hagenia abyssinica* compound leaves

(Photographs taken in October 2011 at Brackenhurst, Limuru, Kenya)



Figure 1.5: A photograph of *Hagenia abyssinica* flowers

(Photograph taken by Jaroslav Stastny; available at <http://www.biolib.cz/en/image/id115804/>)

1.6.2.3 Uses of *Hagenia abyssinica* in traditional medicine

Hagenia abyssinica (Bruce) Gmel is an important medicinal plant that was used traditionally for combating various ailments. *Hagenia* has served as an anthelmintic in ruminants and also against tapeworms in humans. Though all parts of this medicinal plant are important to local communities, the most frequently used and mentioned part are the flowers, which are claimed to have anthelmintic properties (Feyissa T., 2006).

In Kenya, the roots of the plant are cooked with meat and the soup drunk for general illness and for malaria, while the dried and pounded female inflorescence is used as an anthelmintic especially for tapeworm. The bark may be pounded, the paste added to cold

water and the liquid drunk as a remedy for diarrhea and stomach ache. Large quantities can be taken to induce abortions (Gachathi, 1989; Beentje, 1994; Kokwaro, 2009).

In general, plant parts of *Hagenia* are processed either in fresh or dried forms. The medicine is usually taken orally as a decoction or a paste. These can be taken in combination with other plants such as *Girardinia bullosa* (Steudel) Wedd), pumpkin (*Cucurbita pepo* L.) seed, cabbage (*Brassica oleracea* L.) and fruits of *Embelia schimperi* Vatke. The combinations could result in enhanced medicinal effect against taeniasis, help reduce bitterness of the medicine as well as nausea, a common side effect of *Hagenia* preparations. Another means of application includes the utilization of bark in the form of smoke where it is claimed to ease muscle aches and stiff joints, stimulate blood circulation and boost the immune system. In addition, people apply the powdered seeds on wounds and cuts of both humans and livestock (Birukatyet *et al.*, 2010).

1.6.2.4 Non-medicinal use of *Hagenia abyssinica*

Hagenia abyssinica is a good source of firewood and charcoal. It is also used as timber and the wood is dark red, medium soft but not durable. It is used to make furniture, poles, flooring, carvings and cabinets. Young leaflets are spongy and are used as a mattress for mothers who have just given birth. *Hagenia* trees are employed in soil-conservation activities for erosion control. The fire-resistant species can be used as a firebreak. The plant constantly sheds leaves, providing mulch and green manure which improves the soil. As an ornamental, the attractive tree is suitable for planting in amenity areas (Negash, 1995).

1.6.2.5 Previous work done on *Hagenia abyssinica*

Hagenia abyssinica has been researched on from as far back as 1839. Various compounds have been isolated from its dried flowers. Among them are phloroglucinol derivatives which have also been isolated from other plant species. Phloroglucinol derivatives have shown antimicrobial, antidepressant, anthelmintic, anti-tumour, phytotoxic and anti-retroviral activities. They are also used as smooth muscle relaxants (Lounasmaa, 1973; Singh and Bharate, 2006). Examples of phloroglucinol derivatives isolated from *Hagenia* species include kosin (Fig. 1.7), a crystalline compound with a melting point of 142 °C (Hems and Todd, 1937). Other phloroglucinol derivatives include protokosin (Fig. 1.7) which is a colorless crystalline compound with no vermifugal properties. Kosidin and kosotoxin have also been isolated. Kosotoxin and kosin have anthelmintic activity against cestodes. These compounds have been used as a taenicide in veterinary medicine (Lounasmaa *et al.*, 1974, 1993; Abegaz *et al.*, 1999).

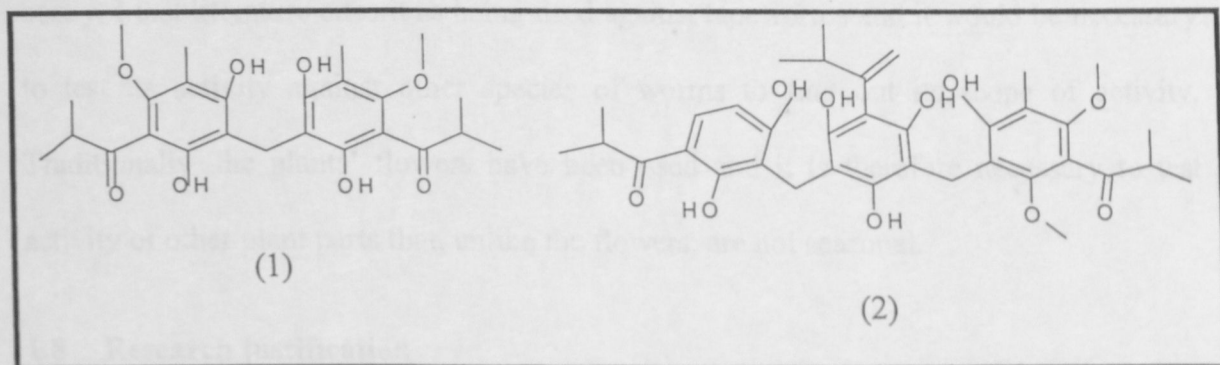


Figure 1.6. Structures of kosin (1) and protokosin (2)

Essential oil from *Hagenia abyssinica* (Rosaceae) was investigated for its chemical composition, trypanocidal and cytotoxic activities. Twenty components were identified from the essential oil of the female flowers with ledol (58.57%) being the principal

volatile oil component. The oils exhibited trypanocidal activities with IC₅₀ values of 42.30 µg/ml (Nibret and Wink, 2010).

1.7 Study problem

The potential of higher plants as a source of new drugs is still largely unexplored. Historically, pharmacological screening of compounds has been a source of many therapeutic agents with random screening being very productive in the area of antibiotics. Among the estimated 250,000 to 500,000 plant species, only a small percentage has been investigated phytochemically or screened for biological activity. Even for the few that have been studied, only a limited spectrum of activity has been investigated (Newman *et al.*, 2000).

The plant *Hagenia abyssinica* has been used traditionally for management of diarrhea, stomach ache and as an anthelmintic. There is need to provide scientific proof to the traditional claim. This will provide an alternative to modern drugs, which are usually costly. Most literature cites it as being used against tapeworms and it would be necessary to test its activity against other species of worms to find out its scope of activity. Traditionally, the plants' flowers have been used and it is therefore necessary to test activity of other plant parts that, unlike the flowers, are not seasonal.

1.8 Research justification

Screening of the plant's extracts for antimicrobial activity may reveal activity not previously reported and this could provide a template for development of new drugs, or even a decoction that can be used as an antimicrobial agent.

In addition, screening of other plant parts besides the flowers may reveal activity and this would mean that the other plant parts can be used when the flowers are not in season.

1.9 Objectives

1.9.1 General objective

The main objective of this study was to investigate the phytochemical composition of *Hagenia abyssinica* and to evaluate its anthelmintic and antimicrobial activity.

1.9.2 Specific objectives

The specific objectives of this study were to:

1. Isolate and characterize phytochemical constituents of *Hagenia abyssinica*.
2. Screen the extracts of *Hagenia abyssinica* for anthelmintic activity.
3. Screen the extracts of *Hagenia abyssinica* for antifungal and antibacterial activities.

CHAPTER TWO: EXPERIMENTAL

2.1 Plant collection, identification and preservation

The stem bark, leaves and flowers of *Hagenia abyssinica* were collected from the Aberdare ranges in Kiburu Forest Station and surrounding area in September 2009. The station is located in Njabini location, Kinangop division of Nyandarua South District. The plant was identified on site and local traditional medicine practitioners provided detailed information on folklore uses. The identity of the plant was confirmed by Mr. Mathenge at the National Museums of Kenya Herbarium. A voucher specimen designated HAP0036 was deposited at the School of Pharmacy, University of Nairobi.

The flowers were separated from stalks, leaves from branches and barks from stem. The stem bark was cut into small pieces. All parts were evenly spread to dry at room temperature. After drying, the plant parts were ground into coarse powder and stored in separate labeled plastic containers at room temperature until used.

2.2 Materials, reagents and equipments

2.2.1 Materials and reagents

Methanol was purchased from Alpha Chemicals Ltd (Nairobi, Kenya), while chloroform, petroleum ether and dichloromethane were obtained from Kobian Kenya Ltd (Nairobi, Kenya). Ethyl acetate and ethanol were from Kanha Laboratory Supplies (Nairobi, Kenya). The solvents which were of general purpose grade were distilled before use. Concentrated nitric acid was also obtained from Kanha Laboratory supplies. Artificial perienteric fluid (APF) was obtained from Fisher Scientific, USA.

Thin layer chromatography (TLC) was performed using aluminium TLC plates pre-coated with silica gel 60F₂₅₄. Silica gel 60F₂₅₄ and iodine were obtained from Merck (Darmstadt, Germany). Column chromatography was carried out using silica gel for column chromatography of porosity 32- 63 µm from Sigma-Aldrich GmbH & Co., Seelze, Germany. Filtration was done using Whatman filter paper No. 1 (Whatman International Ltd, Maidstone, England).

