AN INVESTIGATION OF A TRADITIONAL HERBAL THERAPY USED TO TREAT MALARIA IN KISII

BY

DR. BOSIRE, KEFA OGONYO

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DECLARATION

Thesis is my original work, and it has not been presented for a degree in any other University.

Signature .....

Bosire, Kefa Ogonyo

Supervisors

This thesis has been submitted for examination with our approval as University Supervisors.

PROFESSOR ANASTASIA N. GUANTAI PhD, (Nbi)

Department of Pharmacology and Pharmacognosy

Faculty of Pharmacy

University of Nairobi

.....

SIGNATURE

PROFESSOR ISAAC O. KIBWAGE PhD, (K.U. Leuven, Belgium)

Chairman Department of Pharmaceutical Chemistry

Faculty of Pharmacy

University of Nairobi

.....

SIGNATURE

DEDICATED TO

My wife NAOMI BOSIBORI

Daughter VENESSAH

My Mother MRS. MILKA NYABOKE

My Father DR. ENOCK BOSIRE NYANUSI

And The Herbalist

MR. HENRY MOTANYA

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List of Abbreviations

CMS	Complete media with serum
DCM	Dichloromethane
DMSO	Dimethylsulphoxide
FTIR	Fourier Transform Infrared
GPR	General purpose reagent
HEPES	N- (2-Hydroxyethyl) piperizine-N- (2-ethanosulfonic acid)
HPLC	High performance liquid chromatography
IR	Infrared
LDH	Lactate Dehydrogenase
MeOH	Methanol
NMR	Nuclear magnetic resonance
PABA	Para-amino benzoic acid
RBC	Red blood cell
Rf	Retention factor
TLC	Thin layer chromatography
UV	Ultraviolet
WHO	World Health Organization

ABSTRACT

The plant *Rhamnus prinoides* L. Herit (Rhamnaceae) is used in traditional medicine for the treatment of malaria among other diseases by the Kisii community. By using column chromatographic methods, geshoidin was isolated and tentatively identified. Other isolates were also obtained but were not identified.

All extracts were found to have *in vitro* antimalarial activity. The highest activity was in the hexane and dichloromethane extracts with IC_{50} values of 19.9 µg/ml and 30.3 µg/ml,respectively. None of the extracts showed toxicity in the brine shrimp test.

This study provides scientific basis for the use of the plant part in the treatment of malaria by the herbalist in Kisii.

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1. Malaria

Malaria poses a major health risk to people who are exposed to infection in malaria endemic areas [WHO, 1996; Olliaro *et al.*, 1996]. Despite efforts to contain the disease, it remains one of the greatest causes of morbidity and mortality in the tropical and sub-tropical parts of the world. Estimates by World Health Organization (WHO) place about 300 – 500 million clinical cases of malaria annually with 1.5 – 2.7 million deaths from the disease [WHO, 1990; 1996].

Of the 4 species of plasmodia that infect man, *Plasmodium falciparum* is the most virulent parasite and is responsible for the high morbidity and mortality associated with the disease [Anonymous, 1992; WHO, 1996]. *Plasmodium falciparum* parasite is also the predominant species in sub-Saharan Africa, East Asia, Oceania and the Amazon region. It poses a risk, not only to people living in these areas but also to travelers visiting these areas [Seaton *et al.*, 1998].

1.1.1. Chemotherapy of malaria

Various drugs and drug combinations are currently used for prophylaxis, such as proguanil hydrochloride, mefloquine, doxycycline and

primaquine. Unfortunately, there is increasing resistance to most of these drugs hence the need for novel compounds [Bunag and Harinasuta, 1987; Hoffman, 1992; Nosten *et al.,* 1991; Warhurst, 1986). Antimalarial drug resistance has been reported in nearly all malaria-endemic areas and is most severe in the endemic areas of South East Asia where a daily dose of primaquine and doxycycline seem to be the only reasonable regimen [Looareesuwan *et al.,* 1994]. Mefloquine, initially developed to treat multi-drug resistant malaria, only offers 90% cure rates when given as a monotherapy [Nosten *et al.,* 1991]. Halofantrine is even less effective [Ter Kuile *et al.,* 1993]. Artesunate, an orally administered artemesinin derivative is well tolerated, but treatment requires 5 days and gives 88% cure rates [Looareesuwan *et al.,* 1992]. Presently, compliance to current antimalarials is compounded by side effects caused by these drugs [WHO, 1973; Warrel *et al.,* 1990]. Mefloquine has been associated with neuropsychiatric disturbances [Weinke, 1991; Bem and Kerr, 1992], halofantrine with prolongation of the QT interval [Nosten *et al.,* 1993; Monlun *et al.,* 1995.], and quinine with tinnitus, central nervous system toxicity, and blood dyscrasias [Danielson *et al.,* 1984 Karlsson *et al.,* 1990]. Tetracycline can cause hepatotoxicity in pregnant women and permanent discoloration of teeth as well as stunting of bone growth in children [Werndorfer, 1982]. Pyrimethamine/sulfadoxine has been associated with severe and sometimes fatal cases of Stevens-Johnson syndrome [Hernborg, 1985],

epidermal necrolysis [Miller *et al.,* 1986] and hepatic necrosis [Hernborg, 1985; Zitelli, *et al.,* 1987]. The unsatisfactory choices among antimalarial drugs have prompted considerable scientific investigation aimed at finding newer, more effective and better tolerated agents, demonstrating unique modes of action and a lower propensity to develop resistance.

1.1.2. Malaria prevention and control

A combination of inadequate vector control measures in most areas and increasing parasite resistance has compromised efforts to manage the disease [Trape *et al.,* 1985; Ekanem *et al.,* 1990; Sowumni and Salako, 1992; Ruebush, 1996; Murray and Lopez, 1997]. In endemic areas, children and pregnant mothers suffer higher mortalities than the rest of the population [Verhoeff *et al.,* 1998]. There is a growing need for affordable and efficacious drugs affordable to the third world where malaria is most endemic [Clyde, 1987].

A study conducted to establish the effectiveness of a herbal therapy indicated significant symptomatic improvement and reduction in parasite counts in patients taking the medication [Wilcox, 1998]. Such studies could provide a basis for further fundamental research in designing newer and more potent drugs to target the parasite. One of the most remarkable developments out of this kind of venture is artemisinin and

its derivatives, which were derived from Chinese folklore [Li and Wu, 1998].

Current research trends aim to provide the most effective therapies. Such studies may involve plants as sources of new drugs [Trager and Jansen, 1976; Desjardins *et al.,* 1979; Watkins *et al.,* 1987; Chen *et al.,* 1994; Kirby, 1996; Oketch-Rabah, 1996; Sharma and Sharma, 1998].

1.1.3. The role of research

Herbal therapy contributes the significantly to healthcare in sub-Saharan Africa (WHO, 1978). Currently, over 80% of people in Africa use herbal medicines to treat various illnesses including the management of malaria. This is not surprising considering the level of poverty in the region and the consequent collapse of conventional basic health care systems. Any attempts to alleviate the situation through international programs conducted by WHO and malaria research agencies have not achieved adequate reduction in malaria prevalence. The percentage of modern medicines whose origin can be traced back to plants has continued to increase exponentially over the past four decades (Fellows, 1992). Africa holds one of the widest ranges of plant diversities and has attracted great interest for bioprospective research for novel compounds. These act as lead molecules for new drugs and possibly treatment for

diseases that appear to have failed to respond to medicines in current use (Farnsworth, 1988)

Only a very small percentage of the known plants have been investigated and even among them, not all components have been tested (Hamburger *et al.*, 1990).

1.2. Bioprospecting for new antimalarial drugs

The search for new drugs can be done in three major ways. The medicine may be used in its natural form as obtained from the plant source. An example includes quinine from Cinchona bark and artemesinin from *Artemesia annua*. Others may provide the lead compounds, even though it may not be used directly, could offer new modes of action and therefore be used to synthesize other drugs (Warhurst, 1986). An example is the development of mefloquine from quinine. Others may serve as raw materials for the synthesis of new drugs as is done for oral contraceptives from sapogenins (Evans, 1989).

The incorporation of herbal therapies in primary healthcare is one way of encouraging greater value for plants as sources of safer, cheaper and more effective therapy to treat malaria in Africa. This is already in place in China and Nigeria (Cox, 1990).

1.2.1. Selecting plants for screening

There are estimated to be over 250,000 known species of plants investigated phytochemically. Considering that over 500,000 have not been studied, the prospects are immense (Principe, 1989). Several approaches could be used, some of which are systematic.

1.2.2. Ethnopharmacological approaches

Several studies have shown a significant correlation between folklore and pharmacological activity. In one case, a comparison of 119 useful compounds derived from traditional sources showed a 74% correlation to their use in folklore (Cox, 1990) and hence the greater likelihood of this approach in the search for new medicines. This approach is shown in figure 1.1.

1.2.3. Taxonomic criteria

Recognition of plant taxa was until recently based on plant morphology. There has been growing need for a complementary system that can distinguish even those that appear very close. This has given rise to characterization based on the presence of certain common constituents,

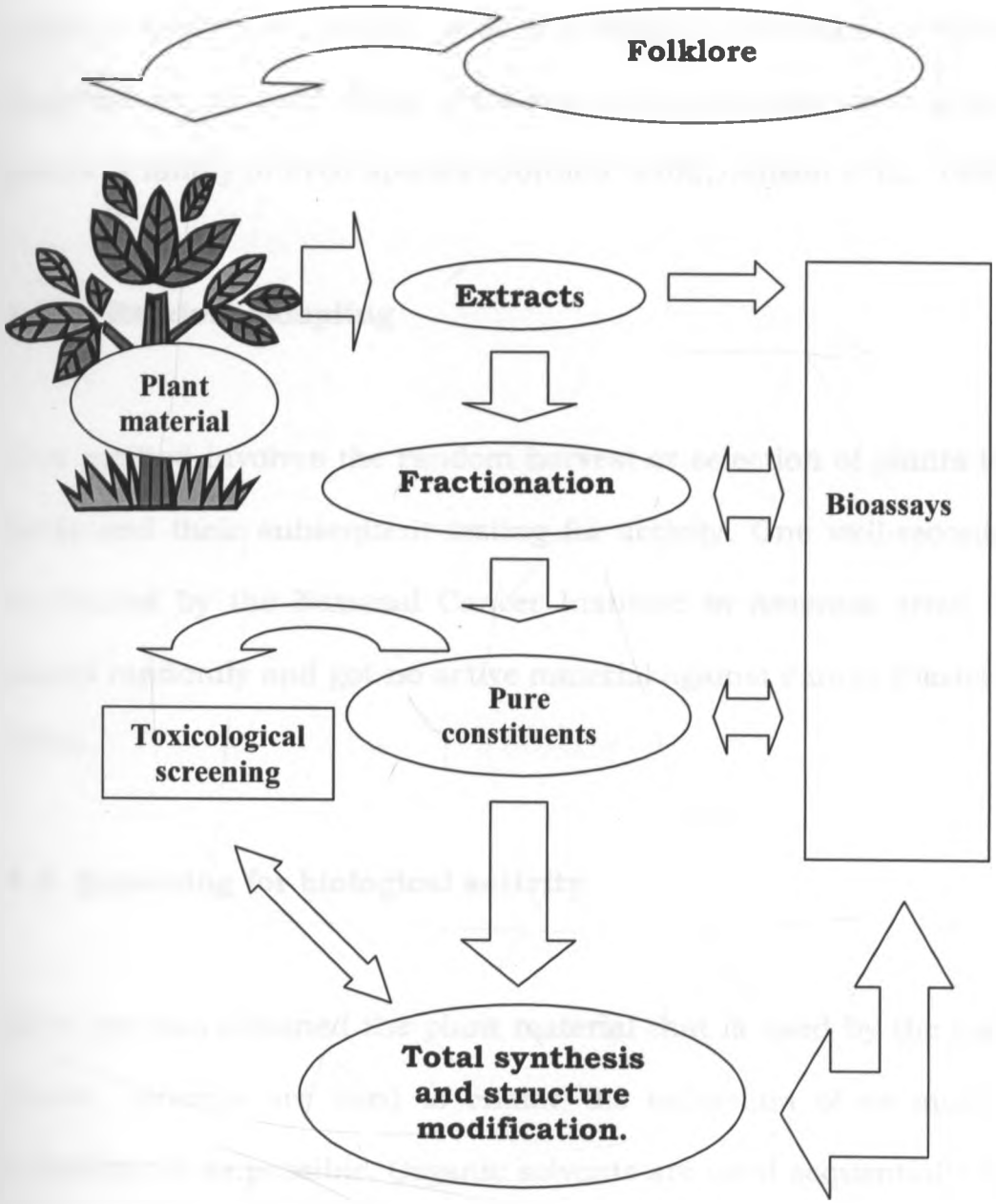


Figure 1.1. Phytochemical sourcing of new drugs

secondary metabolites, which can be used to help select possible sources of the compounds that are being sought e.g. alkaloids or steroids. In order to apply this method, prior knowledge of these constituents would therefore be required. Many metabolites are associated with a particular genus or family or even species (Gottlieb, 1982; Jensen *et al.*, 1989).

