PRELIMINARY ANTIMALARIAL AND PHYTOCHEMICAL STUDIES OF SOME KENYAN MEDICINAL PLANTS

BY

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A thesis submitted in partial fulfilment
for the degree of Master of Science

KENYATTA UNIVERSITY

AUGUST 1995

DECLARATION

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

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DEDICATION

To my parents, Susan and Festus; my brothers

and

To my husband, Watson and our children;

Barnabas, Paul and Esther.

ACKNOWLEDGEMENT

I wish to express my sincere gratitude to Prof. S.C. Chhabra and Dr. B. Khan for their guidance, invaluable suggestions, sound advice and patience during the course of the project and later in the preparation of this report.

I am also indebted to the entire staff of the Chemistry Department, Kenyatta University; Malaria Laboratory, Biomedical Sciences Research Centre, Kenya Medical Research Institute; Phytochemistry Laboratory, Kenya National Museums; and Chemistry and Biochemistry Research Unit, International Centre for Insect Physiology and Ecology.

I wish to thank Mr.S. Mathenge of Botany Department, University of Nairobi, who assisted in the collection and identification of plant materials.

My heartfelt gratitude goes to the Japan International Cooperation Agency and Jomo Kenyatta University of Agriculture and Technology for providing funds for this study.

My deep appreciation goes to Salome Gichimu and Patrick Ahuta for kindly accepting to type the thesis and their patience during the entire period.

ABSTRACT

Malaria, caused by *Plasmodium* species, inflicts over 270 million people and kills two million every year. Herbal medicine is practiced worldwide and has been recognised by WHO as an essential part of primary health care programmes. This thesis gives the results of antimalarial screening of nine Kenyan medicinal plants belonging to the families Combretaceae, Euphorbiaceae, Melastomataceae, Papilionaceae and Moraceae. The antimalarial activity was quantitatively measured by the ability of the extracts to inhibit the uptake of radiolabeled nucleic acid precursor by *Plasmodium falciparum* during the short-term culture in microtitration plates. Using an ID_{50} of 50µg/ml as a cut off dose, the results showed that out of 30 extracts, 57.1% were active against the chloroquine non-sensitive strain (ENT36) while 33.3% against the sensitive strain (K67).

The phytochemical analysis of the stembark of *Terminalia spinosa*, stems of *Dissotis brazzae* and leaves of *Suregada zanzibariensis* and *Phyllanthus reticulatus* indicated the presence of alkaloids, flavonoids, steroids, triterpenoids, tannins, saponins and volatile oils among others. In the last two decades, work on antimalarials of plant

origin other than alkaliods has intensified. This has led to the discovery of antimalarials from classes of compounds such as terpenoids (as sesquiterpene lactones and quassinoids), flavonoids and coumarins. Tannins have been found to be effective anticytotoxic agents.

Chromatographic separations of the methanolic extract of the stembark of *Terminalia spinosa* led to the isolation of some pure compounds which have partially been identified as flavonoids and terpenoids by ultraviolet and infra-red spectroscopy.

CONTENTS

CHAPTER II

EXPERIMENTAL

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

1.1 MALARIA - A SERIOUS HEALTH PROBLEM

Malaria is a serious health care problem in the tropics and subtropics region and has far reaching medical, social and economic consequences. In fact it is the most important infectious disease causing morbidity and mortality (Kondershin and Dixit, 1985). Generally, it is estimated that 2073 million people (40% of world's population) living in more than 100 countries are exposed to the risk of malaria and about 270 million of these are infected with malarial parasites. Of the people infected, Africa accounts for 80%, India 8% while South America and others make up the 12% (Bektimirov, 1990). Among the four *Plasmodium* species (*vivax, ovale, malarae and falciparum*) that infect man via the female Anopheles mosquito, *P. falciparum* is the most lethal. Warrel (1988) reported that the mortality rate of falciparum malaria remains high (varying from 10-50%) inspite of the advances made in medicine in the last four decades. Malaria, when in acute infection causes fevers and serious neurological complications such as celebral malaria. Chronic infection of the disease causes severe anaemia while prenatal infection results in low birth weight (Greenwood et al., 1992).

It was anticipated in the 1950's and 60's that malaria would be eradicated by the turn of the century (Bruce-Chwatt and Zulveta, 1980). But in 1987, it was indicated that over 103 million cases of the disease had been reported world wide and in Africa alone it accounted for the death of 1 million children. Resurgence of malaria is attributed to two major factors namely, the development of resistance of malarial parasite (*Plasmodium falciparum*) to chloroquine, pyrimethamine and other clinical agents, and the resistance of the vector to insecticides (Phillipson and Wright, 1991).

In addition to the above factors, efforts to eradicate or control malaria, especially in the developing countries, have not been successful because of inadequate financial resources to administer antimalarial programmes. So far, no effective vaccine has been found primarily because of the different infestation stages of the parasite. *P. falciparum* is antigenically an extremely complex organism which is capable of changing its pattern of antigens to evade the immune defenses of its host and may exist in immunologically distinct strains in different geographical regions of the world (Carter and McGregor, 1973; Nkunya, 1992.).

1.2 MEDICATION

Peters et al. (1990) reported that the resistance of the malignant tertian malaria parasite, *P. falciparum* to chloroquine and other compounds is a steadily increasing clinical problem which may now be extending also to *P. vivax*. Few new candidate compounds are either available or under development to respond to the situation, therefore, new approaches to the prevention and treatment of malaria are urgently required. Drug development is a very expensive endeavour and has very rigid requirements before a drug is marketed. Reduced sensitivity to the amino quinoline-based drugs calls for new efforts directed in obtaining drugs which have different structural features either synthetically or from natural sources (Nkunya et al., 1991; Farnsworth and Soejarto, 1991). Because of the obvious need for new non-cross resistant and less toxic antimalarial drugs, plants and other natural sources will continue to be investigated (Weenen et al., 1990).

1.3 MEDICINAL PLANTS

The main natural source of therapeutic agents is the plant kingdom. At present, plant resources are unlimited as far as the search for useful phytochemicals is concerned although due to civilization, these resources are fast disappearing before they are fully exploited (Hudson, 1990;

Kokwaro, 1991). Only 5-15% of the 250,000 to 750,000 existing higher plants species have been surveyed for biologically active principles (Marderosen and Liberti, 1988). This indicates that studies on the therapeutic value of plants should be intensified. The human pressure on the natural resources is rising. The tropical rainforest is quickly being converted and depleted through fuel wood, forest farming and commercial logging. There is also the indirect human pressure of environment threat through genetic engineering (Farnsworth and Soejarto, 1991).

Medicinal plants have been used in the treatment of malaria throughout the tropics and subtropics with a folklore often encompassing centuries of practical experience (Noster and Kraus, 1990). Tradition lays down the foundation of materials to be used and also specifies the mode of use. Thus the exact method of preparation of the extract, the part of plant to be used and even the appropriate season for collection, and the details of administration are known (Hudson, 1990). Early records of experience of medical uses of natural products have been found in Babylonian writings, among Egyptian documents and in ancient cultural records of Asia, especially India and China. In recent years, renewed interest in natural products, not just medicines, has been attributed to "back to-

nature" fad and to the notion that natural drugs are perhaps safer than synthetic drugs or can fill many gaps that exist in the physicians medical supply (Marderosen and Liberti, 1988). Indeed herbal medicines as compared to their synthetic counterparts are more readily available in the wild, can be cultivated and are easily biodegraded in the environment (Addae-Mensah and Achenback, 1987).

Modern medicine employs a single drug to a target point. In traditional medicine, holistic approach is used where, generally, a recipe consists of several herbs or at least more than one part of a plant. Medicinal plants extracts are therapeutically succesful because of the virtue of the synergetic effects of two or more compounds that complement each other *in vivo*. In fact in the Chinese traditional medicine, a recipe consists of four different groups of agents, that is, the principal (the principal curative effect), the adjuvant (strengthens the principal effect), the auxiliary (relieves secondary symptoms and decreases the toxicity of the principal) and the conductant (directs the action of the principal to the target organ or site (Bai, 1990). Since plants contain a multiplicity of constituents, it has been often claimed that the use of the whole plant rather than one purified constituent may be more effective therapeutically and also produce fewer adverse

effects (Wright and Phillipson, 1990).

To this end, the World Health Organization (WHO) has acknowledged that its target of providing total health coverage for everybody by the year 2000 can only be met by incorporating traditional medical practices into the overall health care delivery schemes of developing countries (WHO, 1978). In most developing countries where coverage by health services is limited, it is the traditional practioner or folk medicine that the majority of the population turns to in sickness and the treatment is mainly based on medicinal plants. Early in the century, the greater part of medical therapy in the industrialised countries was dependent on medicinal plants. However, with the growth of the pharmaceutical industry, their use fell out of favour. Even so, 25% of all perscriptions dispensed from community pharmacies from 1959 to 1980 in U.S.A., for example, contained plant extracts or active plant principles prepared from higher plants (Akerele, 1991).

Kokwaro (1976), Hedberg et al. (1982, 1983), Chhabra et al. (1987, 1989, 1990a, 1990b, 1991, 1993), Chhabra and Mahunnah (1994) and Watt and Breyer-Brandwijk (1962) have listed more than a thousand plants in East Africa having medicinal properties. From preliminary screenings, it has been indicated that these plants contain secondary

metabolites that may be responsible for curative properties observed (Rukunga et al., 1986).

In the last two decades, work on antimalarials of plant origin other than alkaliods has intensified. This has led to the discovery of antimalarials from classes of compounds such as terpenoids (as sesquiterpene lactones and quassinoids), flavonoids and coumarins. Tannins have been found to be effective anticytotoxic agents.

1.4 NEED FOR SCIENTIFIC EVALUATION OF MEDICINAL PLANTS

The use of crude extracts without any scientific evaluation could lead to serious complications. At least 121 chemical substances of known structure are still extracted from plants that are useful as drugs throughout the world (Farnsworth and Soejarto, 1991).

Of the documented ethnomedical uses of plants, drugs from the temperate lands and subtropical regions form a higher propotion inspite of the tropical region, especially the tropical rainforest leading in biodiversity. This is due to the fact that phytochemical studies and medical advances in the temperate areas have taken place for much longer periods than those for the tropical regions. Another problem is the accessibility, namely, logistical and political problems in the collection of plant samples from the tropics and tropical rainforests

for study (Farnsworth and Soejarto, 1991).

The development and application of scientific methodology to validate the medicinal properties and the documentation of toxicological properties of traditional medicines (drugs) have been stressed as important requirements for improving the quality of traditional medical practices. Thus, investigations on materia medica should attempt to validate the scientific basis for their medicinal properties, to evaluate their clinical usefulness and toxicological potential, and finally to identify active components and their pharmacodynamic/kinetic properties (Kyerematen and Ogunlana, 1987). The Natural Product Alert (NAPRALERT), which is a computerized data base for natural products scientific literature, lists some 152 genera which have folkloric reputation for the treatment of malaria (Wright and Phillipson, 1990).

1.5 OBJECTIVES

1.5.1 General Objectives

To evaluate the efficacy of selected medicinal plants used in Kenya by traditional healers for the cure of malaria *in vitro*.

1.5.2 Specific Objectives

1. To select some medicinal plants used by Kenyan medicine men for the treatment of malaria in

prevalent areas based on the information obtained from literature.

2. To assess the antimalarial activity of different plant extracts *in vitro* on *P. falciparum* using an isotopic semi-automated microtest against a non-sensitive strain (ENT36) and a sensitive strain (K67). The sensitivity is in reference to chloroquine.
3. To phytochemically screen the promising plants for the class of compounds present.
4. To isolate and if possible, fully characterize the active principles of the most active extract.

1.6 LITERATURE REVIEW

1.6.1 Antimalarials of Plant Origin

In the last four decades work on antimalarials of plant origin has intensified. Several antimalarial screening studies of medicinal plant extracts have been and are still being carried out, aimed at identifying plants which have antimalarial activity. The search is giving promising results and investigations are giving leads which may be explored for the eventual synthesis of potent and non-toxic antimalarial drugs. Extensive studies on *Azadirachta indica* A. Juss, locally known as mwarobaini (Kiswahili, Kikuyu) have shown that the extract is active against malaria (Obih and Makinde,

1985). In an ethnopharmacology survey by Labadie et al. (1991), 75% methanolic leaf and bark extract showed a significant oral antipyretic activity in rabbits. The aqueous and ethanolic leaf extracts and nimbolide (a triterpene) showed *in vitro* activities against *P. falciparum* and had EC_{50} of 115, 5 and 0.95µg/ml, respectively. The conclusion from this survey indicates that *A. indica* leaf extracts cause a substantially increased state of oxidation in the red blood cell preventing normal development of the plasmodia. An antimalarial agent gendunin, was isolated from the leaf of *A. Indica* and its structure was determined by one and two dimensional nuclear magnetic resonance (nmr) spectroscopy (Khalid et al., 1991).

Chloroform extract from *Ailanthus altissima* Mill. stem showed high activity against *P. falciparum in vitro* (IC_{50}=0.015µg/ml) and *P. bergheii* (ED_{50}=0.76µg/kg/day) in mice. The activity is due to the quassinoid, ailathone. Fourteen other active quassinoids have been isolated from the Simaroubaceae family and in particular from the plant *Brucea javanica*. The antimalarial potency of the quassinoids is due to the free enolic hydroxyl group in the molecule (O'Neill et al., 1987). The methanol extract of the bark of *Alstonia schloris* L. was found to be weakly active against *P. berghei* in mice (Gandhi and Vinayak, 1990).

Makinde et al. (1990) found that significant antimalarial activity was shown by the chromatographic fractions of chloroform and hexane extracts of the stembark of *Spathodea campanulata* Beauv.

Artemisinin is the antimalarial principle isolated by Chinese scientists in 1972 from *Artmesia annua* L., a medicinal plant from which is derived the traditional medicine qinghaosu. *A. annua,* indigenous to China yields 0.01-0.6% (w/w) artemisinin from the air dried leaves and flowering tops. Although grown in many countries for its aromatic oil, the yield of artemisinin is very low compared to that grown in China. Artemisinin is a sesquiterpene lactone with an unusual peroxide linkage whose activity is enhanced by methoxylated flavones. The peroxide moiety of the molecule appears to be responsible for the antimalarial activity against chloroquine sensitive and chloroquine resistant *P. falciparum*. At present the artemisinin derivatives that are soluble in water or oil, that is artemether and sodium artesinuate, have been developed for clinical use (Klayman, 1985, Nkunya, 1992).

The crude extract of the European medicinal plant, *Artemisia abrotanum* L. yielded two sesquiterpenes, namely isofraxidin and 1(S)-hydroxy-ó-bisabololoxide A acetate which were mildly active

against *P. falciparum in vitro* (Cubukcu et al., 1990). Also from the same genus *Artemesia, A. absinthium* have considerable antimalarial potential (Zafar et al., 1990).

Ficus pyrifolia Burm.f., and *Rhus taratana* Baker. were found to posses antimalarial activity on *P. falciparum* (Uverg et al., 1991). The extracts of these plants also showed low cytotoxity.

The plant, *Bridelia cathartica,* is employed in herbal antimalarial remedies in Southern Mozambique. Crude ethanolic and aqueous extracts of the root and the ethanolic extract of the stem of *B. cathartica* caused a 50% inhibition of parasite growth at an incubation concentration of 0.05µg/ml (Jurg et al., 1991). Upto now antimalarial compounds with diverse chemical structures have been isolated from plants but most compounds include alkaloids, limonoids, quassinoids, sesquiterpenes and dihydrochalcones. Also there are the less common ones like coumarins, naphthaquinones, steroids and flavones (Nkunya, 1992).

Literature on claims of herbal medicine for treatment of various ailments and conditions are numerous. However, it should be kept in mind that they are assertions by the medicine men and most of these uncritical claims have not been scientifically proved. The use of plants by man came about through observation of their definite effects on man and

animals. Herbalists employ both physical and psychological treatments.

When dealing with malaria, it may be difficult for the medicine men to differentiate between malaria and fevers. Thus some antipyretics could be mistaken for antimalarial treatment. However, ethnobotanical data shows that medicinal plants continue to be sought for especially in the more traditional communities of the world.

1.6.2 Medicinal Plants Screened in the Present Study

Nine Kenyan medicinal plants belonging to the families Combretaceae, Euphorbiaceae, Melastomataceae, Moraceae and Papilionaceae used by medicine men for the treatment of malaria were chosen based on the information documented by Watt and Breyer-Brandwijk (1962), Kokwaro (1976), Chhabra et al. (1987, 1989, 1990a, 1990b, 1991, 1993), and Chhabra and Mahunnah (1994). The plants chosen, the parts used and the adminstration of the drugs are listed in table 1.

Table 1: Ethnobotanical data of the plants (parts used, methods of preparation and administration) employed in screening antimalarial activity.

Family	Name	Part Used	Preparation	Method of Administration
Combretaceae	*Terminalia spinosa Engl.*	Leaves	Juice	Drunk
Euphorbiaceae	*Antidesma venosum* Tul.	Roots	Decoction	Drunk
	Phyllanthus reticulatus Poir.	Leaves	Infusion	Drunk
	Suregada zanzibariensis Baill.	Roots	Decoction	Drunk
Melastomataceae	Antherotoma naudinii Hook.f.	Aerial parts	Infusion	Drunk
	Dissotis brazzae Cogn.	Aerial parts	Infusion	Drunk
Moraceae	*Ficus natalensis* Hochst.	Roots	Decoction	Drunk
Papilionaceae	*Kotschya africana* Endl.	Aerial parts	Decoction	Drunk
	Rhynchosia minima (L.) DC.	Roots	Decoction	Drunk

1.6.2.1 The Family Combretaceae

1.6.2.1.1 The Genus *Terminalia*

Terminalia species have hard, durable wood, resistant to termite and ant attack. To this end they are used for fencing poles, hut building and making furniture (Specht and Shaeffer, 1990). Extracts from *Terminalia* species have been found to be active in controlling the growth of *Aspergillus* and its aflatoxin production (Garcia and Garcia, 1988). They are progressively being used as mountain vegetation, arid and semi-arid plantation and energy plantations. The fact that they are fast growing and have hard wood, makes the *Terminalia* species useful as a source of fuel (KENGO, 1992; Ramulus et al., 1989). All *Terminalia* species yield tanning materials and gums of various percentages and are widely used as medicines. *Terminalia spinosa* Engl. is used to treat diarrhoea, dysentry and when powdered with the mylabris beetle treats schistosomiasis (Watt and Breyer-Brandwijk, 1962). The leaf extract is drunk against malaria and is said to contain tannins and proteins (Chhabra et al., 1989). The seed oil of *Terminalia* species are said to contain palmitic, oleic, linoleic and oxalic acids (Prabbu and Theagarajan, 1987). The bark is chewed or added to tea to reduce fevers (Heine and Brezinger, 1980). *T. spinosa* is naturally found in Tsavo National Park, Taita/Taveta district, Kilifi

Forest, Meru and is now being tried as a crop in arid and semi-arid areas of Kenya (KENGO, 1992).

1.6.2.2 The Family Euphorbiaceae

1.6.2.2.1 The Genus *Antidesma*

This genus is considered as rare and newly recorded in Kenya (Waliaula, 1992). The fruit of *Antidesma venosum* Tul. is edible when raw and its taste is sweet/acidic making it useful as a fish bait. An infusion of fruit, leaves and twigs, containing an alkaloid, is drunk for abdominal complaints (Watt and Breyer-Brandwijk, 1962; Peters et al., 1992; Lindsay, 1978). The roots are toxic and are used in magic but when mixed with other plants, the infusion is used for epilepsy (Williamson, 1955). *A. venosum* is a fairly large leafy forest tree about 5m tall found mainly at the South Coast of Kenya and in the drier parts of Meru district. It is also being tried as a crop for arid and semi-arid areas of Kenya (KENGO, 1992).

1.6.2.2.2 The Genus *Phyllanthus*

The *Phyllanthus* species often grow on forest edges and are therefore used as live hedge for cultivated areas. Its thin but strong branches are used in basketry (Waliaula, 1992; Watt and Breyer-Brandwijk, 1962). *Phyllanthus* species are widely used in traditional medicine in the tropics. The

Phyllanthus species are used as diuretics and astrigents in South America and India, was found to inhibit spore germination of *Helminthasporum turcicum*, the incitant of sorghum leaf blight at concentrations below 5µg/ml, to control the growth of *Aspergillus* and its aflatoxin production and is being experimented as a dryland horticultural crop (Bachi, 1984; Khan et al., 1971; Garcia and Garcia, 1988). *P. niruri* and *P. reticulatus* are specifically used for malaria treatment and spasms in the tropics (Chhabra et al., 1990a; Herdberg et al., 1983). The roots of *P. reticulatus* yielded octacosanol, taraxeryl acetate, fredelin, epifriedelinol, taraxerone, betutin, ß-sitosterol, glochidinol and 21-ó-hydroxy-friedelin-3-one. The structures of the compounds were determinded by ultraviolet, infra-red, mass spectroscopy, nuclear magnetic resonance and melting point (Joshi et al., 1981). *Phyllanthus reticulatus* contain a neutral bitter principle and an alkaloid (Chopra et al., 1965).

1.6.2.2.3 The Genus *Suregada*

The root decoction of *Suregada zanzibariensis* Baill. is traditionally drunk against malaria (Chhabra et al., 1990a). The fruit is eaten by humans and the soft tall tree (7m) with a smooth cylindrical trunk is found growing in the forest shade on coral or stony ground.

1.6.2.3 The Family Melastomataceae

1.6.2.3.1 The Genus *Antherotoma*

Antherotoma naudinii Hook.f. is an ever green leafy herb about 1m tall and has a purple fluorescent. It is found growing on the edge of open grasslands in wet forests. Its aerial parts are steeped in water and the resulting infusion is drunk against malaria (Chhabra and Mahunnah, 1994).

1.6.2.3.2 The Genus *Dissotis*

The leaves of *Dissotis* species are generally chewed for relief from colds, coughs and intestinal worms. So far, none of the species of *Dissotis* have been found to be poisonous (Peters et al., 1992). The dry leaves are used as a febrifuge (Williamson, 1955). *Dissotis brazzae* Cogn. is a herb of about 40-60cm height with a yellow fluorescent at the tip of the plant. *D. brazzae* grows in and near the open swampy areas of a wet forest. An infusion of the aerial parts is drunk against malaria (Chhabra and Mahunnah, 1994).

1.6.2.4 The Family Moraceae

1.6.2.4.1 The Genus *Ficus*

There are 800 species of *Ficus* to date. *Ficus* species are generally planted for shade or as ornamental trees. However, due to their shallow feeding roots, they heave up roads, clog sewers,

penetrate buildings via the plumbing while others like *F. aurea* engulfs and kills other ornamental trees like palms. They have polyphenolic constituents and tannins. Most of these are used as anthelmintic due to the presence of ficin, a proteolytic enzyme. They have a bitter resinous bark that is used for marking clothes, mats and ropes. They are able to collect water (water divining) and therefore are regarded sacred by some communities in the world, for example the Kikuyus of Kenya and some tribes in Angola. All indigenous figs have characteristic edible fruits but are too seedy and often infested with insects. They all have a milky latex that has medicinal value. The wood is white and soft and thus does not render itself useful as fuel or timber (Watt and Breyer-Brandwijk, 1962; Williamson, 1955; Peters et al., 1992). The root decoction of *Ficus natalensis* Hochst. is drunk against malaria (Chhabra et al., 1990b). The aqueous extract of the crushed bark is a cure for influenza (Kokwaro, 1976), serves as a galactagogue, is antisyphilitic, and is found to contain flavonoids and sterols (Watt and Breyer-Brandwijk, 1962; and Chhabra and Uiso, 1990).

1.6.2.5 The Family Papilionaceae

1.6.2.5.1 The Genus *Kotschya*

Kotschya africana Endl. is a shrub about 1-2m tall with small, thin green leaves, a feathery (hairy) stem and a long spongy taproot. It is mainly found in swampy plains. Its twigs are used in the treatment of malaria (Chhabra and Mahunnah, 1994).

1.6.2.5.2 The Genus *Rhynchosia*

Some *Rhynchosia* species yield fruit that is edible but the roots of all species are chewed for their sweet/minty flavour. Some species produce tubers that are eaten as famine food (Peters et al., 1992). The roots of *Rhynchosia minima* (L.) DC. are used as antimalarials (Chhabra and Mahunnah, 1994). *R. minima* has been found to be productive and resistant as a tropical legume on alkaline soil. It is, therefore, being experimented on as a pasture legume on alkaline soil in Australia (Conway et al., 1988).

1.7 *IN VITRO* ANTIMALARIAL TEST

There are several *in vitro* methods for antimalarial test. These include short-term culture system, macro-test, microtest, 48h test and the semi-automated microtest. Of all the tests listed, the semi-automated microtest is considered as the best method so far for evaluating antimalarial

activity of crude and refined extracts (O'Neill et al., 1985;WHO,1984). Also, because of the increased occurrence of drug resistant strains of *Plasmodium falciparum* and the difficulties encountered *in vivo* studies, *in vitro* drug sensitivity tests are being used more often to study the epidemiology of drug resistance and contribute to the advance of knowledge of the biology of *P. falciparum* and the development of new drugs (Bras and Deleron, 1983).

The most extensive investigation of higher plants for antiprotozoal activity was published in 1947. Extracts from 600 species distributed among 126 families were tested for their curative potential against avian malaria in chicks and ducklings *in vivo*. The most active extracts were from plants *Hymenocallis caribea, Castela fortusa, Simaba cedron* and *Simaruba amara* (Simaroubaceae) and *Cooperia penduculata* (Amaryllidaceae). The other plants were *Cornus florida* (Cornaceae), *Dichroa febrifuga* (Saxifragaceae), *Gentiana* species (Gentianaceae), *Croton* species (Euphorbiaceae), *Cissampelos pareira* (Menispermaceae), *Aristolochia* species (Aristolochiaceae) *Datisca glomerata* (Datiscaceae) and *Erynguim foetidum* (Umbelliferae). Eleven of these 13 species have been shown to elicit varying degrees of antineoplastic and/or cytotoxic activity (Farnsworth, 1966).

It was not until 1976 when it was reported that

it is possible to cultivate *P. falciparum in vitro* in human erythrocytes (Trager and Jensen, 1976). By 1979 semi-automated microtitre plate assay had been developed (Desjardins et al., 1979) which was later on improved by O'Neill et al. (1985). In the present study, the plant extracts were tested for antimalrial activity *in vitro* on *P. falciparum* using an isotopic semi-automated microtitre plate assay method developed by Desjardins et al. (1979) and improved by O'Neill et al. (1985) with a few modifications.

The adaptation of the automated microtitration equipment used in this study provides a rapid and quantitative measurement of antimalarial activity for a large number of compounds against *P. falciparum* cultivated *in vitro*. Thus the method is valuable as a screen for new antimalarial drugs and gives data on the comparative activity of several analogs within a class of compounds. The use of microtitration technique in a matrix of wells presents an excellent opportunity to evaluate potential synergy or antagonism among compounds and combinations of compounds over a broad range of ratios of their respective concentration generated by sequential dilution in two directions, that is, across rows and columns (Desjardins et al., 1979).

