# "Chemical Studies of Hypoglycemic and anti-HIV Extract of *Lagerstroemia speciosa* Linn. (Lythraceae)"

A dissertation in the partial fulfillment of the requirements

for the degree

of

## Master of Philosophy





Submitted by

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TO LATE ALHAJ
MD. NASIR UDDIN

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# CHAPTER ONE: INTRODUCTION

# 1. Introduction

## 1.1 General:

Natural products are traditionally the cornerstones of drug discovery and research in this field continues to provide tremendous variety of lead structures, which are used as templates for the development of new drugs. Despite tremendous advances witnessed in modern medicine through synthesis, chemotherapy, vaccines and diagnostics, even today medicinal plants continue to hold an important part of pharmacopoeias in both the developed as well as developing countries.

The use of medicinal plants for alleviating diseases had its origin in the activities of the most primitive man of the remote past. Illness, physical discomfort, injuries, wounds and fear of death had forced early man to use any natural substance that he could lay his hands on, without any resistance, for relieving their pains and sufferings caused by these abnormal conditions and preserving his health against diseases and death.

Throughout the ages, nature has provided human with sources of the essentials of life, including food, medicines, and raw materials for the manufacture of clothing and shelters. In particular, higher plants have been the source of medicinal agents since earliest times, and today they continue to play a dominant role in the primary health care of about 80% of more than 6 billion inhabitants of the world rely chiefly on traditional medicines for their primary healthcare needs, and it can be safely be presumed that a major part of the traditional therapy involves the use of plants extracts or their active principles.[1] Natural products, and medicinal agents derived therefrom, are also an essential feature in the healthcare systems of the remaining 20% of the population residing mainly in developed countries, with more than 50% of all drugs clinical use having natural product origin.[2] This is because of various reasons: (i) there is historical legacy of folklore, ethnomedicinal uses and well-documented indigenous system of medicine using natural resources for the treatment of diseases, (ii) they are believed to be comparatively safer than drugs of modern medicine, (iii) they are economical; (iv) they are environmentally better suitable to local conditions, and (v) they possess curatives/ preventives for certain disorders for which modern medicine has nothing to provide.[3]

Plants produce within their cells and tissues, through metabolic activities, not only food materials but also the life sustaining oxygen gas and other substances, such as glycosides, steroids, terpenoids, alkaloids, flavones, tannins, pigments etc. These are usually called secondary metabolites that are responsible for their various therapeutic properties and pharmacological actions. The compounds stored in their bodies may exert both harmful and useful effects on human beings. Traditional healers have been using toxic as well as nontoxic materials for treatment of various diseases. But, unfortunately, the traditional practitioners did not document the active principles of these natural products. So, researchers have been trying to find out the active ingredient(s) of toxic and nontoxic plants.

The study of natural product resulted in many therapeutic agents becoming available and many potentials of useful drugs remain to be discovered. There is an urgent need to identify novel, active chemotypes as leads for effective drug development. It is apparent that whatever progress science might have made in the field of medicine over the years, plants still remain the primary source of supply of many important drugs used in modern medicine. Indeed, the potential of obtaining new drugs from plant sources is so great that thousands of substances of plant origin are now being studied for activity against such formidable foes as heart diseases, cancer, diabetes and AIDS. This type of study is sure to bring fruitful results, because of the fact that the plant kingdom represents a virtually untapped reservoir of new chemical compounds, many extraordinarily dynamic, some providing novel bases on which the synthetic chemist may build even more interesting structures. Powerful new chemical and biological techniques now permit isolation and characterization so that drug design principles can be applied rapidly to 'fast track' natural lead as never before. The structure determination of novel plant constituents can now be performed with minimal delay by using a combination of sophisticated spectroscopic and X-ray crystallographic techniques. High throughput automated bioassay are widely available, so that a detailed biological profile can be obtained easily on just a few milligrams of natural product. It has been estimated[4] that only 5-15% of the approximately 250000-500000 species of higher plants (more than 80,000 are medicinal) has been investigated phytochemically and the fraction submitted to biological or pharmacological screening is even smaller. Thus, there are considerable chances of finding new natural compounds with pharmacological activities, useful for the development of new drugs.