1.2.4. Random sampling

This method involves the random harvest or selection of plants from the fields and their subsequent testing for activity. One well-recorded case conducted by the National Cancer Institute in America, tried 114,000 plants randomly and got no active material against cancer (Oketch-Rabah, 1996).

1.3. Screening for biological activity

After one has obtained the plant material that is used by the traditional healer, solvents are used to enable the extraction of as much of the constituents as possible. Organic solvents are used sequentially from the least polar to the most polar. A water extract could also be included in order to replicate the mixture used by the herbalist. In this case, the procedure followed should be identical to that done in the traditional settings, for example using hot water or fermenting in the sun for a

while. Several factors may contribute to compositional variations in the preparations. Attempts should be made to follow the exact folklore procedure as much as possible.

In the screening for biological activity, several model systems have been devised and established in different fields. The choice of the method to use may be dictated by factors such as cost, speed/quickness, availability of materials, ease of automation and other such factors (Hostettman, 1991; Coombes, 1992). It may be postulated that even though one may wish to conduct various tests, the number is limited in scope and variety of facilities accessible. Constraints include the amount of sample, time available and handling capacity of the laboratory (Coombes, 1992). Technology now offers even ELISA based analysis and microprobe based methods that could be fully automated. Systems have been miniaturized and made more efficient. One should not ignore the initial cost element associated with the development and installation of such hyphenated instrumentation and hence the extent of its use.

1.3.1. Antiplasmodial testing

In vitro and *in vivo* systems have been used to test for antiplasmodial activity. In vitro methods are preferred due to their high throughput.

1.3.1.1. *In vivo* antiplasmodial activity

The plant materials are monitored for activity *in vivo* in a 4-day suppressive test against *P. burghei* infection in mice (Peters, 1980). Mice are inoculated intravenously with *P. burghei* infected red blood cells. After 3 h, they are orally dosed with the plant extract. Further dosages of the drug are given daily for three days. On day 4 the blood smears are made from the tail blood and appropriately stained and parasitaemia assessed. A control group dosed with the vehicle is set up for comparison. The results are then used to calculate the minimum dose required to effect a 90% reduction in parasitaemia in the infected rats as compared to the controls.

1.3.1.2. Lactate dehydrogenase based assay

The principle of this method is based on the role played by lactate dehydrogenase in the metabolism of the parasite (Basco *et al.*, 1995). Lactate dehydrogenase (LDH) is the terminal enzyme in Embden-Meyerhoff glycolysis. It plays an important role in the metabolism of the human malaria parasite. The method is based on the ability of the malarial LDH to use 3-acetylpyridine nicotinamide adenosine dinucleotide (NAD) as a coenzyme in the reaction leading to the formation

of pyruvate from lactate (Merkler and Hinrichs, 1993; Merkler and Reis 1993) The enzymatic activity of fresh isolates of the parasite, *P. falciparum* are determined in relation to the incubation time, asexual stages, and parasitaemia and applied to a drug susceptibility assay. Lactate dehydrogenase activity is detected at a parasitaemia of more than 0.4% and a haematocrit of 1.5% and increases with increasing parasitaemia. Maximal lactate dehydrogenase activity is detected after 36-48 h when the trophozoites and schizonts predominate. Optimal results are obtained when fresh clinical isolates are used and the initial parasitaemia is at least between 1-2 % at a haematocrit of 1.5 %. Other workers have made modifications to this basic concept especially in the approach to assaying. Delhaes *et al.,* (1999) for example, compared a method involving the inclusion of sodium-2, 3-bis- (2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxalide in a colorimetric method. In a reaction involving this compound, a chromophore is generated. The reading in UV at 450 nm is determined and used to give the value of optical density.

1.3.1.3. The tritium-labeled hypoxanthine test

This test relies on the inhibition of the incorporation of ^{3}H-hypoxanthine into the parasite *in vitro*, (Desjardins *et al.,* 1979; Fairlamb *at el.,* 1985). The details of these are given by O'Neill (O'Neill *et al.,* 1985; 1986). This

involves setting up test solutions in 2-fold dilutions in multiples and also their controls using the 96-well microtitre plate. This is the test procedure adopted in the present study due to availability.

1.3.2. Toxicity screening - the brine shrimp test

There are a variety of tests that could be used to evaluate the general toxicity of substances one of which is the brine shrimp test (Solis, 1972). It is simple and easy to implement. The brine shrimp are sensitive to a large variety of toxic compounds and hence their adoption in preliminary screening. In tests, a solution of known concentration is made of the extracts using a solvent such as DMSO. Shrimp hatchlings are introduced and after 24 h, the larvae are counted and the number of dead or immobile larvae recorded as a fraction of the initial number. Percentage survival is calculated and recorded for each set.

1.4. Set backs in the screening of phytochemicals

Plants, like all other living organisms have over the evolutionary cycle developed mechanisms for self-preservation. Some of these include the tendency to accumulate certain basic molecules, which confer on the plant the ability to deal with various stress factors in its environment (Mukherjee, 2001). A simple illustration is given in figure 1.2.

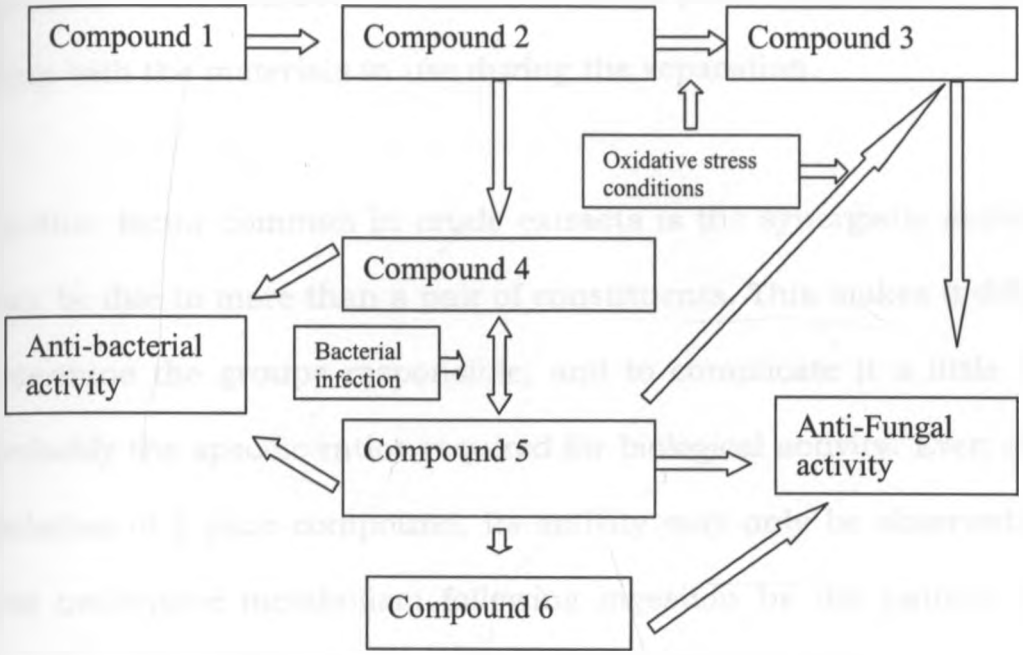


Figure 1.2. Elaboration of secondary metabolites by a plant in response to environmental stress.

Using the above simplified flow chart to illustrate the concept, one would observe an increase in certain plant metabolites during different stress circumstances. In this example, the plant is likely to increase the levels of compound 3 and 5 in the event of undergoing oxidative stress. More of compounds 4 and 6 will be synthesized from 2 in response to bacterial infection. In the event of a fungal infection, compounds 3, 5 and 6 would all be increasingly elaborated.

The number of chemicals that a plant synthesizes for its own use may sometimes be closely related and could therefore interfere with the extraction and isolation of some of them as pure compounds. Some may react with the materials in use during the separation.

Another factor common in crude extracts is the synergistic activity that may be due to more than a pair of constituents. This makes it difficult to determine the groups responsible, and to complicate it a little further, probably the specific ratios required for biological activity. Even after the isolation of a pure compound, its activity may only be observed after it has undergone metabolism following ingestion by the patient. Such a factor may mean that one could probably miss out the active component from an *in vitro* method of assay. This is one of the reasons why models used for biological activity testing would best include an *in vivo* model.

Plant constituents are known to vary for various reasons such as the soil they grow on, the time of day harvested, the season, the life stage, and the plant part. An example is the level of coniceine in the hemlock (*Conium maculatum*) that vary by as much as 500% in the cycle of the day or the levels of morphine, which approach 200% between
8:00 – 10:00 hrs daily (Evans, 1989). The slow growing higher plants serve as the most common sources of secondary metabolites of concern to ensure continued availability. Their replacement becomes an issue.

Cell cultures may sometimes be employed to help in the mass production where possible, as is the case in antibiotics such as Penicillin (Stockigt *et al.*, 1989; Kreis and Reinhard, 1990).

1.5. Antimalarials from natural sources

Several plants from a wide range of families and species have been screened for antimalarial activity. Some important examples include *Artemesia annua, Cinchona ledgeriana, Rhamnus prinoides, Warbugia volkensii, Melia azadirachta, Kaya senegalensis, Azadirachta indica* and *Ajuga remota.*

Out of these a number of antimalarial drugs have been fully developed for use in clinical management of malaria. Typical examples include quinine from *Cinchona ledgeriana* and artemesinin from *Artemesia annua* from which several highly active derivatives have been synthesized.

In the Kisii community, the genus *Rhamnus* has been used by herbalists in the management of malaria. *Rhamnus prinoides* L. Herit (Rhamnaceae) is a widely used species in a number of communities. The plant is known by different names among East African communities namely, Omomisi Komonyaigena (Kisii), Kosisitiet (Kipsigis), Ol-kokola, Ol-konyil (Maasai),

Kokilai (Samburu), Mshibamba (Chaga), Gesho (Amharic-Ethiopia) as searched in the NAPRALERT database.

The genus *Rhamnus* is in the Rhamnaceae family and comprises of 150-200 species. It is widely distributed throughout tropical, subtropical and temperate regions (Thulin, 1988). There were as many as 35 species of Rhamnaceae cited in the Chemical Abstracts by 1995 (Bezabih and Abegaz, 1998). *R. prinoides* and *R. staddo* are the only species found in Africa. The genus is relatively widely studied. In these reports the isolation and characterization of various secondary metabolites have been described, the genus has been a source of a variety of flavonoids that are based on quercetin and kaempferol and their glycosides, several anthrones. Anthraquinones and their glycosides have also been reported. Glucose and rhamnose are the common sugars in glycosides from the genus *Rhamnus* (Salgues, 1962; Abegaz and Degane, 1988; Abegaz and Kabede, 1995)

R. prinoides is a tree that grows to a height of about 1.2 to 9 M as depicted in figure 1.3 (whole plant) and figure 1.4 (sample shoot). It rarely scrambles. The bark is pale gray with characteristic gray lenticels. The spotting helps identify the stem at close range.

Figure 1.3 Rhamnus prinoides tree.

Figure 1.4 Shoot of Rhamnus prinoides.

The leaves are commonly glabrous with a rounded or cuneate base and shiny on the dorsal side. They range from obovate to elliptic with glandular-serrulate margins measuring 2-10 cm long and 1.5-4 cm wide. The flowers are pale green occurring in fascicles of about 8 having 2-3 flowers each 2 mm long. The fruits are red when young, but turn dark purple upon ripening. They are 3-lobed sub-globose with an average diameter of 5-6 mm (Beentje, 1994)

The tree has a tap root system with numerous roots of shallow spread in thin soils. The cork of the roots is red brown. Scrapping its thin outer coat reveals a brilliant red surface that just covers the cortex of the root bark. The bark can easily be sloughed off the thinner branches of the root but stripping is the easier way to get it off the broader sections. The cream inner bark gradually turns pale green when exposed to air probably due to a degradation of some of its constituents. Care should be taken to dry it as quickly as possible after harvest (Abegaz, 1995)

Rhamnus prinoides is known to occur in Cameroon, Sudan, throughout East Africa to South Africa and Angola (Thulin, 1988). In Ethiopia, it is cultivated on a large scale for sale in the open market.