The use of a radiolabeled nucleic acid precursor [6-^{14}C]orotic acid as an indicator of

parasite growth in an *in vitro* antimalarial screen had been reported. Several other compounds were tried as precursors. [G-^{3}H]hypoxanthine had earlier been used as an indicator of drug activity in the *in vitro* studies on Babesia parasites. This isotopic method had been studied and assessed, and found that hypoxanthine is capable of crossing the malaria parasite membrane and is ultimately incorporated into both ribonucleic acid and deoxyribonucleic acid and therefore provides a reasonably broad index of parasite metabolism (Desjardins et al., 1979). Thus in the present study, the isotope [G-^{3}H] hypoxanthine has been used as an indicator of drug activity.

1.8 PHYTOCHEMICAL SCREENING

Initially phytochemical surveys used to be random, viz a viz using random sampling approach where large numbers of plants were surveyed with an aim of establishing whether certain compounds were present in the plant. The major compounds of interest were alkaloids and saponins since by then it was known that the above compounds had some biological activity, medicinal usefulness and the test methods are simple, rapid and reliable. Other less common naturally occurring phytochemicals surveyed were flavonoids, leucoanthocyanins, tannins, cyanogenetic compounds, cardenolides, unsaturated sterols, triterpenoids, organic acids

and phenols, essential oils, choline, glycocides, pigments and rubber (Farnsworth et al., 1965).

A method for use in phytochemical screening should be simple, rapid, designed for a minimum equipment and reasonably selective for the class of compounds under study. The method should also be quantitative in so far as having a knowledge of the lower limit of detection is concerned and if possible should give additional information as to the presence or absence of specific members of the group being evaluated (Farnsworth, 1966). In the present study, the phytochemical method used is the one developed by Chhabra et al. (1984), and Chhabra and Uiso (1990).

1.9 CHROMATOGRAPHY

Chromatography is a separation technique which can be achieved by adsorption, partition, exclusion and even by ion exchange principle. Of all the different types of separation methods, chromatography has the unique position of being applicable to all types of problems in all areas of science and has undergone explosive growth in the last 40 years. All chromatographic processes involve a mobile phase which passes over a stationary phase. The chromatographic procedures can be used for quantitative determination, qualitative identification and purification (Pietrzyk and Frank,

1979). The feature that distinguishes chromatography from most other physical and chemical methods of separation is that two mutually immiscible phases are brought into contact, one phase is stationary and the other mobile. A sample introduced into a mobile phase is carried along through a column containing a distributed stationary phase. Species in the sample undergo repeated interaction (partitions) between the mobile phase and the stationary phase. When both phases are properly chosen, the sample components are gradually separated into bands in the mobile phase. At the end of the process, separated components emerge in order of increasing interaction with the stationary phase. The least retarded component emerges first, the most strongly retained component elutes last. Partition between the phases exploits differences in the physical and/or chemical properties of the components in the sample (Willard et al., 1988).

1.9.1 Column Chromatography (CC)

In column chromatography, the stationary phase is placed as a slurry in a cylindrical tube that is plugged at the bottom by a piece of glass wool or an inert porous disk. The sample is dissolved in a minimum of solvent, applied to the column and passed into the column with the liquid mobile phase. With appropriate eluting conditions, the individual

solutes of the mixture emerge at the bottom of the column as a function of elution time or elution volume.

The solid material acting as the stationary phase in adsorption chromatography is called the adsorbent where as the inert solid used to retain a liquid film in partition is called the support. Passing of the mobile phase over the adsorbent or support is called development and during this process, the sample components separate themselves to bands or zones. The process of development is elution and the mobile phase used for the development is called the eluting agent. Eventually the zones are detected or visualized.

Flow of the mobile phase is controlled by a stopcock pinch clamp employing gravity or pumps and the tubing carrying the mobile phase to the column and the effluent away from the column should be inert with respect to the mobile phase. Common materials for the tubing are stainless steel, glass, teflon and polyethylene. If the column is too long, too much mobile phase is used, if the flow is slow, zone broadening occurs and if the flow is too fast then extensive tailing occurs. Thus optimum flow conditions have to be predetermined (Pietrzyk and Frank, 1979). When pressure is applied to the column using an inert gas like nitrogen, helium or argon the technique is then referred to as Flash

Column Chromatography.

1.9.2 Paper Chromatography (PC)

Paper chromatography is an example of sheet method chromatography and can be considered as an open thin column. Sheet methods, that is, paper and thin-layer chromatography have the advantages of being simple, rapid, inexpensive and provide excellent resolving power. The sheet methods are mainly for qualitative identification but can also be used for quantitative results. Paper chromatography is essentially partition chromatography on cellulose papers. However, the papers can be modified by impregnating them with different adsorbents and thus alter the chromatographic behaviour. Successful separation by sheet methods are carried out in developing chambers, that is, chambers whose atmosphere is saturated by the mobile phase. The mobile phase can be made to pass from reservoir up the sheet, this is called ascending chromatography while passage down the sheet is called descending chromatography (Pietrzyk and Frank, 1979).

1.9.3 Thin-Layer Chromatography (TLC)

This also is a sheet method which is quite unique in that all chromatographic principles

functioning in solid-liquid and liquid-liquid systems can be utilized. The choice of the chromatographic principle is determined by the chemical nature of the compounds and by the desired pattern of fractionation. Thin-layer chromatography (TLC) is valuable in that two or more principles of chromatography can be applied consecutively on one plate, the equipment used is of low cost, separations are sharp, sensitivity of detection of the fractions is high and it is rapid and simple (Welcher, 1966). Sample separation on the layer, which is a thin stationary phase (silica gel, alumina or polyamide) coated on a glass, metal or plastic are effected by adsorption, partition, exclusion or ion exchange process (Pietrzyk and Frank, 1979).

1.9.4 Preparative Thin-Layer Chromatography (PTLC)

Thin layer is often used as a preparative tool when difficult separation cannot be accomplished by other methods or if the material to be fractionated prohibits handling large amounts.

To scale up the process, thin layer chromatography is carried out on larger plates and layers of 0.2-2mm thickness are prepared by special applicators. The plates are air dried for 24 hours and then placed in the oven for 1 hour at 110^0C for

activation (Pietrzyk and Frank, 1979).

1.9.5 High Performance Liquid Chromatography (HPLC)

This is similar to column liquid chromatography except it is at high inlet pressure. Consequently the instrument uses a pump that is capable of producing pulse-free inlet pressures of 1000-6000psi (100-400 atmospheres) and the column is a narrow bore metal (stainless steel) tube containing the stationary phase as micro particles. In HPLC, adsorption, partition, ion-exchange and exclusion chromatography can be carried out. HPLC is suitable for all organic compounds separation. The results are represented as peaks, where, the time the peak takes to appear is characteristic of the compound (that is, qualitative analysis) and the area under the peak is directly proportional to the concentration (quantitative analysis) of the compound under the given conditions. Presently, HPLC has been developed so that it can be used for preparative and for ascertaining the level of purity of the samples (Pietrzyk and Frank, 1979).

When the stationary phase is polar and the mobile phase is less polar then the process is referred to as normal-phase liquid-liquid chromatography and is used to separate mainly low or moderately polar homologous series, oil-soluble

vitamins, nitrophenols and essential oils. When the stationary phase is non-polar and the mobile phase is polar, the process is referred to as reverse-phase chromatography (Willard et al., 1988).

1.10 ULTRAVIOLET-VISIBLE SPECTROSCOPY (UV)

Molecular absorption in the ultraviolet (UV) and visible region of the spectrum depend on energy which is quantized, resulting in the elevation of electrons from the orbital in the ground state to higher energy levels in the excited state. UV spectrum is a plot of wavelength (or frequency) of absorption versus the absorption intensity (that is transmittance or absorbance) but the data is frequently converted to a graphical plot of wavelength versus the molar absorptivity (E_{max} or log E_{max}). The wavelength is expressed in nanometers (nm). 1nm = 10^{-9}m) or Angstrom units, Å, where 1Å = 10^{-10}m. The UV range which the organic chemist uses is between 200nm and 380nm or 50000cm^{-1} and 26300cm^{-1} (Silverstein et al., 1991; Kemp, 1986).

The samples can be determined when in gaseous or liquid state. For gases, quartz cells are used while for liquids, quartz cuvettes are used.

1.11 INFRA-RED SPECTROSCOPY (IR)

Whereas IR includes the electromagnetic spectrum between visible and microwave regions, the

part that is practically useful to the organic chemist is the portion between 4000-400cm^{-1}. Although IR is characteristic of the entire molecule, only certain groups of atoms give rise to bands at or near the same frequency regardless of the structure of the rest of the molecule. It is the persistence of their characteristic bands that permits the chemist to obtain useful structural information. Samples in the IR spectroscopy can be studied in gaseous, solution and solid states. A wide range of techniques have been developed and is available for use in mounting the samples into the beam of the IR spectrophotometer (Kemp, 1986; Silverstein et al., 1991).

CHAPTER II

EXPERIMENTAL

2.1 GENERAL EXPERIMENTAL PROCEDURES

2.1.1 Glassware

All the glassware used during this work was cleaned using chromic acid followed by washing in detergent. They were then rinsed with distilled water followed by acetone and kept in the oven to dry.

2.1.2 Solvents and Reagents

The solvents (n-hexane, acetone, methanol) used were distilled and were of laboratory reagent grade. All the solvents and reagents were purchased from our local suppliers.

2.2 PLANT MATERIAL

2.2.1 Sampling

The plant materials for the present study were collected from three different provinces. For each plant species, the place of collection and district are given in table 2. The species were taken to the Herbarium, Botany Department, University of Nairobi for identification. A voucher specimen of each plant used in the present study has been kept in the herbarium for future reference.

Table 2: The Place of Collection of the Plant Materials.

Plant Name	Vernacular Name	Tribe	Location
Terminalia spinosa	Mwangajemi	Giriama	Arabuko Sokoke Forest, Kilifi
Antidesma venosum	Mziwaziwa Mutonye	Giriama Meru	Shimba Hills, Kwale
Phyllanthus reticulatus	Mkwamba	Digo	Mida Creek, Kilifi
Suregada zanzibariensis	Mdimutsaka	Digo	Arabuko Sokoke Forest, Kilifi
Antherotoma naudinii	--	--	Kakamega Forest, Kakamega
Dissotis brazzae	Kwavashieni	Luhya	Kakamega Forest, Kakamega
Ficus natalensis	Nuumo	Kamba	Chiromo Campus, Nairobi
Kotschya africana	Amatumusi	Luhya	Mumias plains, Kakamega
Rhynchosia minima	--	--	Kenyatta University, Nairobi

Each plant sample, after collection, was separated into stems, stembarks, roots, rootbarks and aerial parts. The roots and stems were cut in smaller pieces to facilitate drying. The plant materials were air dried at room temperature under shade and ground to powder using a grinding mill (Christy and Norris Co. Ltd., England).

2.2.2 Extraction

For each sample, a methanolic and an aqueous extract were prepared.

An exact weight between 10-100g (depending on the bulkiness of sample) was obtained and refluxed with 600ml distilled water for 3hrs. The extract was

then filtered through Whatman filter paper no. 40 and the residue was again extracted with 400ml distilled water for another 3hrs and filtered. This process was repeated upto four times. The combined extracts were then freeze dried to powder.

For methanolic extracts, the weights varied between 10-60g. The extraction was done by a soxhlet apparatus. The extracts were then filtered through Whatman filter paper. The solvent was removed under reduced pressure using a rotary evaporator at 50^0C. Some yielded dry solids while others remained as gums or very viscous liquids.

All the samples (aqueous and methanolic extracts) were then preserved in a freezer at -20^0C.

2.3 *IN VITRO* ANTIMALARIAL SCREENING

2.3.1 Preparation of Parasites

The parasites used were two strains of *P. falciparum*: ENT36 - originally from Entosopia, Rift Valley, Kenya, a chloroquine non-sensitive strain, and K67 - originally from Kisumu, Kenya, a chloroquine sensitive strain. The two strains of parasites were grown continuously in stock cultures. A 6% suspension of human type O+ erythrocytes was prepared in culture medium which consisted of powdered RPMI 1640 (GIBCO Laboratories, New York) diluted in sterile water with 25mM HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid,

Calbiochem, California), 32mM $NaHCO_3$ (GIBCO Labarotories, New York) and 10% heat inactivated (40min at 56^0C and then clarified by centrifugation) human fresh frozen plasma in acid-citrate-dextrose (ACD) anticoagulant. Stock cultures were maintained in 5.0ml of the 6% erythrocytes suspension in 25ml tissue culture flasks (Corning Glass Works, New York). The flasks were flushed with a gas mixture consisting of 5% O_2, 3% CO_2 and 92% N_2 (East African Oxygen, Nairobi) screw topped and incubated at 37^0C.

Best results for antimalarial activity are obtained when the growth rate is between 3-4 per day or more. This was accomplished by daily changes of the culture medium and by dilution with fresh erythrocytes (O+) every 2 days so that less than 2% of the cells were infected at any time (Sixsmith et al., 1982). For each experiment, samples of the stock cultures were further diluted in culture medium containing sufficient non-infected type O+ human erythrocytes to yield a final heamatocrit of 1.5% and parasitemia of 0.4% in preparation for addition to the microtitration plates.

2.3.2 Preparation of Drugs

Only those extracts that were soluble in water were used. This included twenty seven of the water extracts that had been freeze dried and three methanol extracts that yielded powders on

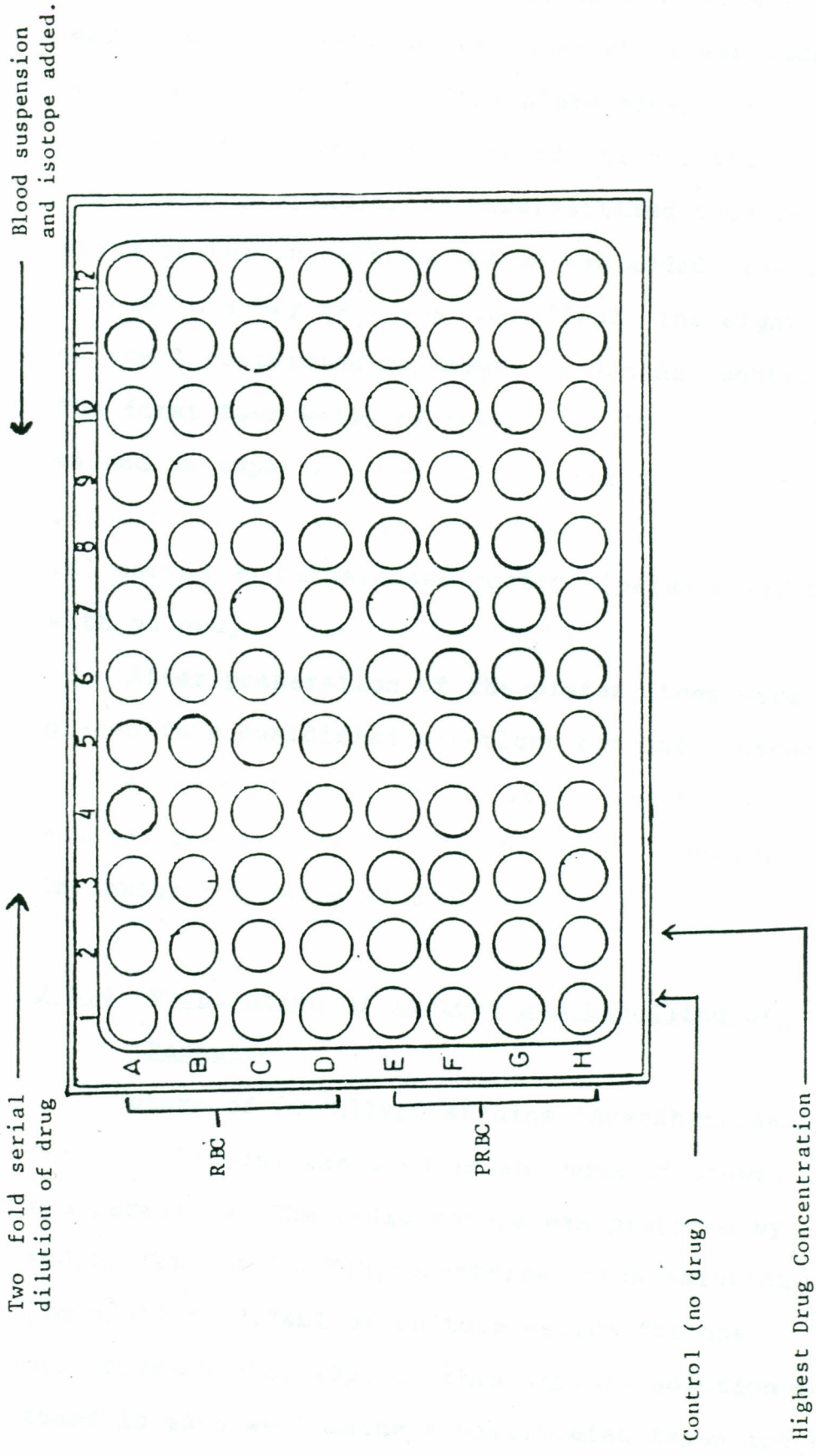
evaporation at reduced pressure. Known weights of the drugs were dissolved in known amounts of sterile water, that is, distilled autoclaved water (DAW) and then filtered through 0.45µm and then 0.22µm microfilters under a laminar hood. The prepared drugs were then sealed and stored at 4^0C.

2.3.3 Preparation of Microtitration Plates

Microtitration techniques were used to measure the activity of the plant extracts. The microtitre plate (Costar Glass Works, Cambridge) used consisted of 96 flat-bottom wells arranged in a matrix of twelve rows (1 through 12) and eight columns (A through H) as shown in Figure 1. The plates were prepared and cells harvested in the following sequence. An Eppendorf pipette was used to place 25µl of the culture medium in each well of the microtitre plate. 25µl of each drug solution (prepared as described above) was then added to two adjacent wells in the second row (2) of the plate. Four compounds were thereby accommodated by each plate. After the drugs were added to the wells of row 2, an automatic diluter (Titertek Laboratory Products, Virginia) was used to make serial two fold dilutions across the plate in each column. When this was complete, row 1 remained free of any drug and each of the drugs was present in duplicate columns at eleven concentrations over a 1024 fold range in

rows 2 through 12

Figure 1: Design of Microtitration Plate



A constant volume (200µl) of the parasitized erythrocyte suspension described above was added to each well of the microtitre plate except the first four wells of row 1, to each of which 200µl of an equivalent suspension of unparasitized type O+ human erythrocytes in culture medium was added. The total volume in every well was then 225µl. The eight wells of row 1 containing no drugs, served as control. The first four wells of row 1 (1A, 1B, 1C and 1D) served as unparasitized control (no drug and no parasite) while the last four wells (1E, 1F, 1G and 1H) served as parasitised control (parasitized blood with no drug).

After preparation of the plates, they were placed in a humidified air-tight box and flushed with a gas mixture of 5% O_2, 3% CO_2 and 92% N_2 and sealed. The box was then placed in an oven draft incubator for 48hrs at 37^0C.

2.3.4 Preparation of Isotope and Labelling of Parasite

Uptake of [G-^{3}H]hypoxanthine (Amersham/Searle Corp., Illinois) was used as an index of growth of the parasites. The radioisotope was prepared by adding 56µl of [G-^{3}H]hypoxanthine stock solution (1mCu/ml) to 2.74ml of culture medium for one microtitre plate. 25µl of this isotope solution was added in each well using a Hamiltonian Repeating

Dispenser (HRD) with a 1.25ml barrel. The plates were then returned to the humidified boxes flushed with the same gas mixture and placed in an incubator at 37^0C for an additional 18 hours.

2.3.5 Harvesting Parasites and Scintillation Counting

At the end of the second incubation period, each plate was harvested on a Skatron automated cell harvester (Skatron, Lier). This instrument aspirated and deposited the particulate contents of each of the wells onto filter strips in disc shapes which were washed with copious amounts of distilled autoclaved water. The strips were then dried for 2 hours at 25-37^0C. Discs from the filter strips were placed into 96 glass scintillation vials into which was added 1ml toluene-based scintifluor for counting. The counting was done by a Beckmann multisample and multichannel scintillation counter for one minute. The counts per minute (cpm) were recorded on computer paper.

The computation of the antimalarials' ID_{50} values for each isolate was done in duplicate. The parasitized control mean cpm values were used to estimate the midpoint (Y_{50}) as shown in the formula:-

Midpoint (Y_{50})=(PRBC - UPRBC)/2 + UPRBC,

where PRBC is parasitized red blood cells and UPRBC is unparasitized red blood cells.

The ID_{50} value for each drug was then determined by interpolation between one data point above and below the Y_{50}. Interpolated ID_{50} values were obtained after logarithmic transformation of both concentration and cpm values, using the formula:-

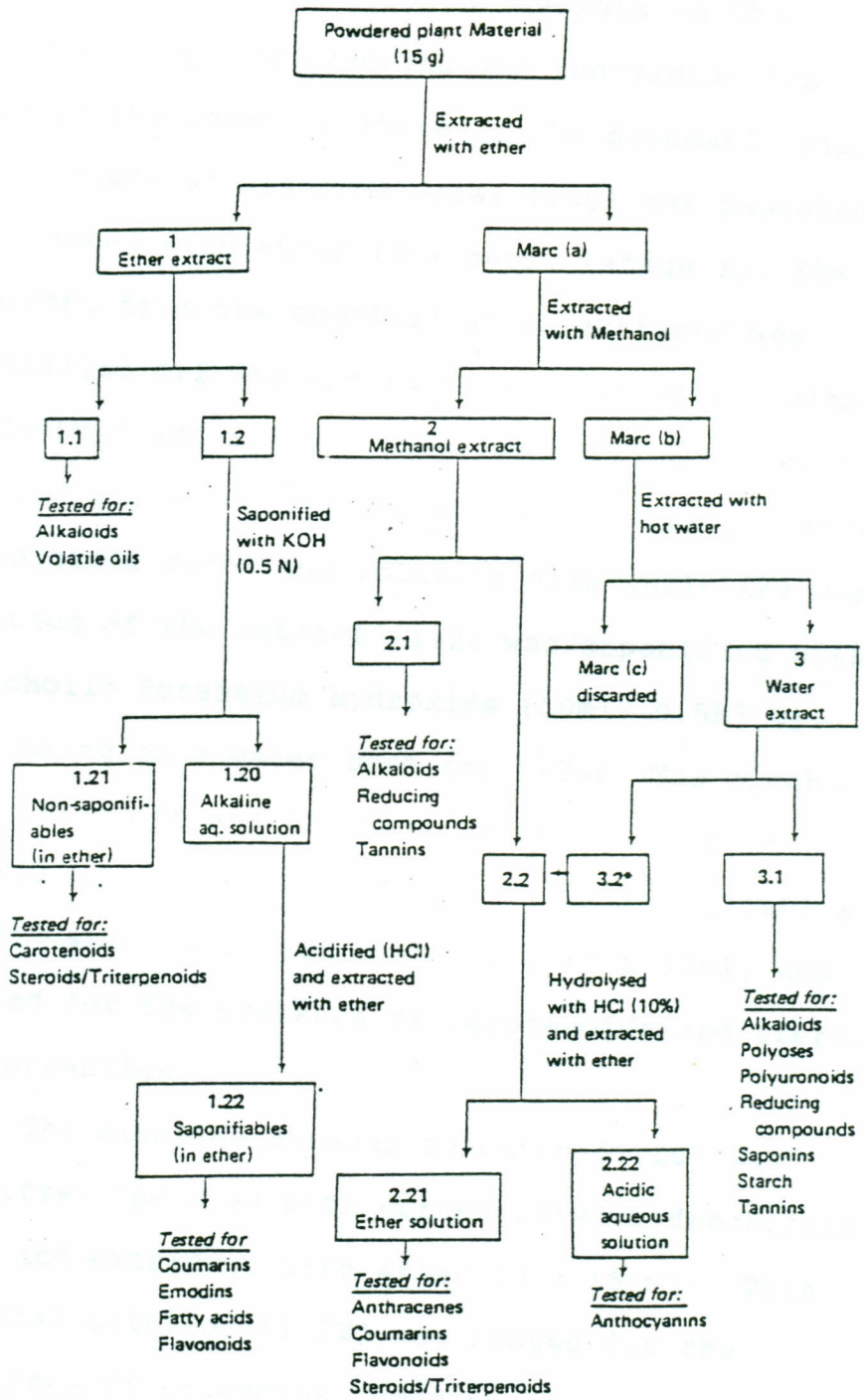
$$ID_{50} = antilog(logX_1) + \left\{ \frac{(logY_{50} - logY_1)(logX_2 - logX_1)}{(logY_2 - logY_1)} \right\}$$

where X_1, Y_1, X_2, Y_2 are the concetrations and cpm values, respectively, for the data points (Sixsmith et al., 1982, 1984).

2.4 PHYTOCHEMICAL SCREENING

Following the *in vitro* test, four extracts were identified for preliminary chemical analysis. These were aqueous extracts of *Phyllanthus reticulatus* leaves, *Suregada zanzibariensis* leaves, and *Terminalia spinosa* stembark, and the methanolic extract of *Dissotis brazzae* stems. These samples were subjected to phytochemical screening as shown in the schematic diagram (Figure 2).

Figure 2: Schematic Diagram of the Method Used for Phytochemical Screening



*3.2 is treated in the same manner as 2.2.

The method employed for phytochemical screening of plant materials is the one described by Chhabra et al. (1984). The numbers in brackets in the description of extraction and individual test procedures refer to those in the schematic diagram.

Powdered plant material (15g) was repeatedly extracted with ether (3 x 50ml) (stage 1). The solvent from the combined ether extracts was distilled off and the residue dissolved in ethanol (30-40ml) and divided into two portions. A portion of the extract (1.1) was tested for the presence of alkaloidal bases and volatile oils while the other portion of the extract (1.2) was saponified with alcoholic Potassium hydroxide (10ml, 0.5N) by refluxing on a water bath for 1-2hr. The alcohol was distilled off and the residue redissolved in hot distilled water (10-15ml). The non-saponifiables (1.21) were extracted with ether (3 x 10ml) and tested for the presence of carotenoids and steroids/ triterpenoids.

The alkaline aqueous solution (1.20) was acidified (pH 3-4) with concentrated hydrochloric acid and extracted with ether (3 x 15 ml). This etherial solution (1.22) was tested for the presensce of coumarins, emodins, fatty acids and flavonoids.

The plant material, marc (a), exhausted with ether was repeatedly extracted with hot methanol (3

x 50ml) (stage 2). The combined extracts were concentrated under reduced pressure to one third volume and divided into two portions. To a portion of the extract (2.1), chemical reactions were performed for the detection of alkaloidal salts, reducing compounds and tannins while the other portion (2.2) was hydrolysed with hydrochloric acid (10ml, 10%) by refluxing on a water bath for 30min. The contents were cooled, distilled water added (15ml) and extracted with ether (3 x 10ml). The ether extract (2.21) was tested for the presence of anthracenes, coumarins, flavonoids, steroids/ triterpenoids while the acidic aqueous solution (2.22) was tested for the presence of anthocyannins.