**1.2 Medicinal importance of plant materials:**

From ancient times plants have been used as a source of medicines that form the backbone of human healthcare. The use of medicinal plants as a source for relief from illnesses can be traced back over five millennia to written documents of the early civilization in China, India and the near east, but it is doubtless an art as old as mankind.[4] In "*Rig Veda*", which was composed by Aryans in India as early as 3000 years BC, there is mention of the use of 'Soma Rasha' which is the juice of the soma plant (*Amanita muscaria*) and which is used as the elixir of life.[5]

The evidences of the importance of natural products are provided the following facts:

a) Of 119 plant-derived drugs commonly in use in one or more countries, 77% were discovered as a result of chemical studies directed at the isolation of the active constituents of plants used in traditional medicine.[1]

b) Farnsworth *et al.* reported that over 25% of US prescriptions dispensed in 1959-1973 contained active ingredients derived from plants.[6]

b) The cost of plant drugs produced in USA, OECD countries and Japan has been rising and expected to reached $100 billion soon.[7]

c) In 1980 the United States alone paid over US$ 8000 million for prescription drugs in which the active ingredients are still derived from plants.[1] Indeed the total world annual turnover of the industry utilizing medicinal plants have been recently estimated to be of the order of US$ 20,000 million.[8]

d) 47% of some 300 million new prescriptions written by physicians in America in 1961 contained, as one or more active ingredients, a drug of natural origin.[9]

e) About 33% of the drugs produced in the developed countries are derived from plants.[9]

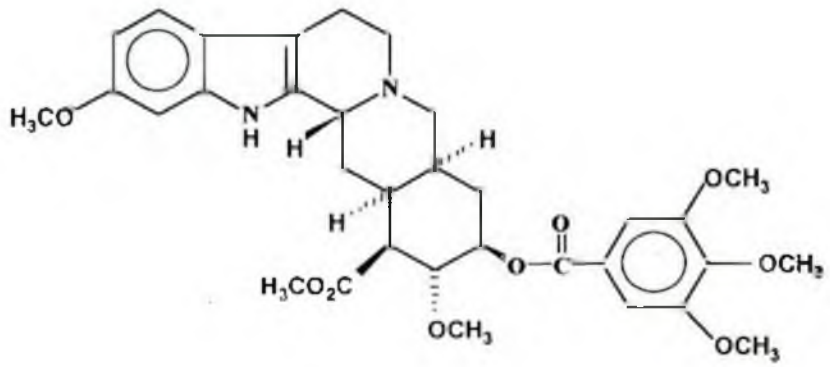
f) More than 47% of all drugs used in Russia are obtained from botanical sources.[9]

g) If microbes are added, 60% of the modern medicinal products are of natural origin.[9]

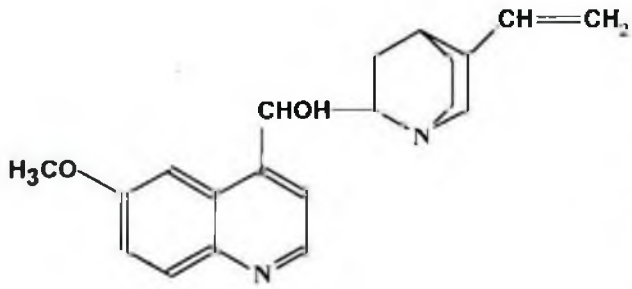
h) In 1967, 824 out of 3354 of the trade name products, which appeared in the 10.5 billion prescriptions filled the US, contained one or more ingredients derived from higher plants.[10]

i) Of the world 25 best-selling pharmaceutical agents, 12 are natural product-derivatives.[11]

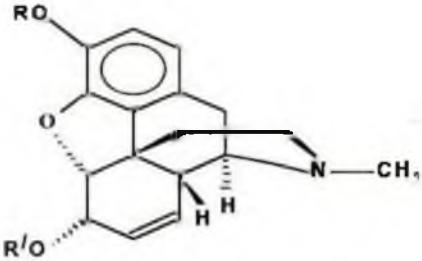
In the modern age, the scientists are endeavoring to isolate different chemical compounds from plants having biological properties. Well-known examples include the cardiac glycosides from *Digitalis purpurea* **L,** the antihypertensive agent and tranquilizer, reserpine (**1**), from the East Indian snakeroot, *Rouvolfia serpentina* (**L**) Bentham ex Kurz; the antimalarial agent, quinine (**2**), from *Cinchona* sp.; the analgesics codeine (**3**) and morphine (**4**), from *Papaver somniferum* **L**; ninbadin from, *Azadirachta indica*; hyosine and atropine (**5**), from *Datura metel*; strychine (**6**), from *Strychnos nux-vomica*, emitine (**7**), from *Cephaelis ipecacuanha* etc.