Rhamnus prinoides is popularly known by the Amharic name *gesho*. The leaves and stems of *gesho* are indispensable ingredients in the making of

home brewed alcoholic beverages *Tella* and *Tej*. Tella is a malt beverage, like beer. Sun dried leaves and stems are pounded to powder before they are introduced in the brewing process. *Tej* is also a fermented beverage based on honey as the sugar source. Only stems of *gesho* impart the characteristics bitterness of these beverages, more precise understanding of the scientific role of this plant in the brewing process is emerging only very slowly. The role of *gesho* in the fermentation process has been investigated and it is claimed that the plant regulates the micro-flora responsible for the fermentation process. These reports further indicate that the bitterness of the brew is directly related to the amount of *ghesho* added (Bezabih and Abegaz, 1998; Nindi *et al.,* 1999).

It has also been reported that extracts from *gesho* can be used as a commercial hopping agent for beer (Kleyn and Hough, 1971). The fruits of *R. prinoides* are carefully avoided in the preparation of leaves and stems for the beverages. The fruits, however, on their own have an important role in the health care system of Ethiopians. They are used for the treatment of ringworm infections, suggesting possible antifungal activity (Bezabih and Abegaz, 1998).

In Tanzania, the entire plant is used as a liniment to relieve pain. (Chhabra *et al.,,* 1984). A leaf decoction is also taken orally and used as a sedative (Chhabra and Uiso, 1991). The dried leaf is used to treat

pneumonia in adults (Gebre-mariam *et al.,* 1993) The dried root is used to treat gonorrhoea and colics and in some cases, as a mixture with *Erythrina abyssinica.* (Chhabra, *et al.*, 1984; Chhabra and Uiso, 1991(a); Kokwaro, 1993).

Rhamnus prinoides is widely distributed in Kenya and thrives best at altitudes of 1500-3150 m above sea level. It is found in the Rift Valley and the surrounding highlands (including Kericho, Nandi hills, around Mount Elgon and Transmara), hills bordering lake Victoria basin and small pockets near mount Kilimajaro. It's found mostly along forest edges, but rarely in the secondary bush land or bamboo/heath areas (Beentje, 1994)

Due to destruction of habitat to pave way for agriculture, the plant is no longer available in such places as Kisii. The Kisii herbalists source it from other sites, especially in Kericho and Transmara.

1.6. *Rhamnus prinoides*

The first report on the chemical content of *R. prinoides* is that of Salgues (1962) in which he reported the presence, in the leaves, of inorganic cations, organic acids and the flavonoid derivative rhamnetin (Figure 1.5).

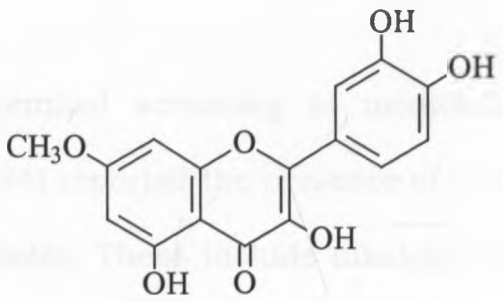


Figure 1.5. The structure of rhamnetin

Abegaz and Kebede (1995) worked on the fruit and leaves of *Rhamnus prinoides* and isolated eleven compounds from the leaves (Figure 1.6). These include the anthracene derivatives named and numbered as follows: emodin (**1**), chrysophanol (**2**), physcion (**4**), and 10-oxoprinoidin.

Other compounds isolated included the flavonoids: rhamnocitrin (**7**), rhamnazin (**8**), quercetin (**9**) and 3-O-methylquercetin and naphthalene

derivatives: β-sorigenin-8-O-β-D-glucoside (**10**), β- sorgenin (**11**), and musuzin (**12**).

β-Sorigenin-8-O-β-D-glucoside was given the trivial name geshoidin. In the same report, it was mentioned that an organoleptic evaluation of geshoidin had revealed that it is a bitter principle. It was also found to be nontoxic to brine shrimp (*Artemia salina*) (Abegaz and Kebede, 1995).

During a phytochemical screening of medicinal plants in Tanzania Chhabra *et al.,* (1984) reported the presence of a variety of compounds in the leaf of *R. prinoides*. These include alkaloids, coumarins, flavonoids, quinones, sterols/triterpenes, and tannins.

1.7. Biological activities of *Rhamnus prinoides*

The dichloromethane extract of the leaves, flowers and stem were found to be active against *Salmonella typhimurium* at 600 mcg/plate using the agar plate method (Wall *et al.,* 1988). This study was done in South Africa and the extracts of the plant were also found to exhibit antimutagenicity induced by 2-aminoanthracene. A concentration of 50 mg/ml of the dried leaf was found to have no insecticidal activity against the tick *Rhipicephalus appendiculatus*.

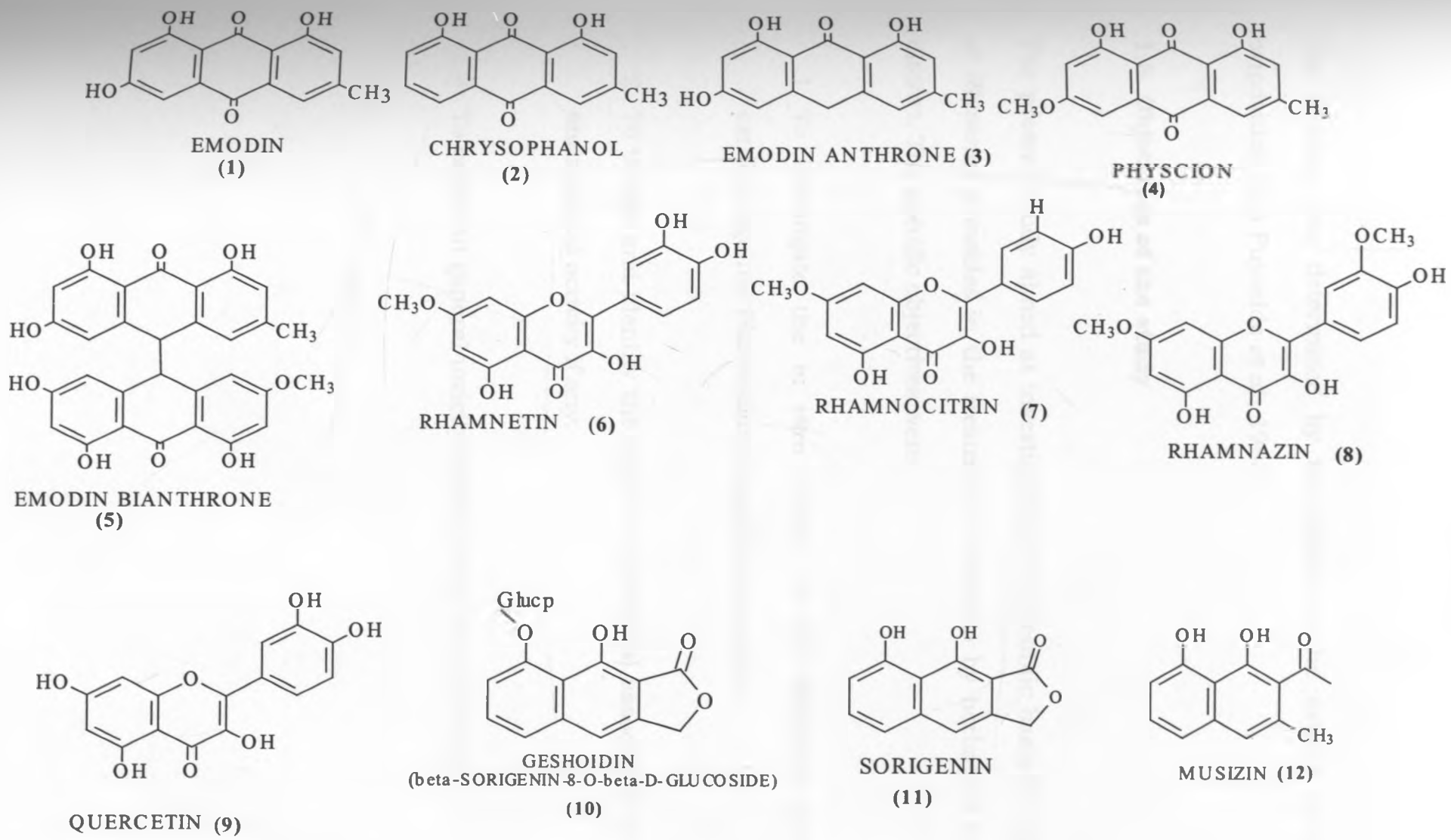


Figure 1.6. Compounds previously isolated from *Rhamnus prinoides*

The activity was determined by the ability of the extract to inhibit oviposition (van Puyvelde *et al.*, 1985).

1.8. Objectives of the study

The present study aimed at investigating the scientific basis for the use of *Rhamnus prinoides* in the treatment of malaria by herbalists in Kisii district. The specific objectives were:

1. To investigate the *in vitro* activity of the *Rhumnus prinoides* extracts against *Plasmodium falciparum* parasites.

2. To isolate and identify the major compound(s) associated with the antimalarial activity if any.

3. To carry out general toxicity testing using brine shrimp.

CHAPTER 2: EXPERIMENTAL

2.1. Materials and equipment

2.1.1. Plant material

The plant specimen was provided by a herbalist, Henry Motanya, residing in Bonchari location, Central Kisii district. However, the specimen was collected from Transmara district. The sample was identified as *Rhamnus prinoides* L. Herit (Rhamnaceae) at the Department of Botany University of Nairobi. A voucher specimen was deposited at the Faculty of Pharmacy herbarium, University of Nairobi. However, to cut down on costs, plant material for research was collected from Kijabe escarpment about 50 km due West of Nairobi City with the help of a taxonomist from the Department of Botany, University of Nairobi. About 2 kg of *R. prinoides* root was collected on 18th December 2000.

The fresh root material was chopped and dried in an oven at 45°C for 48 h. It was then ground into a fine powder and stored at room temperature until used for extraction.

2.1.2. Solvents and reagents

General purpose reagent (GPR) grade n-hexane, dichloromethane (DCM), ethyl acetate, and methanol (MeOH) were obtained from Kobian Kenya Ltd (Nairobi, Kenya). These solvents were glass distilled before use. Analytical grade dimethylsulphoxide (DMSO), sulphuric acid, and n-propanol were from BDH (Poole, England). Water was freshly distilled before use.

The sugars arabinose, glucose, galactose, rhamnose and xylulose were a kind donation from Department of Biochemistry, University of Nairobi. Iodine crystals were from May and Baker (Dagenham, England). HPLC grade methanol and analytical grade potassium bromide were obtained from Merck (Darmstadt, Germany). Activated carbon was purchased from Laboratory Chemicals (Nairobi, Kenya). Sea salt and brine shrimp eggs were purchased from Aquapet (Nairobi, Kenya).

2.1.3 Materials for chromatography

Analytical pre-coated thin layer chromatography (TLC) aluminum plates (silica gel 60 GF_{254}) and silica gel for column chromatography (Licroprep 15-25 μm) were from Merck. Silica gel 60 GF_{254} (Merck) was used for preparative TLC. For this purpose glass plates (20 x 20 cm) were coated

in the laboratory using a TLC spreader to form an even layer of 1.5 mm thickness. Filter paper MN 615 (Macherey-Nagel, Duren, Germany) was used for all filtrations.

2.1.4 Equipment

A UV lamp (Camag, Berlin, Germany) was used for visualization of spots on TLC plates. The high performance liquid chromatography (HPLC) system consisted of a Merck Hitachi L 7100 pump and a L 7400 Detector equipped with a Hewlett Packard 3396 Series II Integrator (Shanghai, China). A Mermmet Oven (Schwabach, Germany) was used to dry the plant material.

A Laborata 4000 rotary evaporator (Heidolph, Germany) equipped with a Laboport KNF Neuberger Vacuum Pump (Freiburg, Germany) was used for solvent recovery from the extracts. For UV and IR spectroscopic analysis, Lambda 1.2 UV-Visible (Berlin, Germany) and FTIR 1000 spectrophotometer (Beckensfield, England) from Perkin Elmer were employed. NMR spectroscopy was run on an AM 200 spectrometer (Bruker, Tokyo, Japan) at 200 or 90 MHz using deutrated methanol (CD_3OD), chloroform ($CDCI_3$) or DMSO as solvents

2.2 Methodology of extraction of plant material

The powdered root material was subjected to sequential solvent extraction in order of increasing polarity as follows: hexane, DCM, DCM-MeOH (50:50) and MeOH. Hexane was used to defat the root material prior to the extraction using the more polar solvents.