A 1% w/v vanillin was prepared using vanillin powder (BDH Chemicals Ltd., Poole, England) in concentrated sulphuric acid (Loba Chemie, PVT Ltd. Mumbai, India). Tween 80 was obtained from BDH Chemicals (Poole, U.K), while dimethylsulfoxide (DMSO) was from Fischer Scientific (Leicester, U.K.). Sodium chloride and Ferric chloride were purchased from Associated Chemical Enterprises (Glenvista, South Africa). Potassium iodide was from Sigma Chemicals Company (Louis, U.S.A.). Glacial acetic acid was obtained from Famitalia Carlo Erba (Milan, Italy)

2.2.2 Equipment

A grinding mill (Muharata Food Company, Nairobi, Kenya) was used to powder the dried plant material. A Sartorius top loading balance, Sartorius (Goettingen, Germany) was used to weigh samples above 500 mg and samples weighing up to 500 mg were weighed using an analytical balance (AUW220D Shimadzu Corporation, Kyoto, Japan).

The Chemical solvents were distilled using electrothermal laboratory distiller (Wassermangelsicherung GFL® GmbH Burgwedel, German) at Drug Analysis and Research Unit (DARU), University of Nairobi, Kenya.

Extractions were done using a Soxhlet apparatus (Quickfit, Birmingham, U.K.). The organic solvent extracts were reduced using a Rotary Vacuum Evaporator, (Heidolph Electro GmbH & Co. KG, Kelheim, Germany) connected to a cooler (Polyscience, Niles, USA), a WB2000 water bath (Heidolph Electro GmbH & Co. KG, Kelheim, Germany) and a diaphragm vacuum pump (KNF Neuberger GmbH, Freiburg, Germany). The reduced extracts were stored in a Samsung refrigerator (Samsung electronics, Seoul, South Korea) maintained at 2-8 °C.

A fractionating column with internal diameter of 25 mm and 1 m in length was used for fractionation. Fractions were collected using Superfrac automatic fraction collector from Pharmacia LKB Biotechnology (Uppsala, Sweden). A Min UV/Vis box (Desaga GMBH, Heidelberg, Germany) was used for visualising TLC plates.

The Nuclear Magnetic Resonance (NMR) spectroscopic data was obtained using a Varian-Mercury 200 MHz spectrometer (Varian Inc. Palo Alto, California, USA) with a magnet from Oxford Instruments (Oxford, UK). The data was acquired using an on-line computer (Sun Micro-Systems, California, USA) and analysed using Varian software. Mass spectrometric data was obtained by electron impact mass spectrometry using 175eV. A Fourier Transform infrared (IR) spectrophotometer (IRPrestige-21, Shimadzu Corporation, Kyoto, Japan) was used for infrared spectroscopy. A hydraulic press machine (Perkin-Elmer GmbH, Germany) was used to prepare the IR discs and IR solution software was used for analysis and recording.

2.3 Extraction of plant materials

About 600 g of the stem bark powder was subjected to Soxhlet extraction with chloroform for 48 h. The dried marc was then extracted by cold maceration (due to a breakdown of the Soxhlet apparatus) with methanol for a further 48 h.

About 1000 g of plant powder was packed into a percolating column and extracted with a 50:50 Methanol/ Dichloromethane mixture. Another 700 g of stem bark powder was subjected to Soxhlet extraction with petroleum ether for 48 h. All the extracts were filtered through Whatmann filter paper and reduced *in vacuo* to dryness and then stored at 2-8 °C.

2.4 Preparation of reagents

2.4.1 Dragendorff's reagent

Bismuth nitrate 1.7 g, 20 g tartaric acid and 20 ml glacial acetic acid were dissolved in 80 ml of distilled water to obtain solution A. About 16 g potassium iodide was dissolved in 40 ml distilled water to obtain solution B. Stock solution was obtained by mixing solution A and B in ratio 1:1 (v/v). To make Drangendorff's spray reagent, 5 ml of the stock solution was added to a solution of 10 g tartaric acid in 50 ml water (Brain and Turner, 1975).

2.4.2 Mayer's reagent

Around 1.355 g of mercuric iodide was dissolved in 60 ml water and 5 g potassium iodide dissolved in 20 ml water. The two solutions were then mixed and made to 100 ml

with water to make Mayer's reagent also known as potassium mercuric iodide (Brain and Turner, 1975).

2.4.3 Iodine

Iodine vapor was prepared by placing 5 g of iodine at the bottom of a glass chromatographic tank which was covered on top and the iodine allowed to sublime and saturate the tank with iodine vapor for 1 h. Developed TLC plates were then introduced in the closed tank for 5 min. It was used for detection of compounds containing conjugated double bonds.

2.4.4 Vanillin in concentrated sulphuric acid

One percent w/v vanillin in concentrated sulphuric acid was prepared by dissolving 0.5 g of vanillin in 50 ml concentrated sulphuric acid. This was sprayed on the developed TLC plates and the plates heated in an oven at 110 °C for 5 minutes for visualization.

2.5 General phytochemical tests

These tests were carried out to confirm the phytochemical constituents of *Hagenia abyssinica*.

2.5.1 Test for Cardiac glycosides

The stem bark and inflorescence were screened for cardiac glycosides using Keller-Kiliani test (Brain and Turner, 1975) and Kedde test (Evans, 2002). About 1 g of the stem bark powder was heated with 10 ml of 70 % alcohol in a water bath for 2 min and filtered. To the filtrate, 10 ml of water and 5 drops of a strong solution of lead subacetate were added and the solution filtered. This precipitated chlorophyll and other pigments

which were filtered off. To the filtrate, 10 % v/v H_2SO_4 was added dropwise until no further precipitation occurred. The filtrate was then extracted with chloroform and the chloroform solution divided into two portions, A and B. Both portions were evaporated to dryness over a water bath. Portion A was dissolved in 3 ml of 3.5 % $FeCl_3$, in glacial acetic acid. The solution was then left to stand for 1 min and then transferred to a test tube. Around 1.5 ml of concentrated H_2SO_4 acid was added slowly so that it ran down the wall of the tube and formed a separate layer at the bottom. On standing, a brown color formed at the interface indicating the presence of deoxy sugars; and a pale green color in the upper layer indicated the presence of a steroidal nucleus.

To portion B, 1 drop of 90 % alcohol and 2 drops of 2 % 3, 5-dinitrobenzoic acid in 90 % alcohol were added, and then made alkaline with 20 % sodium hydroxide solution. A purple color showed the presence of a five-membered lactone ring.

The three tests taken together indicate presence of a cardiac glycoside.

2.5.2 Test for alkaloids

Acid alcohol was made by mixing 1 ml of 1 % acetic acid with 99 ml of 75 % v/v ethanol. About 1 g of the bark powder and inflorescence powder was boiled with 10 ml of the acid alcohol in a boiling tube for 1 min and cooled. The supernatant was filtered into another tube. To about 1 ml of the filtrate, 3 drops of Dragendorff's reagent was added. Precipitation suggested presence of alkaloids (Brain and Turner, 1975).

2.5.3 Test for saponins

A little of the bark powder and inflorescence powder was shaken with distilled water. A persistent froth suggested the presence of saponins (Brain and Turner, 1975).

The extracts were dissolved in water and Tween 80 used to help solubilise chloroform extract. Two ml of 1.8 % w/v sodium chloride solution was added into each of the two test tubes. To one tube 2 ml distilled water was added and to the other 2 ml of the extract to make the concentration of sodium chloride in each tube isotonic with blood serum. One drop of blood was added into each tube and the tubes gently inverted to mix the contents. Haemolysis in the tube containing the extracts but not in the control indicated the presence of saponins.

2.5.4 Test for tannins

Around 0.1 g of the plant powder was extracted with 10 ml hot water for 5 min and filtered while hot. The filtrate was cooled and to a 2 ml portion three drops of ferric chloride solution was added. To another 2 ml portion, 1 ml of lead subacetate was added. Development of a brown-green precipitate with ferric chloride and a creamy-brown precipitate with lead subacetate suggested the presence of tannins (Evans, 2002).

2.6 Screening for anthelmintic activity

The plant extracts were first screened for anthelmintic activity against roundworms. Most literature cited refers to the plant's activity against tapeworms and this screen was to assess if the plant also has activity against roundworms.

2.6.1 Screening for activity against roundworms

The free living nematode *Panagrellus redivivus* served as a model organism to determine lethality of plant extracts to roundworms. This species is advantageous for natural products screening because it is easy to grow and maintain, and possesses no human health threat.

In this qualitative assay, one hundred microlitres of worm suspension were placed in 96 well plates and 5 μ l of 60 % ethanol was added to the first two wells to serve as the negative control and 5 μ l of CuSO_4 (160 mg/ml) was added to the next 2 wells to serve as positive control. Five microlitres of each of the plant extracts was added to the other wells and plate incubated for 4 hours. The plate was observed from beneath by holding a light to it. If worms were killed by the plant extract, they stopped wriggling and collected in the bottom of the u- shaped well. Extracts found to be active would be subjected to assays using *Caenorhabditis elegans*.