Marc (b) exhausted with ether and methanol was repeatedly extracted with hot water (3 x 50ml) (stage 3). The combined water extracts were concentrated under reduced pressure to one third volume and divided into two portions. To a portion of the water extract (3.1), chemical reactions were performed for the presence of alkaloidal salts, polyoses, polyuronoids, reducing compounds, saponins, starch and tannins. The other portion (3.2) was hydrolysed with hydrochloric acid and screened as hydrolysed methanol extract (2.2).

2.4.1 INDIVIDUAL TESTS

Tests for detection of each group of compounds (Chhabra et al., 1984) were carried out using 4-5ml of the extract under examination and the observations recorded in table 3.

2.4.1.1 Alkaloids

Alkaloids were tested in all three extracts, ether, methanol and water. In ether extract, ether solution (1.1) was evaporated and the residue macerated with hydrochloric acid (2%, 1.5ml). The resulting acidic solution was divided in three parts. To one part was added Mayer's reagent, to another Wagner's reagent while the third acted as blank. In alcoholic extract, methanol solution (2.1) was evaporated and the residue macerated with hydrochloric acid (2%, 1.5ml), filtered, basified with ammonium hydroxide (10%) and extracted with ether. The ether soluble portion was evaporated, dissolved in hydrochloric acid (2%, 1.5ml), divided in three parts and tested as in the ether extract (1.1). In water extract, aqueous solution (3.1) was basified with ammonium hydroxide (10%) and extracted with ether. The ether solution was extracted with hydrochloric acid (10%) and the acidic aqueous solution was divided into three parts and tested as in the ether extract (1.1).

A + reaction was recorded if the addition of one or both reagents produced a faint turbidity; a ++ reaction was recorded if a light opalescence precipitate was observed; and a +++ reaction was recorded if a heavy yellowish-white precipitate was observed.

Alkaloid detection in plants showing preliminary positive test by the above screening was further confirmed by the procedure described by Harborne (1976).

Dried plant material (15-25g) was extracted with 10% acetic acid in ethanol (60ml) at room temperature for 4hrs. The extract was concentrated by distillation to one-quarter of the original volume and alkaloids were precipitated by dropwise addition of concentrated ammonium hydroxide. The precipitate was collected by centrifugation, washed with 1% ammonium hydroxide and dissolved in ethanol. An aliquot was chromatographed on sodium citrate-buffered paper (previously buffered with 5% sodium dihydrogen citrate in n-butanol/aqueous citric acid (870ml/4.8g citric acid in 130ml water) while another aliquot was chromatographed on silica gel G plates in methanol/concentrated ammonium hydroxide (200:3). The presence of alkaloids was detected on the paper and plate, first by UV fluorescence (λ = 254nm) and then by the application of Dragendorff, iodoplatinate and Marquis (only on plates) reagents.

2.4.1.2 Anthocyanins

Anthocyanins were tested in methanol and water extracts. The appearance of red colour at pH 3-4 and the change of colour with pH modification (pH 8-9) in the acidic aqueous solution (2.22) of methanol (and/or water) extract indicated the presence of anthocyanins.

2.4.1.3 Anthracene Glycosides

Anthracene glycosides were tested in methanol and water extracts. The appearance of red colour by the addition of ammonium hydroxide (25%) to a portion of the ether solution (2.21, of methanol and water extracts) indicated the presence of anthracene glycosides.

2.4.1.4 Carotenoids

The presence of carotenoids was detected in the ether solution (1.21) by Carr price's reaction.

2.4.1.5 Emodins

Emodins were tested in the ether extract. When the alkaline aqueous solution 1.20 was red in colour, a portion of the ether solution, 1.22 was evaporated and the residue dissolved in benzene. The appearance of red colour by the addition of ammonium hydroxide (25%) indicated the presence of emodins.

2.4.1.6 Flavonoids

Flavonoids were tested in all three extracts, ether, methanol and water. The ether solution 1.22 and 2.21 (of methanol and water extracts) were evaporated and dissolved in aqueous ethanol and tested for flavonoids by Shinoda's reaction.

2.4.1.7 Polyoses

Polyoses were tested in the water extract. The appearance of red colour by the addition of a few drops of concentrated sulphuric acid and alcoholic thymol to a portion of the evaporated extract, 3.1 indicated the presence of polyoses.

2.4.1.8 Starch

Starch was tested in the water extract. The appearance of blue colour by the addition of Lugol solution in water extract, 3.1 indicated the presence of starch.

2.4.1.9 Steroids and Triterpenoids

Steroids and triterpenoids were tested in the ether, methanol and water extracts. The appearance of greenish blue/reddish violet colour by Libermann-Burchard's reaction in ether solutions, 1.21 and 2.21 (of methanol and water extracts) indicated the presence of steroids and/or triterpenoids.

A + reaction was recorded in the tests for anthocyanins, anthracene glycosides, carotenoids, emodins, flavonoids, polyoses, starch and steroids and/or triterpenoids when a slight colouration was observed; a ++ reaction was recorded when a medium colouration was observed; and a +++ reaction was recorded when a strong colouration was observed.

2.4.1.10 Coumarins

The presence of coumarins was tested in the ether, methanol and water extracts. The ether solutions, 1.22 and 2.21 (of methanol and water extracts) were evaporated and dissolved in a small amount of distilled water. UV fluorescence (at λ = 254nm) of these aqueous solutions and the increase in intensity after the addition of ammonium hydroxide (10%) indicated the presence of coumarins.

A + reaction was recorded when a slight flourescence was observed ; a ++ reaction was recorded when a medium fluorescence was observed ; a +++ reaction was recorded when a strong fluorescence was observed.

2.4.1.11 Fatty Acids

Fatty acids were tested in the ether extract. A portion of the ether solution, 1.22 was evaporated on a piece of filter paper and the transparency was noted.

A + reaction was recorded when a slight transparency was observed; a ++ reaction was recorded when a medium transparency was observed; and a +++ reaction was recorded when a strong transparency was observed.

2.4.1.12 Polyuronoids

Polyuronoids were tested in the water extract. To a portion of the water extract, 3.1, an equal volume of haematoxylin solution was added. The formation of a persistent violet precipitate insoluble in ethanol showed the presence of polyuronoids.

2.4.1.13 Reducing Compounds

Reducing compounds were tested in methanol and water extracts. To a portion of the extract 2.1/3.1, a few drops of Fehling's solution (A and B) were added. The appearance of red precipitate on heating the mixture indicated the presence of reducing compounds.

2.4.1.14 Tannins

Tannins were tested in the methanol and water extracts by gelatin-salt block test. Methanol and water extracts (2.1 and 3.1) were separately divided in three parts. A sodium chloride solution was added to one portion of each test extract, 1%

gelatin solution to each second portion, and the gelatin-salt reagent to each third portion. Precipitation with the latter reagent, or with both the gelatin and gelatin-salt reagents was indicative of the presence of tannins.

Precipitation with the salt solution (control) indicated a false-positive test. Positive tests were further confirmed by the addition of a few drops of dilute ferric chloride to the test extracts which gave blue black/green black colouration.

A + reaction was recorded in the tests of polyuronoids, reducing compounds and tannins when a slight precipitate was observed; a ++ reaction was recorded when a medium precipitate was observed; and a +++ reaction was recorded when a heavy precipitate was observed.

2.4.1.15 Saponins

The presence of saponins was tested in the water extract. The honey comb froth in a portion of the water extract, 3.1 which persists after shaking for 10 seconds and a positive Libermann-Burchard's reaction in the hydrolysed solution, 2.21 (of water extract) indicated the presence of saponins.

A + sign was indicated when froth reached a height of 0.5cm; a ++ sign with a height up to 1cm; a +++ sign with a height more than 1cm.

2.4.1.16 Volatile Oils

Volatile oils were detected in the ether extract. Aromatic smell in the evaporated ether extract, 1.1 indicated the presence of volatile oils.

A + sign indicated a slight aromatic smell; a ++ sign indcated a moderate aromatic smell; a +++ sign indicated a strong aromatic smell.

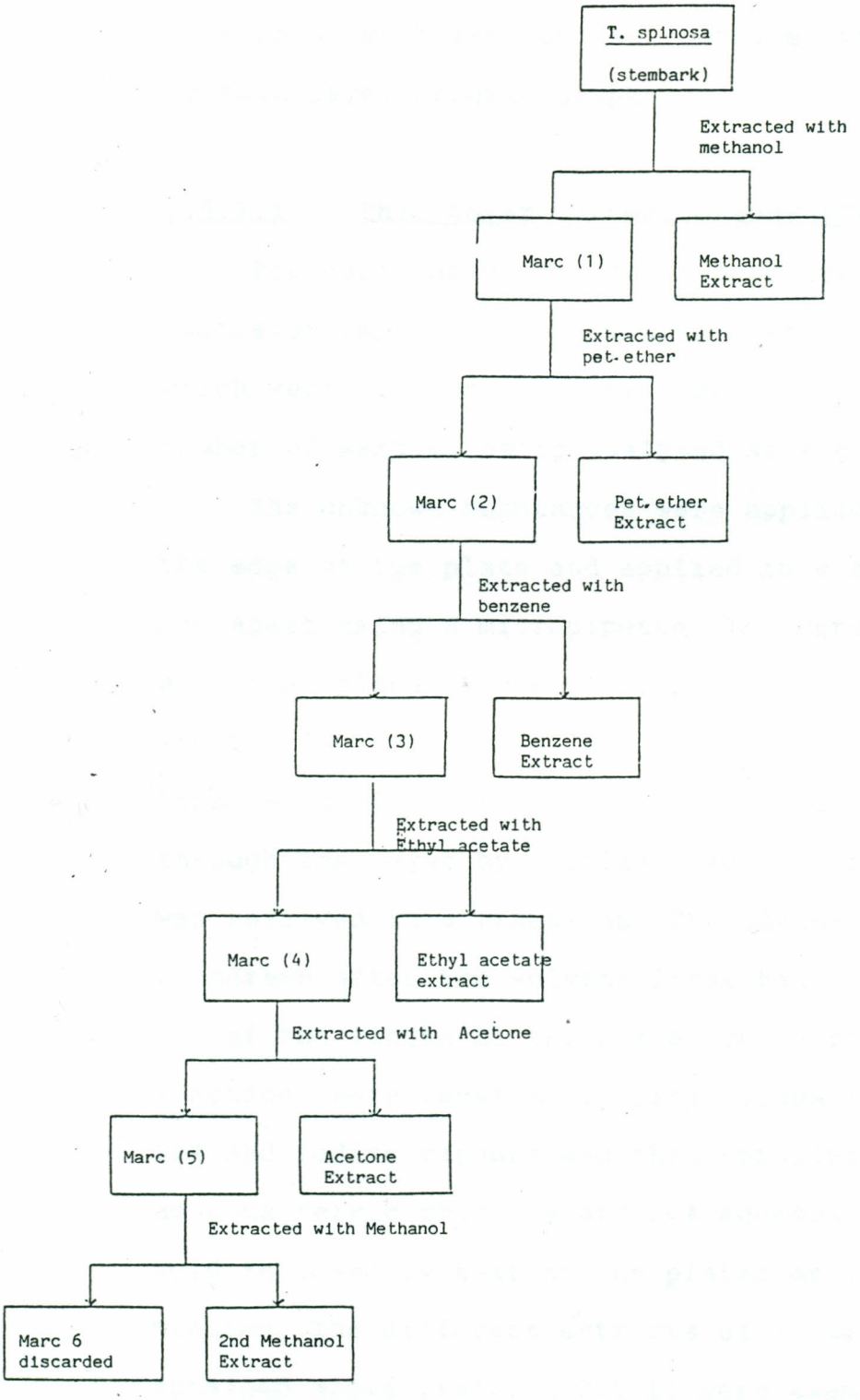
2.5 *TERMINALIA SPINOSA*

From the results of the phytochemical screening *T. spinosa* was chosen for further chemical analysis.

2.5.1 Extraction

T. spinosa stembark powder (1kg) was repeatedly extracted four times with laboratory grade methanol (4 x 2.5l) at room temperature. The combined methanol extract thus obtained was evaporated to dryness using a rotary evaporator at 50^0C under reduced pressure. The weight of the extract was recorded of which 3.1g was removed and kept aside. The remaining extract was successively extracted under reflux with petroleum ether ($40-60^0$), benzene, ethyl acetate, acetone and methanol, respectively (Figure 3).

Figure 3:: Schematic Diagram of the Extraction of T. spinosa



After each extraction, the solvent was evaporated under reduced pressure, the weight of the dry extract recorded, a small portion removed and kept aside before extraction by the next solvent. A portion of each extract thus obtained was examined by thin layer chromatography.

2.5.1.1 Thin Layer Chromatography (TLC)

For qualitative analysis, precoated and indicator impregnated aluminium plates were used which were cut into various sizes to accommodate the number of samples being analysed at a given time.

The unknown substances were applied 1cm from the edge of the plate and applied in a row of spots 1cm apart using a micropipette. The chromatoplates were then placed in developing tanks containing about 0.3-0.5cm high of developing solvent. The tanks were closed with a lid. As the solvent rose through the layer by capillary action, the sample was resolved into fractions. The plates were withdrawn after the solvent front had migrated about 0.9 of the length of the plate. The various fractions were revealed by first visualising in UV-254 and iodine vapours and then spraying with 3% aqueous ferric chloride and 30% aqueous sulphuric acid followed by heating the plates at 110^0C for 10 minutes. The different extracts of *T. spinosa* obtained above (section 2.5.1) were examined by TLC

on silica gel using the following solvent systems- butanol:acetic acid:water (BAW) - 4:1:5 (upper layer) , 5% ethyl acetate in benzene, 15% ethyl acetate in methanol, 5% methanol in ethyl acetate and 25% methanol in ethyl acetate. The acetone and ethyl acetate extracts showed better separation and responded to colour reactions for flavonoids and triterpernoids, and were thus chosen for further studies.

2.5.2 Acetone Extract

2.5.2.1 TLC of Acetone Extract

From various trials with different solvent systems, 5% methanol in ethyl acetate and BAW - 4:1:5 (upper layer) were found to be the best solvent systems for the separation of different components of the acetone extract. The developed plates were visualised in ultraviolet light, iodine vapours, 3% aqueous ferric chloride, and 30% aqueous sulphuric acid followed by heating the plates at 110^{0}C for 10 minutes.

2.5.2.2 Flash Column Chromatography of Acetone Extract.

50ml of the upper layer of butan-1-ol:acetic acid:water (BAW) mixed in the ratio of 4:1:5, respectively was added to a column, 1000mmx80mm i.d. Into this column was then added a slurry upto a

height of 600mm using a funnel. The slurry was prepared by mixing silica gel 60 (0.04 - 0.065mm, 230-400 mesh ASTM) powder with the solvent BAW in the ratio 1:2 weight by volume. The tube was tapped during the addition of the slurry so that the silica gel was tightly packed at the bottom of the column. Any slurry remaining on the sides was washed down by the solvent. Excess solvent was drawn off until approximately 5mm of solvent remained above the level of the silica gel. The surface of the silica gel was kept covered with solvent throughout the chromatographic procedure. 10g of the acetone extract, weighed to an accuracy of ±0.0001g in a weighing boat, was placed in a 50ml beaker. About 20ml of the solvent (BAW) was added and dissolved by a stirring rod. The rod was removed and washed with about 5ml of the solvent. The sample in the solvent was transferred into the column and the beaker washed thoroughly with an additional 20ml of the solvent. The top of the column was also washed with two successive 10ml washes, while the solvent was left to drain up to 5mm remaining above the silica gel. The solvent was then added upto 2cm below the top of the column. The column was then connected to an argon gas cylinder to provide pressure. The pressure was maintained at 1 bar and the percolate was collected at a rate of 2 to 4ml per minute into conical flasks. When the volume reached 200ml of

eluent, the flask was changed. Fourteen fractions were collected this way. A sample of the fraction was tested for phenolic compounds by adding ferric chloride solution. The fractions were then concentrated by rotary evaporation and spotted on thin layer chromatography sheets for visualization and detection. The solvent system employed was BAW (4:1:5) which caused maximum separation.

From the results of the TLC, fractions 2-5 referred to as $2A_5$ inclusive were mixed and fractions 6 and 7 were combined and referred to as S6. The two samples, $2A_5$ and S6 were then subjected to paper chromatography (PC) and column chromatography (CC). After the initial study, only sample $2A_5$ was studied on a larger scale.

2.5.2.2.1 Flash Column Chromatography of $2A_5$

$2A_5$ (1g) was further column chromatographed using fresh BAW and twelve fractions (100ml each) were collected. After spotting the concentrated fractions, only the 3rd, 4th and 7th fractions were retained for further analysis. They are herein referred to as A1, A2 and A3.

2.5.2.2.2 Paper Chromatography of $2A_5$

Whatman no. 1 paper was cut into sheets measuring 23x28cm. With a hard pencil, an origin line was ruled 2cm from the bottom edge. The sample

continued for 2-3 minutes longer. Crystallization of the product from the red coloured solution commenced immediately. The reaction beaker was cooled in an ice bath for 2 hours. The product thus formed was collected by suction filtration, washed with a little ice-cold water, and dried in the air. The yield of the colourless acetylmethylurea (m.p.178-180^{0}C) was 50g.

A mixture of acetylmethylurea (4.9g) and concentrated hydrochloric acid (50ml) was heated with hand stirring on a steam bath until saturation but the heating continued for 3-4 minutes longer. The total time of heating on the steam bath was about 10 minutes. The resulting solution was diluted with 50ml water and cooled below 10^{0}C in an ice bath. A cold saturated solution of sodium nitrite (38g) in water (55ml) was added slowly while stirring. The mixture was kept in the ice bath for 10 minutes. The solid was filtered at the pump and washed with ice-cold water (8-10ml). The nitrosomethylurea (pale yellow crystals) thus obtained was dried first in the air and then in a vacuum desiccator. The yield was 34g (m.p.123-124^{0}C).

2.5.2.2.2.1.2 Preparation of Diazomethane and Methylation of Sample AP

An etheral solution of diazomethane was prepared by adding a cold solution of aqueous sodium hydroxide (1M, 200ml) to a 500ml Erlenmeyer

flask containing a cold suspension of nitrosomethylurea (10g) in diethyl ether (100ml). The upper etheral solution of diazomethane thus obtained was washed with cold water and then added to a cold etheral solution of sample AP. The contents were mixed thoroughly and left in a refrigerator overnight. The crude methylated product obtained after distillation of ether was crystallized from ethyl acetate. The crystallized methylated product was designated as APE. APE was studied chromatographically and colour reactions were performed.

2.5.3 Ethyl Acetate Extract

2.5.3.1 TLC of Ethyl Acetate Extract

The best solvent systems were found to be 5% methanol in ethyl acetate and BAW (4:1:5:) upper layer. After development, the plates were visualised using ultraviolet light, iodine vapours, 3% aqueous ferric chloride and 30% aqueous sulphuric acid followed by heating the plates at 110^0C for 10 minutes.

2.5.3.2 FCC of Ethyl Acetate Extract

Ethyl acetate extract (5g) was column chromatographed as the acetone extract mentioned above with the weights and volumes reduced proportionately. The column measured 1000mm x 25mm.

The solvent used was distilled ethyl acetate and the adsorbent was silica gel 60 (0.04-0.065mm and 230-400 mesh ASTM). Ten fractions of 100ml each were collected and their concetrates spotted on TLC. The results are shown in table 10. Fraction 6 and 7 were combined and coded E.

2.5.3.3 Preparative TLC of Sample E

A methanolic solution of sample E was applied to the plate as a row of closely spaced spots using a micropipette. Development of the plates dipped in BAW 4:1:5 (upper layer) was carried out. When the solvent reached a height of about three quarters of the plate, the plate was withdrawn and the solvent left to evaporate. UV-254nm was used to visualise the spots. The different bands thus obtained were scrapped from the plates and eluted with methanol through a glass tube. The eluents were concentrated separately and checked for purity on TLC using a number of solvent systems. The upper band, R_f 0.78 of the PTLC of the sample E showed a single spot on TLC and is hereby reffered to as E1.

2.6 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

The purity of the samples obtained through PC, FCC and TLC were determined by HPLC. HPLC was carried out on Hitachi HPLC (model 332) fitted with Beckman pumps (model 110) and controlled by a

microprocessor system (Altex Scientific Inc., model 420). The sample was introduced through sample injector (Beckman Instruments, model 210) equipped with a 20µl loop. Separation was achieved by reverse phase column (Varian M-CH 10 Micro Pak ; 1030cm long x 4mm i.d.). The packing material used in the column was bonded C-18 (Octadecyl group) and the mobile phase was methanol:water, 50:50.

The column effluents were monitored by UV(λ= 254nm) absorption using variable detector (Hitachi, Model 100-40). The pressure was 4000 psi while chromatographic peaks were recorded on a Kipp and Zonen BD 41 recorder that was also controlled by the microprocessor system.

In the present study five samples, A1, A2, A3, E1 and APE were examined for their purity. 0.1mg of the concentrate in methanol of each sample was diluted to 5ml with methanol and then tested for purity. The HPLC chromatograms of the above samples are represented in figures 4 to 8.

2.7 ULTRAVIOLET-VISIBLE SPECTROSCOPY

UV spectra were recorded on Beckman DU-50 spectrophotometer at a scan speed of 750nm/min. 0.1mg of each sample (APE, A1, A2, A3 and E1) was dissolved in methanol (4ml) and the spectra recorded. Shift reagents were then added to the samples and the behaviour of the peaks observed. The

shift reagents used were sodium acetate, sodium acetate and boric acid, aluminium chloride, aluminium chloride and hydrochloric acid, and freshly prepared sodium methoxide designated R_1, R_2, R_3, R_4 and R_5, respectively. The results are given in figures 9 to 33.

2.8 INFRA-RED SPECTROSCOPY

IR spectra of the samples were recorded on a Perkin Elmer (Model 598) Spectrophotometer. A 1% by weight of each sample was ground with dried potassium bromide (KBr). This mixture was compressed under vacuum and high pressure (10 bars) to form transparent discs which were mounted into the IR spectrophotometer beam. The absorption spectra are shown in figures 34 and 35.

CHAPTER III

RESULTS AND DISCUSSION

3.1 PER CENT YIELD OF DIFFERENT EXTRACTS

Different parts of the nine plants were extracted separately with water and methanol and gave the following per cent yields (table 3).

Table 3. Per Cent Yield of Different Extracts.

Plant Name	Code	Part	EXTRACTION WITH			
			WATER		METHANOL	
			Nature	% Yield	Nature	% Yield
P. reticulatus	Pr	Roots	Powder(p)	10.8	Liquid(b)	21.4
		Leaves	Powder(g)	12.7	Liquid(g)	44.4
		Stems	Flakes(p)	6.1	Powder(b)	17.5
S. zanzibariensis	Sz	Roots	Powder(y)	8.5	Liquid(b)	24.7
		Leaves	Woolly(g)	13.9	Liquid(g)	16.4
		Stems	Powder(y)	3.8	Liquid(g)	31.5
A. venosum	Av	Rootbark	Powder(b)	6.3	Liquid(b)	35.6
T. spinosa	Ts	Stembark	Powder(y)	9.3	Liquid(y)	15.8
		Stemwood	Powder(y)	4.4	Powder(g)	18.7
F. natalensis	Fn	Roots	Powder(b)	9.6	Powder(y)	22.4
		Rootbark	Powder(b)	14.7	Viscous(b)	29.8
		Leaves	Woolly(b)	8.8	Powder(b)	10.1
K. africana	Ka	Roots	Powder(y)	14.7	Viscous(y)	34.1
		Stemwood	Powder(g)	18.3	Viscous(g)	23.2
		Aerial	Viscous(g)	29.2	Viscous(g)	36.1
R. minima	Rm	Roots	Powder(b)	24.7	Liquid(b)	21.7
		Leaves	Powder(b)	25.7	Liquid(g)	36.8
		Aerial	Powder(g)	24.8	Viscous(g)	17.9
D. brazzae	Db	Roots	Powder(g)	6.9	Viscous(g)	11.8
		Leaves	Powder(g)	12.6	Liquid(g)	21.8
		Stems	Powder(g)	9.0	Flakes(g)	5.3
		Aerial	Powder(g)	8.5	Viscous(b)	14.9
A. naudinii	An	Roots	Powder(b)	26.9	Liquid(b)	31.4
		Aerial	Powder(g)	22.2	Liquid(g)	28.5

KEY:

b - brown; g - green; r - red; y - yellow; p - pink

3.2 *IN VITRO* ANTIMALARIAL SCREENING

The results of the *in vitro antimalarial* test of 30 aqueous and/or methanolic extracts of different parts of the nine plants are given in table 4.

Table 4. ***In Vitro*** **Antimalarial Activity of the Nine Medicinal Plants.**

S.No.	Species	Part	IC_{50} (µg/ml) ENT36	K67
1	*P. reticulatus*	Roots	159.8	165.1
2		Leaves	10.1	1.7
3		Stems	23.9	7.7
4		Stems*	21.5	52.2
5	*S. zanzibariensis*	Roots	291.4	378.8
6		Leaves	1.5	1.5
7		Stems	95.5	>412.8
8	*A. venosum*	Rootbark	N.D.	>369.2
9	*T. spinosa*	Stembark	29.5	9.9
10		Stemwood	49.2	35.9
11	*F. natalensis*	Roots	119.0	>396.3
12		Rootbark	31.4	355.2
13		Leaves	>390.0	>390.0
14	*K. africana*	Roots	32.2	60.0
15		Stems	44.0	>411.8
16		Stemwood	N.D.	267.5
17		Aerial	19.7	33.9
18	*R. minima*	Roots	182.8	>376.9
19		Roots*	349.7	>385.7
20		Leaves	82.2	10.7
21		Aerial	168.2	>405.4
22	*D. brazzae*	Roots	218.8	131.7
23		Roots*	40.8	43.9
24		Leaves	14.7	28.2
25		Stems	6.4	>468.4
26		Stems*	14.5	>377.9
27		Aerial	38.0	>393.0
28		Aerial*	25.0	>432.2
29	*A. naudinii*	Roots	66.8	39.4
30		Aerial	56.3	191.1

KEY:

*N.D. - Not Determined; * - Methanol Extract*

Results from table 4 show that extracts from some of the plants do posses antimalarial activity. Those having ID_{50}<50µg/ml are 57.1% against ENT36 strain and 33.3% against K67 strain. These results also seem to indicate that extracts having antimalarial properties against ENT36 strain are not necessarily the same as for K67 strain and the potency of each extract on the two strains also varied. Out of the thirty extracts only three (10%) of them had almost similar activity or potency against both strains. These were aqueous extracts of leaves of *S. zanzibariensis* and *F. natalensis;* and the methanol extract of roots of *D. brazzae* although the extract of *F. natalensis* was not effective. In seven cases (23.3%), the aqueous extracts of the leaves and stems of *P. reticulatus,* the stembark and stemwood of *T. spinosa,* the leaves of *R. minima, the* roots of *D. brazzae* and of *A. naudinii* were at least 1.4 times more potent against K67 strain than on ENT36 strains. The activity of the aqueous extracts of stemwood of *K. africana* and the rootbark of *A. venosum* were not determined against ENT36 strain and therefore, comparisons could not be made. The rest of the extracts (66.7%), whether effective or non-effective, showed more potency to ENT36 strain than the K67 strain. The plant species under this study contain more extracts that are potent to the ENT36 strain. These observations are consistent with other

literature. An example is tetrandrine, an alkaloid from *Stepahania tetranda* S. Moore (Menispermaceae) which is three times more potent against chloroquine resistant strains than against chloroquine sensitive strains *in vitro,* having IC_{50} of 5.1 X 10^{-7}M for the senstive strain and 1.5 X 10^{-7}M for the resistant strain (Ye and van Dyke, 1989).