This material (1.25 kg) was first soaked and then topped with an additional 200 ml of solvent with occasional shaking and then allowed to stand for 1 h. The crude extract was then filtered and concentrated using a rotary evaporator. The material was further extracted using 200 ml of solvent to exhaustion. The extracts were pooled and evaporated to dryness. Plant material was allowed to dry at room temperature on aluminum foil before introducing the next solvent.

This extraction procedure is outlined in Figure 2.1

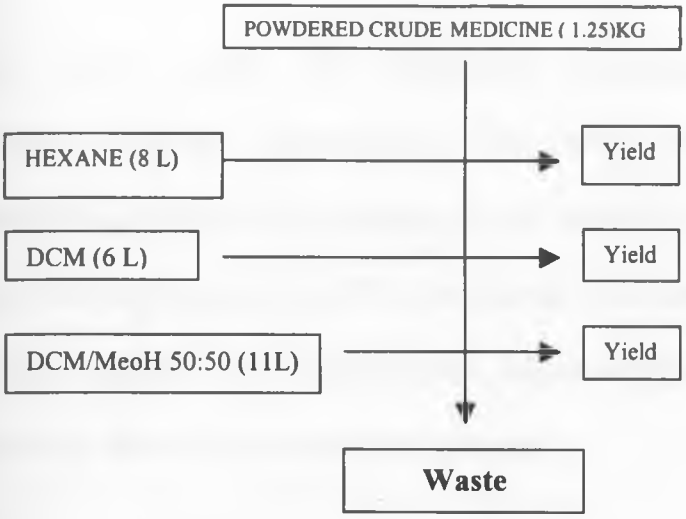


Figure 2.1. Sovent extraction of *Rhamnus prinoides* root

2.3. Isolation of compounds from the extracts

The extracts were profiled using TLC and isolates were sought from the major fractions especially the DCM/MeOH (50:50) and the MeOH extract that showed greatest similarity to the hot water extract used by the herbalist.

Extracts and fractions were examined on TLC using normal phase silica gel 60 F_{254} pre-coated aluminum plates. The plates were first visualized

under UV (254 and 366 nm) and then developed in the iodine chamber for detection of UV inactive compounds.

The TLC profile so obtained supported further preparative chromatographic procedures. To this effect, TLC and column chromatography were performed to achieve separation of compounds. The mobile phase of the TLC profiling were used in determining the best solvent system for preparative chromatography and for monitoring fractions during the isolation process.

2.3.1. Column Chromatography

Low-pressure column chromatography was carried out in suitable glass columns packed with silica gel. The amount of silica used was 10-12 times the weight of the sample to be loaded. The size of column used was chosen based on the size that would support the amount of silica and headspace for additions of the mobile phase.

2.3.2. Isolation of compounds from the dichoromethane-methanol extract

Two grams of the DCM/MeOH crude extract were chromatographed on a column packed with 20 g silica deactivated with methanolic oxalic acid

(1.5%). The column was eluted with 100 ml batches of solvent as follows: from 100% n-hexane followed by n-hexane-DCM mixtures of increasing DCM composition of 1, 2, 4, 10, 20, 40, 60, 80 and 100%. The column was then eluted with DCM-MeOH mixtures of increasing MeOH composition of 1, 2, 4, 10, 20, 30,40, 50, 60, 70, 80 and 100% MeOH. Fraction 14 corresponding to 10% MeOH in DCM was found to contain only one compound that was recovered and coded 7K (0.01% yeild). Purity was checked using TLC normal silica using MeOH:ethyl acetate:H_2O (10:85:5).

When the DM extract was allowed to settle during storage, a precipitate formed. A portion of the precipitate was washed with DCM then a DCM - MeOH (50:50) mixture. The washed solid was dissolved in water and filtered through filter. A pale green solution was obtained. This was further filtered through 9.3 g of activated carbon to yield a clear solution. This solution was evaporated using a rotary evaporator to obtain a dry solid, MEP 1.

2.3.3. Isolation of compounds in the methanol extract

The TLC profile of the methanol extract showed 5 components. The methanol extract (3 g) were chromatographed on a column packed with 30 g silica gel deactivated with methanolic oxalic acid. The column was

eluted with 100 ml of DCM followed by 4 X 100 ml DCM-MeOH mixtures with increasing MeOH content (1, 2, 4, 10, 20, 30,40, 50, 60, 80 and 100%). Fraction 25, corresponding to 30% MeOH in DCM, contained only one compound that was coded 25K (0.02% yeild). The purity of the compound was determined as 99.9% by HPLC (Appendix 1).

2.3.4. Fractionation of hexane extract

A portion of hexane extract (3.0 g) was chromatographed on a column packed with 30 g silica deactivated with methanolic oxalic acid using 410 ml of hexane (Fraction 1) to afford 210 mg of a faint yellow viscous oil (AO). The column was subsequently flushed with one litre of DCM - MeOH (50:50) and concentrated to obtain (2.5 g) extract, RHE.

A portion of AO (10 mg) was suspended in 2 ml of MeOH for 72 h. A white wax-like solid was formed. The MeOH was pipetted off and the residue washed with three 2 ml portions of fresh MeOH to obtain AO1.

The RHE extract (2.0 g) was chromatographed through a silica column using DCM as the elution solvent. The eluent was collected in 100 ml fractions. The sixth and seventh (600-700 ml) fractions were shown by means of TLC using Hexane:DCM (5:1) to contain a single component. They were pooled and reduced to dryness giving a solid denoted as HXF.

Separately, a portion of RHE (500 mg) was subjected to preparative thin layer chromatography on laboratory-spread silica plates using DCM: hexane (10:90) as the mobile phase. Three of the well-separated bands were scraped off and extracted with DCM to obtain isolates RPH2, RPH3 and RPH4. Using DCM:hexane (10:90) as mobile phase, each of the isolates showed one principal spot on TLC. However the quantities were insufficient for spectral analysis (yield of 0.01%).

A chart showing extracts and compounds isolated is given in figure 2.2

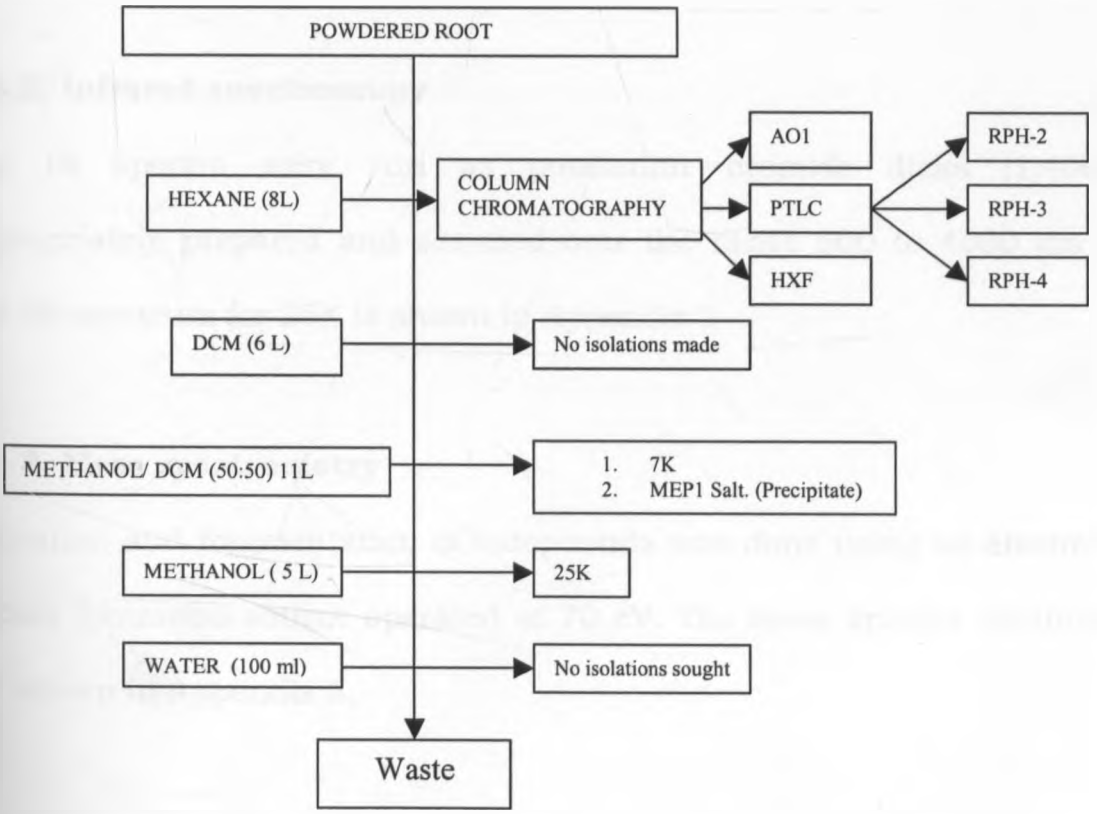


Figure 2.2. Extraction and isolation of compounds

2.4. Spectroscopic analysis

Based on the quantities of individual isolates obtained from the N-hexane, DCM-MeOH (50:50), and methanol extracts, further spectroscopic studies were performed on 7K and 25K in an attempt to identify their structures.

2.4.1. Ultraviolet spectroscopy

UV spectra were obtained in water by scanning over the wavelength range 200 nm- 400 nm. The spectrum obtained is shown in Appendix 1.

2.4.2. Infrared spectroscopy

The IR spectra were run as potassium bromide disks (1:400) appropriately prepared and scanned over the range 600 to 4000 cm^{-1}. The IR spectrum for 25K is shown in Appendix 2

2.4.3. Mass spectrometry

Ionization and fragmentation of compounds was done using an electron impact ionization source operated at 70 eV. The mass spectra obtained are shown in Appendix 3.

2.4.4. Nuclear magnetic resonance

Chemical shifts are given in δ values relative to internal standard TMS (δ = 0 ppm). ^{13}C NMR spectra were recorded at 90 MHz, with $CDC1_3$, DMSO and CD_3OD as solvents. Chemical shifts are given in δ values relative to the internal standard $CDCI_3$ or TMS. Appendices 4-8 are recordings of the NMR spectra of the compounds 7K and 25K.

2.4.5. Melting point determination

Melting points were determined by using the Buchi Melting Point device B- 540(Hiedelburg, Germany) by loading a small amout of sample in a sealed capillary tube and measurents recorded automatically.

2.5. Biological screening of extracts

The crude extracts were used for performing an *in vitro* antiplasmodial activity assay and a general toxicity (brine shrimp) test as described below.

2.5.1. Test for antiplasmodial activity

These tests were carried out at the Danish School of Pharmacy in Denmark. The method used to estimate the antiplasmodial activity of the

extracts is the radioisotope method described by Desjardins *et al.*, (1979), with minor modifications. One ml of thawed blood was transferred to a 15 ml centrifuge tube, and centrifuged at 1500 rpm for 5 min. The plasma and buffy coat were removed and the sedimented erythrocytes re-suspended in 1.5 ml of a wash medium consisting of RPMI 1640 medium containing para-aminobenzoic acid (PABA) or folic acid, HEPES buffer 25 mmol/L, and bicarbonate 25 mmol/L.

After centrifugation, the wash was repeated, and fresh wash medium added to produce a 50% v/v red blood cells (RBC) suspension. Cultures were initiated in 25 ml culture flasks by adding a pellet of parasitized erythrocytes together with 0.3 ml of 50% haematocrit RBC containing washed 12 to 28-day-old non-parasitized erythrocytes and 4.7 ml complete medium with serum (CMS).

The culture flasks were placed in a gas-tight box, flushed for 2 min with a 5% O_2, 3% CO_2, 92% N_2 gas mixture and incubated at 37°C. The supernatant in each well was renewed daily, and the culture mixed by gently rotating the plate on a level surface, before regassing and returning to the incubator. Parasitemia was assessed every 2 days on Giemsa-stained thin films. When the parasitemia exceeded 3%, well cultures were transferred to a 25 ml flask with additional RBC and CMS to give a 5 ml culture at 6% hematocrit, which was maintained as

described above. Parasites were considered adapted to *in vitro* culture when they achieved a growth rate of more than 3-fold per 48 h cycle.

Aliquots of 200 µl of culture were diluted with fresh RBC and CMS to a parasitemia of 0.4% and hematocrit of 1.5%. These were added to each well of a 96 well microculture plate pre-dosed with 12 drugs in single columns of 7 well each. The first 4 wells on each plate were drug-free controls, while 8 wells received parasitized RBC.