2.6.2 Assays using *Caenorhabditis elegans*

2.6.2.1 Introduction

The free living nematode, *Caenorhabditis elegans* was used as the model for anthelmintic activity testing. Unlike many parasitic worms, *C. elegans* of any stage can be obtained in large quantities by virtue of their simple and inexpensive cultivation method and their short generation time. It is maintained in the laboratory on nematode growth medium (NGM) agar which has been aseptically poured into petri dishes and seeded with *Escherichia coli* OP50 as a food source. Despite its wide use as a model organism, *C. elegans* cannot provide information about the adaptations essential for or associated with parasitism. The cuticle of *C. elegans* is a permeability barrier to xenobiotics and the objective of the cut worm assay is to overcome this barrier.

2.6.2.2 Synchronizing *Caenorhabditis elegans* worm cultures

In this assay, *C. elegans* was grown in NGM plates with OP50 bacteria for about 3 days when there were a large number of gravid adults. The worms were transferred into a

sterile falcon tube using sterile M9 buffer. The tube was centrifuged at 1000 rpm at room temperature for 30 seconds. The supernatant was discarded and alkaline bleach solution (5 ml) added to the tube. The tube was incubated at room temperature with occasional shaking for 3 minutes. The tube was centrifuged and the supernatant discarded. The worms were washed in M9 buffer three times to ensure all the alkaline bleach had been removed. The worms were re-suspended in M9 buffer and left on a rocker at room temperature for 2 days.

2.6.2.3 Whole worm assay

The media was prepared by addition of 7 ml OP50 to 62.3 ml of sterile M9 buffer. To the mixture, 0.7 ml ampicillin was added and 2 ml of the media was pipetted into each well of a 48 well plate.

The worm suspension was prepared by introducing the synchronized cultures into nematode growth media plates with OP50 and incubated at room temperature for 44 h. The plates were then washed with M9 buffer and the worms allowed to pellet by gravity. The worms were then re-suspended in 5 ml M9 buffer. One hundred microlitres of the worm suspension was pipetted into a microscopic slide and the worms counted. The same procedure was repeated using 50 μ L of the worm suspension.

Fifty microlitres of the worm suspension was pipetted into each well and the worms in each well were counted. Five microlitres of the test compound (10 mM for pure compounds and 20 mg/ml for extracts) were introduced into the wells and the plates were incubated for 24 h at 22 °C. The anthelmintic activity of test extracts against the model organism was determined by enumeration of living and dead nematodes under a

microscope and the percentage of worms surviving after 24 h incubation period was recorded.

2.6.2.4 Cut worm assay

Worms were grown on nematode growth media plates as described above. Adult worms were picked and transferred onto a slide containing artificial perienteric fluid (APF). Extracts were diluted at a final concentration of 1mg/ml in DMSO. The positive control (levamisole) was dissolved in artificial perienteric fluid, APF, to a final concentration of 100 μ M. The negative control was 5 % DMSO in APF. The worms were cut transversely between the anterior and middle segments, making sure that the cut was clean then transferred into 100 μ L of the test solution in APF. The worms were observed under the microscope and the time taken to cause paralysis noted.

The time to paralysis was analyzed using Survival Analysis Model. The software used to achieve this was STATA[®] Version 12 (Corp, Texas, USA). The hazard ratio (HR) and their 95% confidence intervals (C.I) were displayed. P values of < 0.05 were regarded as statistically significant.

2.7 *In vitro* testing for antimicrobial activity

Bacterial test microorganisms: *Staphylococcus aureus*, *Escherichia coli* and *Bacillus subtilis* were subcultured onto slants of Tryptone Soy Agar and incubated at 37 °C for 24 hours while the fungal test microorganisms, *Saccharomyces cerevisiae* was subcultured on Sabourauds Dextrose Agar at 25 °C for 48 hours.

All the plant extracts were weighed and dissolved in dimethyl-sulphoxide (DMSO) to a final concentrations of about 50 mg/ml solutions. Erythromycin and nystatin solutions were used as standards at concentrations of 10 µg/ml and 100 µg/ml respectively.

The plate agar diffusion method was adopted according to Kavanagh (1972) to assess the antibacterial activity of the prepared extracts. Nutrient agar was prepared according to manufacturer's instructions. It was sterilized in a steam autoclave at 121 °C for 20 mins and left to cool to about 50 °C on the bench. A loopful of the standardized microorganism stock suspensions was thoroughly mixed with 100 ml of the sterile nutrient agar to produce an inoculum of approximately 1×10^6 colony forming units per ml. Twenty millilitres of the inoculated nutrient agar were distributed into sterile petri dishes to form uniform thickness of about 3 mm. The agar was left to set. In each of these plates, 8 bores of 8 mm in diameter were cut using a sterile cork borer and the agar discs removed. Alternate bores were filled with 50 µl of each extracts, DMSO and standards using microtiter pipettes and allowed to diffuse at room temperature for 0.5 h in a laminar flow cabinet. The plates were then incubated in the upright position at 37 °C for 18 hrs. Two replicates were carried out for each extract against each of the test organism. After incubation the diameters of the growth inhibition zones were measured, averaged and the mean values were tabulated.

2.8 Isolation of compounds

Thin layer chromatography was performed to determine the suitable mobile phase for fractionation of the extracts. Fractionation was done by column chromatography using silica gel as the stationary phase. Two chromatographic columns were ran. In the first column, the petroleum ether extract was subjected to gradient elution with n- hexane,

dichloromethane, ethyl acetate and methanol. In the second column, the chloroform extract was eluted isocratically with chloroform.

Every other tenth tube of the fractions from each of the columns was profiled by thin-layer chromatography using 90:10 % v/v chloroform / methanol as the mobile phase. The crude extract was used as reference. The spots in the chromatograms were detected by visualization in ordinary light, observing under both short and long UV light (254 nm and 366 nm respectively), exposure to iodine and spraying with 1% vanillin. The fractions showing similar TLC profile were pooled to give seven fractions, HA1, HA2, HA3, HA4, HA5, HA6 and HA7. All the fractions were reduced *in vacuo* to a volume of about 10 ml and left to evaporate at room temperature.

Fraction HA1 formed clear crystals on the sides of the wall of the test tube. The crystals were scrapped off and redissolved in methanol before recrystallising in acetone. This recrystallized portion was designated as compound **KWE02**. The quantity of KWE02 obtained was 3 mg.

Fraction HA4 formed a crystalline deposit after four days. The deposit was washed with methanol and dissolved in chloroform and left overnight and recrystallised in acetone yielding 23 mg. The compound obtained was designated **KWE01**. Thin layer chromatography of the crystals was carried out and then further recrystallisation carried out by dissolving in 10 % v/v methanol in chloroform and slowly evaporating the solution.

The other fractions formed very little amounts of deposits and thus no further investigations were carried out on them.

The two compounds, KWE01 and KWE02, were subjected to mass spectrometry in an attempt to elucidate their structure. Further, the NMR and IR spectra of KWE01 were obtained.

The results of the phytochemical tests, as depicted in Table 3.1, show that the leaves, stem bark and root bark of *Moringa stenoptera* contain cardiac glycosides, saponins, tannins and alkaloids.

Table 3.1. Phytochemical groups present

Phytochemical	Stem bark	Flowers	Leaves
Alkaloids	present	present	Present
Cardiac glycosides	present	present	Present
Tannins	present	present	Present
Saponins	present	present	present

The phytochemicals previously reported in this plant are flavonoids such as quercetin and rutin, chlorophylls, glycosides, saponins, phenols and phenolic compounds as ellagic acid (Choudhary et al., 2012).

The results of the alkaloids, saponins and cardiac glycosides have been reported from this plant.

3.1. Yields of plant extracts

The yields obtained from the extraction of the plant are shown in table 3.2.

CHAPTER THREE: RESULTS AND DISCUSSION

3.1 Phytochemical composition of *Hagenia abyssinica*

The results of the phytochemical tests, as depicted in Table 3.1, show that the leaves, flowers and stem bark of *Hagenia abyssinica* contain cardiac glycosides, saponins, tannins and alkaloids.

Table 3.1. Phytochemical groups present

Phytochemical	Stem bark	Flowers	Leaves
Alkaloid	present	present	Present
Cardiac glycosides	present	present	Present
Tannins	present	present	Present
Saponins	present	present	absent

The phytochemicals previously reported in this plant are flavonoids such as quercetin and rutin, phloroglucinol glycosides, cyanidin glycosides and phenolic acids such as ellagic acid (Thomsen *et al.*, 2012).

This is the first time that alkaloids, saponins and cardiac glycosides have been reported from this plant.

3.2 Yields of plant extracts

The yields obtained from dried plant material extraction are as shown in table 3.2.