3.3 PHYTOCHEMICAL SCREENING

Based on the *in vitro* antimalarial activity, the four plants, *Phyllanthus reticulatus, Suregada zanzibariensis, Terminalia spinosa* and *Dissotis brazzae* were chosen for phytochemical screening. Results of the phytochemical screening are shown in table 5.

Table 5. Results of Phytochemical Sc

Class of Compounds	Pr (leaves)			Sz (leaves)			Ts (stembark)			Dd (stems)		
	E	M	W	E	M	W	E	M	W	E	M	M
Alkaloids	++	-	-	++	-	-	-	-	-	++	-	-
Anthocyanins	-	+++	+++	-	-	-	-	+++	+++	-	+++	+++
Anthracene glycosides	-	-	+	-	-	-	-	-		-	++	-
Coumarins	-	-	-	-	-	-	++	++	-	++	+	-
Emodins	++	-	-	-	-	-	+	-	-	+++	-	-
Fatty acids	-	-	-	-	-	-	-	-	-	-	-	-
Flavonoids	-	-	-	+	-	-	++	+	-	-	-	-
Polyoses	-	-	+	-	-	++	-	-	++	-	-	-
Polyuronoids	-	-	+	-	-	+	-	-	+++	-	-	-
Reducing compounds	-	+++	+++	-	+	-	-	++	+++	-	+	++
Saponins	-	-	-	-	+	+	-	+++	+++	-	+++	+++
Starch	-	-	+++	-	-	-	-	-	-	-	-	-
Steroids/ triterpenoids	+	-	-	-	++	-	-	+	+	+++	+	++
Tannins	-	-	-	-	+	++	-	+++	+++	-	+++	+++
Volatile oils	-	-	-	-	-	-	-	-	-	-	-	-

KEY:

E - Diethyl ether; M - Methanol; W - Water

Table 5 shows the class of compounds detected in separate parts of these plants. The compounds common to these four extracts are steroids, triterpenoids and reducing compounds. Alkaloids have been detected in the ether extracts of the leaves of *P. reticulatus*, *S. zanzibariensis* and stems of *D. brazzae*. The ether and methanol extract of *T. spinosa* stembark and the ether extract of *S. zanzibariensis* leaves showed the presence of flavonoids. These extracts containing flavonoids have shown antimalarial activity (IC_{50} < 50µg/ml) against both the strains, ENT36 and K67. Saponins and tannins were common in three of the four plant extracts. Most of the compounds mentioned above have some pharmacological potential and in this study show an activity specific to malaria. Their presence could possibly potentiate the activity of some antimalarials. It is documented (Nkunya, 1991) that artenitin, a flavonoid from *Artemisia annua* is antimalrial at high doses and at low doses potentiates the *in vitro* activity of artemisinin. Alkalloids combat malaria by intercalation with DNA, quassinoids by inhibition of protein synthesis while quinones by causing alkylation or oxidant stress (Nkunya, 1991). Tannins are well known as cytotoxic agents and several of them have antimutagenic effect and DNA breaking activity (Kashiwada et al., 1992).

3.4 *TERMINALIA SPINOSA*

3.4.1 Extraction

The stem bark of *T. spinosa* was successively extracted with different solvents and the extracts thus obtained are listed in table 6.

Table 6. Different Extracts from the Stembark of *T. spinosa*.

Solvent	Colour of extract	% Yield	Extract
Methanol	black/brown flakes	18.68	1
Petroleum ether	creamy white powder	0.12	2
Benzene	creamy yellow flakes	0.39	3
Ethyl acetate	orange/brown powder	1.41	4
Acetone	shiny brown beads	5.93	5
Methanol	dark brown flakes	11.13	6

3.4.2 Thin Layer Chromatography (TLC)

Qualitative TLC of *T. spinosa* extracts was done using 5% methanol in ethyl acetate and butanol:acetic acid:water (BAW) - 4:1:5 upper layer as the mobile phases on silica gel and the results obtained are shown in (tables 7 and 8). The other solvent systems (mobile phases) did not give better results.

Table 7. TLC Results of Extracts from *T. spinosa* using 5% Methanol in Ethyl Acetate as Mobile Phase.

Extract	No. of Spots	R_f	UV (f)	$FeCl_3$	Nature	H_2SO_4	Nature
1	5	0.05	+ve	-ve	--	+ve	brown
		0.10	+ve	-ve	--	+ve	light grey
		0.14	+ve	-ve	--	-ve	--
		0.18	+ve	-ve	--	-ve	--
		0.45	+ve	+ve	blue/black	+ve	brown/yellow
2	2	0.55	+ve	-ve	--	+ve	brown
		0.75	+ve	-ve	--	+ve	light - brown
3	3	0.45	+ve	-ve	--	+ve	light - brown
		0.55	-ve	+ve	blue/black	+ve	light - grey
		0.75	-ve	-ve	--	+ve	light - brown
4	5	0.05	+ve	-ve	--	+ve	dull - grey
		0.20	+ve	-ve	--	+ve	dull - grey
		0.45	+ve	-ve	--	+ve	intense - brown
		0.55	+ve	-ve	blue/black	+ve	intense brown/orange
		0.65	+ve	+ve	light purple	+ve	intense - grey
5	4	0.10	-ve	-ve	--	+ve	intense - brown/orange
		0.20	+ve	-ve	--	+ve	light - blue/black
		0.45	+ve	-ve	--	+ve	dull - brown/grey
		0.55	+ve	+ve	intense-blue/black	+ve	dull - grey

KEY

f - fluorescent

Table 8. TLC Results of Extracts from *T. spinosa* using BAW (4:1:5) as Mobile Phase.

Extract	No. of Spots	R_f	$FeCl_3$	Nature
1	2	0.37 0.54	+ve +ve	intense - blue/black light - blue/black
2	None	---	---	-------
3	1	0.38	+ve	light - blue/black
4	2	0.58 0.38	+ve +ve	dull - blue/black intense - blue/black
5	2	0.58 0.78	+ve +ve	intense - blue/black light - blue/black

3.4.3 Acetone Extract

3.4.3.1 Flash Column Chromatography (FCC) of Acetone Extract

Twelve fractions were collected from the column and after TlC, fractions 2-5 were combined and coded $2A_5$, fractions 6 and 7 were combined and coded S6 and the rest remained as collected. Results are shown in table 9.

Table 9. TLC Results (in BAW) of the Twelve Fractions of Acetone Extract.

Fraction	Spots	R_f	H_2SO_4	Nature
1	None	---	---	---
2	2	0.14 0.32	+ve +ve	milky/grey milky/grey
3	2	0.24 0.35	+ve +ve	milky/grey milky/grey
4	2	0.24 0.35	+ve +ve	milky/grey milky/grey
5	2	0.22 0.31	+ve +ve	milky/grey milky/grey
6	1	0.22	+ve	milky/grey
7	1	0.22	+ve	milky/grey
8-12	no separation	---	---	---

Sample $2A_5$ (1g) was further column chromatographed wherefrom 12 fractions were collected. After spotting the concentrates on TLC, three samples were selected and coded A1, A2 and A3 whose R_f values were 0.14, 0.23 and 0.35 respectively.

3.4.3.2 Paper Chromatography of $2A_5$

When $2A_5$ was subjected to paper chromatography using BAW as a mobile phase, two yellow bands emerged at R_f. 0.2 and 0.4 which intensified on exposure to ammonia fumes. The compound obtained from the upper band of the paper, coded as AP tested positive for flavanones (Shibata's reaction) although the yellow colouration also indicates the

presence of flavanols or flavones (Harborne, 1964). The compound AP also gave positive ferric chloride test.

Methylation of AP with diazomethane gave a solid which on crystallization gave a compound coded APE. APE did not respond to Liebermann-Burchard's reaction showing that it was not a terpenoidal compound. The compound APE had a melting point in the range of 118.5-119.9^{0}C, gave a sooty reddish orange flame on burning and was soluble in warm chloroform. On PC, APE gave a single spot at R_f 0.45.

3.4.4 Ethyl Acetate Extract

3.4.4.1 FCC of Ethyl Acetate Extract

Ten fractions were collected from the column and after TLC, fraction 6 and 7 were combined and coded E. The TLC results of the different fractions are shown in table 10.

Table 10. TLC Results (in BAW) of the Ten Fractions of FCC of Ethyl Acetate Extract.

Fraction	Spots	R_f	H_2SO_4	Nature	$FeCl_3$	Nature
1	2	0.7 0.8	+ve +ve	grey grey	-ve	l. green
2	2	0.7 0.8	+ve +ve	yellow pink	-ve	l. green
3	3	0.6 0.7 0.8	+ve +ve +ve	yellow grey d. red	-ve	green
4		0.6 0.8	+ve +ve	grey grey	-ve	d. green
5	2	0.6 0.7	+ve +ve	yellow grey	+ve	d. blue
6	3	0.6 0.7 0.8	+ve	brown grey grey	+ve	d. blue
7	3	0.5 0.7 0.8	+ve +ve +ve	d.pink brown d. red	+ve	d. blue
8	4	0.5 0.6 0.7 0.8	+ve +ve +ve +ve	d. red grey grey brown	+ve	d. blue
9	4	0.5 0.6 0.7 0.8	+ve +ve +ve +ve	d. red grey grey brown	+ve	d. blue
10	4	0.5 0.6 0.7	+ve +ve +ve	brown grey brown	+ve	d. blue

KEY

d - dull **l - light**

3.4.4.2 Preparative TLC of Sample E

Sample E showed three bands whose R_f values were 0.62, 0.71 and 0.80 on PTLC (silica gel, 5% methanol in ethyl acetate).

The major and upper band of PTLC of sample E on elution, gave a compound which was coded E1.

E1 gave a single spot on TLC at R_f 0.78 in BAW-4:1:5 (upper layer) which turned brownish pink when sprayed with 30% aqueous sulphuric acid and heated at 110^{0}C for 10 minutes. It also gave a positive Liebermann-Burchard's test indicating the presence of a terpenoidal compound.

E1 together with A1, A2, A3 and APE (section 3.4.3) were then subjected to high performance liquid chromatography (HPLC) to ascertain their purity for further analysis.

3.5 High Performance Liquid Chromatography (HPLC)

The percentage purity by HPLC of different compounds isolated above are indicated in table 11 and the chromatograms are reproduced in figures 4 to 8.

Table 11. Percentage Purity of Different Compounds by HPLC.

Sample	% Purity
APE	99.1
A1	85.8
A2	76.0
A3	98.7
E1	60.0

APE, A1 and A3 have quite high levels of purity at the conditions given showing that column chromatography can efficiently be used as a method of separation. Sample E1 has very many prominent peaks indicating that it could be a mixture of compounds and not just impurities. However, since the purity was low, the sample was omitted in the subsequent analysis.

3.6 Ultraviolet and Visible Spectroscopy

The UV/Visible spectra of different compounds with different reagents are indicated in table 12.

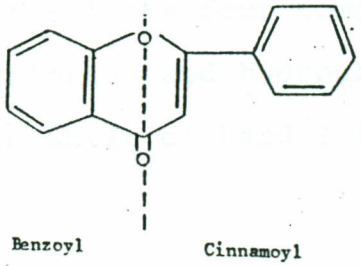
Table 12. Results of UV\Visible Spectra.

Sample	Reagent	Absorption	
		Band I	Band II
APE	MeOH	412, 392	240
	R_1	410, 389	242
	R_2	--	245, 299
	R_3	392	248
	R_4	359	--
	R_5	359	--
A1	MeOH	372	244
	R_1	374	248
	R_2	390	248
	R_3	395	269
	R_4	374	--
	R_5	371	--
A2	MeOH	372	240
	R_1	359	245
	R_2	377, 302(s)	248
	R_3	386, 302(s)	248
	R_4	359	--

	R_4	359	--
	R_5	359	--
A3	MeOH	368	235,298(s)
	R_1	365	245
	R_2	380	242,298(s)
	R_3	--	242
	R_4	365	242
	R_5	365	245
E1	MeOH	242	--

KEY:
R_1 - Sodium Acetate, R_2 - Sodium acetate/Boric acid
R_3 - Aluminium Chloride, R_4-Aluminium chloride/ Hydrochloric acid; R_5 -Sodium methoxide.

Table 12 shows that all compounds have at least two bands, I and II in the range 300-500nm and 240-285nm, respectively except E1 which consistently exhibited one peak at 242nm. The presence of two bands in the above ranges indicates a flavonoid structure (Goodwin, 1976). Band I arises from a cinnamoyl system (ring B) while band II arises from a benzoyl system (ring A) as shown below.



Benzoyl Cinnamoyl

The two bands also suggests a fully conjugated compound.

APE, A1, A2 and A3 exhibited a small shift (+2 to +10) in band II while band I remained unaffected by sodium acetate. Sodium acetate is a weak base that tends to ionize the more acidic phenolic groups. The behaviour thus observed suggests a free 7-hydroxyl group in all the four compounds.

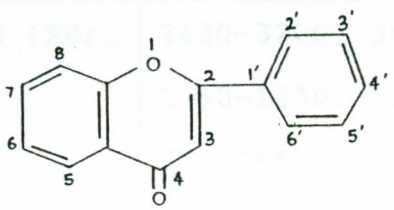
A mixture of sodium acetate and boric acid caused a large bathochromic shift (>12nm) in band I of A1 and A3 indicating the presence of orthodihydroxyl groups in ring B. The absence of an appreciable shift of band II as compared to sodium acetate alone indicates the absence of orthodihydroxyl groups in ring A of all the compounds, APE, A1, A2 and A3.

Aluminium chloride chelates with functional groups such as 5-hydroxy-4-keto, 3-hydroxy-4-keto and orthodihydroxyl systems. Addition of this reagent therefore, causes a shift in one or both bands. Compound APE and A3 showed a small bathochromic shift in band II only while A1 and A2 showed a small shift in band II but a strong shift in band I. This information indicates the presence of a 5-and/or 3-hydroxyl group in the flavonoid structure of all the four compounds. A mixture of aluminium chloride and hydrochloric acid caused a hypsochromic shift of band I in all the four

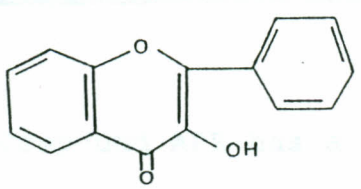
compounds again indicating the presence of orthodihydroxyl groups in ring B of the compounds.

Only A3 showed a small shift in band II when sodium methoxide was added to it. This confirms the presence of replacable hydrogens in A3.

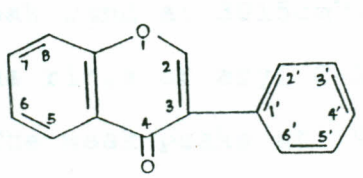
These UV\Visible spectral results suggest that A3 is most probably a flavanol, A1 and A2 are flavones and APE an isoflavone (Goodwin, 1976; Finar, 1975; Harborne, 1964).



Flavone



Flavonol



Isoflavone

Compound E1, with a band at 242nm, did not respond to any of the shift reagents. This is a strong indication that it is not a flavonoid. Its absorption value and colour reactions (section 3.4.4.2) suggest it to be a terpenoid (Finar, 1975).

3.7 Infra-Red Spectroscopy

Table 13 lists the IR absorption spectra of samples APE and A3.

Table 13. Results of IR Spectra.

Sample	Absorption
APE (KBr)	3430-3200, 3015, 2940(s), 2260, 1910, 1760-1650, 1250-1180, 990-940, 840, 775-765, 695, 600, 490, 420.
A3 (KBr)	3400, 2860, 1640-1560, 1405, 1375, 1260, 1230, 1070-1015, 770, 650.

Compound APE has a broad peak in the interval 3400-3200cm^{-1}implies an aromatic OH stretch while the the weak band at 3015cm^{-1}shows the presence of benzene rings or aryl C-H stretch.

The weak peaks at 1910cm^{-1} and 1710cm^{-1} probably represent overtone bands and weak combination bands for aromatic compounds.The broad peak in the interval 1760-1650cm^{-1} indcates the presence of

either ketonic C=O stretch or a C-C stretch within the ring.

The weak broad peak in the interval 1250-1180cm^{-1} shows an in-plane ring C-H bend while the peak at 1085cm^{-1} indicates a C-O stretch.

Between 900 and 600cm^{-1} there are several sharp peaks. The spectra in this region shows out-of-plane C-H bending for polynuclear aromatics. There is even a pair of doublets at 775cm^{-1} and 755cm^{-1} to confirm the presence of polynuclear aromatic compounds.(Silverstein et al., 1991).

Compound A3 has a broad but definite peak at 3400cm^{-1} shows an aromatic O-H. The stretch in the interval 2960-2840cm^{-1} shows C-H bonds are present. The weak peaks between 1600cm^{-1} and 1300^{-1} indicate C==C ring stretching (skeletal bands) weak bands at 1260^{-1} and 1230cm^{-1} show the presence of a C-H bond.(in plane). The shoilder in the interval 1070-1015cm^{-1} shows a C-O stretch. (phenolic) There is an out of plane bonding at 770cm^{-1}. A broad, less intense peak at 650cm^{-1} indicates the presence of six membered rings.

CONCLUSION

In the present study, at least one extract (irrespective of the part of plant) of eight out of nine plants was active against one of the *Plasmodium falciparum* strains. These observations reveal that the medicinal plants used by Kenyan medicinemen have acceptable efficacy levels. In total, 60.0% of the thirty extracts were active on one or both of the malaria parasites.

With values as low as ID_{50} = 1.5µg/ml exhibited by leaves of *Suregada zanzibarensis* indicates that these plant extracts are effective treatments for malaria.The observations also show that it is feasible to obtain an antimalarial drug from the crude extracts tested.

At this juncture, a study on dose-dependence using mice and human subjects would be highly recommended.In addition to the above, a toxicity study on the plant extracts would go a long way in enhancing their effectiveness and marketability to the `enlightened' fraternities of our country. The thesis also suggests that in the search for ~~for~~ bioactive compuonds, bioassay-guided serendipity, would be preferable over straight serendipity, that is, exhaustive analysis of a plant sample for all types of natural products. It is evident, therefore, that natural products have been and continue to be an important source of biologically active

substances.Thus the search for new bioative phytoconstituents presents unique challenges and problems.

Variation in the parasite strain causes a variation in the response to the drug concerned as shown by two strains, ENT36 and K67 of *Plasmodium falciparum*. Of the thirty extracts, 57.1% were active against ENT36 and 33.3% were active against K67. This means that not one antimalarial drug can be administered to all malaria cases in a given locality. Wherever possible, malaria treatment or prophylactic antimalarials should be administered after the strain of *Plasmodium falciparum* has been identified at some rudimentary level.

The ability of the malaria parasite to evade the defences of the host by changing its pattern of antigens enables it to quickly develop resistence to the ever increasing line of synthetic malaria drugs. Since traditional medicines are holistic in their approach to treatment,as discussed in the first chapter, they would considerably reduce the rate at which resistance is developed.

Incorporation of traditional medicine in primary health care programmes would be a cost-effective endevour as proposed by the World Health Organisation.

The phytochemical screening of the four plant extracts, that is, the leaves of *Phyllanthus*

reticulatus and *Suregada zanzibarensis*, stems of *Dissotis brazzae* and the stembark of *Terminalia spinosa* revealed a total of fourteen compounds. Of these, eleven classes were secondary metabolites. To date, the drugs used or under study for malaria treatment are secondary metabolites of plant origin such as limonoids, terpenoids and alkloids to name just a few.

The chromatographic separations achieved a significant level of pure flavonoidal and terpenoidal compounds from the stembark extract of *Terminalia spinosa*. Exact characterization was not achieved due to time constraint on the project.

However, spectral data helped to reveal that, of the five chromatographic fractions, sample A1 and A2 are most probably flavones, A3 a flavanol and APE an isoflavone. Sample E1 is a terpenoidal compound.

Isolation and characterization of the bioactive principles, although not completely achieved in this study, would be important in two ways. It would aid in the treatment of malaria and would also serve as a pointer to other plants containing the same compounds that could be used as antimalarials.

In conclusion, therefore,it is important to note that traditional medical practices should not be done away with but rather improve their quality. This can be done by developing and applying

scientific methodology to validate the medicinal value and document toxicological properties of the traditional drugs. For the above to be achieved, there should be a working relationship between scientists and traditional medicine practitioners.

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Figure 4. High Performance Liquid Chromatography Spectrum of Sample APE.

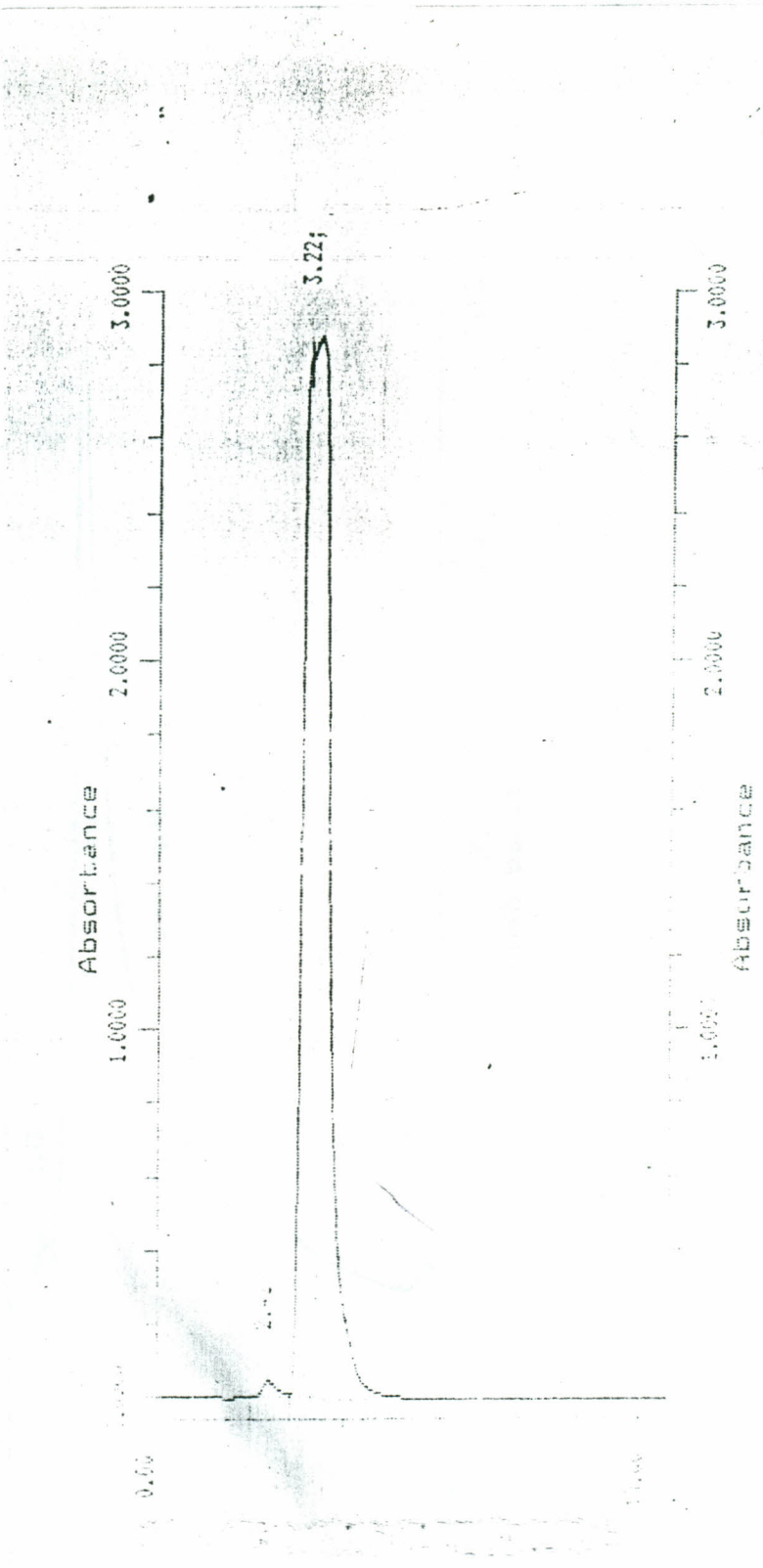

Figure 5. High Performance Liquid Chromatography spectrum of Sample A1

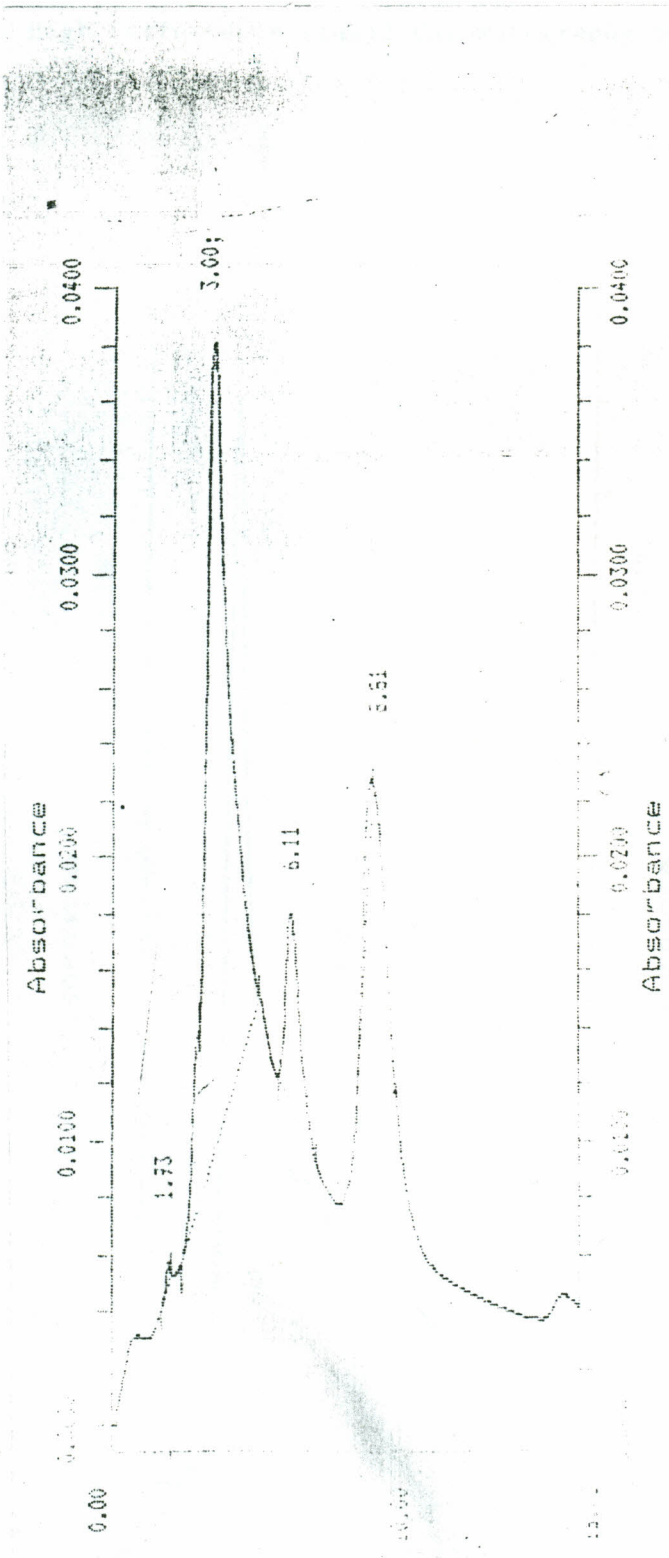

Figure 6. High Performance Liquid Chromatography Spectrum of Sample A2.