After 48 h incubation at 37°C in the gas mixture, test plates were labeled by adding 25 µl of 1 µCi/ml ^{3}H-hypoxanthine solution and re-incubated for 18 h. The plates were then harvested and ^{3}H-hypoxanthine incorporation was measured. The drug concentration causing 50% inhibition of radioisotope uptake (ID_{50}) was calculated by interpolation after logarithmic transformation of both drug concentration and label uptake. The percentage inhibition was calculated by using the following formula:

$$\text{Inhibition \%} = \frac{\text{(Mean NTPE - mean DTPEcpm)}}{\text{(Mean NTPE - meanNPE)}} \text{ X } 100$$

Where

- Mean NTPE: mean cpm for non-treated parasitized erythrocytes
- Mean NPE: Mean cpm for non-parasitised erythrocytes
- Mean DTPE cpm: Mean cpm for drug treated parasitised erythrocytes.
- cpm : counts per minute

Preliminary tests were carried out using the crude plant extracts of hexane, DCM, and methanol. However the yield of the isolates was too low and insufficient to carry out the biological assays.

2.5.2. The brine shrimp test

The test was done as per the method described by Solis *et al.,* (1972). The extracts were dissolved in DMSO (300 µl) to give clear solution. A volume equivalent to 5 mg was drawn from a stock solution by means of a micropipette and diluted to 5 ml using sea salt solution. An average of ten shrimp hatchlings harvested within 48 h were drawn from a hatchery and transferred into each vial. The minimum water level in each vial was just adequate to prevent the shrimp from desiccation. The vials were divided into 2 sets each consisting of 3 vials. To the first and second were added 1 ml and 0.5 ml of the diluted extracts to achieve a final concentration of 1 and 0.5 mg per ml respectively. The vials were then filled with salt water and kept in the dark at room temperature for 24 h. The larvae were counted and the number of dead or immobile larvae recorded. The percentage survival for each set was calculated.

CHAPTER 3: RESULTS AND DISCUSSIONS

3.1. Phytochemistry

3.1.1. Solvent extraction

Extraction of plant material was achieved by use of solvents of varying polarity: n-hexane, DCM, DCM-MeOH (50:50) and MeOH. However, initially greater interest was in the isolates of the latter two, which represented the more polar fraction comparable to the preparation administered by the herbalist.

Results of the TLC profiles of these extracts were used as a guide in determining the compounds to be targeted during the isolation procedures. For example, using ethyl acetate as the mobile phase, TLC was run comparing the profile of the DCM extract with that of the hexane or the DCM - MeOH extracts. It was shown that six spots were present in the DCM extract. These spots were also present in either the hexane or the DCM - MeOH (50:50) extracts as indicated by the Rf values of the spots.

A comparison of the TLC results of the DCM-MeOH (50:50), the MeOH and the water extracts showed that they had similarity in composition. In one case, a spot coded HXF in the hexane extract was found to have an

identical Rf to the second least polar and a less intense spot in the DCM extract. Isolation was therefore made from the hexane extract.

Due to the large quantities of extracts obtained, the isolation was carried out on the DCM-MeOH (50:50), MeOH and hexane extracts. A summary of the yields is as shown in the table below:

Table 3.1. Extraction yields of powdered root of *Rhamnus prinoides*

Extract	Yield %	% Yield of isolates
n-hexane	0.4	
DCM	0.02	
DCM-MeOH (50:50)	21	0.01(K7)
MeOH	20	0.02 (K25)

It is evident from the results of the sequential extraction, that the greater proportion of phytoconstituents present in the root are polar and may explain why the herbalist uses a hot water extract.

3.1.2. Spectroscopic investigations

Spectroscopic investigations were conducted on the isolates 7K and 25K. This involved the application of NMR, IR, UV, and Mass Spectrometry. Details of the results are presented and discussed here below.

3.1.2.1. Spectral data of 25K

The isolate 25K was obtained as a fluffy light gray non-crystalline solid with the following spectral data for UV, IR, ^{1}H NMR and ^{13}C NMR.

Melting point °C: 157.8-158.8

UV (H_2O): λ_{max} 214, 244, 296, 309, 360.

IR(KBr): ν_{max} cm^{-1} 3361(OH), 2900, 1742 (Lactone ester COO), 1643 (Lactone CO), 1606, 1453, 1376, 1339, 1205, 1081.

^{1}H NMR (DMSO/CD_3OD) δ: 3.30, 3.34, 3.48, 3.52, 3.59, 3.63, 3.71, 3.77, 3.93, 3.99, 4.66, 5.13, 5.19, 5.35 (CH_2 Lactone), 7.33 (Ar-H), 7.42 (Ar-H), 7.51 (Ar-H), and 7.55 (Ar-H).

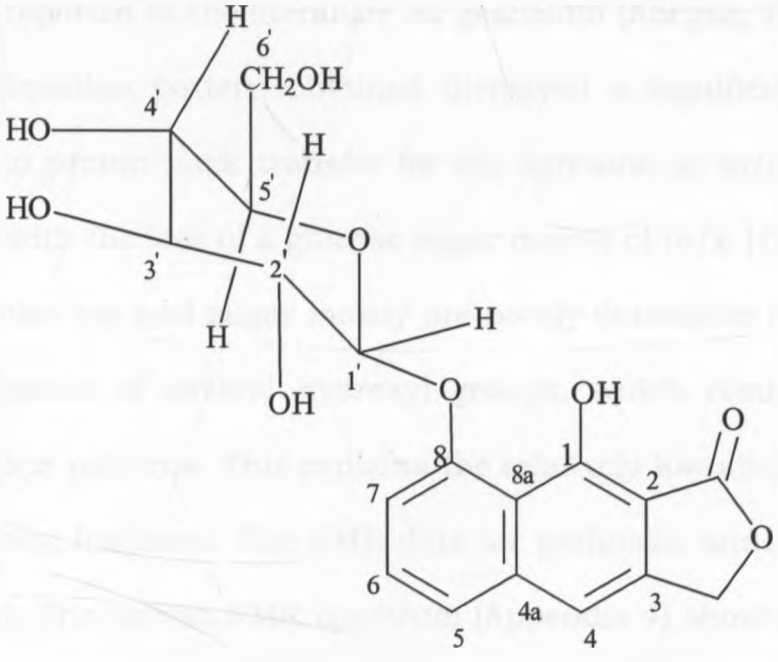
^{13}C-NMR (DMSO/CD_3OD): (C_1) 156.178, (C_2) 114.894, (C_3) 140.336, (C_4) 111.678, (C_{4a}) 143.612, (C_5) 123.751, (C_6) 130.319, (C_7) 111.527, (C_8) 156.565, (Lactone CH_2) 106.370, (Lactone CO) 168.771, (C_1) 103.428. Glucose: (1') 103.428, (2') 73.875, (3') 78.152, (4') 70.303, (5') 76.757, (6') 61.271.

M.S. data: 390.8 (100%), 312.8 (25%), 239.7 (20%), 217 (95%).

The details of the spectra are shown in appendices 4, 5 and 6

3.1.2.2. Tentative structure of 25K

The assigned structure of 25K (Figure 3.1) was based on comparison of its spectral data with literature values for geshoidin (Abegaz, 1996). UV spectrum of 25K (Appendix 1) showed λ_{max} values characteristic of benzenoid compounds. They are associated with the E 180-220, K 220-250, B 250-290 and the R 275-330 benzenoid bands (Finar, 2001). This is evidence that the structure of the isolated compound contains an aromatic moiety.



beta-SORIGENIN-8-O-beta-D-GLUCOSIDE (GESHOIDIN)

Figure 3.1. The structure of geshoidin

Several characteristic peaks are notable in the IR spectrum (Appendix 2). The broad peak at 3361 cm^{-1} denotes presence of several hydroxyl groups. The peak at 1742 cm^{-1} is usually associated with carbonyl ester of a lactone and that at 1643 cm^{-1} for a bonded carbonyl group suggesting the possibility of a lactone ring. There are numerous peaks in the region 600–1400 cm^{-1} that are characteristic of substituted aromatic benzyl structure (615, 642, 755, and 1081 cm^{-1}).

The mass spectrum of 25K (Appendix 3) showed a molecular ion at m/z 378.6. This mass corresponds to the molecular formula $C_{18}H_{18}O_9$. This formula is reported in the literature for geshoidin (Abegaz, 1995).

The fragmentation pattern obtained displayed a significant M+1 base peak due to proton back transfer for the aglycone at m/z 217. This is consistent with the loss of a glucose sugar moiety of m/z 163.

The molecular ion and sugar moiety are poorly detectable in the MS due to the presence of several hydroxyl groups, which result in variable fragmentation patterns. This explains the relatively low abundance of the corresponding fragment. The NMR data for geshoidin and 25K are given in table 3.2. The proton NMR spectrum (Appendix 4) shows the presence of many protons in the structure. The shift at δ 5.34 ppm is assigned to a methylene group protons of the lactone ring. Four other shifts at δ 7.33, 7.42, 7.52, and 7.55 are assignable to aromatic protons ($C_{4\text{-}7}$).These shifts support the presence of two substitution sites on the sorgenin

structure (Figure 3.1). The shifts at δ 4.3-5.0 are generally associated with the hydroxyl groups of the sugars. These shifts have also been obtained for glycosides (Abegaz, 1995; Okamura, 1997).

The ^{13}C NMR (Appendix 5) spectrum indicates that the compound has 18 carbons. The shifts in the naphthalenic component of the structure showed identical shift patterns. The shifts at positions C_4 and C_7 indicate that the sugar must be attached to position C_8.

The DEPT spectrum (Appendix 6) indicates the presence of two CH_2, nine CH, seven quaternary carbons and the absence of CH_3 group. This is similar to the findings reported for geshoidin (Abegaz 1995). The ^1H-NMR (δ 5.34 for CH_2) and 106.37 ^{13}C NMR suggested a naphthalenic lactone glycoside.

Comparison of the NMR spectra of 25K and geshoidin shows similarity in all but one carbon atom (5'). The ^{13}C-NMR shift for such carbons in other glycosides is about 80 ppm (Abegaz, 1995), which is closer to 76.9, obtained for 25K. The possibility of an alteration in the spatial orientation of the sugar moiety may explain the difference observed in some shifts between 25K and geshoidin. There is evidence that an inversion of the sugar could account for a shift difference between stereoisomers involving the glycosidic sugar. For instance, it has been

shown that in one case, the difference in shifts for three carbons at positions 4, 6, 5a of 8-O-methyl-7-hydroxyaloin A differs from that of 8-O-methyl-7-hydroxyaloin B by δ 1.8, 1.4 and 4.0, respectively.

Based on these observations 25K may tentatively be geshoidin as given by Figure 3.1

Table 3.2. ^{13}C NMR spectra geshoidin and 25K

CARBON	**δ values**	
	25K	GESHOIDIN
1	156.178	155.3
2	114.894	114.3
3	140.336	139.5
4	111.678	111.0
4a	143.612	142.7
5	123.751	123.0
6	130.319	129.5
7	111.527	111.0
8	156.565	155.8
8a	106.370	105.8
Lactone CH2	68.771	67.9
Lactone CO	169.374	168.8
1'	103.428	102.9
2'	73.875	73.4
3'	78.152	77.8
4'	70.303	69.9
5'	76.757	72.3
6'	61.271	60.8
* Melting point °C	*157.8-158.8*	*160-162*

*The melting points shown are not the corrected values.

3.1.2.3. Spectral data for 7K

The isolate 7K was obtained as a cream colored solid with the following NMR data:

^{1}H NMR 200 MHz (DMSO/CD_3OD): δ 7.645, 7.605, 7.588, 7.546, 7.509, 7.418, 7.381, 5.345, 5.136, 5.103, 5.054, 5.047, 4.620, 3.807, 3.814, 3.760, 3.553, 3.496, 3.448, 3.408, 3.371, 3.251, 2.482.

^{13}C NMR (CD_3OD): δ (C_1) 156.178, (C_2) 114.977, (C_3) 143.794, (C_4) 123.842, (C_{4a}) 140.336, (C_5) 111.868, (C_6) 130.648, (C_7) 103.648, (C_8) 156.694, (C_{8a}) 106.673 (Lactone CH_2) 68.741 (Lactone CO) 168.903 Glucose: (C_1) 103.594, (C_2) 74.140 (C_3), 78.577, (C_4) 70.523 (C_5) 76.939, (C_6) 61.521, (C_1) 156.080, (C_2) 115.137, (C_3) 77.030, (C_4) 70.644.