Table 3.2. Yields of crude plant extract

Extract	Stem bark	Flower
Dichloromethane/ methanol	9.26%	7.8 %
Petroleum ether	0.345%	0.2%
Chloroform	2.9%	2.43%
Methanol	9.81%	7.02%

Sohxlet extraction was the method used except for dichloromethane/ methanol extracts

3.3 Anthelmintic activity

3.3.1 Activity against roundworms

This was a qualitative assay where the activity against roundworms was tested against *Panagrellus redivivus*. A positive result was deduced if worms stopped wriggling and collected in the bottom of the U- shaped wells.

The methanol and the dichloromethane/ methanol crude extracts of all parts of the plant exhibited anthelmintic activity while the petroleum ether and chloroform extracts showed no activity. The isolated compounds exhibited no activity. This suggests that anthelmintic activity resides in the polar components since non polar extracts showed no activity.

3.3.2 Whole worm assay

At the beginning of assay there were 13 worms in each well. Table 3.3 shows the percentage of live worms after a 24 h incubation period with the drug under test.

Table 3.3. Percentage of worms alive after incubation at room temperature for 24 h

Test extract/ Compound	Adult worms alive after 24 h (%)
M9 Buffer	88
5 % DMSO	77
Levamisole 10mM	15
<i>H. abyssinica</i> 20 mg/ml MeOH extract	33

The anthelmintic activity of *Hagenia* methanol extract was determined by enumeration of living and dead nematodes using a microscope. The light stimulation during the microscopic investigation induced movement of living nematodes where agile nematodes were considered alive and immotile ones considered being dead. 5 % DMSO was the negative control and was not expected to affect the viability of worms. The buffer in which the worms were maintained was also not expected to affect their viability. However, 12 % of the test worms died in the buffer and 23 % died in the negative control. This may have resulted from the drying effects caused by light during microscopic investigation.

The worms incubated with the known anthelmintic, 20 mg/ml levamisole served as a positive control. Incubation resulted in 85 % death of all the worms while incubation with the test drug resulted in 67 % death. This shows that *Hagenia abyssinica* stem bark methanol extract has anthelmintic activity but it is less active than levamisole.

3.3.3 Cut worm assay

The results obtained for cut worm assay are as presented in table 3.4.

The time taken for the worms to be completely paralyzed was shortest for the positive control, levamisole, and longest for the negative control. Hagenia extract showed moderate activity with the time to paralysis being shorter than that of the negative control except for worm number five which can be considered as an outlier.

Table 3.4: Time (min) taken for *C. elegans* worms to be completely paralyzed after administration of drug compounds/ extracts

Worm	Levamisole 100 μ M (Positive control)	5% DMSO in APF (Negative control)	1mg/ml <i>Hagenia</i> <i>abyssinica</i> methanol extract
1	1.50	6.40	5.52
2	1.55	6.35	2.10
3	1.57	6.41	2.52
4	1.37	7.02	5.38
5	3.00	6.38	8.07
6	3.20	12.5	7.50
Mean	2.03	7.51	5.18

The mean time taken to paralyze the worms by *Hagenia* methanol extract was 1.7 times less than that taken by the negative control, DMSO. However, the evidence to support that was weak (HR 1.73, 95% C.I 0.51- 5.84, P= 0.38).

3.4 Antimicrobial activity

Table 3.5 presents the mean diameters of the zones of inhibition produced by the stem bark extracts of *H. abyssinica* in comparison with standard antimicrobials. The extracts were used at a concentration of 50 mg/ml. There was a significant difference in antibacterial activity for extracts obtained using different solvents.

The petroleum ether and dichloromethane/ methanol extracts each at a concentration of 50 mg/ml were found to be active against all the bacterial micro-organisms and exhibited a high degree of antibacterial activity. The dichloromethane/ methanol extract at a concentration of 50 mg/ml, showed activity against *Staphylococcus aureus* (with a zone of inhibition of 19 mm), *Escherichia coli* (20 mm) and *Bacillus subtilis* (18 mm) whereas the petroleum ether extract at the same concentration showed activity against *Staphylococcus aureus* (with a zone of inhibition of 17 mm), *Escherichia coli* (16 mm) and *Bacillus subtilis* (16 mm) while that of erythromycin at a concentration of 0.01 mg/ml was 22 mm. However the methanol extract exhibited no antibacterial activity.

The antibacterial activity against both the gram positive and gram negative bacteria exhibited by the dichloromethane/ methanol and the petroleum ether extracts could be due to the phenolics, alkaloids or the phytosterols present in the plant.

All four extracts did not exhibit any antifungal activity against the test micro-organism. The results for antifungal activity are unlike what was obtained in a previous study by Abera *et al.*, 2011 in which *Hagenia abyssinica* ethanolic and aqueous extracts showed

mild activity against *Colletotrichum kahawae*, a fungus which causes Coffee Berry Disease.

Table 3.5: Zones of inhibition (mm) of bacterial and fungal growth by *Hagenia abyssinica*

Test solution	Diameters of zones of inhibition (mm)				
	<i>Staphylococcus aureus</i>	<i>Escherichia coli</i>	<i>Bacillus subtilis</i>	<i>Saccharomyces cerevisiae</i>	<i>Candida albicans</i>
50 mg/ml					
dichloromethane/ methanol extract	19.0	20.0	18.0	8.0	8.0
chloroform extract	8.0	8.0	9.0	8.0	8.0
methanol extract	8.0	8.0	8.0	8.0	8.0
petroleum ether extract	17.0	16.0	16.0	8.0	8.0
Erythromycin 0.01mg/ml	22.0	20.0	20.0	-	-
Nystatin 100mg/ml	-	-	-	10.0	12.0

Zones of inhibition included 8.0 mm well diameter

3.5 Structure elucidation of isolated compounds

3.5.1 KWE-01

The compound was isolated as colorless needle-like crystals in acetone with a melting point of 135 – 137°C. The compound could not be visualized under UV but gave a yellow spot when the TLC plate was put in a saturated iodine tank and a purple colour when sprayed with 1 % vanillin in concentrated H₂SO₄. In addition, KWE-01 gave the following spectroscopic data:

IR λ_{\max} (KBr) cm^{-1} 3456.4(O-H str), 2935.6 (aliphatic C-H str), 2860.4 (CH₂ C-H str), 2333.8, 1720.5, 1629.8(C=C str), 1466.9 (CH₃ C- H bend), 1373.3, 1261.4, 1103.2, 1049.2, 958.6(=C-H bend), 729.1, 472.5

¹H-NMR δ H (200MHz, acetone- d₆): 8.14 (s, 1H), 5.31 (d, 2H, J = 5.2 Hz, H-6), 4.73 (s, 1H), 3.71 (d, 1H, J= 4.8 Hz, H- 3), 3.38 (m, 2H, J= 5.4Hz), 2.88 (d, 24H, J= 6.2 Hz), 2.18 (d, 4H, J= 4.0 Hz), 0.85, 0.87, 0.90 (3H, d, CH₃-21), 0.95, 1.02 (3H, s, 64 CH₃-19), 1.13, 1.16, 1.24, 1.45, 1.53, 1.61, 1.85, 1.87, 1.99, 2.23,

¹³C NMR(acetone,50MHz): δ 141.7, 120.9, 71.0, 57.0, 56.3, 50.5, 46.0, 42.7, 42.4, 39.9, 37.6, 36.6, 36.3, 34.0, 32.1, 32.0, 31.8, 30.3, 28.3, 26.1, 24.3, 23.1, 21.1, 19.4, 19.1, 18.7, 18.6, 11.6, 11.6

MS m/z 437.19 (M+Na)⁺

Infrared spectrum showed the presence of hydroxyl group at 3456.4 cm^{-1} and an aliphatic system 2935.6 cm^{-1} (CH₃ C-H str), 1466.9 cm^{-1} (C-H bend). Peak at 1654 cm^{-1} was due to the double bond between C5 and C6.

The compound was identified as β -sitosterol based on spectroscopic data which is in agreement with literature. Infrared spectrum showed hydroxyl (3456 cm^{-1}) and a double-bond (1630 cm^{-1}). The mass spectrum gave a molecular ion at $m/z\ 437.19\ (M + Na)$, corresponding to the molecular formula, $C_{29}H_{50}O$, for β -sitosterol. The H-NMR spectrum showed 2 tertiary methyl groups at $\delta\ 0.68$ and 1.02 , corresponding to C-18 and C-19. Three secondary methyls appeared at $\delta\ 0.92$, 0.82 and 0.84 , corresponding to C-21, C-26 and C-27, respectively. C-29 appeared as triplet at $\delta\ 0.85$.