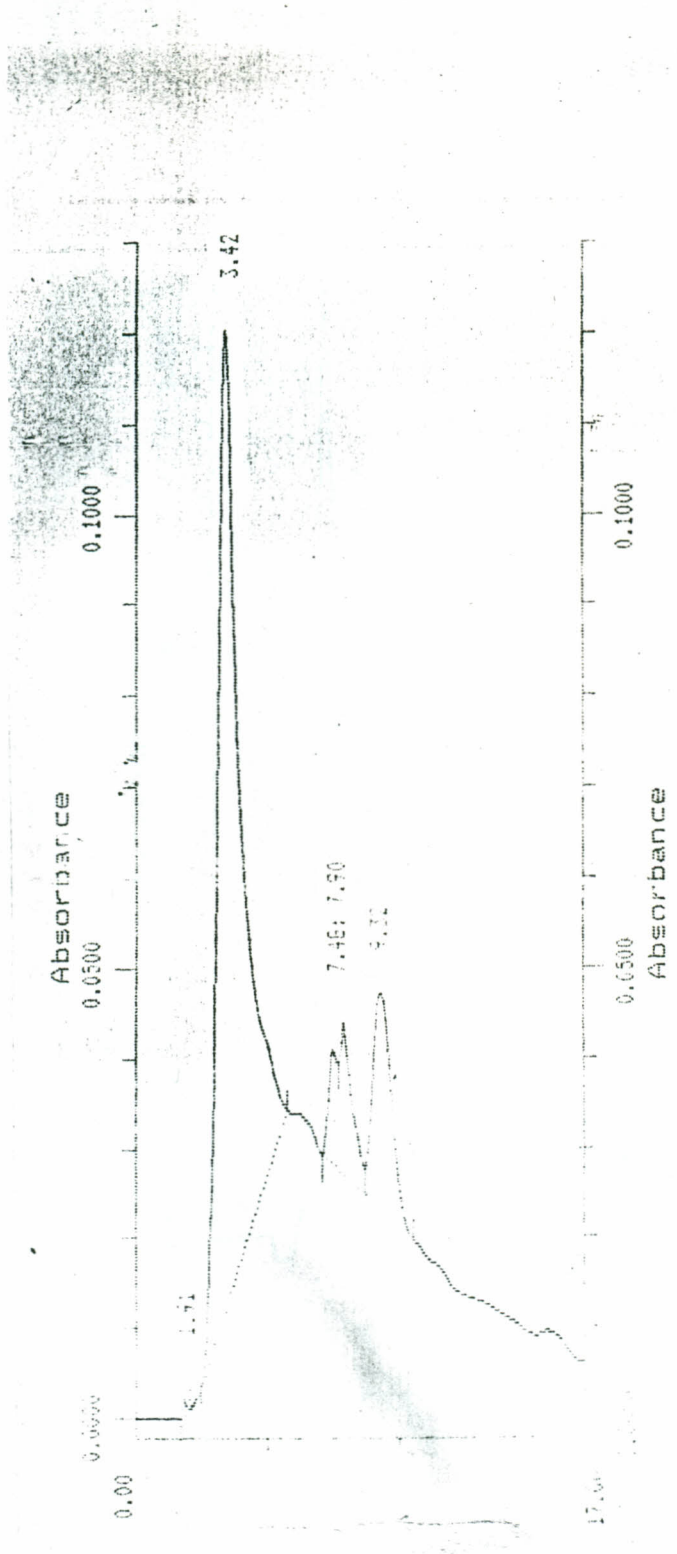

Figure 7. High performance Liquid Chromatography Spectrum of Sample A3

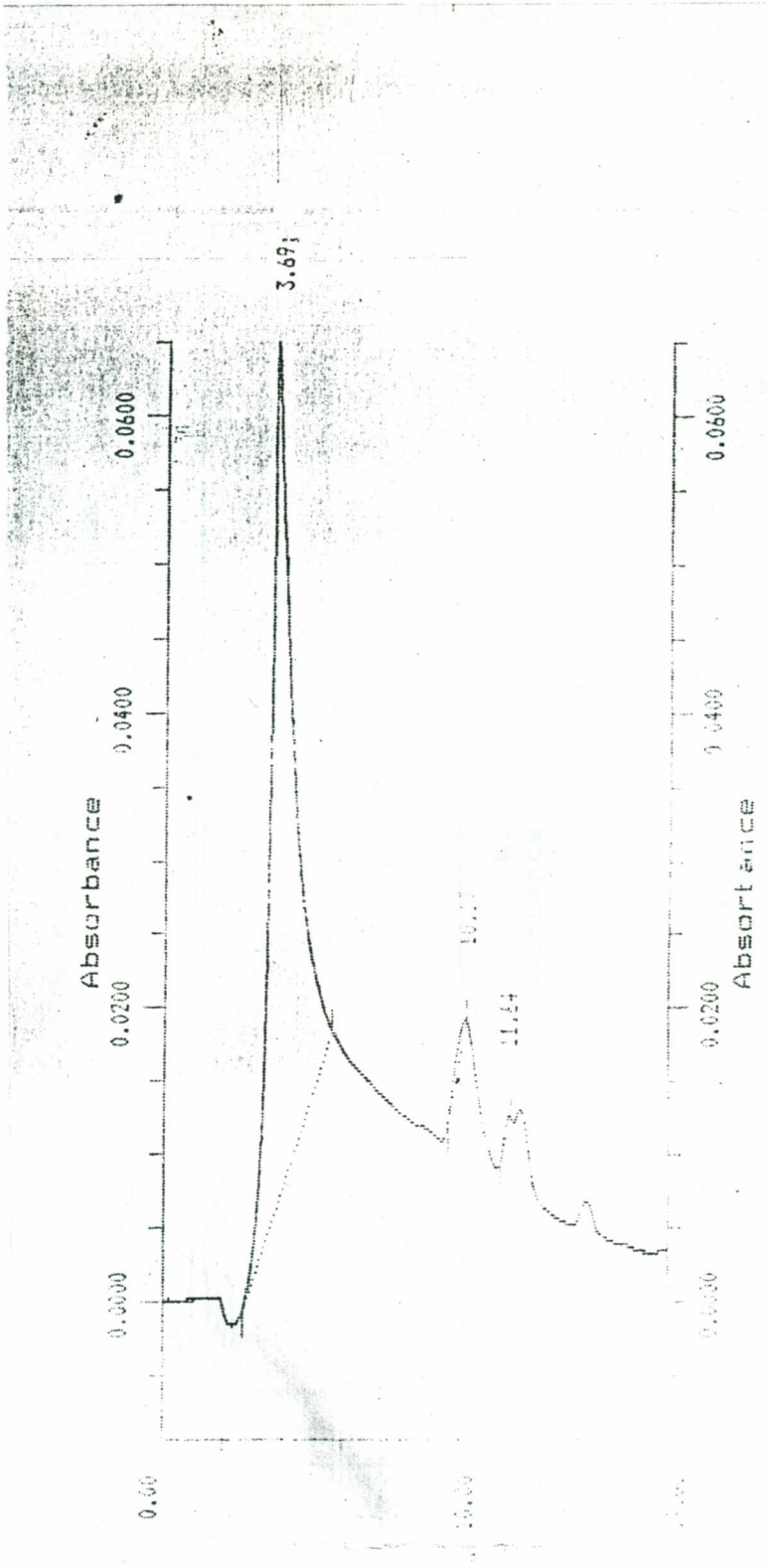

Figure 8. High Performance Liquid Chromotography Spectrum of Sample E1.

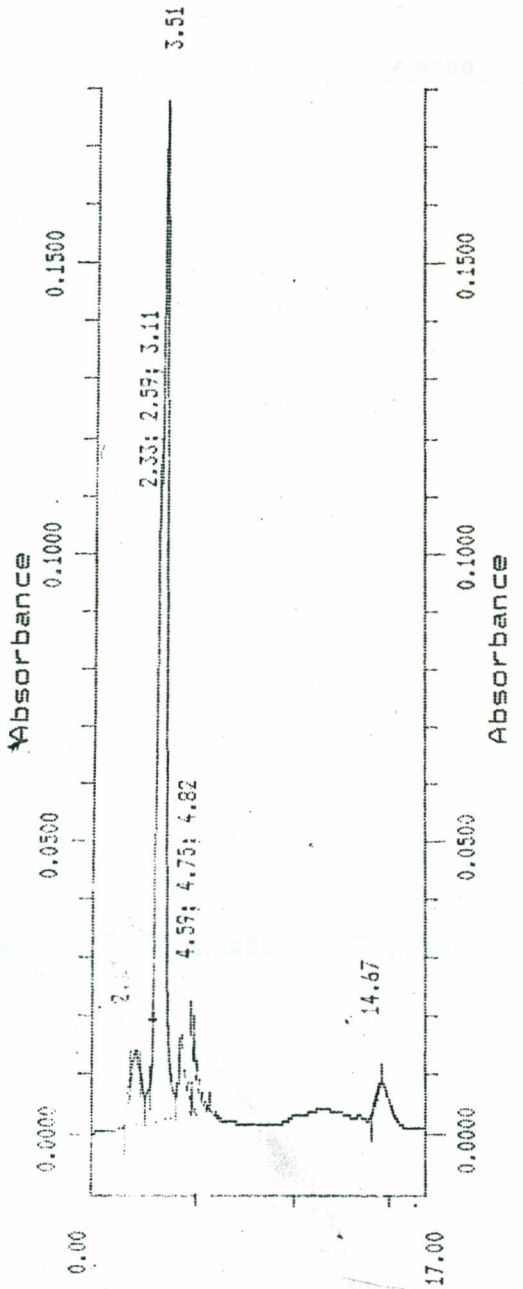

Figure 9. Ultra-violet Spectrum of Sample APE.

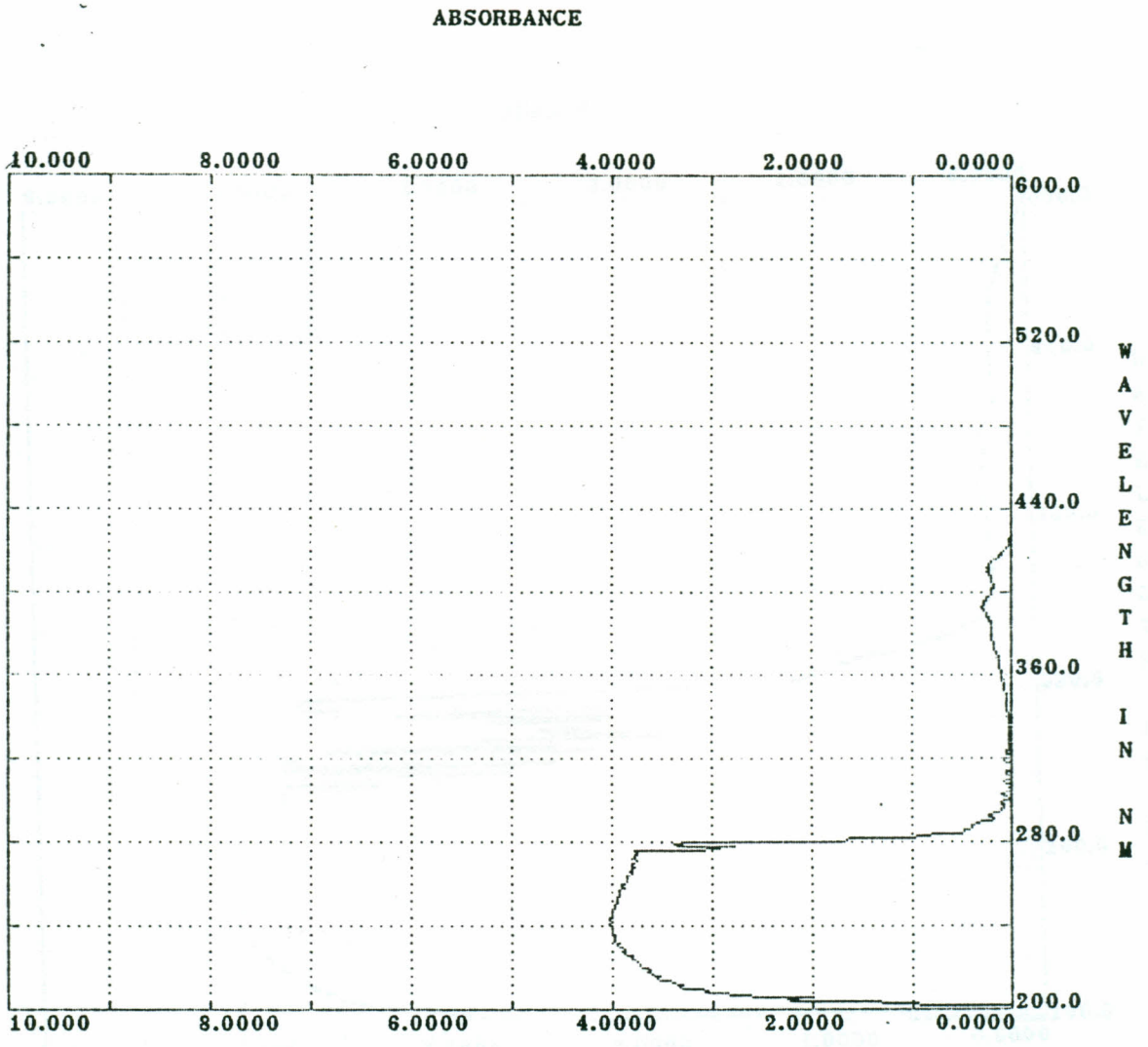

Figure 10. Ultra-violet Spectrum of Sample APE in Sodium Acetate Solution.

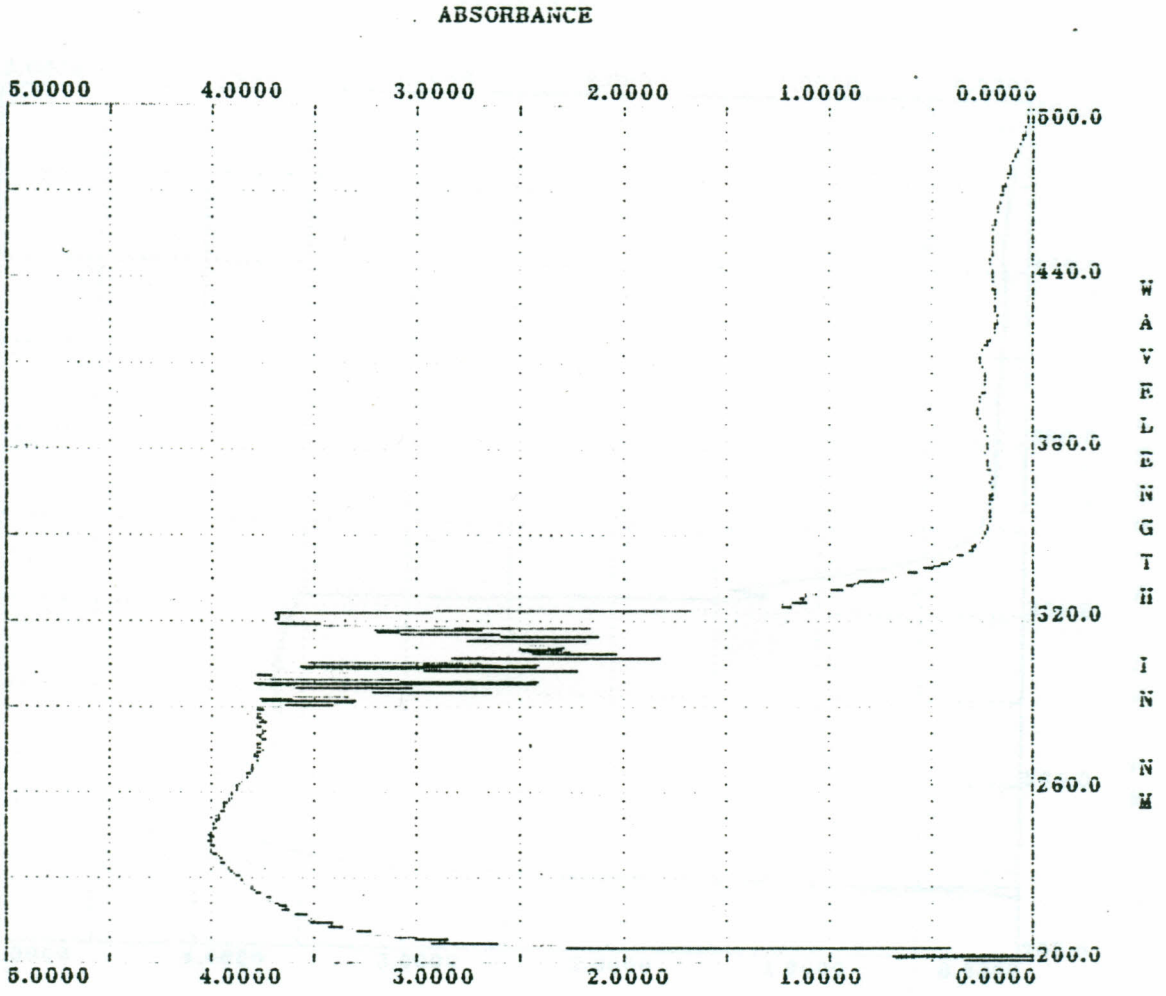

Figure 11. Ultra-violet Spectrum of Sample APE in Sodium Acetate/Boric Acid Solution.

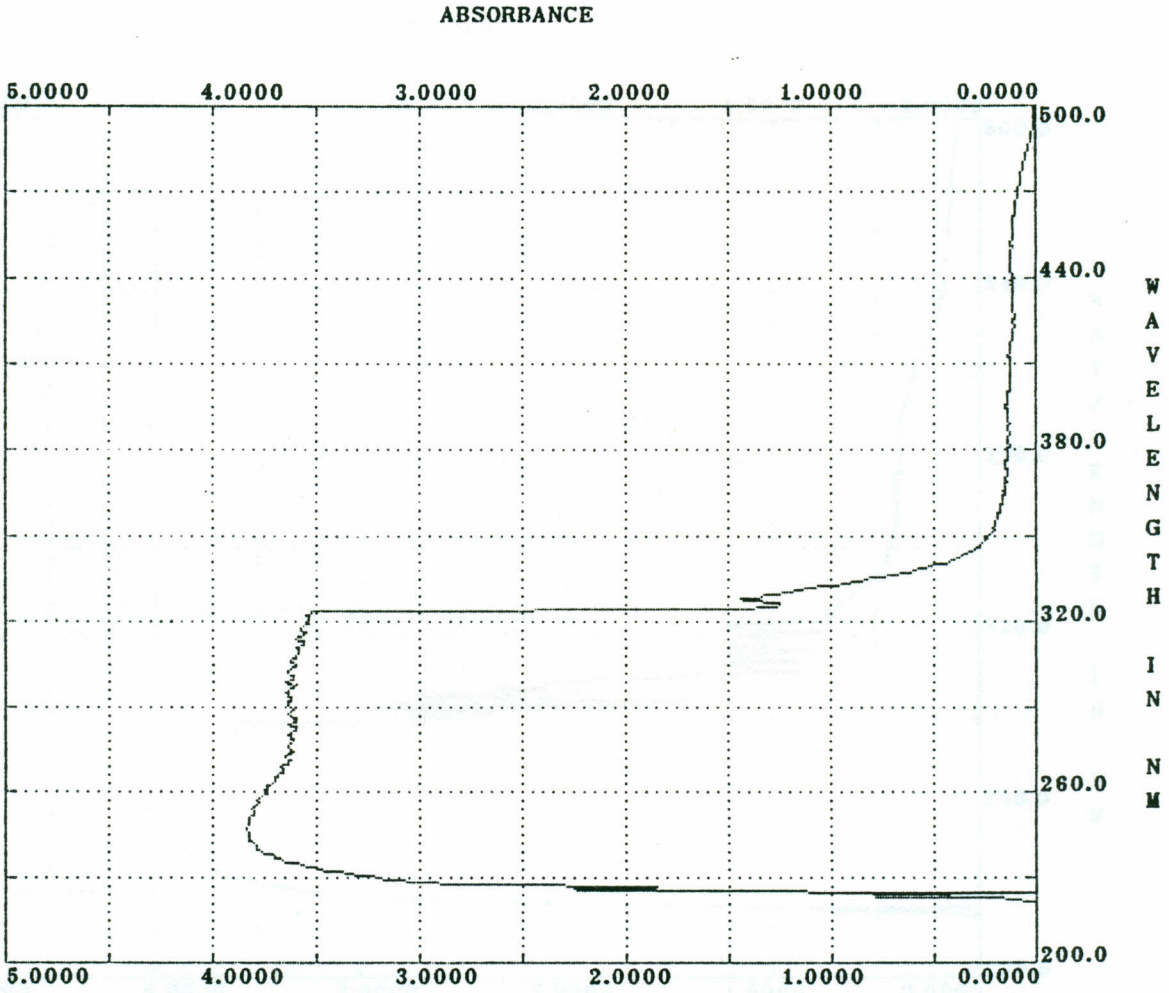

Figure 12. Ultra-violet Spectrum of Sample APE in Aluminium Chloride Solution.

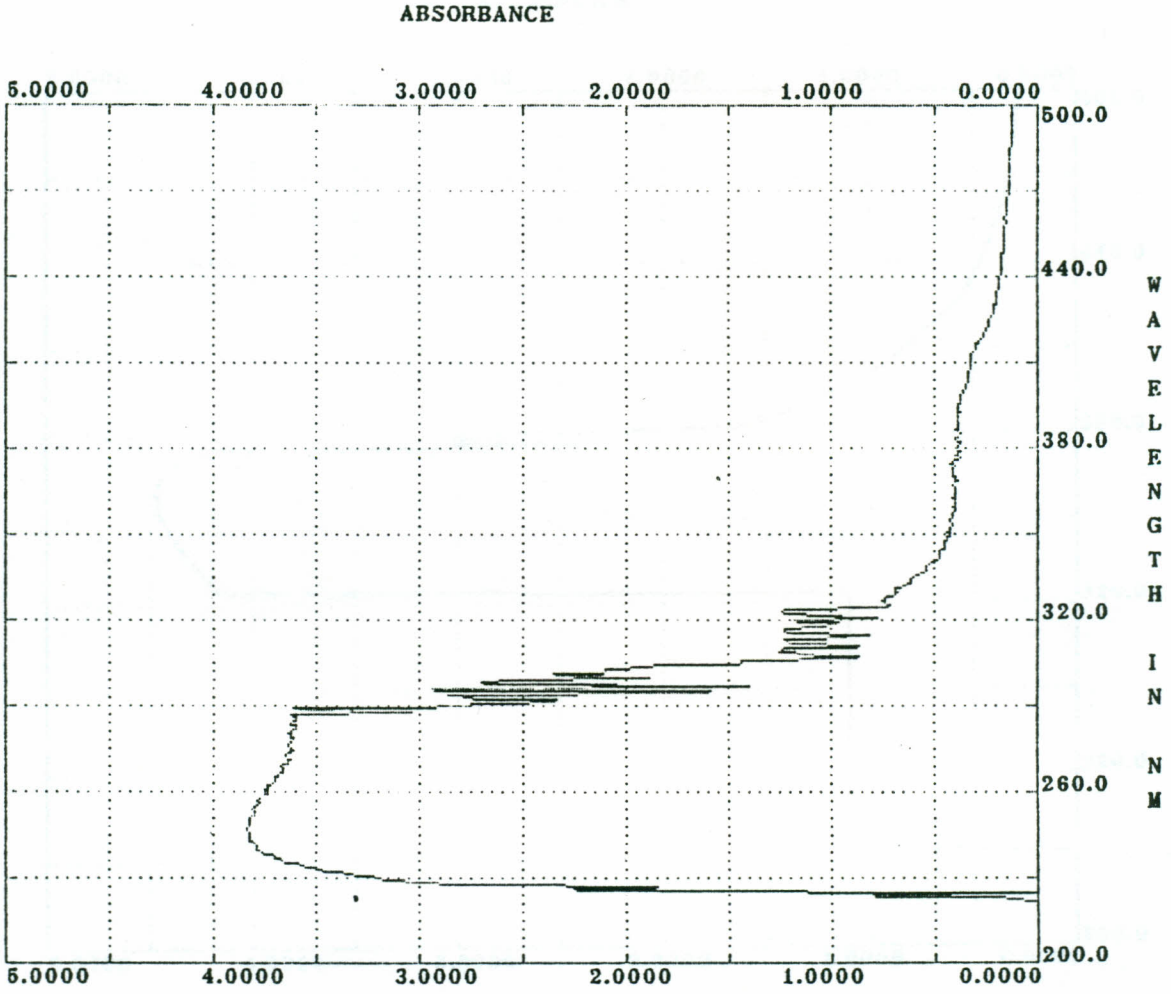

Figure 13. Ultra-violet Spectrum of Sample APE in Chloride/Hydrochloric Acid Solution.