Reserpine (**1**)

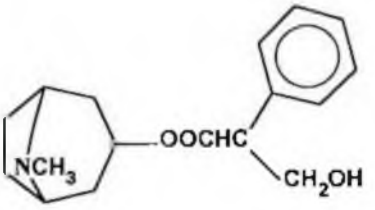


Quinine (**2**)

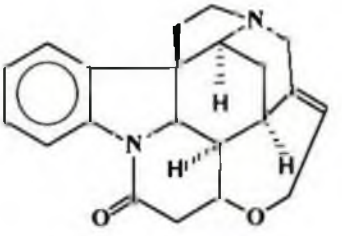


R=R$^{/}$=H Morphine (**4**)

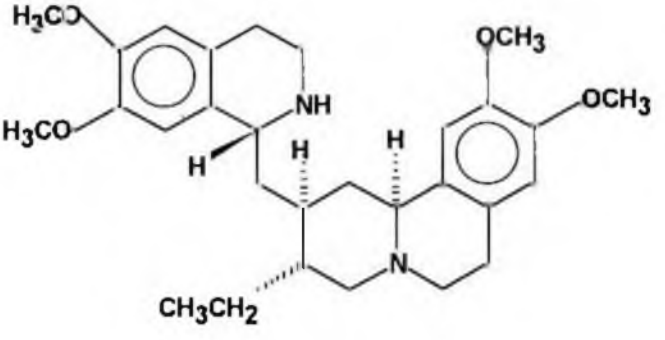
R=Me R$^{/}$=H Codeine (**3**)




Atropine **(5)**

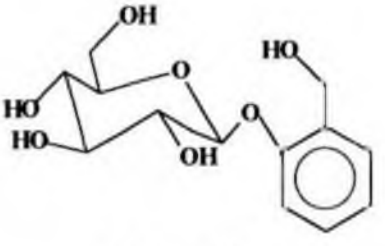



Strychine **(6)**



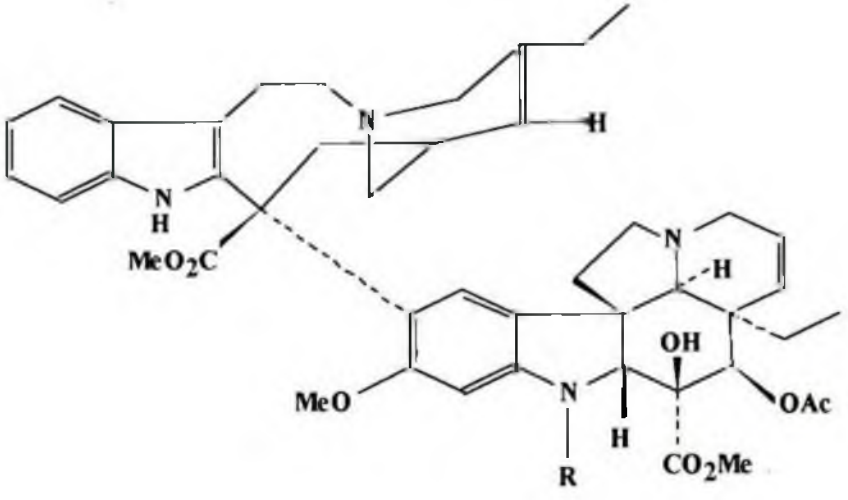

Emetine (**7**)

Around at 500 BC the Greek physician Hypocrites recommended the use of willow bark extracts to alleviate the pain of childbirth and treat eye disease. Salicin **(8)** is the natural substance responsible of the willow tree for the pain relief.[12]




Salicin (**8**)

A strong effort has been put into the search for novel anticancer agents since the early 1950s. The first clinically useful compounds to be isolated were the *Catharanthus* alkaloids. The antineoplastic activity of *Catharanthus roseus* G. Don (Apocynaceae) was independently discovered by Canadian and American research teams. Combined efforts led to the isolation and structure elucidation of the active bis-indole alkaloids vinblastine (**9**), vincristine (**10**), leurosine and leurisidine in the late 1950s and early 1960s.[4] Two of them, vinblastine and vincristine have been developed as commercial drugs. The two compounds differ in their clinical utility and toxicity. The major use of vinblastine is the treatment of patients with Hodgkin's disease, non-Hodgkin's lymphomas and renal, testicular, head and neck cancer.