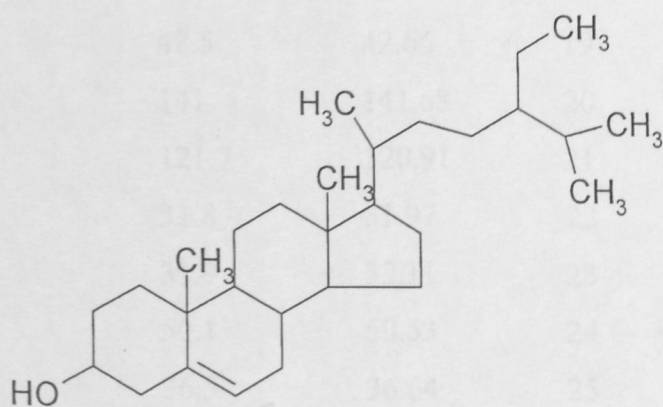


Figure 1.7: Chemical structure of beta-sitosterol

Table 3.6: Comparison of KWE-01 ¹³C chemical shifts to the literature values for β-sitosterol

Carbon	¹³ C chemical shifts		Carbon	¹³ C chemical shifts	
	β- sitosterol	KWE-01		β- sitosterol	KWE-01
1	37.2	37.57	16	28.2	28.31
2	31.6	31.84	17	56.0	
3	71.8	71.04	18	11.9	11.58
4	42.5	42.66	19	19.4	19.14
5	141	141.68	20	36.1	36.27
6	121.7	120.91	21	18.8	18.56
7	31.8	31.97	22	33.9	34.02
8	31.9	32.11	23	26.0	26.06
9	50.1	50.53	24	45.8	46.03
10	36.5	36.64	25	29.1	30.33
11	21.1	21.14	26	19.8	19.44
12	39.7	39.99	27	19.1	18.67
13	42.3	42.42	28	23.0	23.07
14	56.7	56.9	29	12.0	11.62
15	24.3	24.28			

3.5.2 KWE-02

The melting point of KWE02 was above 289 °C. IR $\lambda_{\max}(\text{KBR}) \text{ cm}^{-1}$ 3423.65(O-H str), 2862.36(CH₃ C-H str), 2335.80, 1463.97, 1375.25, 1051.20, 956.69(=C-H bend), 796.60, 476.42.

The compound was obtained in insufficient quantity thus NMR was not carried out. However, the results for mass spectrometry indicated it could be a mixture of sterols.

CHAPTER FOUR: CONCLUSIONS AND RECOMMENDATIONS

4.1 Conclusion

Phytochemical studies showed that the plant powders contained alkaloids, saponins and cardiac glycosides. The extraction was done by maceration and percolation. Thin layer chromatography using pre-coated aluminium plates was used to monitor the fractions. Two crystalline compounds were isolated and analyzed using spectroscopic methods. One of the compounds was identified as β -sitosterol. The second was found to be impure and thus could not be identified. The *in vitro* antimicrobial and anthelmintic activities of crude extracts of the stem bark and flowers were investigated. Some extracts of the plant were prepared and screened for both antibacterial and antifungal activities using agar diffusion method. Petroleum ether extract and dichloromethane/methanol extracts showed activity against all the bacteria microorganisms tested namely; *Staphylococcus aureus*, *Esherichia coli* and *Bacillus subtilis*. The chloroform and the methanol extracts lacked antibacterial activity. All the extracts lacked any antifungal activity at a concentration of 50 mg/ml.

The MeOH and DCM/MeOH stem bark and flower extracts exhibited anthelmintic activity while the petroleum ether and chloroform extracts showed no activity. This shows that anthelmintic activity resides in the polar components. It also shows that *Hagenia abyssinica* has broad spectrum of anthelmintic activity as earlier literature cites it as a taeniocide.

4.2 Recommendations

The isolated compounds were from the nonpolar fraction which did not exhibit anthelmintic activity. The polar fraction should be further worked on to isolate the compounds responsible for this activity.

The extracts from the stem bark had antibacterial and anthelmintic activity. Previously it was the flowers that were known to have anthelmintic activity. The current study has shown that the bark also possesses similar activity. Thus the use of the plant does not have to be limited to the time of the year when the flowers are available in season as the bark can be harvested all year round.

The petroleum ether and dichloromethane/ methanol stem bark extracts had significant antibacterial activity. Further work is recommended in order to isolate the active compounds.

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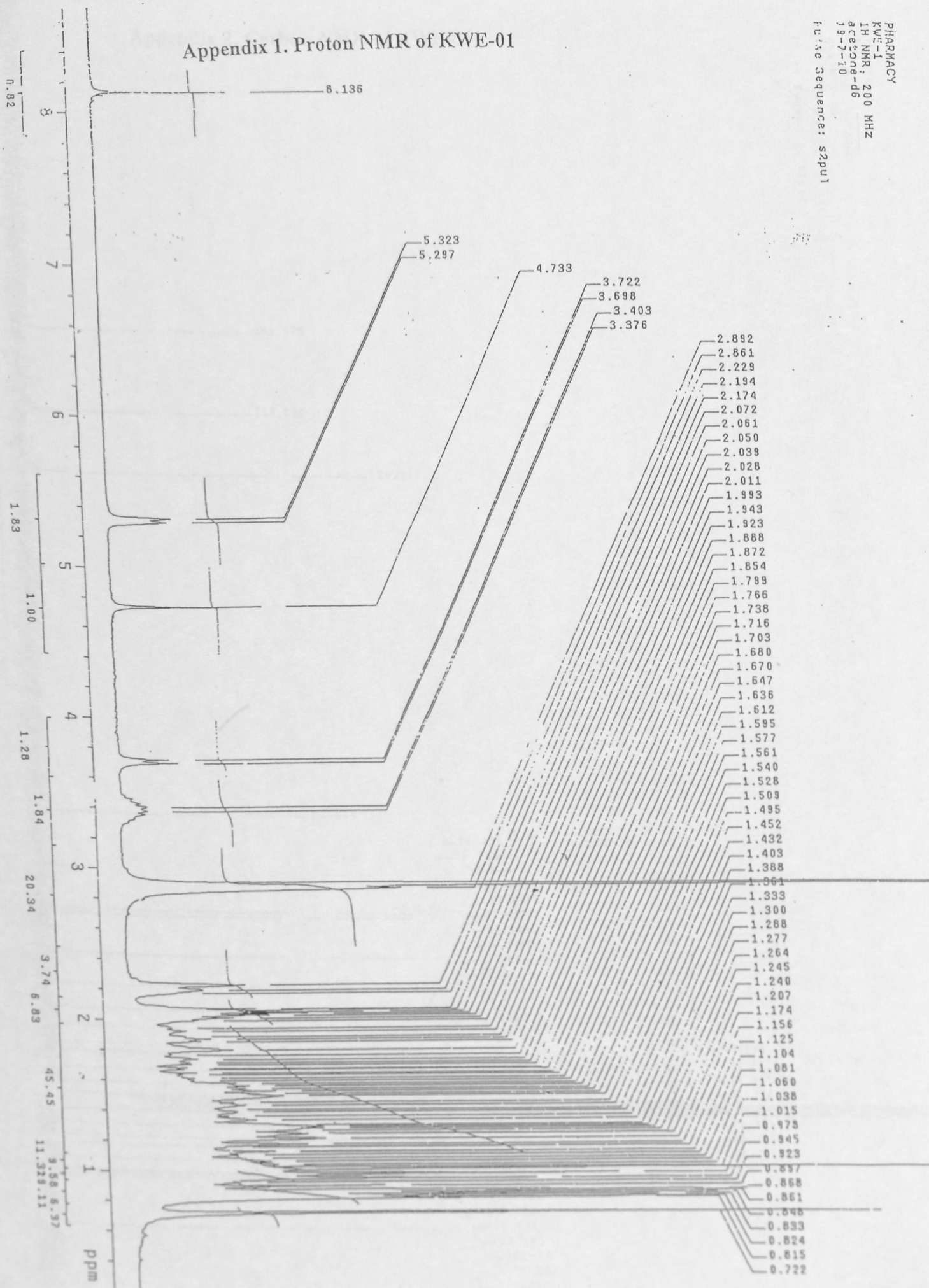
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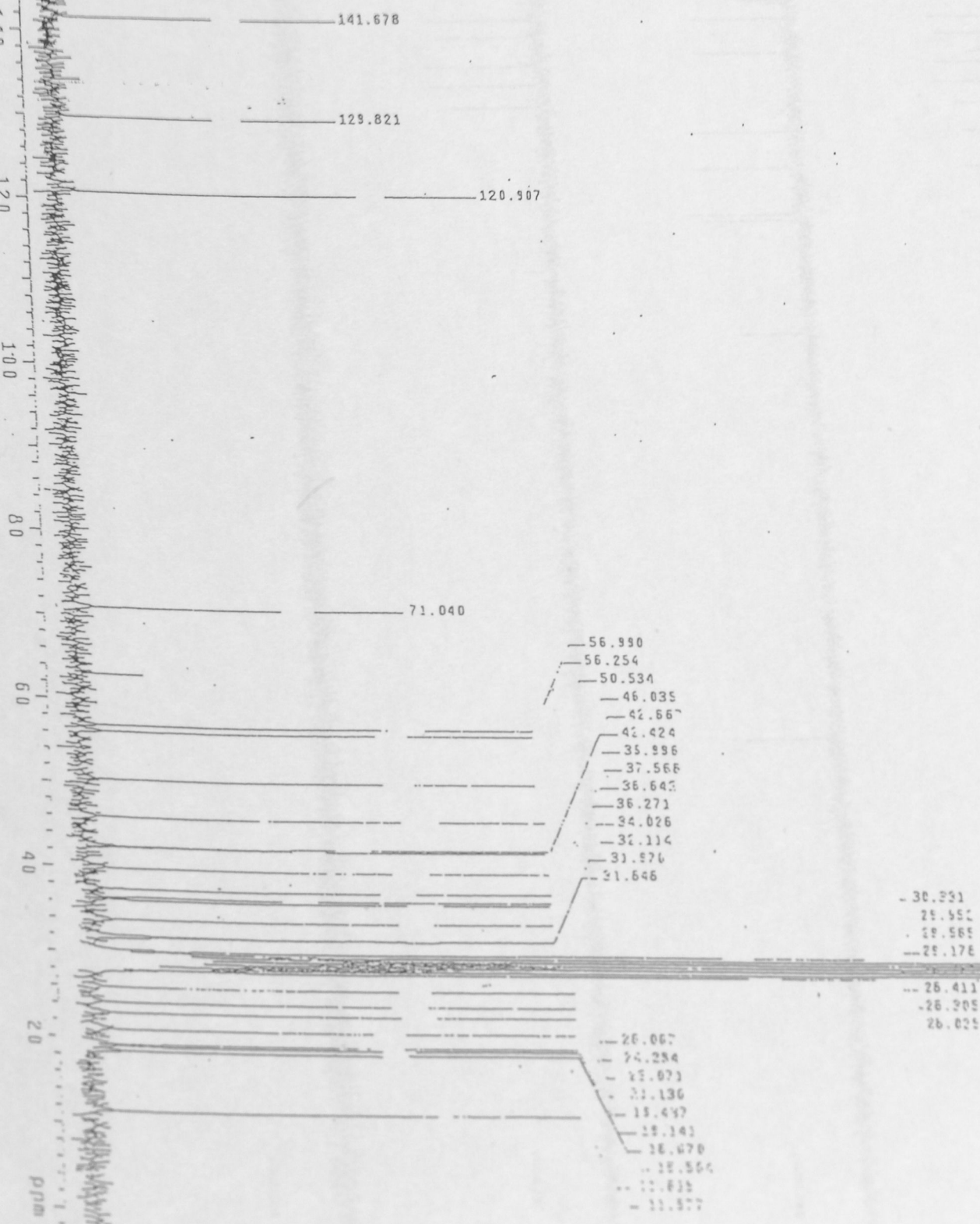
Appendix 1. Proton NMR of KWE-01