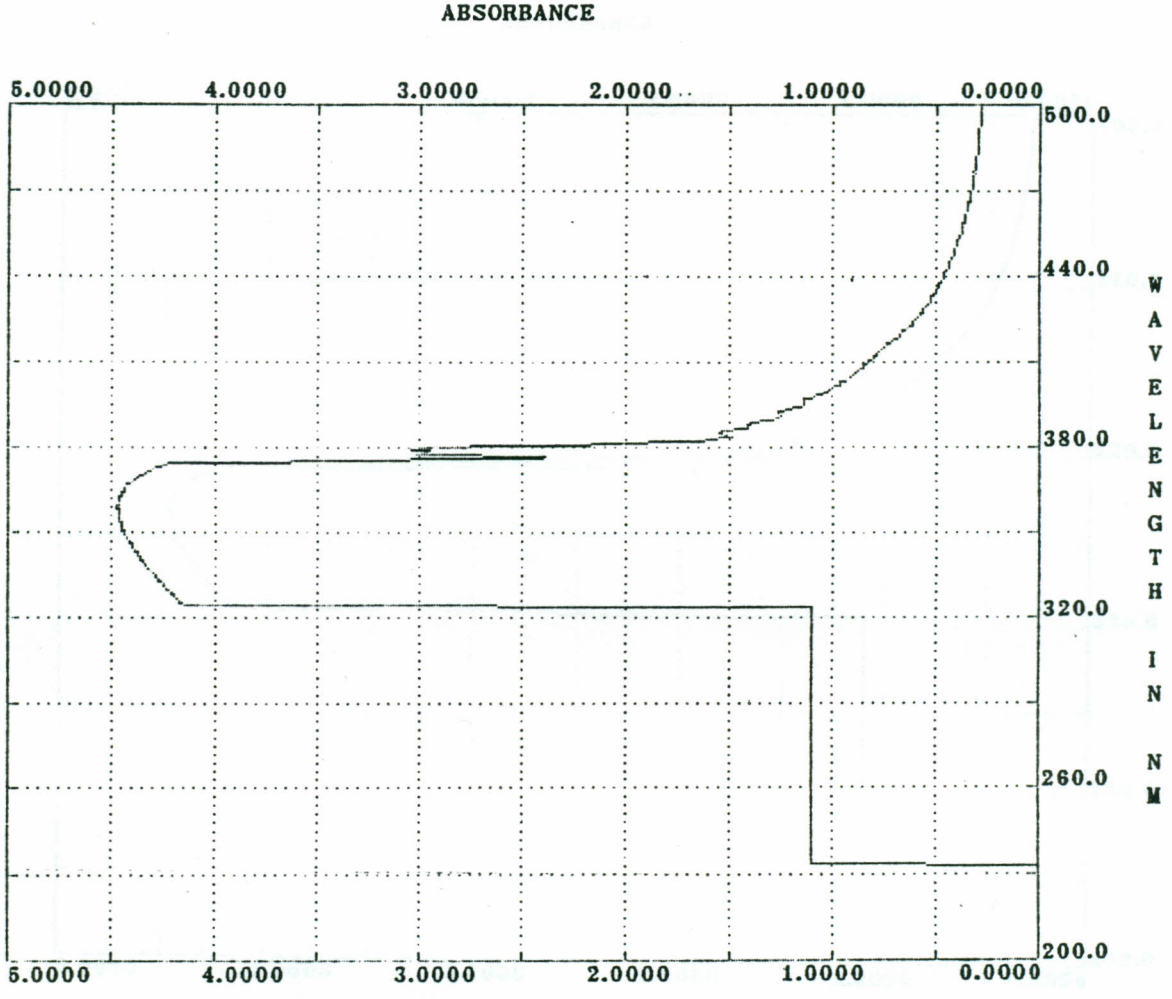

Figure 14. Ultra-violet Spectrum of Sample APE in Sodium Methoxide.

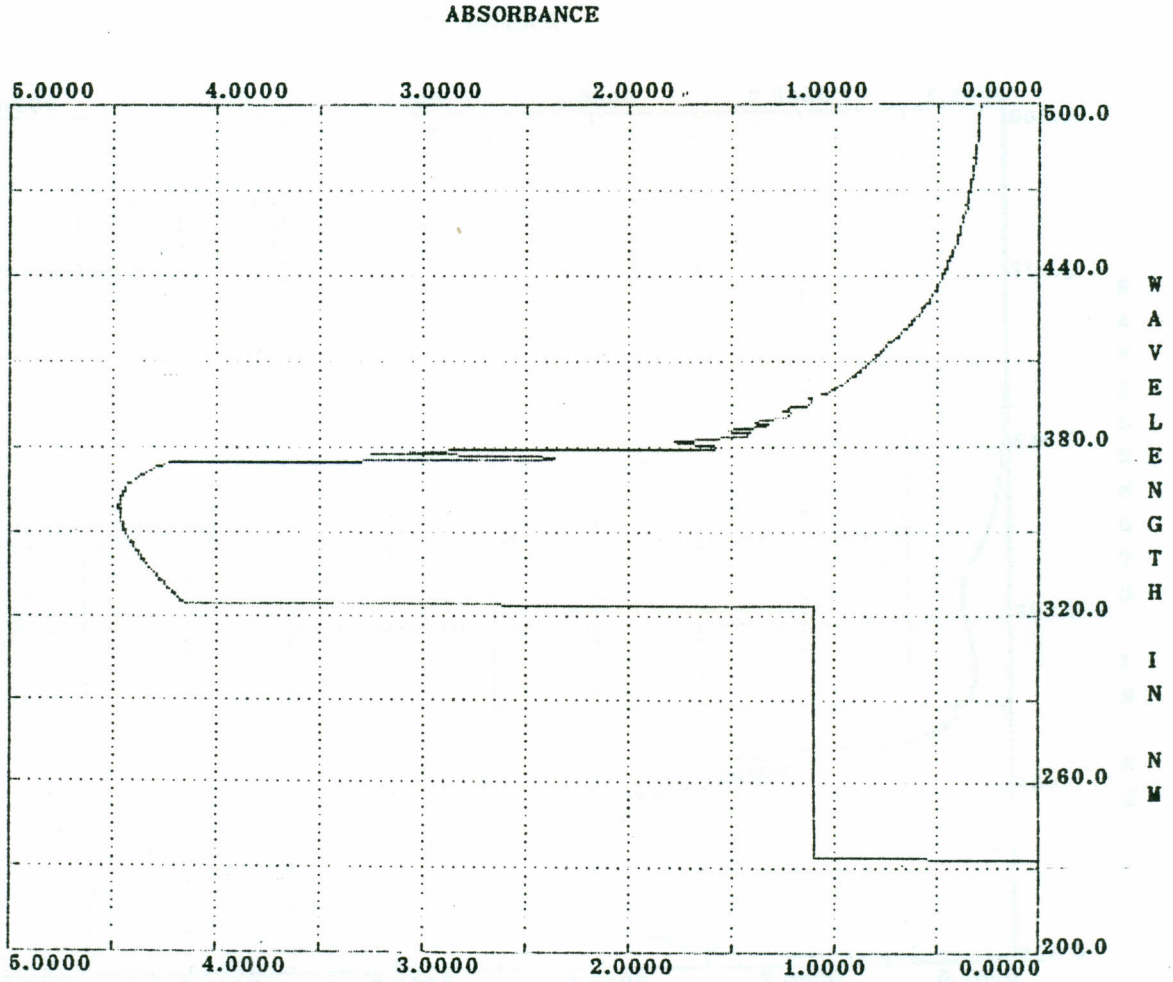

Figure 15. Ultra-violet Spectrum of Sample A1.

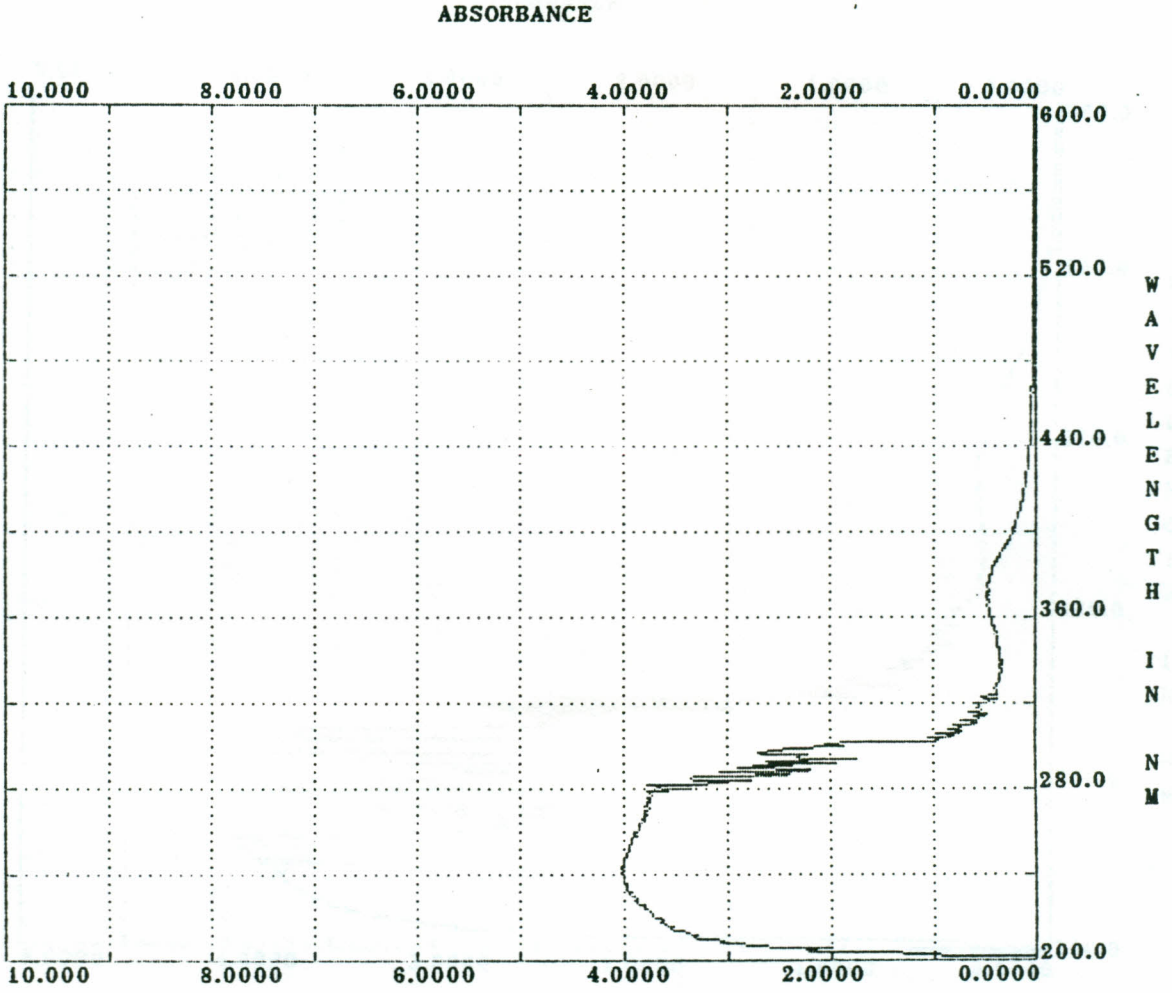

Figure 16. Ultra-violet Spectrum of Sample A1 in Sodium Acetate Solution.

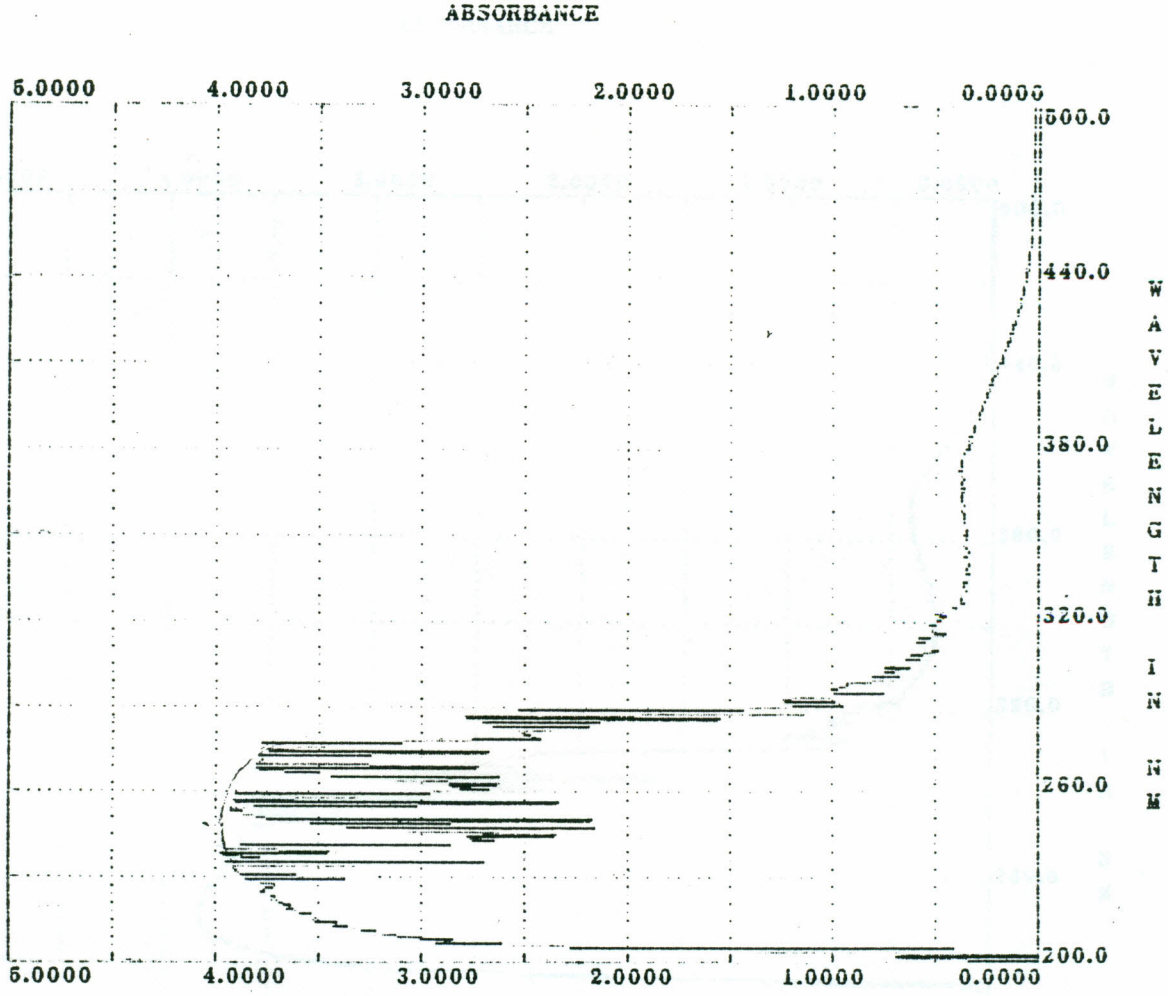

Figure 17. Ultra-violet Spectrum of Sample A1 in Sodium Acetate/Boric Acid Solution.

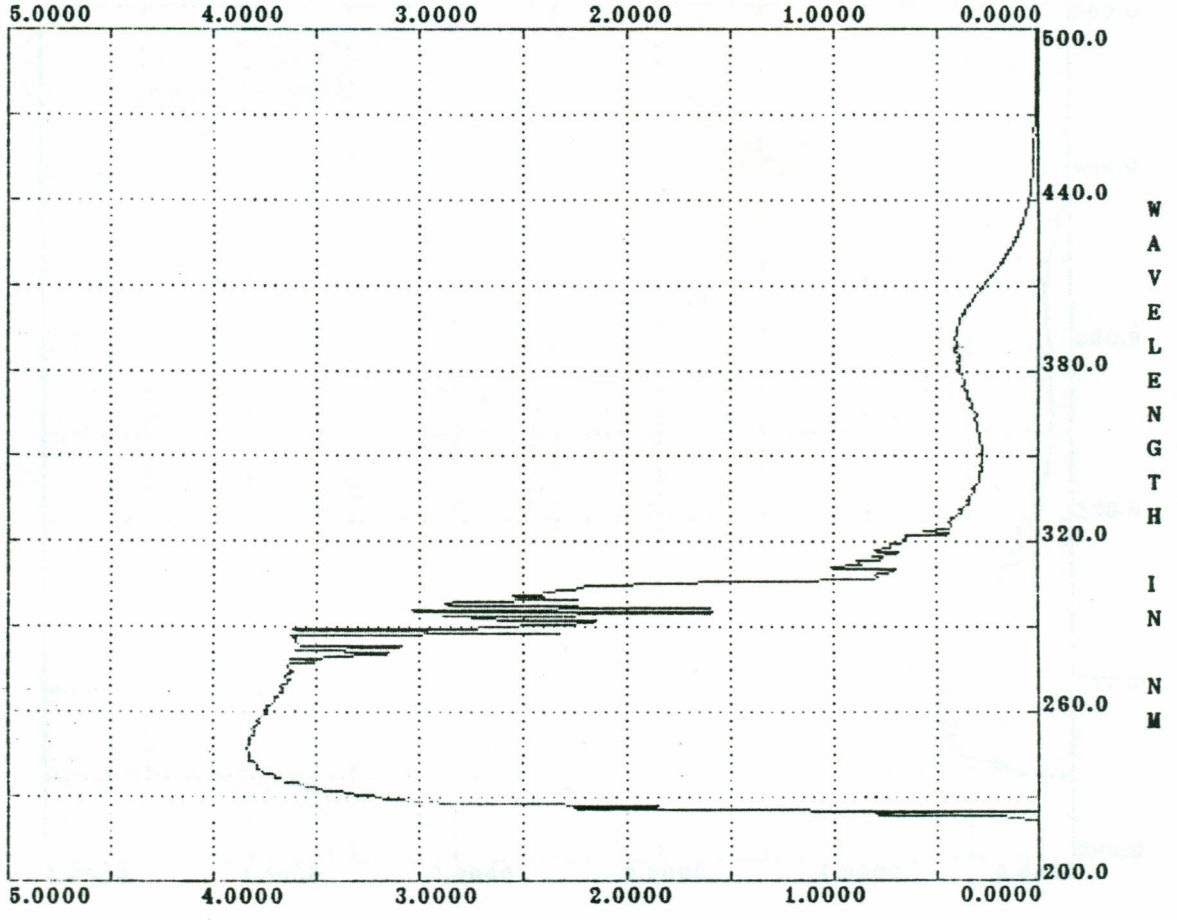

Figure 18. Ultral-violet Spectrum of Sample A1 in Aluminium Chloride Solution.

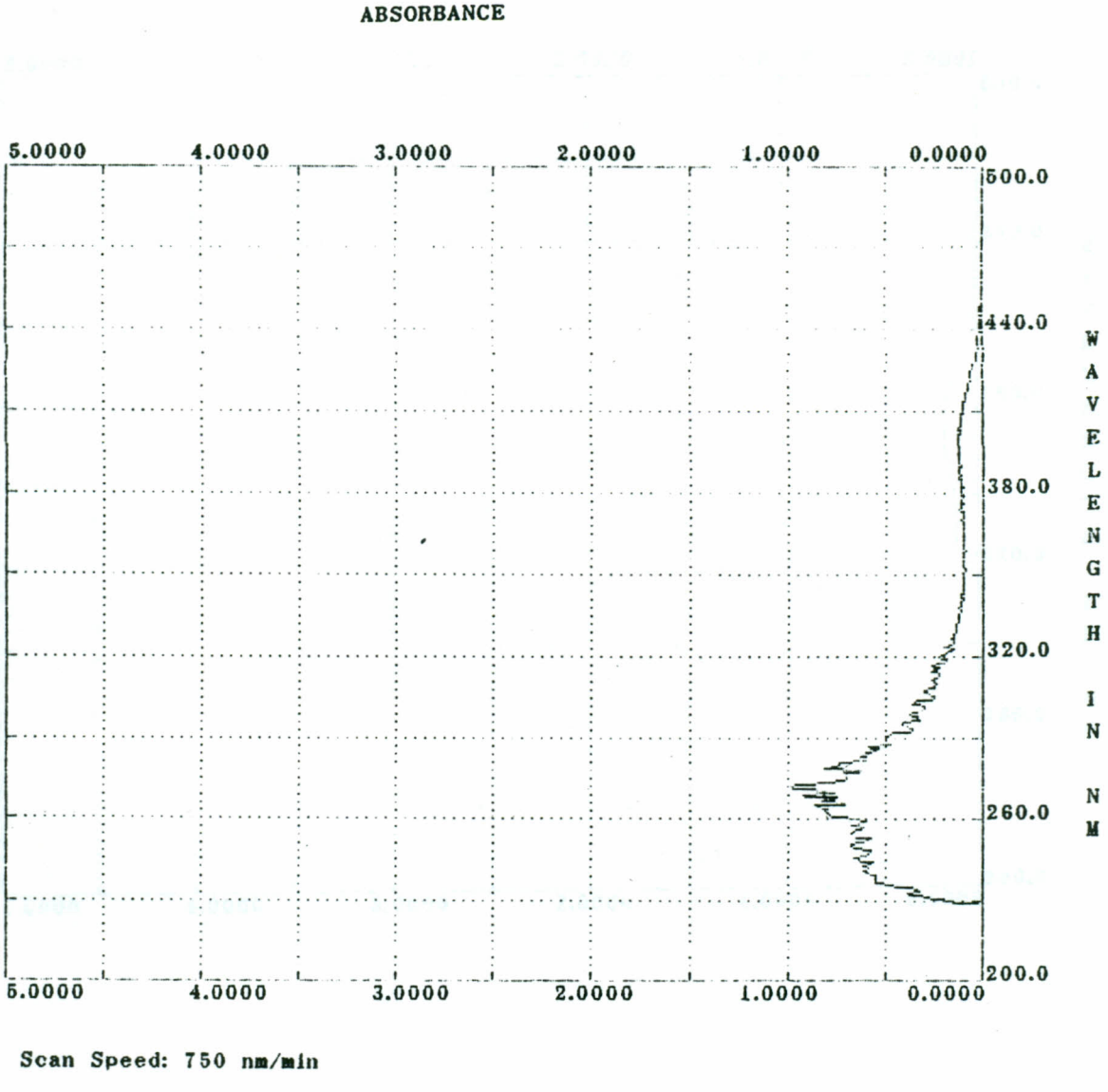

Figure 19. Ultra-violet Spectrum of Sample A1 in Aluminium Chloride/Hydrochloric Acid Solution.

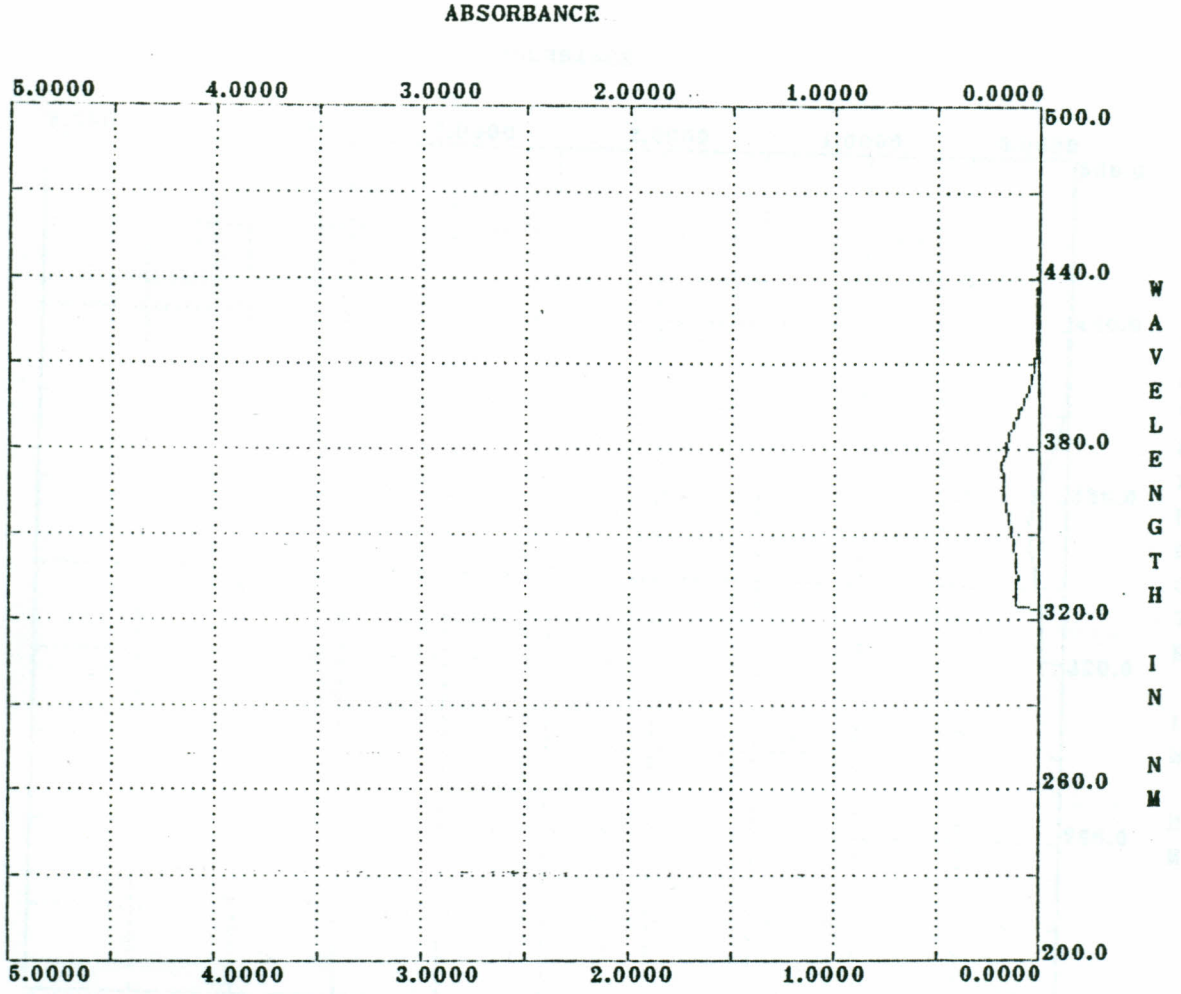

Figure 20. Ultra-violet Spectrum of Sample A1 in Sodium Methoxide.

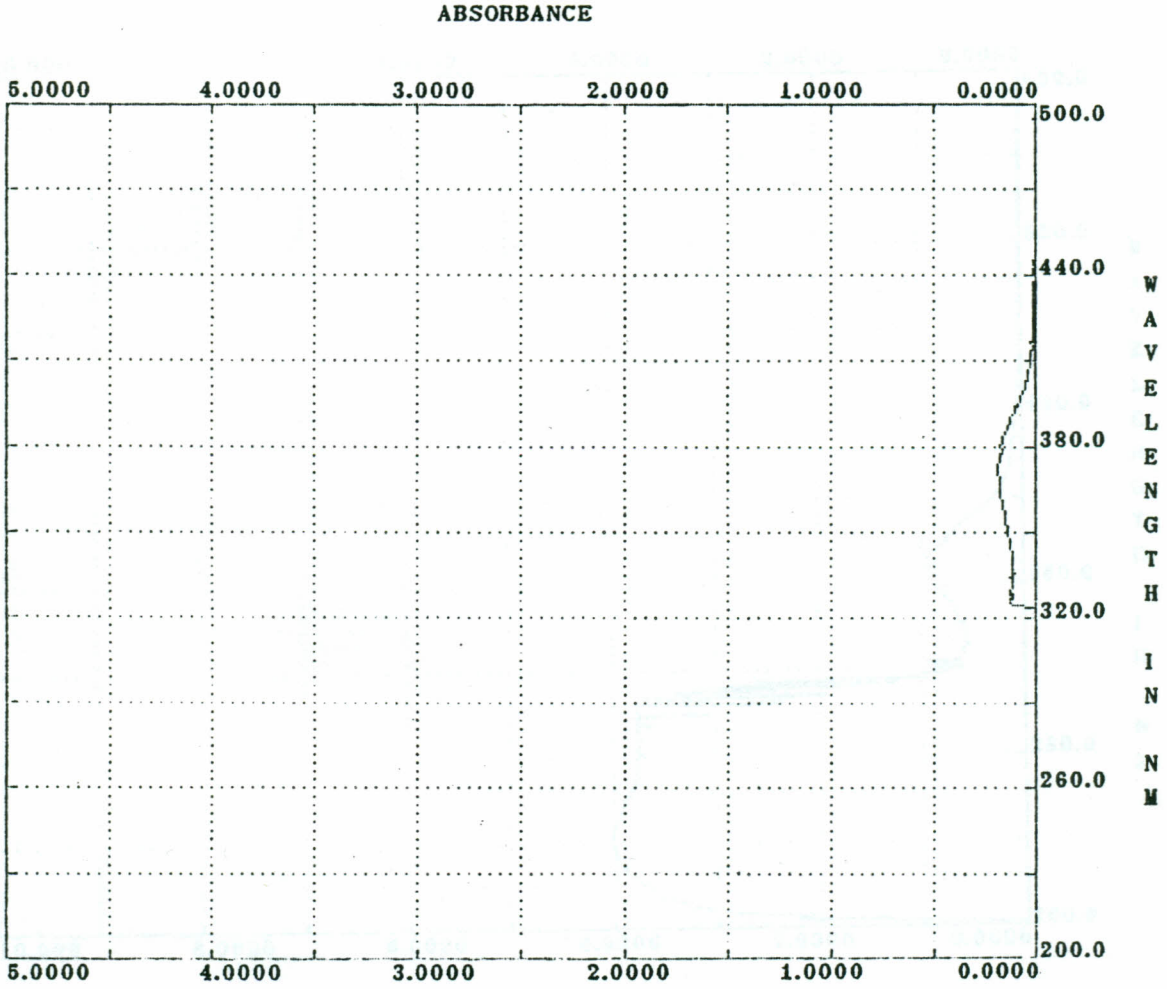

Figure 21. Ultra-violet Spectrum of Sample A2.