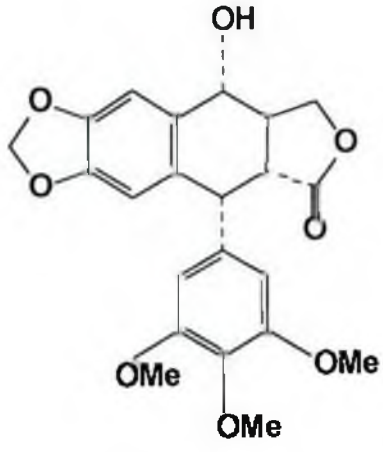



Vinblastine, R=$CH_3$ (**9**)

Vincristine, R=CHO (**10**)

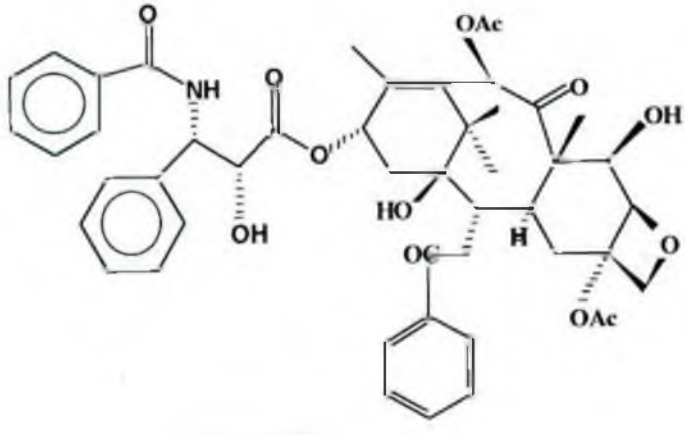
Vincristine is widely used, in combination with other anticancer agents, in the treatment of acute lymphocytic leukemia in childhood, and for certain lymphomas and sarcomas, small cell lung cancer, cervical and breast cancer.[13] Navelbine® (noranhydrovinblastine) is currently used, worldwide in the treatment of non-small cell lung cancer and of breast cancer. It is orally active and its use will almost certainly be extended to the treatment of other cancerous diseases.

Pondophyllotoxin (**11**), isolated from mayapple (also known as American mandrake) *Podophyllum peltatum* L, and its modified form are used as antineoplastic agents. These are also useful in the chemotherapeutic treatment of refractory testicular carcinomas, small cell lung carcinomas, and nonlymphocitic leukemia and non-Hodgkin's lymphomas.[4]




Podophyllotoxin (**11**)

In 1971, a novel compound, taxol (**12**) (also known in the literature as paclitaxel) isolated from the bark of the northwest Pacific yew tree, *Taxus brevifolia* Nutt., demonstrated moderate *in vivo* activity against the P-388, P-1534, and L-1210 murine leukemia, the Walker 256 carcinosarcoma, sarcoma 180, and Lewis lung tumor test systems.[14] In the more than twenty years since the initial report of its isolation, structure elucidation, and bioactivity, taxol has garnered support as an anticancer agent, culminating in recent FDA approval of its use against breast and ovarian cancers.

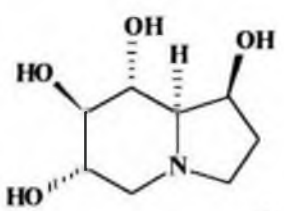



Taxol (**12**)