PHARMACY
 KWE-01
 1H NMR; 200 MHz
 acetone-d6
 19-7-10
 File Sequence: s2pu1



Appendix 2. Carbon NMR of KWE-01

PROGRAM: X
 KWE-1
 13C NMR, 50 MHz
 Acetone-d6
 19-7-10
 Pulse Sequence: s2pu1



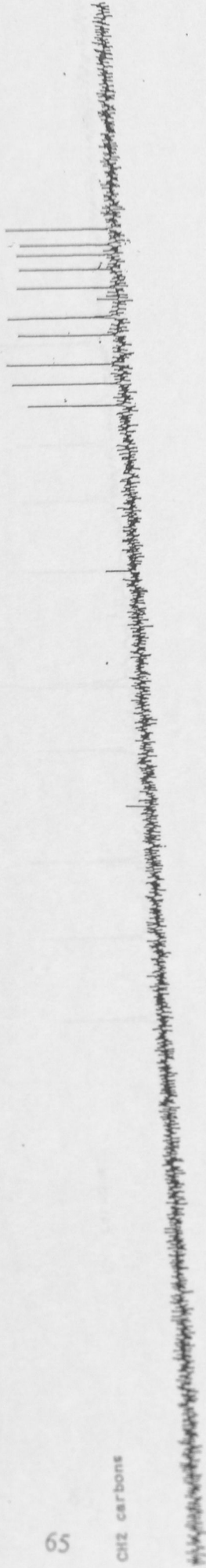
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-7-10