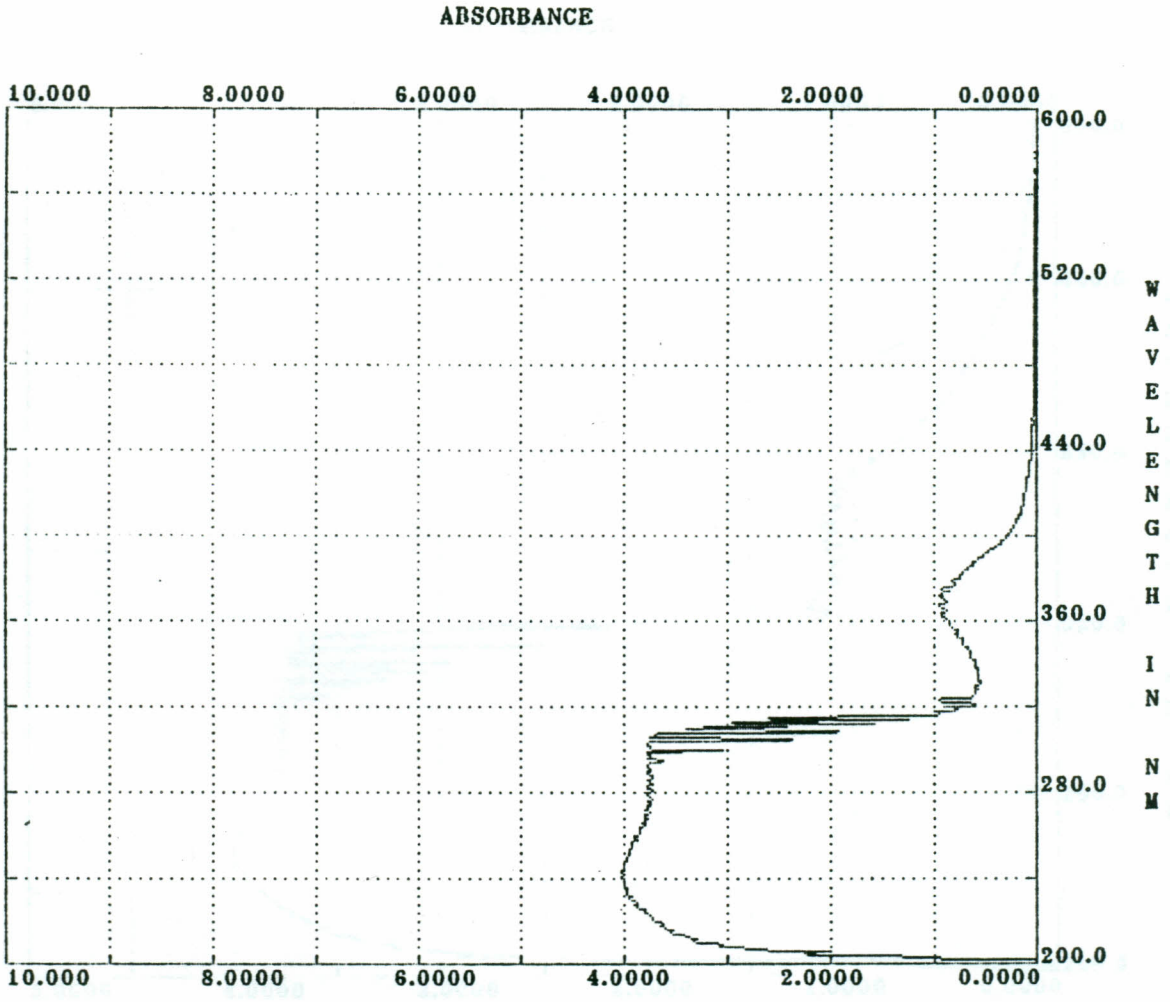

Figure 22. Ultra-violet Spectrum of Sample A2 in Sodium Acetate Solution.

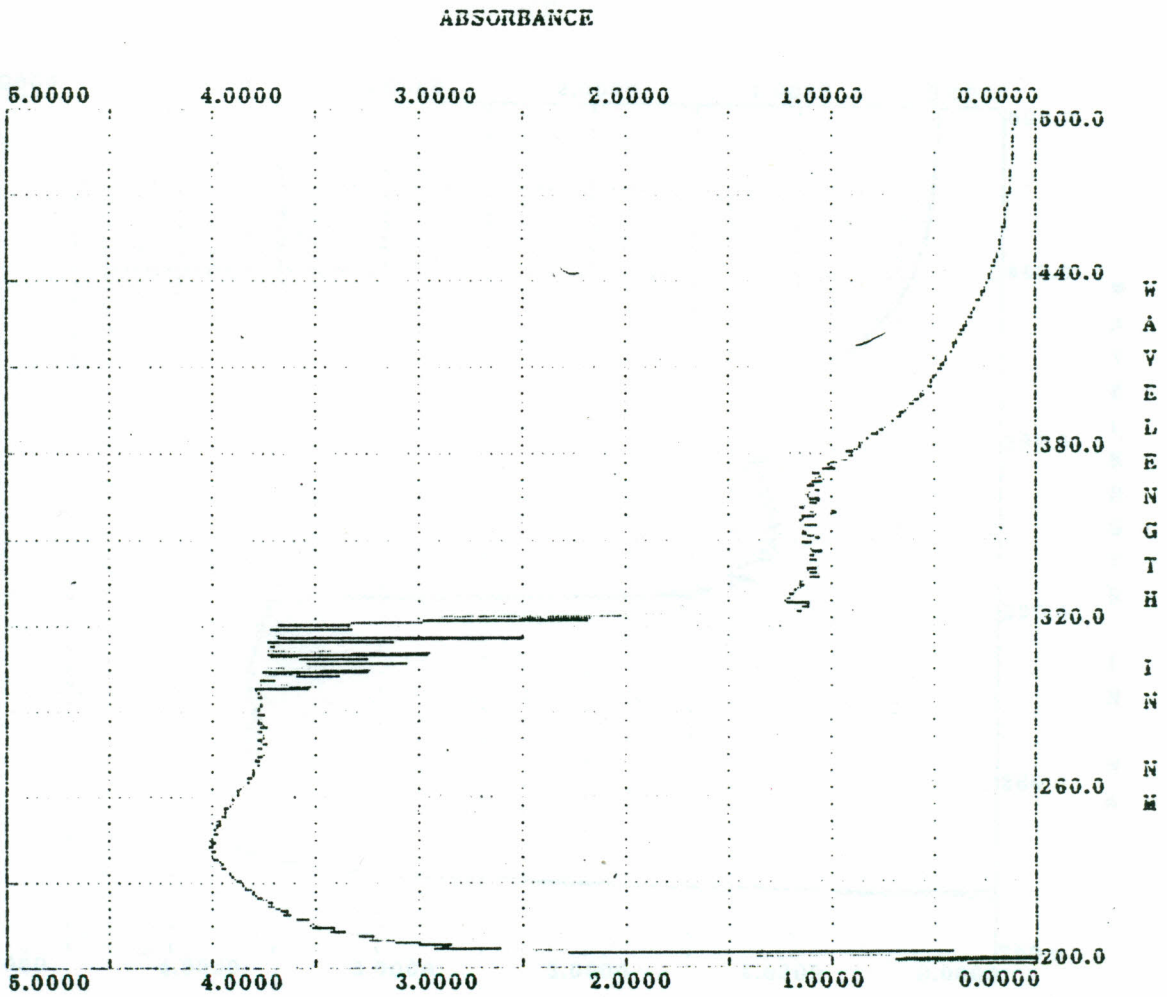

Figure 23. Ultra-violet Spectrum of Sample A2 in Sodium Acetate/Boric Acid Solution.

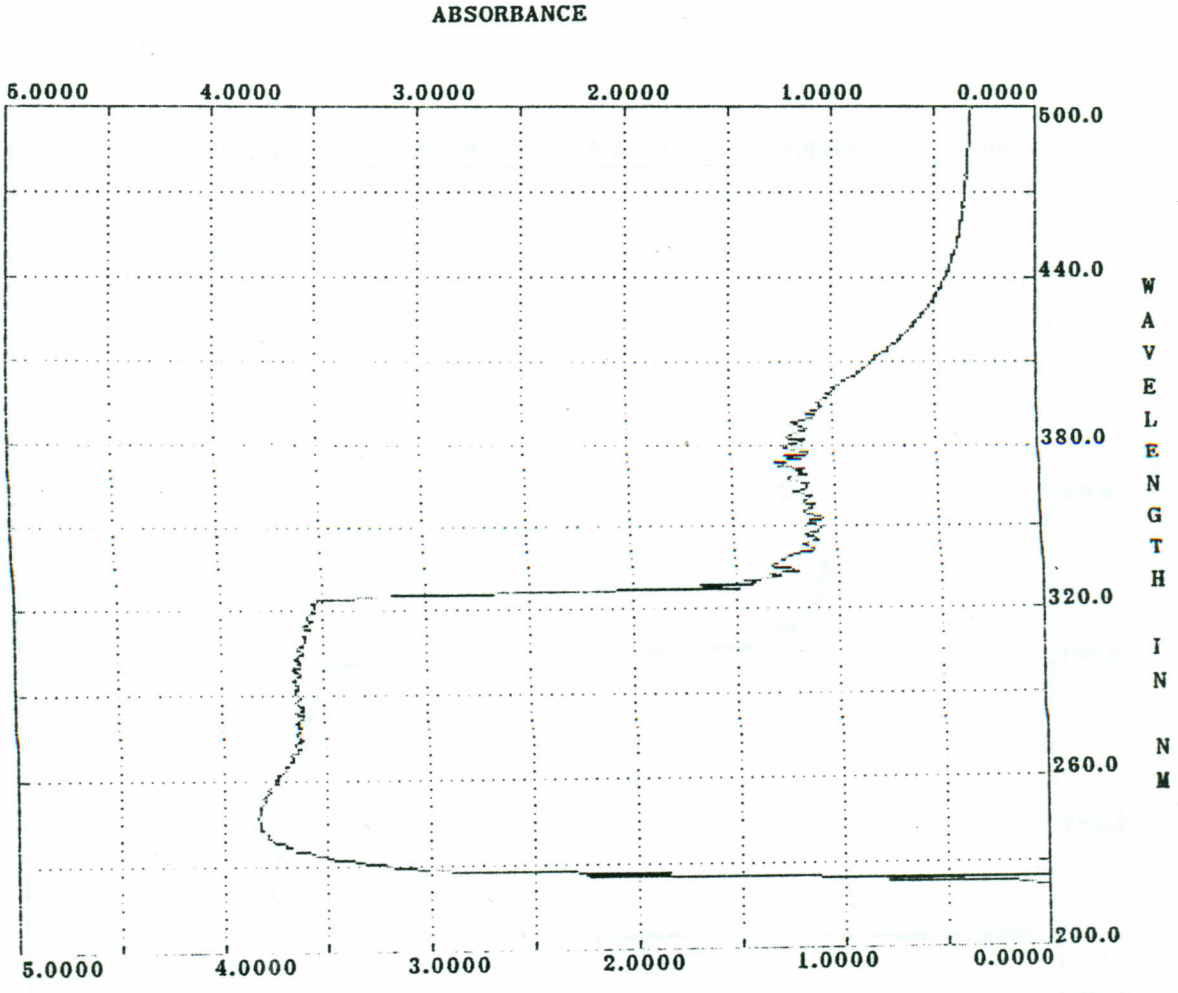

Figure 24. Ultra-violet Spectrum of Sample A2 in Aluminium Chloride.

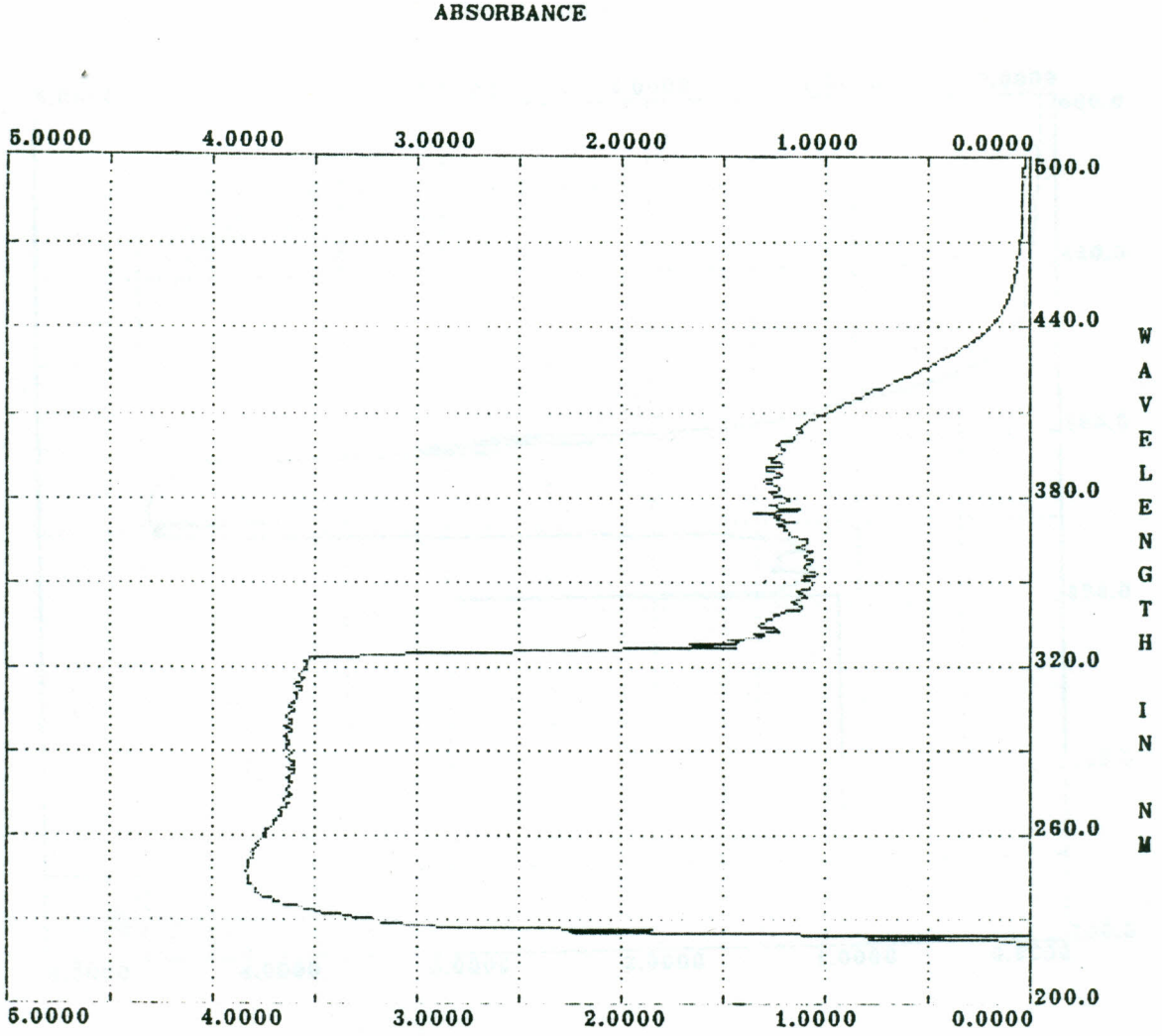

Figure 25. Ultra-violet Spectrum of Sample A2 in Aluminium Chloride/Hydrochloric Acid Solution.

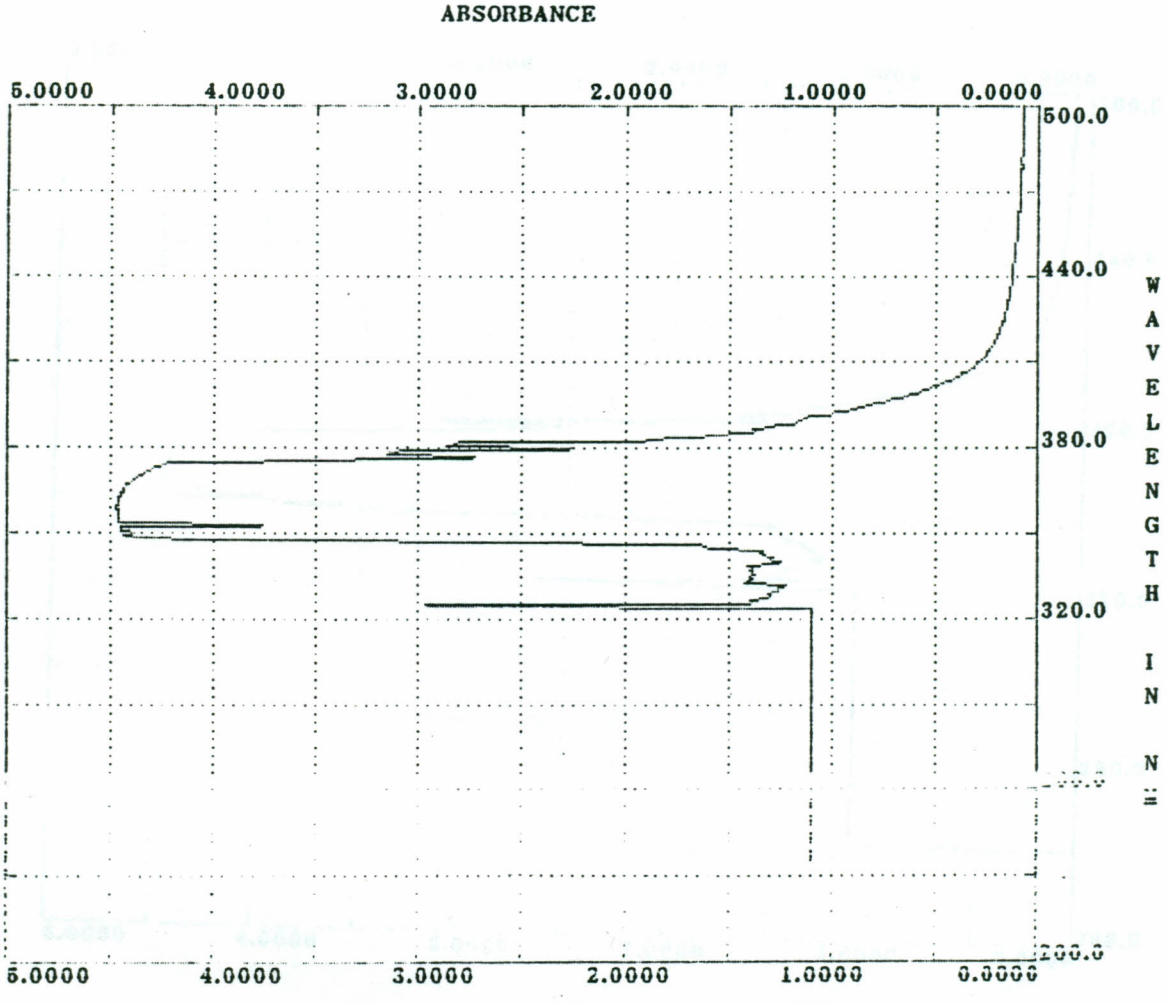

Figure 26. Ultra-violet Spectrum of Sample A2 in Sodium Methoxide.

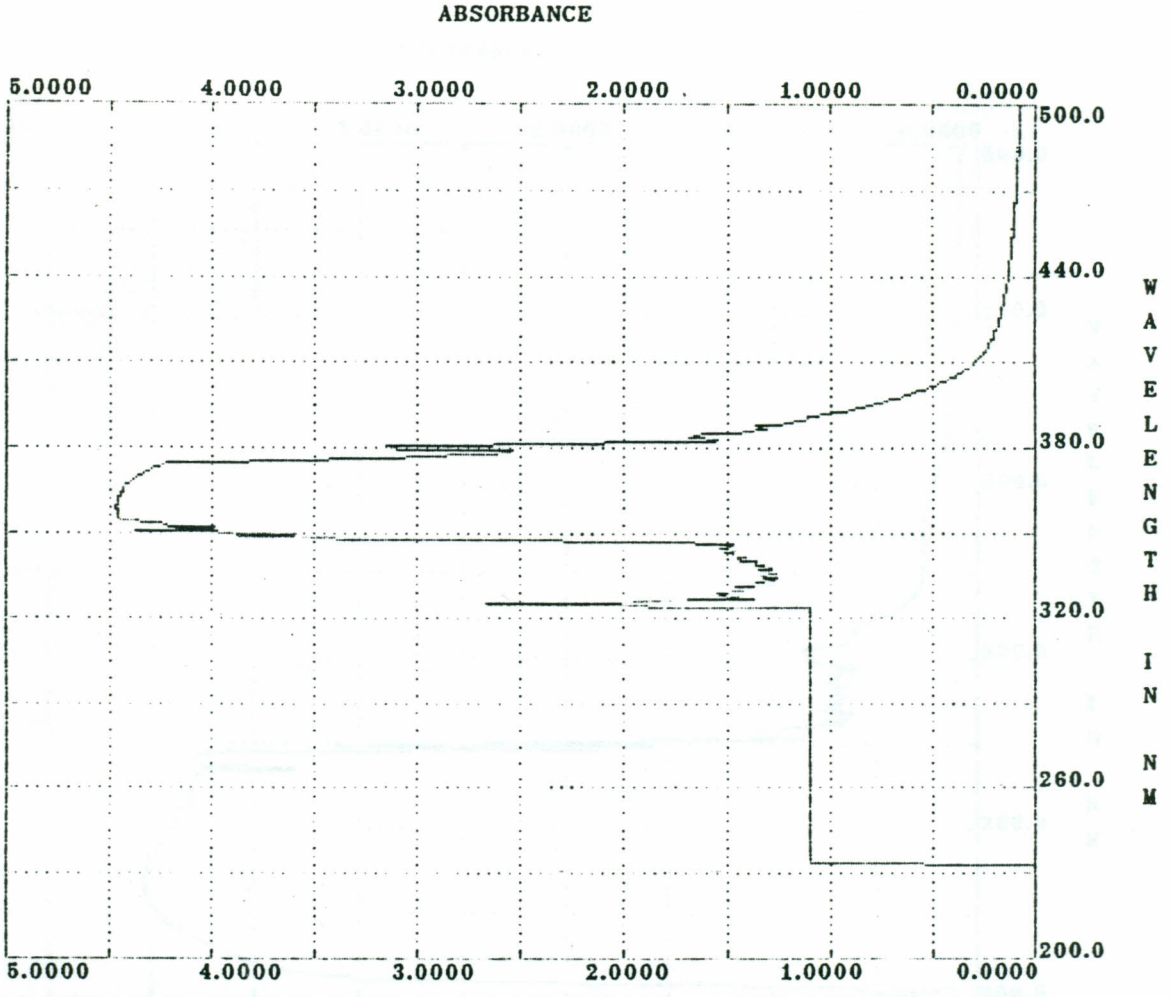

Figure 27. Ultra-violet Spectrum of Sample A3.

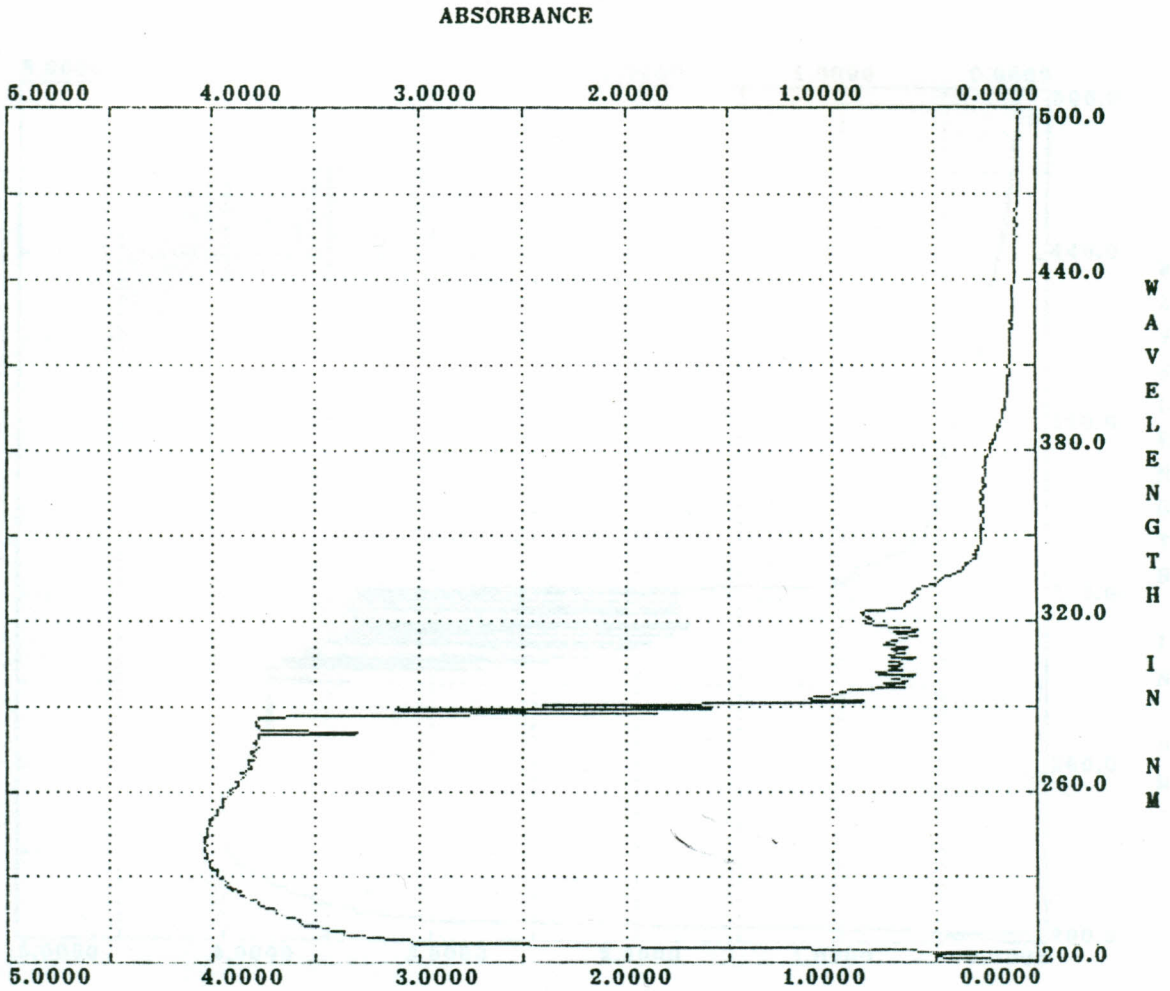

Figure 28. Ultra-violet Spectra of sample A3 in Sodium Acetate Solution.