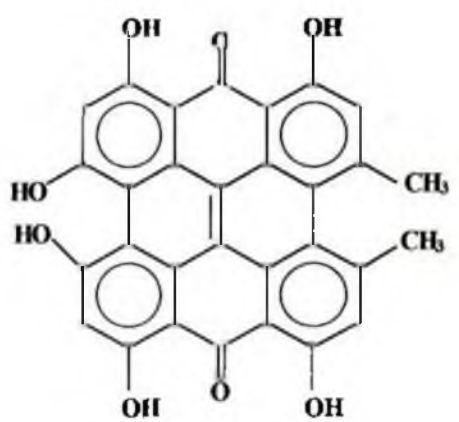
There are two main reasons for the attention directed towards this drug. First, it shows promise against refractory breast and ovarian cancers, which are difficult to treat and which are responsible for the deaths of 60,000 women every year[14,15]. Second, it exhibits a model of action unprecedented among cancer chemotherapeutic agents. Taxol targets microtubule formation, but in a unique fashion. Unlike known antimicrotubule agents, which block microtubule production, taxol promotes tubulin polymerization and stabilizes microtubules against depolymerization. Microtubules are an important subcellular target for chemotherapeutic agents[16,17].

The tetrahydroxyindolizidine alkaloid castanospermine[18] **(13)** occurs in the toxic chestnut-like seeds of the evergreen Australian tree *Castanospermum australe* A. Cuun et Fras. (Leguminosae) was found to inhibit replication of HIV. The compound is a potent inhibitor of $\beta$-glucosidase, $\beta$-glococerebrosinas and lysosomal $\alpha$- and $\beta$ - glucosidases[19].

Hypericin **(14)**, a potential anti-AIDS drug, is a red-colored natural compound belongs to the class of napthodianthrones. Hypericin is a constituent of a number of plants of the genus *Hypericum*, widely distributed around the world, the most common of which is *Hypericum perforatum*. It also appears in other Hypericum species, like *H. hirsutrum, H. maculutum, H. nummularium*, and *H. triquetrifolium*[20].




Castanospermine **(13)**

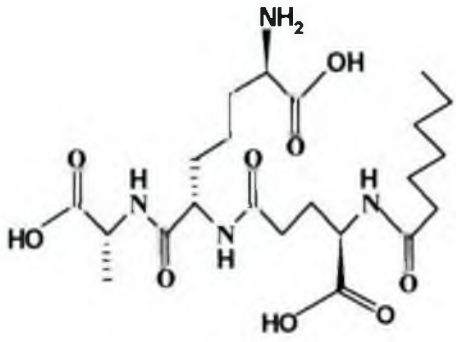



Hypericin **(14)**

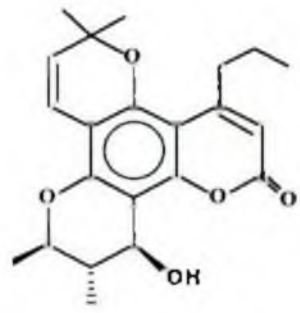
Calanolide A (**15**) is a reverse-transcriptase inhibitor discovered from the Malaysian rainforest tree, *Calophyllum lanigerum* by the U S. National Cancer Institute.

In vitro studies of (+) calanolide A have demonstrated the compound to be effective against HIV-1, including strains resistant to AZT and other non-nucleoside reverse-transcriptase inhibitors. It also exhibited synergistic anti-HIV activity in combination with nucleoside reverse-transcriptase inhibitors, including AZT, ddI (dideoxyinosine), and ddC (dideoxycytidine)[21] (AZT, ddI, ddC are drugs that inhibit HIV through inhibition of reverse transcription).

The immunostimulating peptide FK-156, a metabolite of *Streptomyces* sp. was discovered through its antitumor activity against murine P388 leukemia by Fujisawa. The activity appeared to be a host-mediated effect. FK-565 (**16**), a synthetic tripeptide analogue of FK-156, was shown to act by phagocyte, especially macrophage, activation,



FK-565 (16)

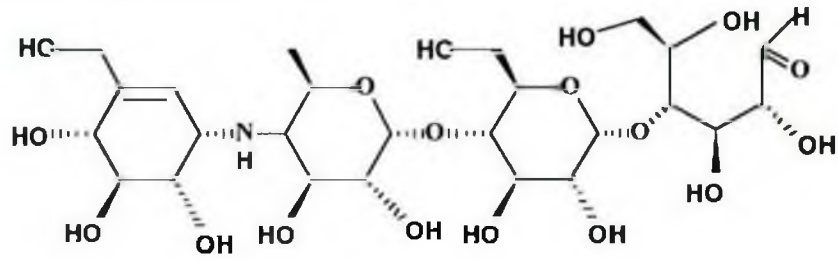


Calanolide A (15)

as it induced IL-6 and granulocyte macrophage colony-stimulating factor (GM-CSF) released from lipopolysaccharide-stimulated peritoneal macrophages, and augmented natural killer cells, macrophage, and T cells *in vivo*. FK-565 was brought into phase II clinical trials in the U.S. for the treatment of cancer and AIDS[22].