CH3 carbons

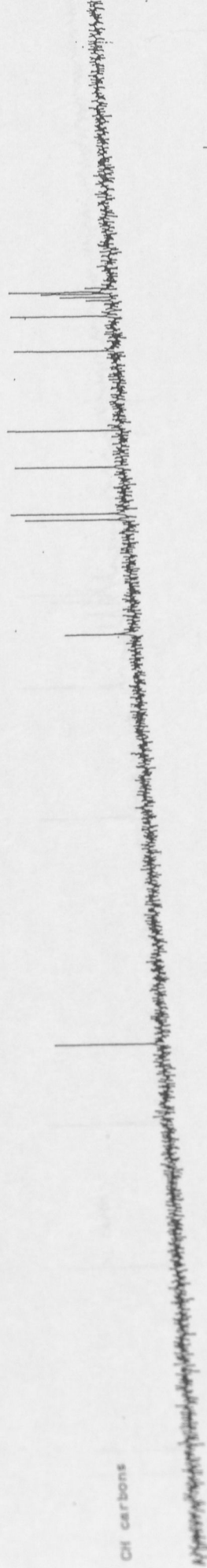


65

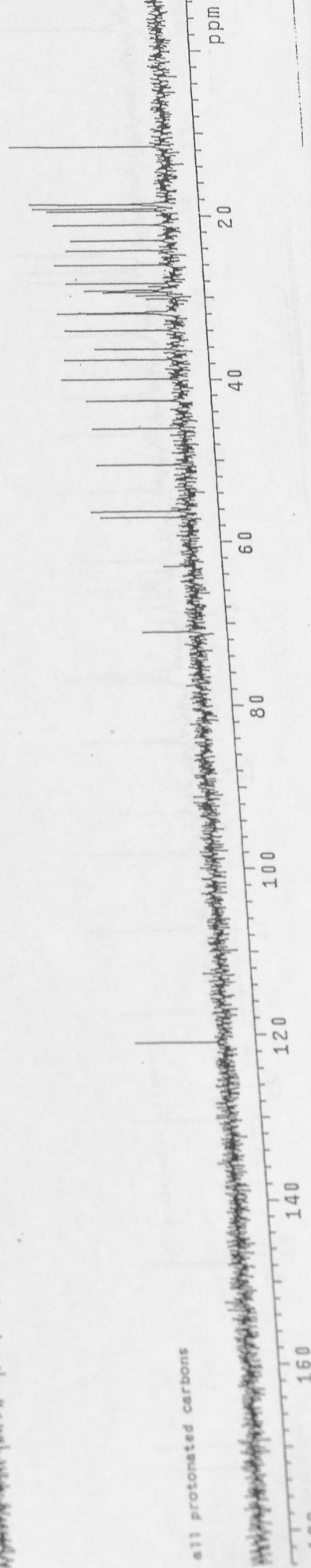
CH2 carbons

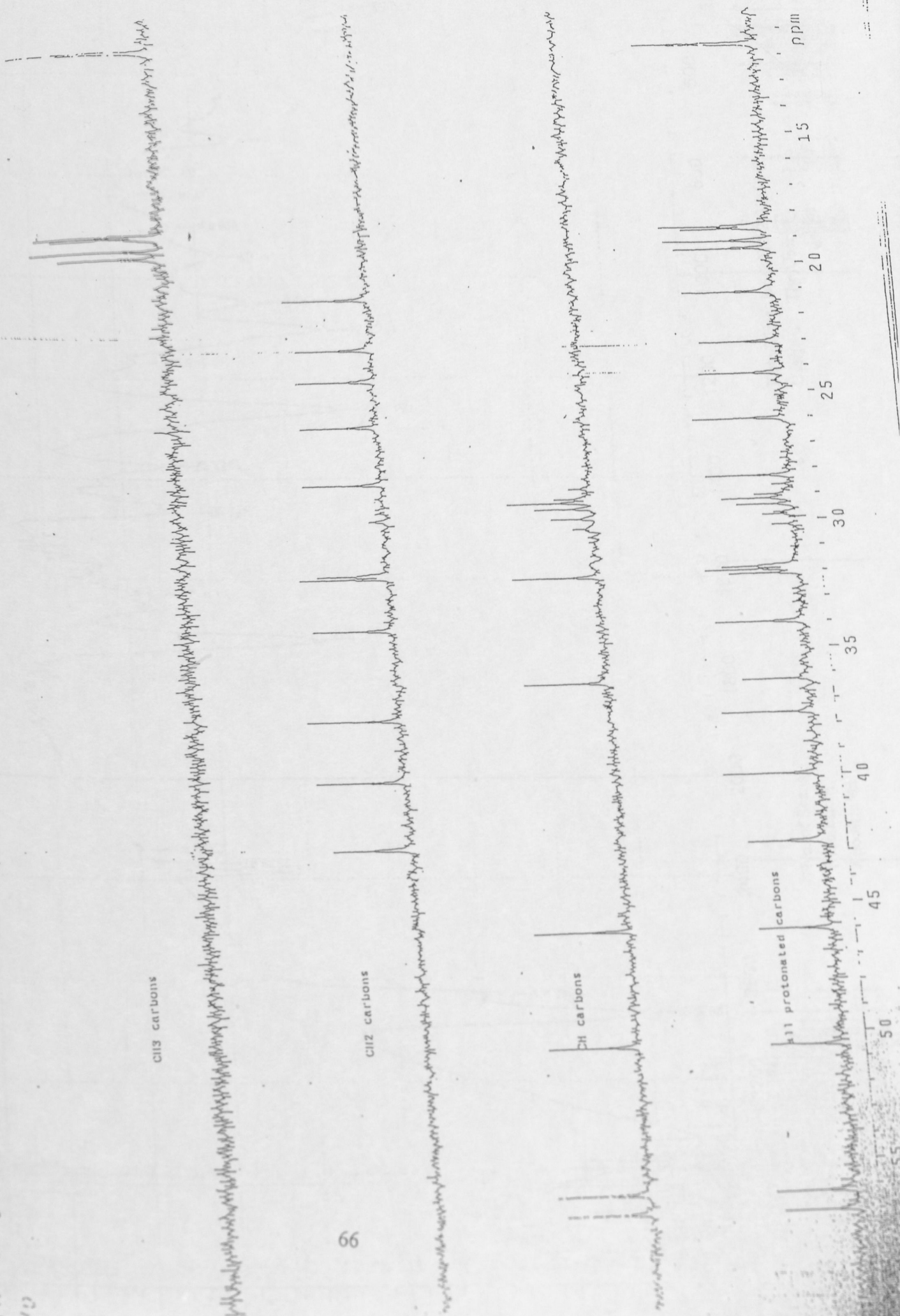


CH carbons



all protonated carbons

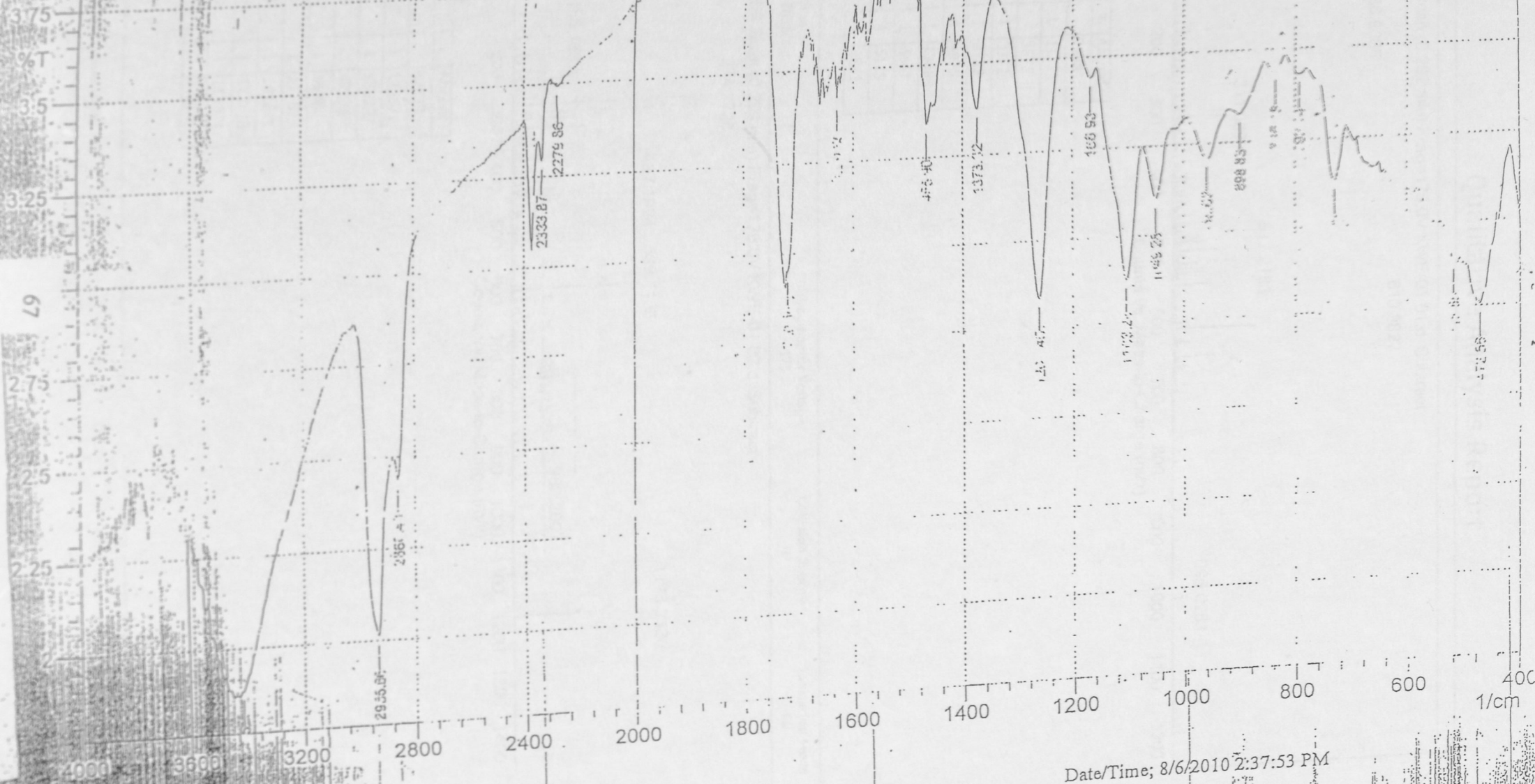




66

7-10

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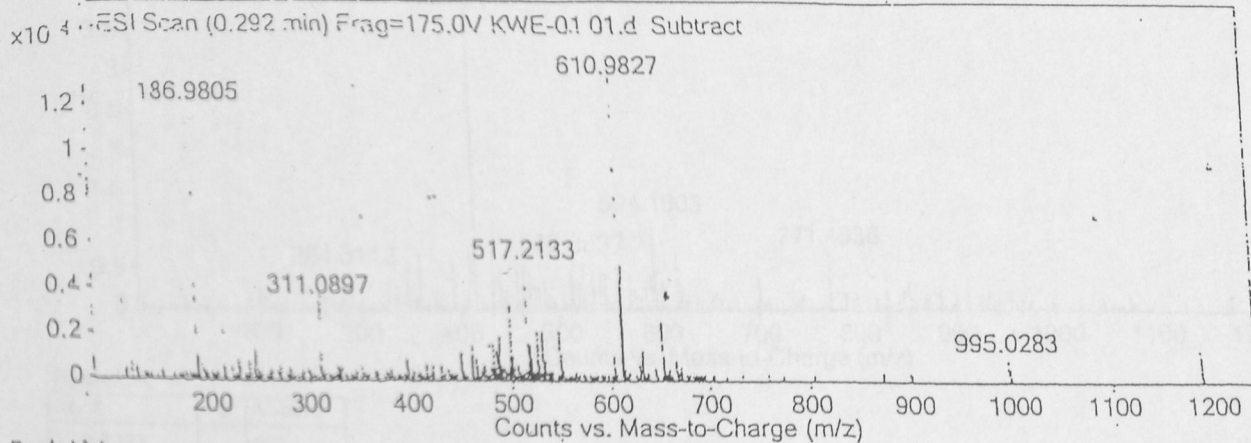
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Appendix 3. Infrared absorption spectrum of NWE-01

Qualitative Analysis Report

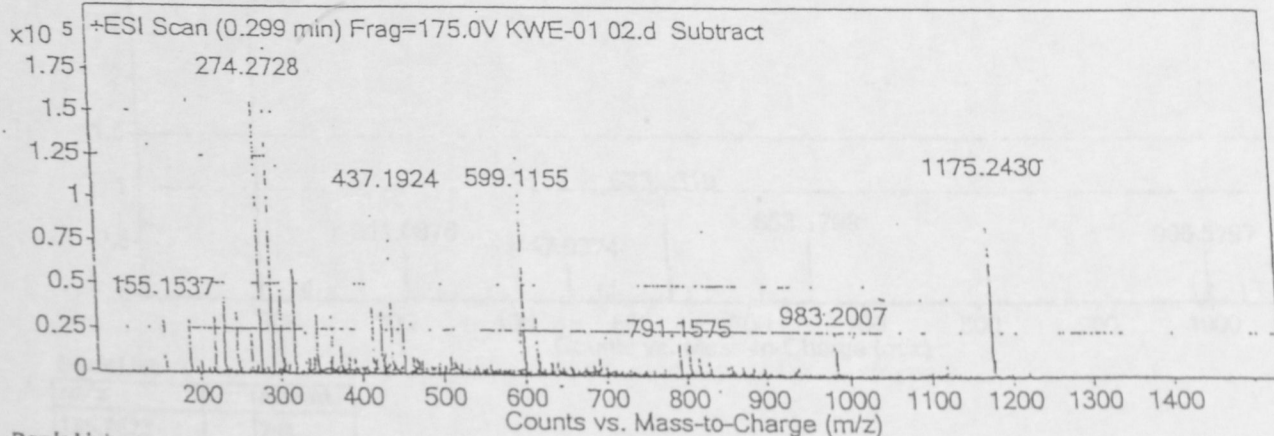
Qualitative Analysis Report



Peak List

m/z	z	Abund
185.9842		2488
186.9805	1	11773
213.8978		3575
311.0897	1	3372
501.219	1	3232
517.2133	1	4975
610.9827	1	13686
611.9857	1	4461
612.981	1	5119
653.1827	1	2541