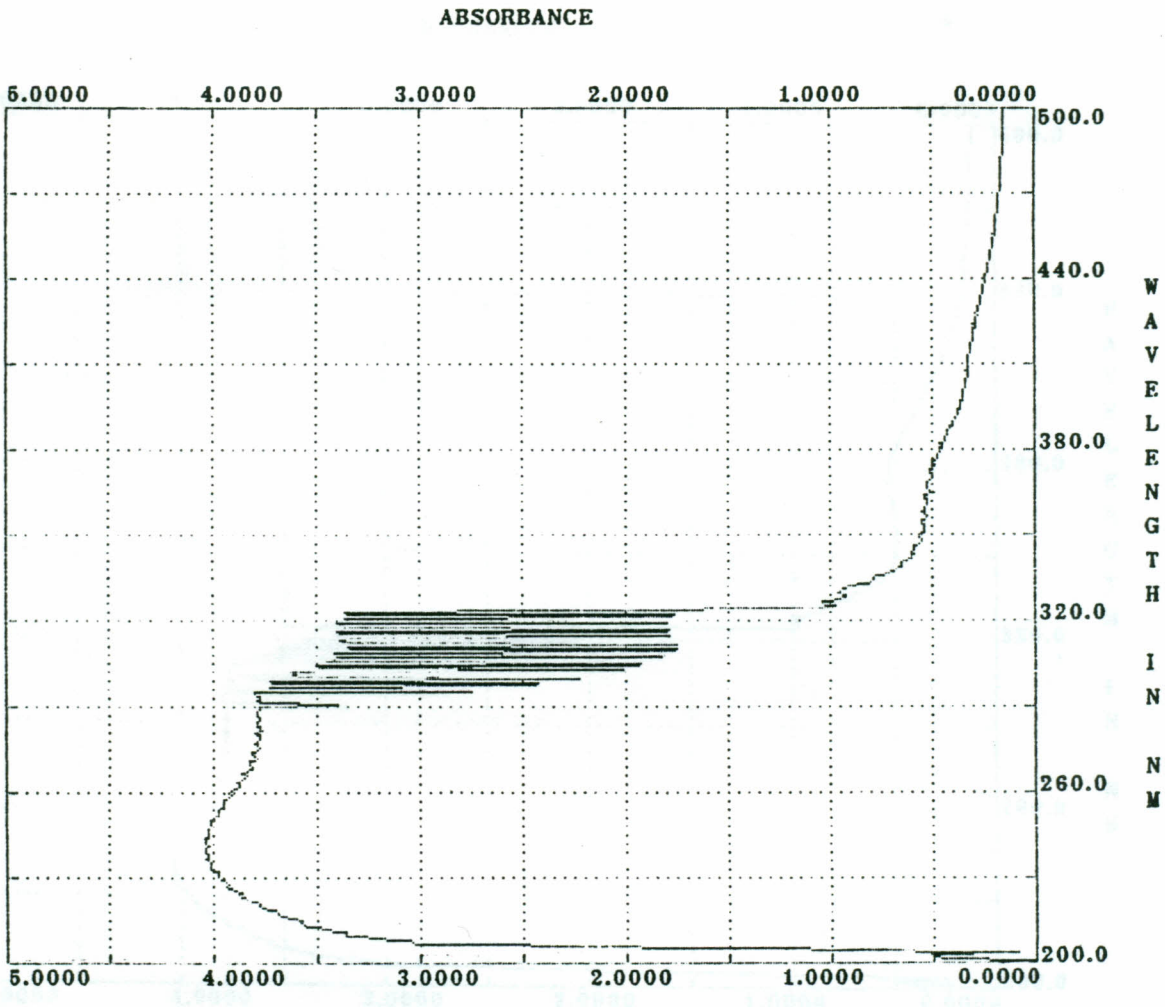

Figure 29. Ultra-violet Spectrum of Sample A3 in Sodium Acetate/Boric Acid Solution.

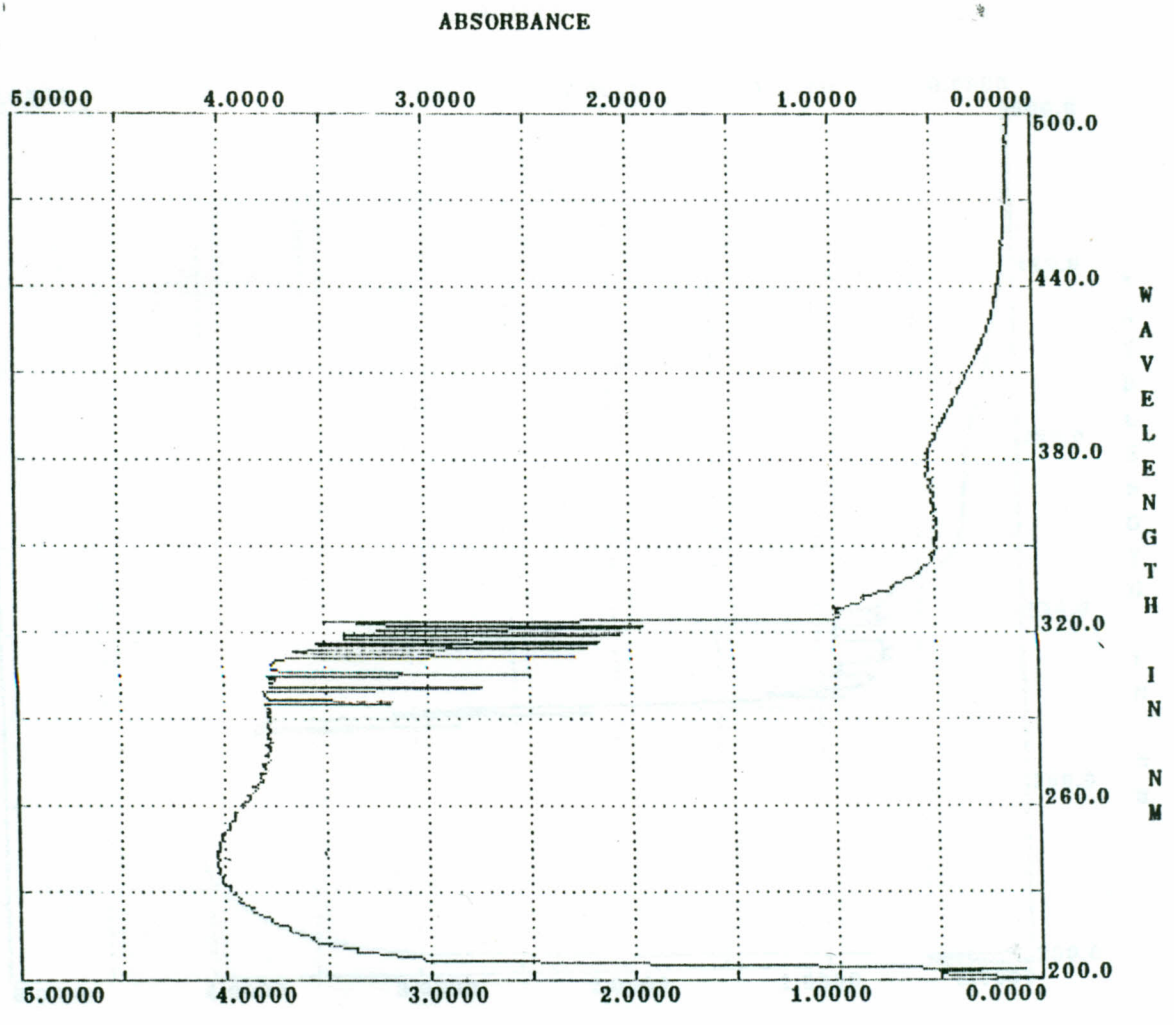

Figure 30. Ultra-violet Spectrum of Sample A3 in Aluminium Chloride Solution.

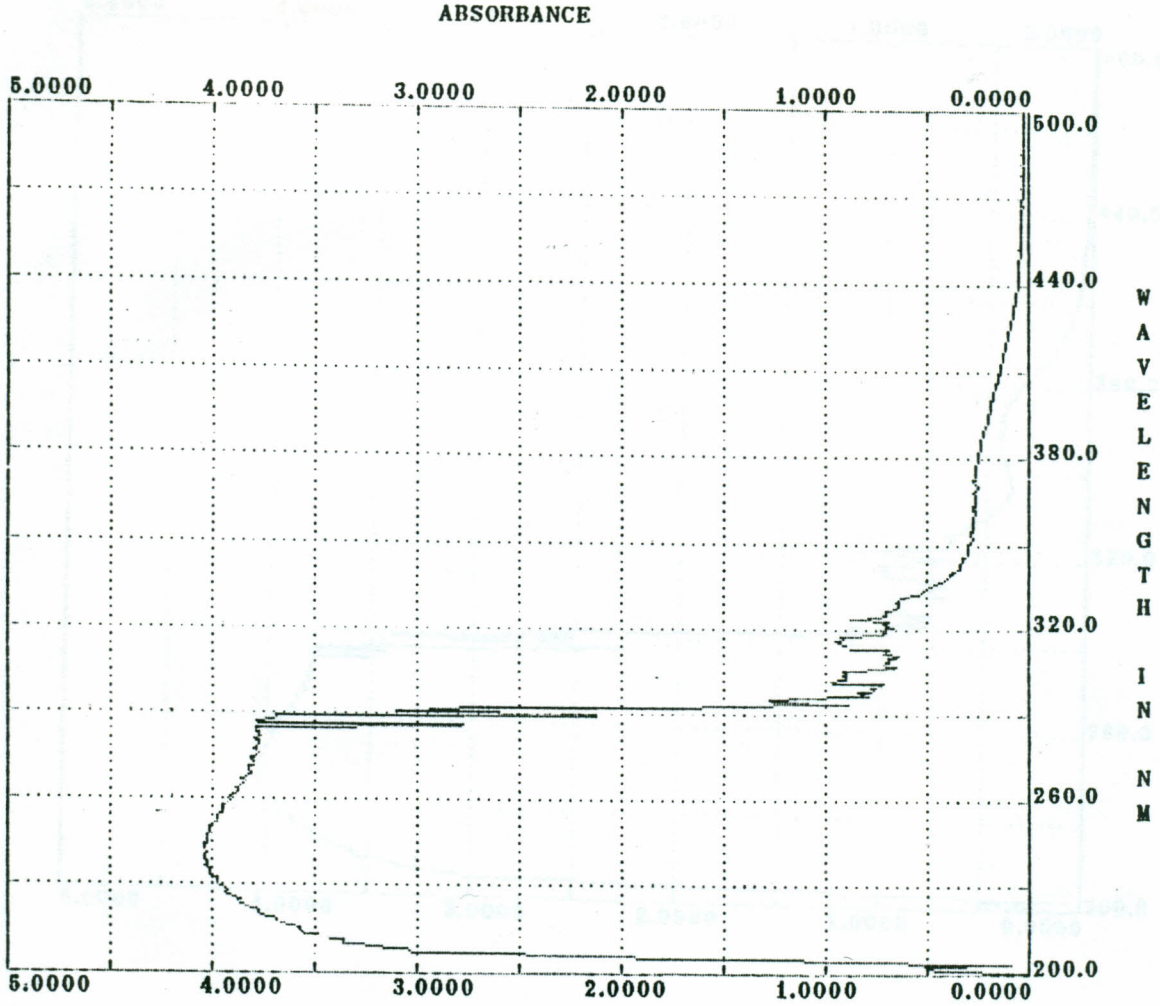

Figure 31. Ultra-violet Spectrum of Sample A3 in Aluminium Chloride/Hydrochloric Acid Solution.

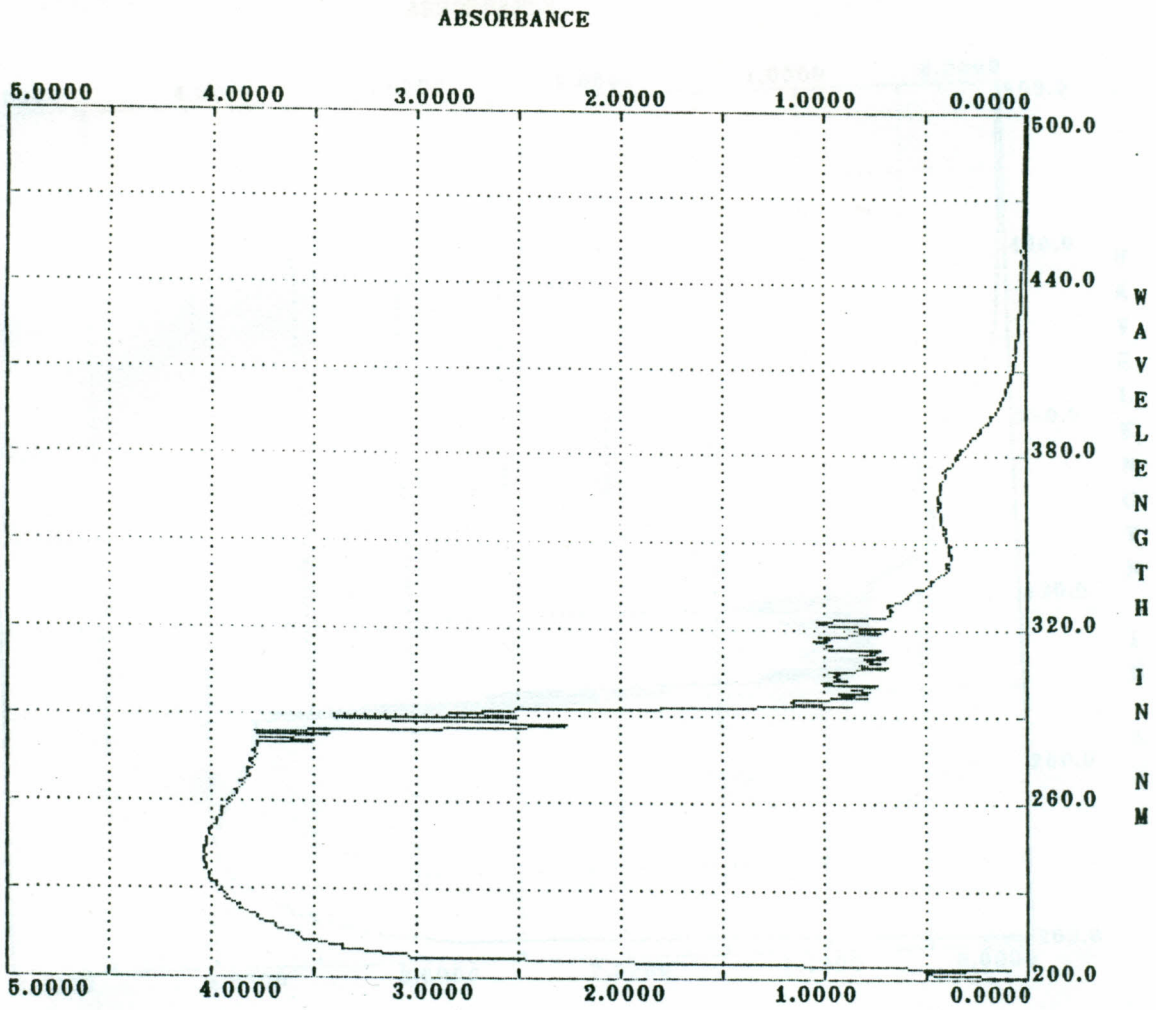

Figure 32. Ultra-violet Spectrum of Sample A3 in Sodium Methoxide.

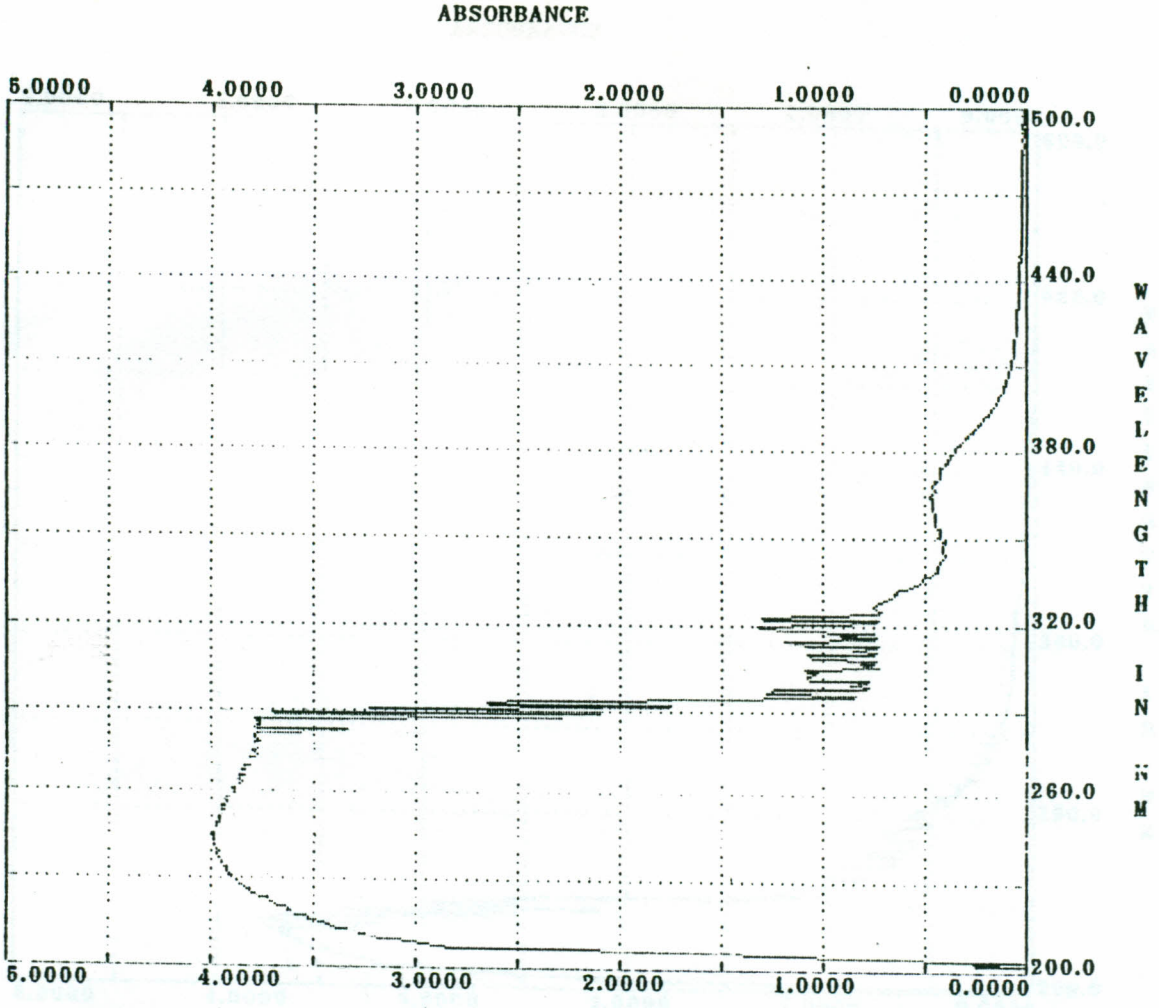

Figure 33. Ultra-violet Spectrum of Sample E1.

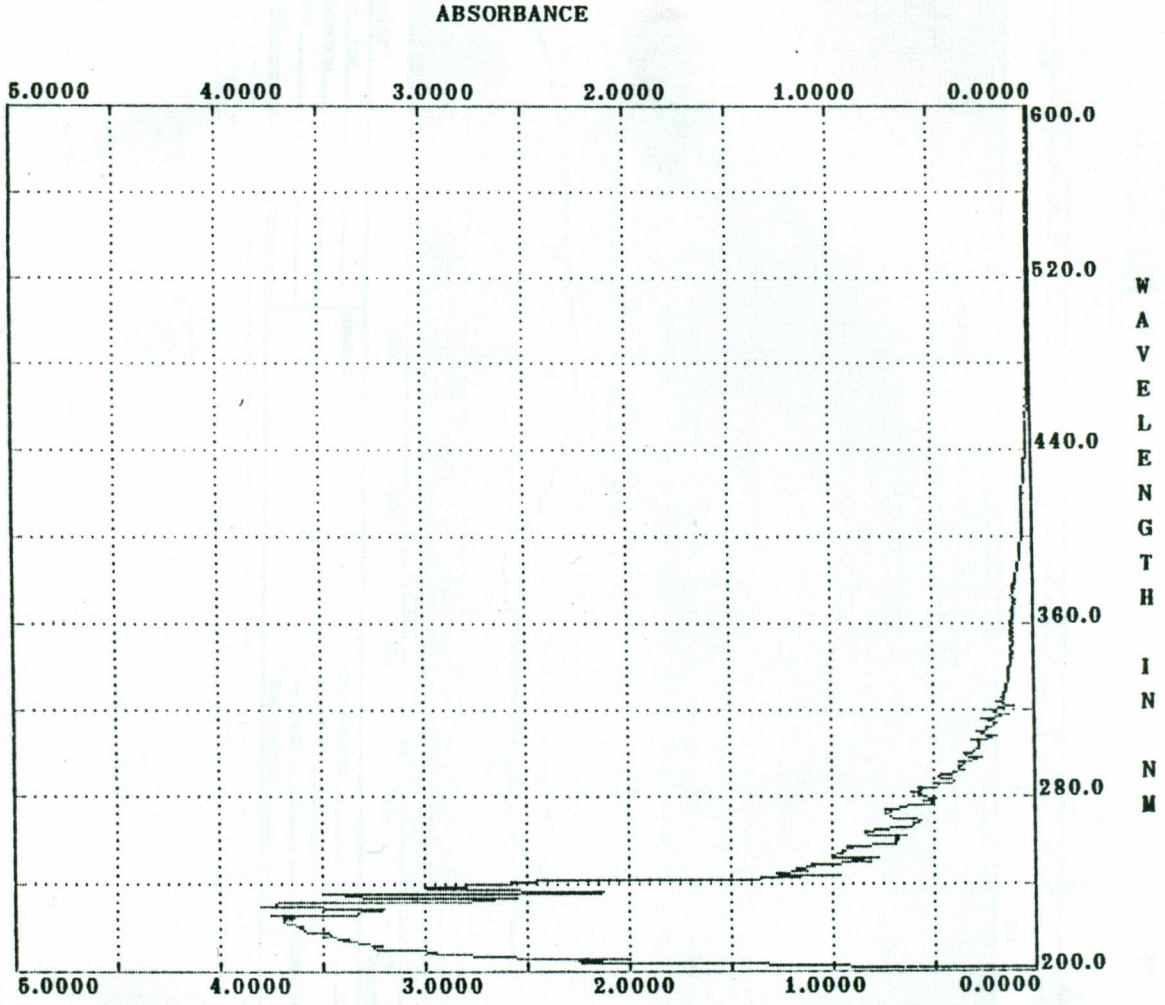

Figure 34. Infra-Red Spectrum of Sample APE in KBr Disc.

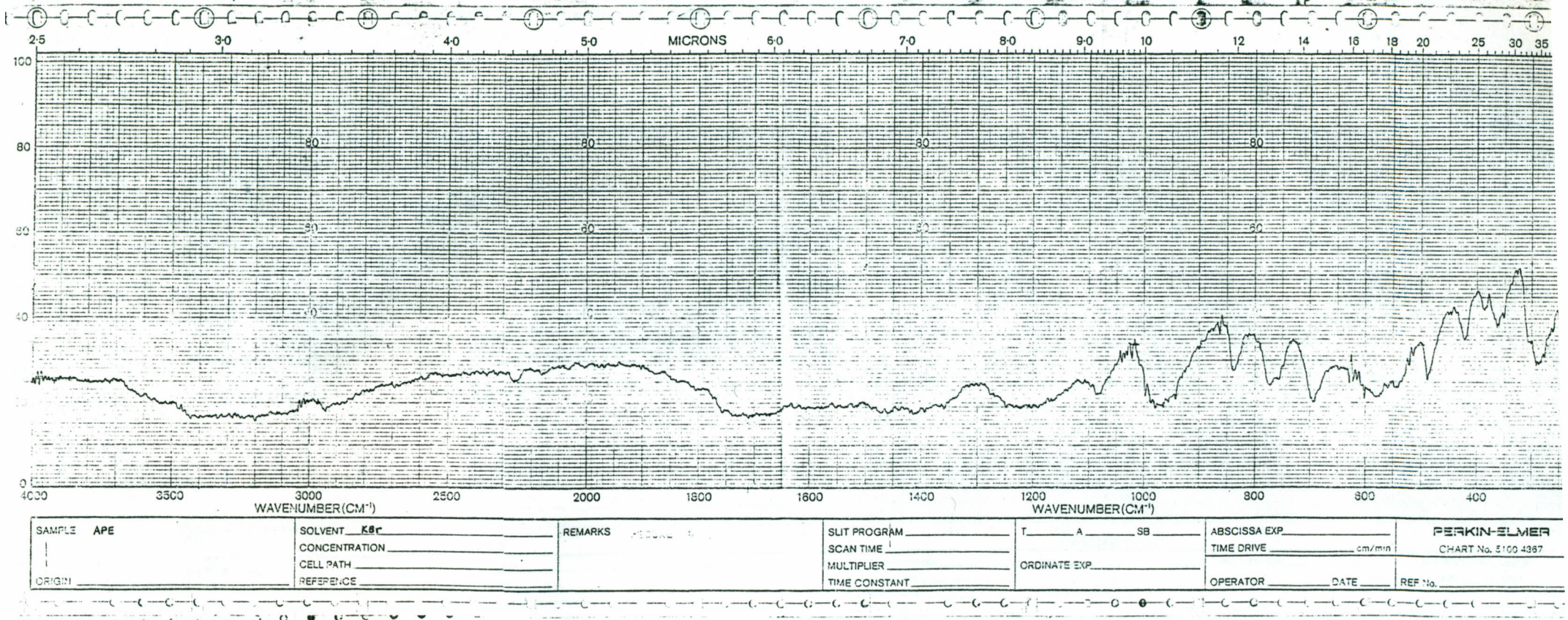

Figure 35. Infra-Red Spectrum of Sample A3 in KBr Disc.