Details of these spectrums are as per appendix 7 and 8. Structural elucidation was not done for lack of sufficient sample to complete the spectroscopic investigations.

3.1.2.4. The isolate MEP1

The isolate was screened for the sugars of arabinose, glucose, galactose, rhamnose and xylulose sugars using normal silica pre-coated plates and propanol-water 85:15 as mobile phase. The plate was sprayed with 10%

sulphuric acid solution where MEP1 did not give any observable spot with the reagent. Comparison was made using the Rf values of the spots.

An aqueous solution of the isolate gave no characteristic peaks in the UV region and the NMR spectroscopy revealed no carbons suggesting an inorganic salt. The plant *Rhamnus prinoides* has been reported to have large amounts of inorganic cations and organic acids (Salgues, 1962).

3.2. The brine shrimp test for general toxicity

Toxicity of solutions was determined from survival of the brine shrimp larvae. The results are shown in table 3.4.

Table 3.3. Results for the brine shrimp test

Extract	Concentration mg/ml	% Survival	Toxicity
Hexane	1.0	100	None
	0.5	100	None
DCM	1.0	100	None
	0.5	86	Low
DCM-MeOH	1.0	100	None
	0.5	100	None

No toxicity was exhibited by the extracts at concentrations of

0.5 mg/ml-1.0 mg/ml. These results also conform with observations by other workers (Abegaz,and Dagane, 1988; Abegaz and Kebede, 1995) using leaf extracts of *R. prinoides*.

Salgue's report (1962) claims that the leaf extract of *R. prinoides* is toxic to rabbits. However, his claim is not consistent with the age long Ethiopian practice of using the plant in the preparation of their traditional brew or in treatment of various ailments by several communities in Eastern Africa. The extracts did not show any toxicity against brine shrimp.

3.3. Antiplasmodial assays

Table 3.5 below shows the activity of the extracts against the *P. falciparum* .

Table 3.4. Results for the antiplasmodial test

Solvent	**IC_{50}** (µg/ml)
Hexane extract	19.9
DCM	30.3
Methanol	277.7

In determining the level of activity, the standard applied is stated to be for any sample that shows an IC_{50} of 50 μg/ml (Aketch-Rabah, 1996). From the results obtained, it was discernible that significant activity was in the n-hexane, and DCM extracts which merit further evaluation. The activity was noted to decrease with increasing polarity of the extract. The hexane extract exhibited the greatest activity and the MeOH extract gives the lowest IC_{50} value.

However, considering that the herbalist utilizes the hot water extract, the contribution of the polar extract should not be ignored as it may have been hydrolyzed to give the non-polar compounds, probably extracted by the less polar solvents.

Another possibility is that the water-soluble components may have some limited effectiveness *in vitro* but may undergo metabolic changes in the body to produce the active form of the mixture. This would require an *in vivo* system (such as *P. berghei* in mice) in which the actual effectiveness may be ascertained.

3.4. Conclusion

The antiplasmodial test results indicate that the various extracts of *Rhamnus prinoides* may have direct inhibitory activity against malaria parasites *in-vitro*. Since the herbalist instructs his patients to take half a teaspoonful of the powdered root in hot water twice a day for five days, it may well be that the isolates 7K and 25K, being the major constituents and soluble in water contribute significantly to the activity associated with this medication.

3.5 Recommendations

In vivo studies using the crude extracts and individual isolates need to be conducted to confirm the effectiveness of *Rhamnus prinoides* against clinical human malaria.

The phytochemical findings may also help in the identification of a new molecule or mixture of compounds that could act as lead compounds in the design of new drugs against malaria.

Acute and chronic toxicity studies should be carried out in animals to map out the safety profile of the plant extract and isolates.

Armed with these conclusive data efforts be made to standardize the dosage and encourage the cultivation of the tree in commercial farms for large-scale production of the material.

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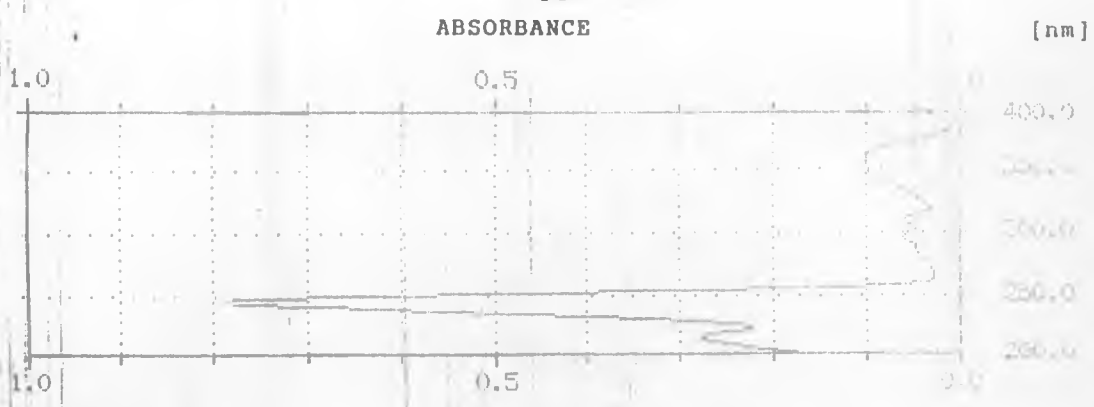
Appendix 1



THRESHOLD : 0.000

BATCH: 001

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		309.6 nm (MAX)	0.062 ABS
		303.2 nm (MIN)	0.050 ABS
		296.4 nm (MAX)	0.063 ABS
		287.6 nm (MIN)	0.048 ABS
		285.2 nm (MAX)	0.048 ABS
		265.6 nm (MIN)	0.029 ABS
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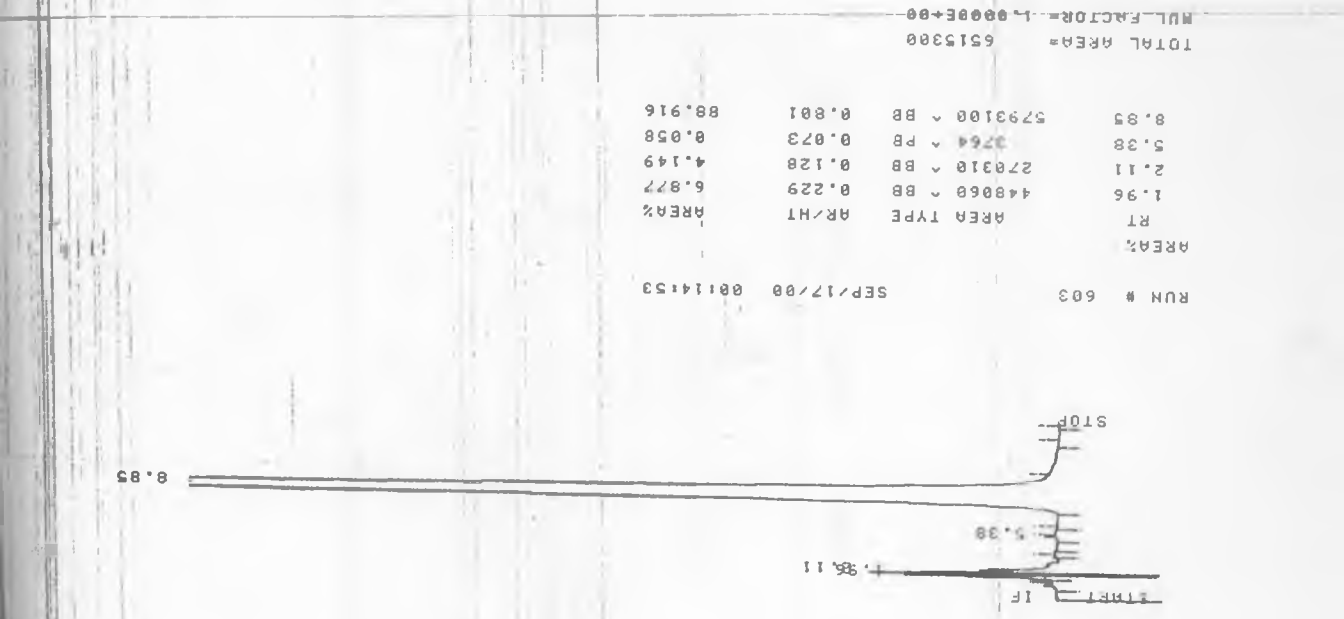

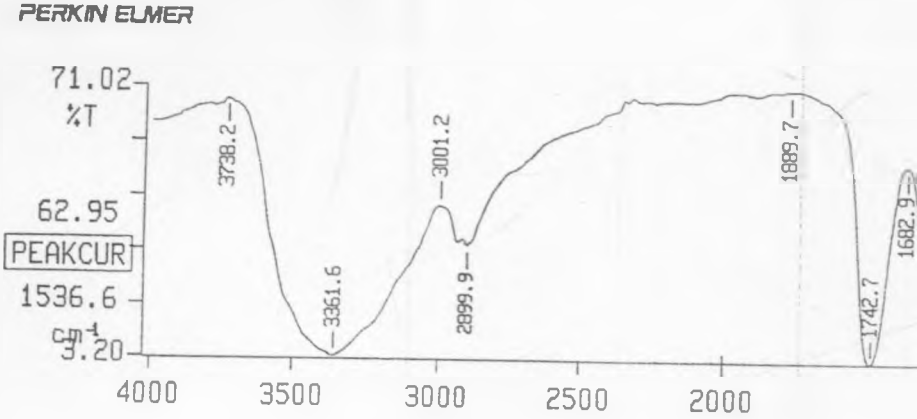
PERKIN ELMER
71.02
%T
62.95
PEAKCUR
1536.6
cm-1
3.20
3738.2
3361.6
3001.2
2899.9
1889.7
1742.7
1682.9
4000
3500
3000
2500
2000

1623.8
1453.5
1429.3
1297.1
1176.2
1160.7
1147.4
1081.5
929.4
893.9
879.9
794.5
755.3
724.4
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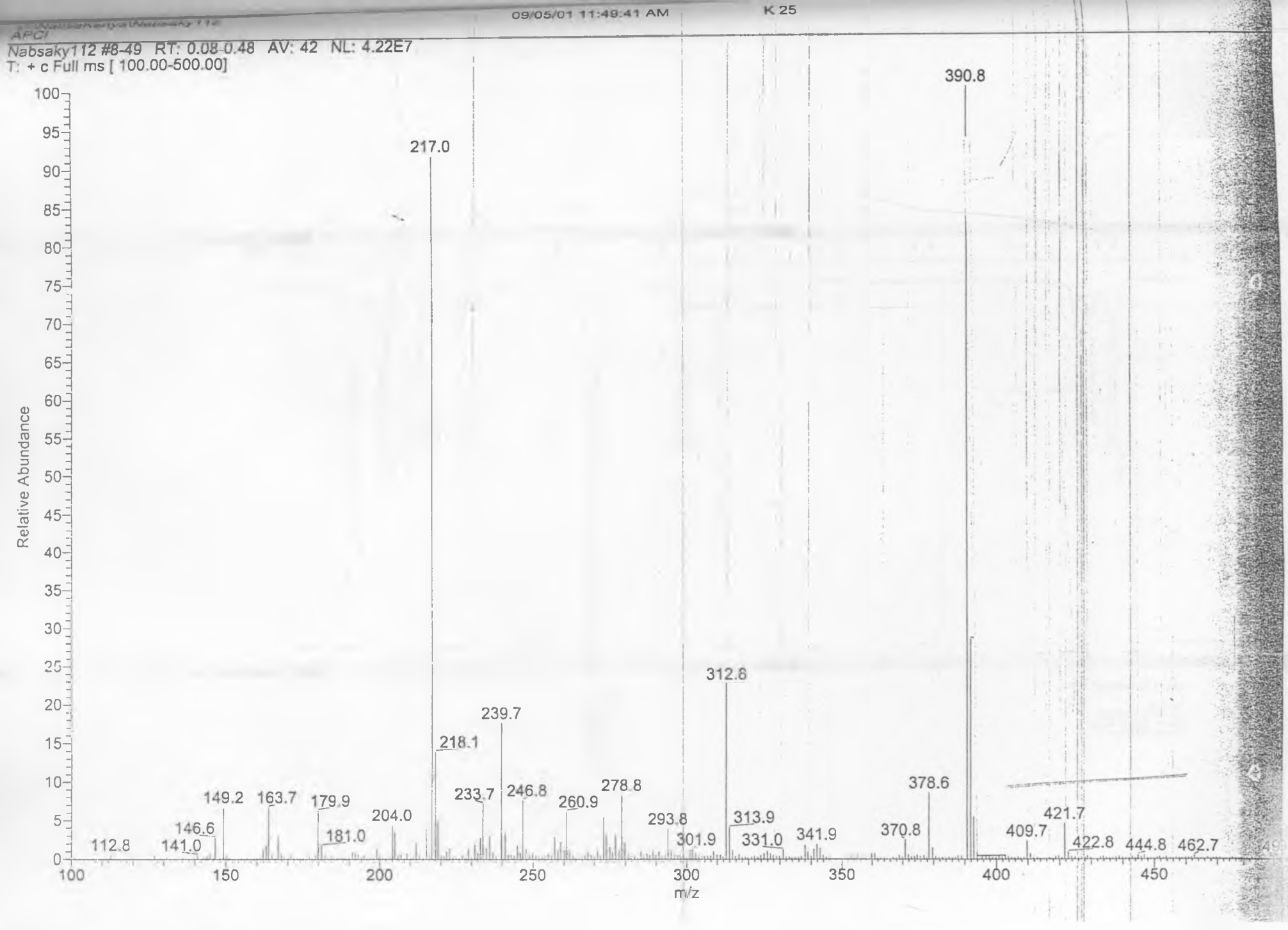
691.0
642.2
629.2
615.1
592.9
543.3
1500
1000
500
cm-1