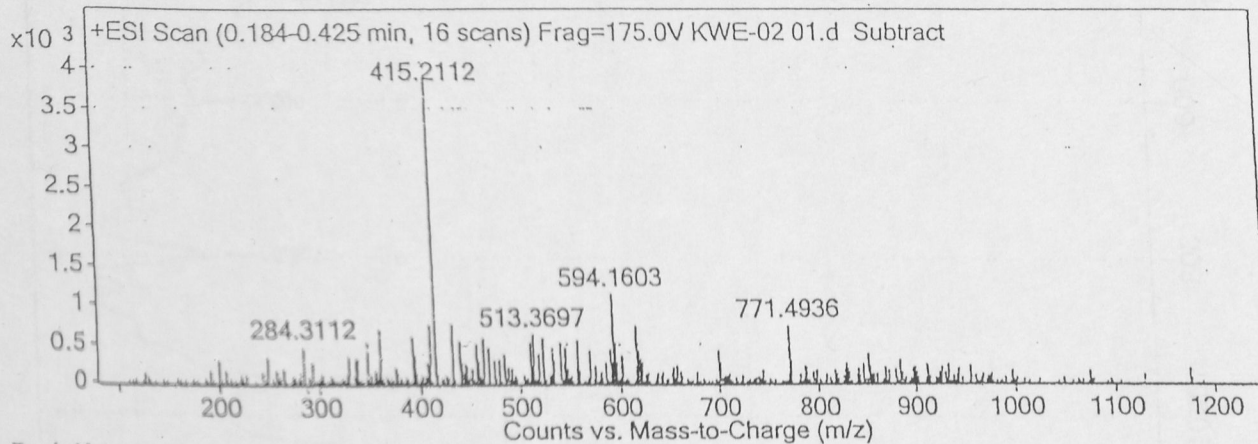
Spectrum Source: Peak (1) In + TIC Scan
 Fragmentor Voltage: 175
 Collision Energy: 0
 Ionization Mode: ESI



Peak List

m/z	z	Abund
231.1191		42523
274.2728	1	175671
288.2882	1	155227
302.3039		47776
316.3193	1	59286
318.2994	1	117873
437.1924	1	101681
599.1155	1	102613
1175.243	1	111371
1176.2461	1	72986

Qualitative Analysis Report



Peak List

m/z	z	Abund
360.3383		691
393.2818		586
411.3521		756
415.2112	1	3937
433.2447		740
513.3697		617
523.3297	1	585
594.1603	1	1120
617.4764	1	719
771.4936	1	735

Spectrum Source

Peak (1) in "- TIC Scan"

Fragmentor Voltage

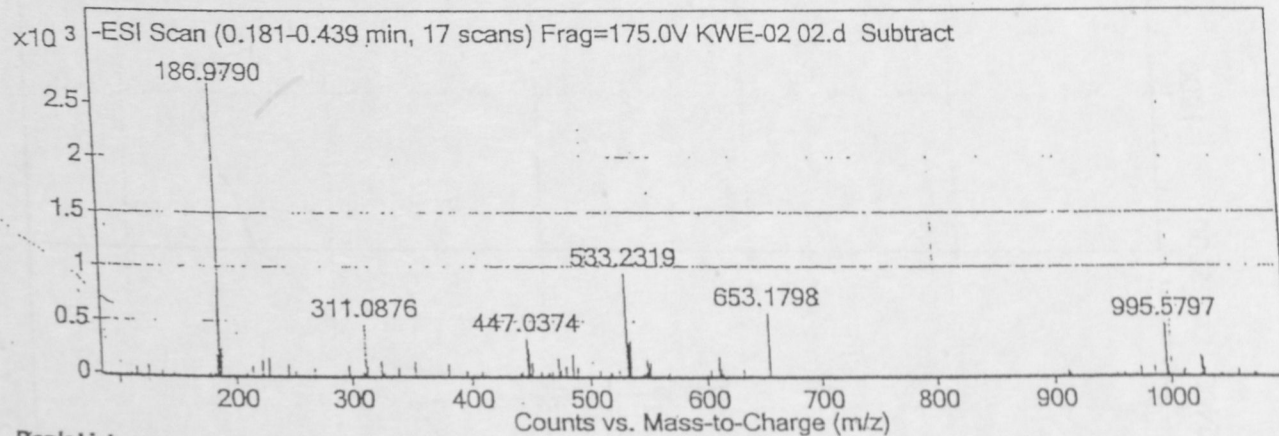
175

Collision Energy

0

Ionization Mode

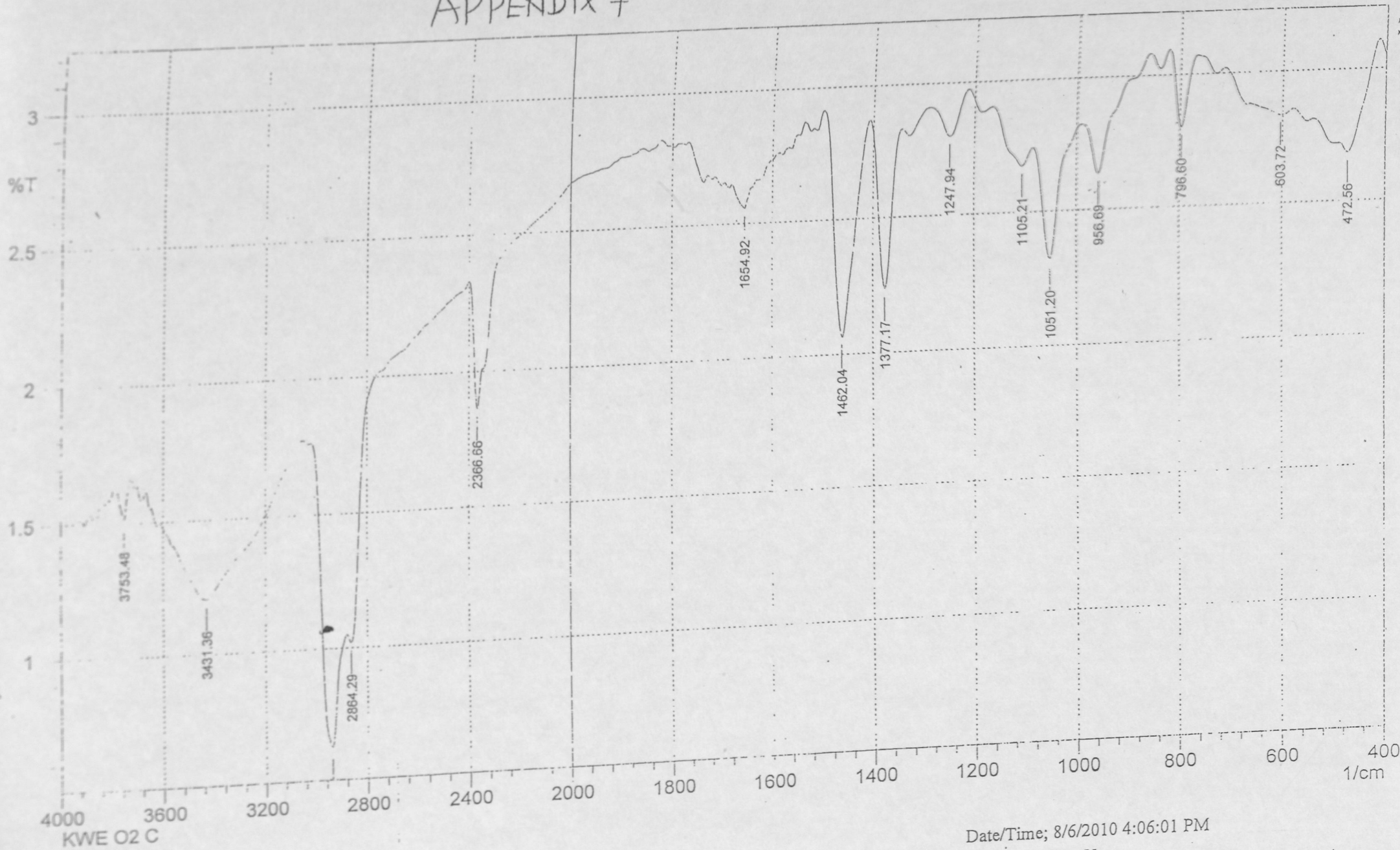
ESI



Peak List

m/z	z	Abund
185.9823		741
186.979	1	2788
311.0876		465
447.0374		337
533.2319	1	943
534.2352	1	281
535.23	1	323
653.1798	1	575
995.5797	1	465
996.5833	1	335

APPENDIX 7



Appendix 7. Infrared absorption spectrum of KWE O2 C

Comment;
KWE O2 C

No. of Scans;
Resolution;
Anodization:

Date/Time; 8/6/2010 4:06:01 PM
User; The User