Appendix 2


09/05/01 11:49:41 AM
K 25
APCI
Nabsaky112 #8-49 RT: 0.08-0.48 AV: 42 NL: 4.22E7
T: + c Full ms [100.00-500.00]
Relative Abundance
m/z
390.8
217.0
312.8
239.7
218.1
149.2
163.7
179.9
233.7
246.8
260.9
278.8
204.0
146.6
181.0
293.8
313.9
301.9
331.0
341.9
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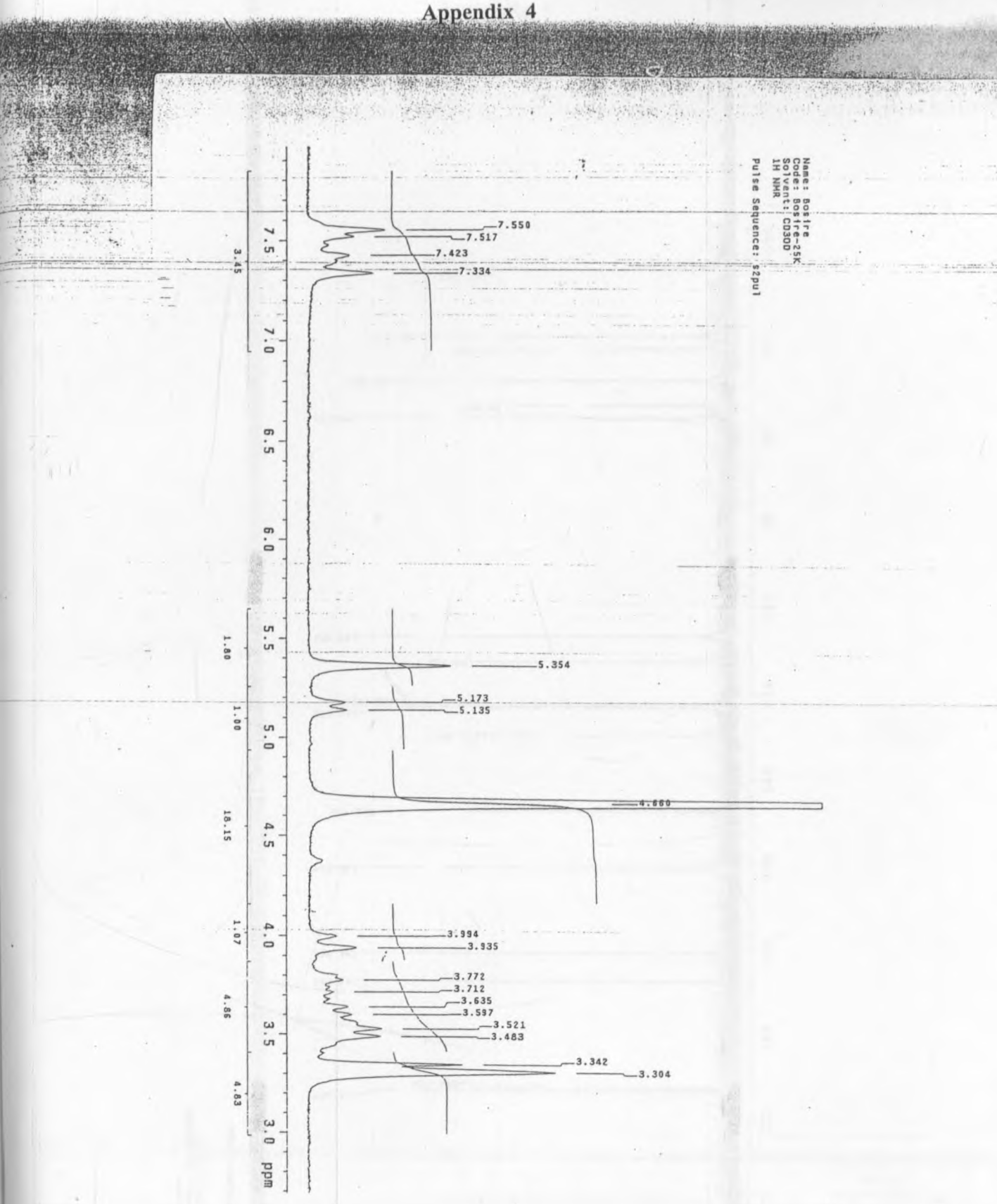
370.8
421.7
409.7
422.8
444.8
462.7
112.8
141.0

Appendix 3

Appendix 4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Code: Bosire-25K
Solvent: CD3OD
1H NMR
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7.517
7.423
7.334
5.354
5.173
5.135
4.660
3.994
3.935
3.772
3.712
3.635
3.597
3.521
3.483
3.342
3.304
3.45
1.80
1.00
18.15
1.07
4.86
4.83
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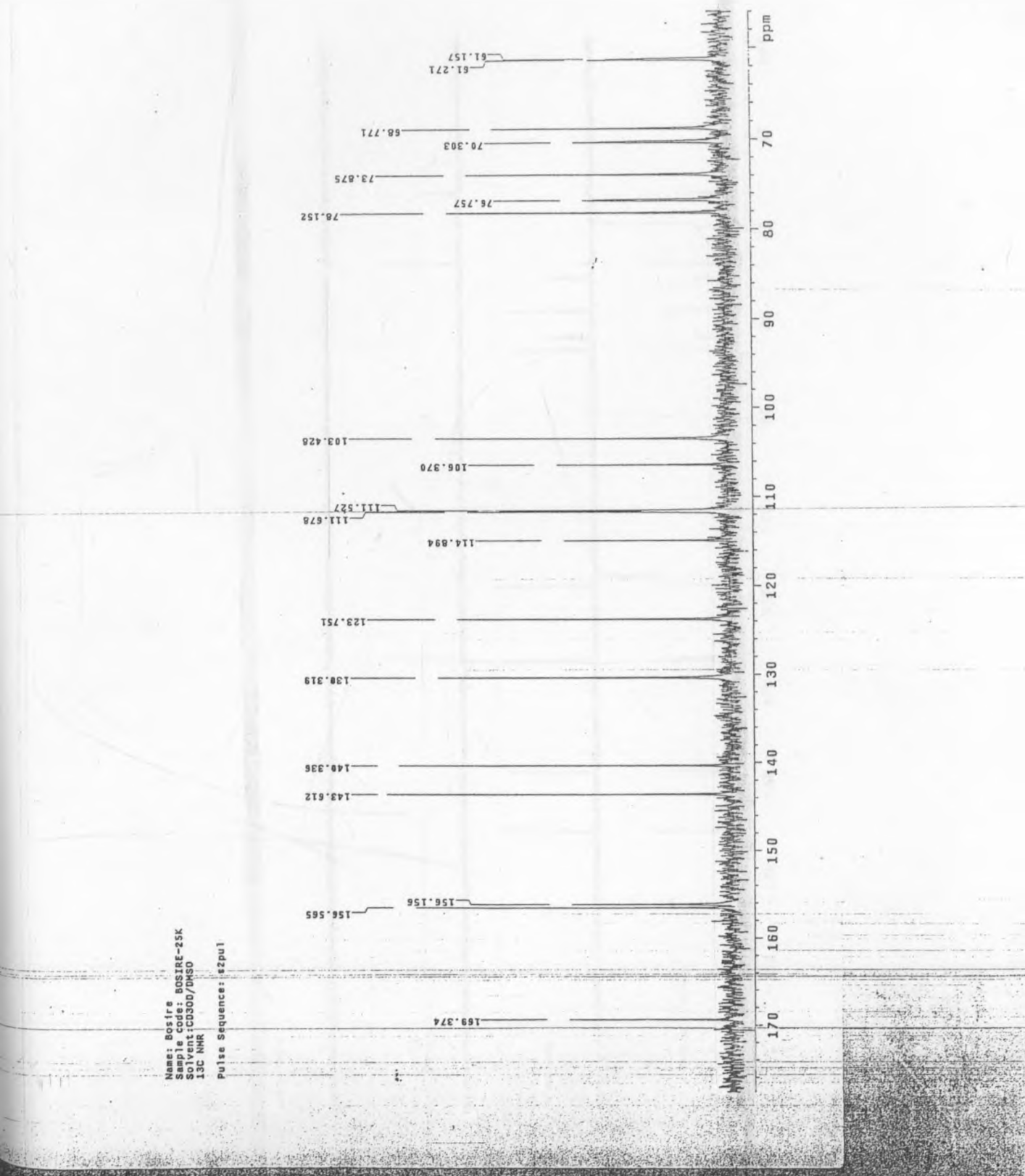
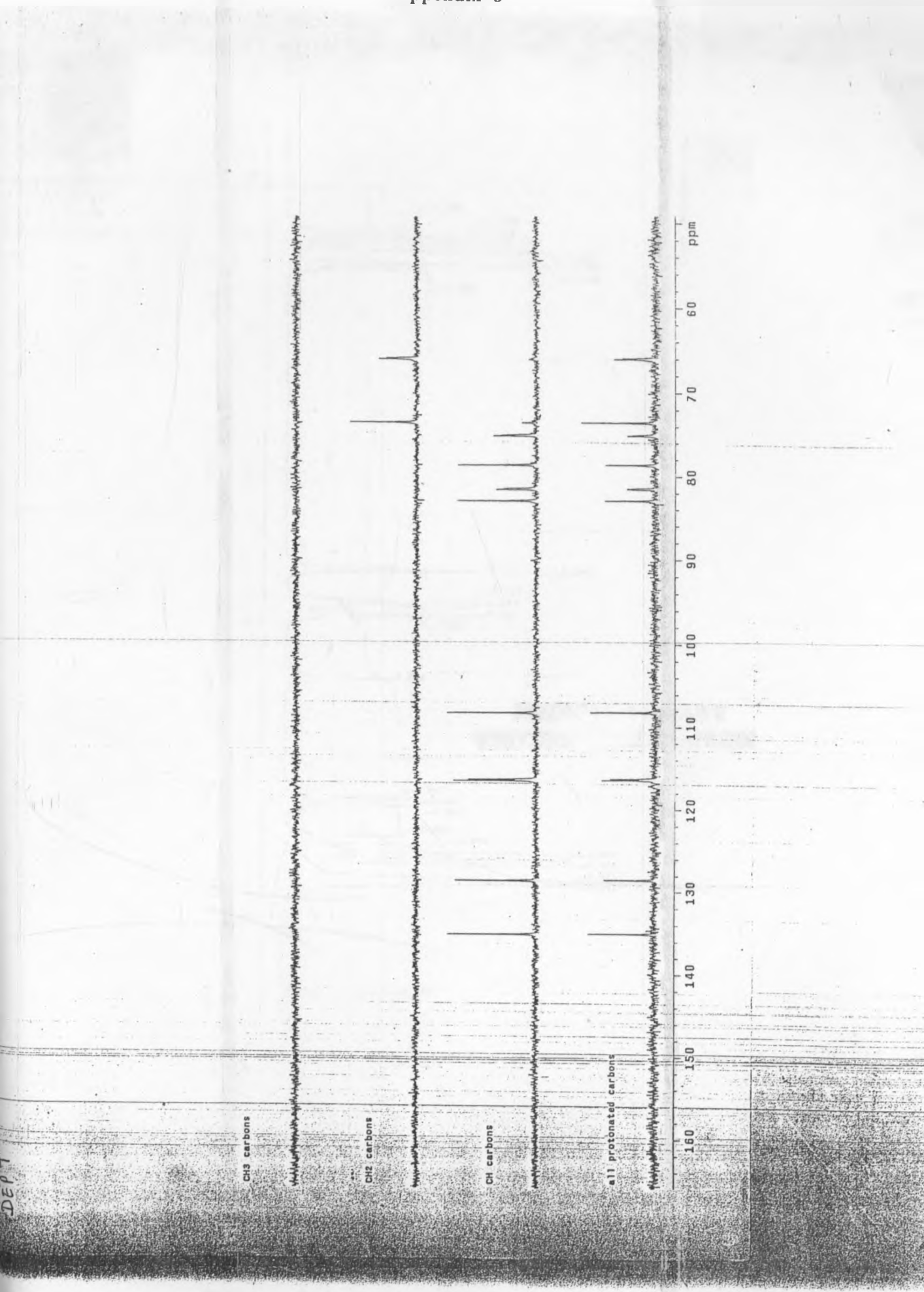
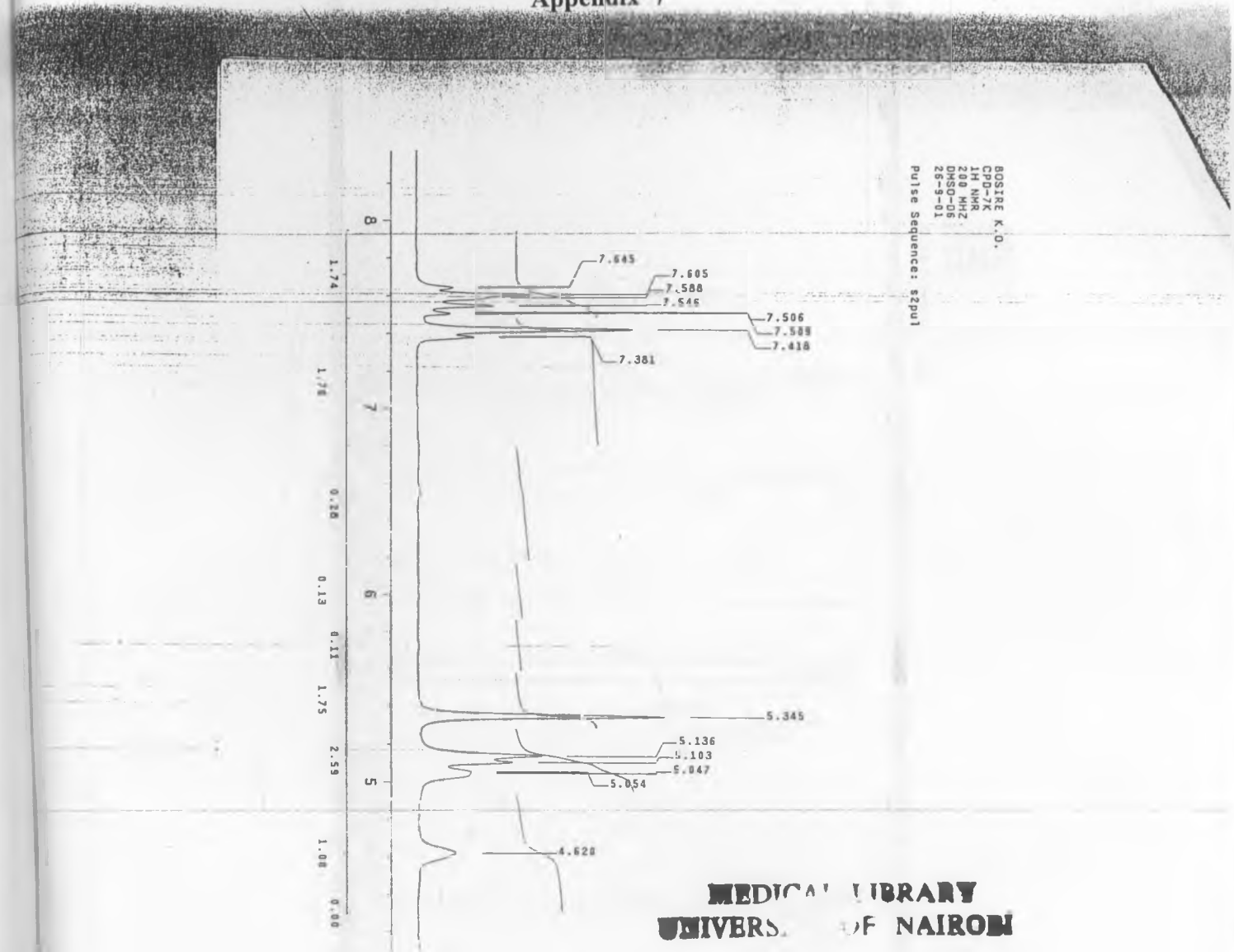
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0 ppm

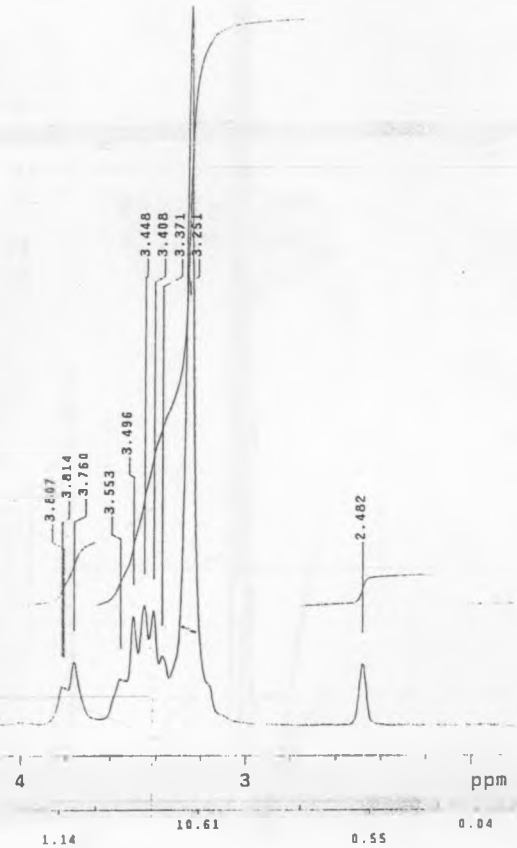
Appendix 5




DEPT
CH3 carbons
CH2 carbons
CH carbons
all protonated carbons
160
150
140
130
120
110
100
90
80
70
60
ppm

Appendix 7

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3.760
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3.408
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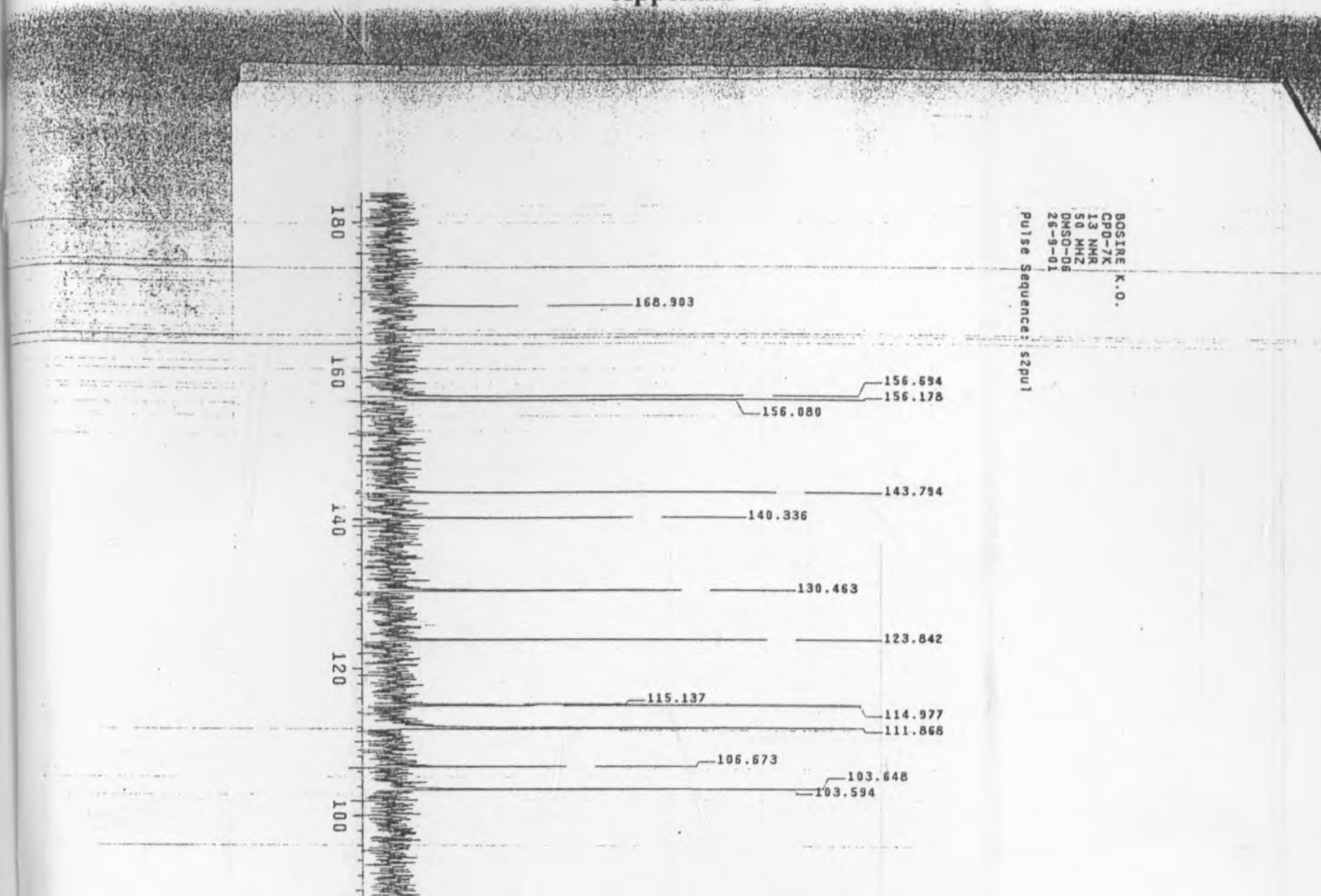
3.251
2.482
4
3
ppm
1.14
10.61
0.55
0.04

Appendix 8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DMSO-D6
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156.178
156.080
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103.648
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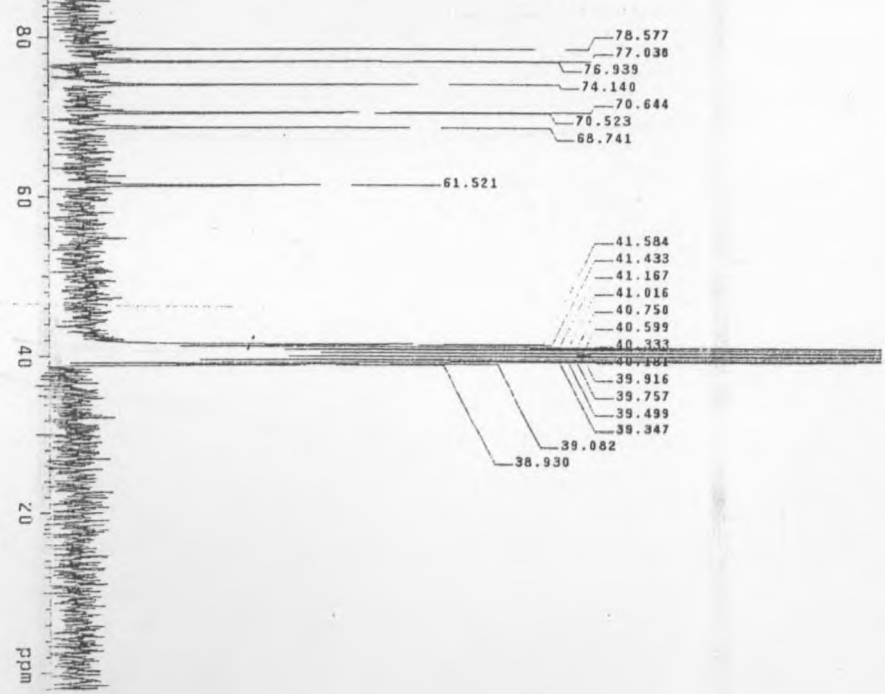
180
160
140
120
100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39.757
39.499
39.347
39.082
38.930
80
60
40
20
ppm