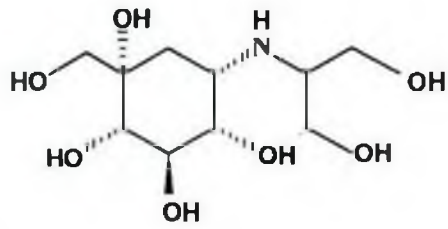
Galegine, a guanidine-type alkaloid, was found to be the active principle of *Galega officinalis* L., and was used clinically for the treatment of diabetes. Before the discovery of this, it had been known that guanidine itself had anti-diabetic properties, but was too toxic for human use. After hundreds of synthetic compounds were prepared, metformin, a close relative to galegine, was developed and marketed as a useful anti-diabetic drug[23].

In the antidiabetes area, the past decade has witnessed the market introduction of several $\alpha$-glucosidase inhibitors derived from natural products. Acarbose (**17**), a complex of oligosaccharides isolated from *Actinoplanes* sp., was discovered at Bayer from a search for $\alpha$-glucosidase enzyme inhibitors.

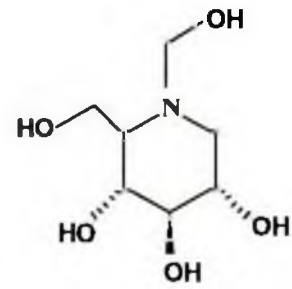


Acarbose (**17**)

By inhibiting $\alpha$-glucosidase, acarbose decreases the release of glucose from ingested carbohydrate and slow the increase of food-induced blood glucose levels. Acarbose is now approved in Germany, Japan, the U.S. and other countries and has been used as adjuvant therapy in diabetes[24,25]. Several known aminoglycoside and pseudoligosaccharide antibiotics were also found to exhibit potent inhibitory effects against intestinal $\alpha$-glucosidase. Takeda's voglibose (**18**) is the synthetic analogue of valiolamine, a *Streptomyces* aminocyclitol; voglibose was introduced in Japan in 1993



Voglibose (**18**)



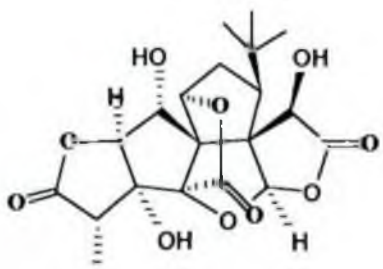
Miglitol (**19**)

for the treatment of diabetes and obesity, and it is much more potent and has fewer side effects than acarbose[26]. Miglitol (**19**) is a hydroxyethyl derivative of 1-deoxynojirimycin synthesized at Bayer for which satisfactory phase III results were obtained, and Bayer has submitted the NDA of miglitol for regulatory approval for the treatment of type I/II diabetes and expects to market it in 1998[27].

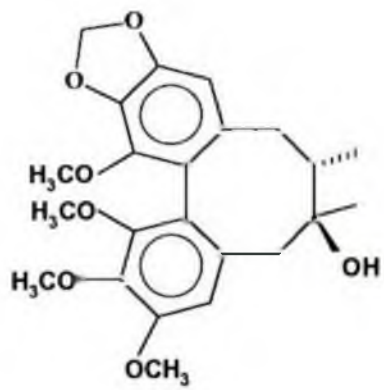
Shaman Pharmaceuticals has focused its discovery of oral antihyperglycemic agents for the treatment of Type II diabetes primarily on the screening of medicinal plants in animal models[28]. A number of orally active natural products were shown to reduce blood glucose in these in vivo models of Type II diabetes.

In 1967, when K. Nakanishi and his group at Columbia University established the elegant structure of four closely related substances ginkgolide A, ginkgolide B, ginkgolide C and ginkgolide D, known collectively as the ginlgolides, a class of unique diterpene caselike molecule was isolated from the leaves of *Ginkgo biloba*. It was found in the late 1980s that ginkgolides represent a group of highly selective platelet-activating factor (PAF) receptor antagonists. Among them, ginkgolide B (BN-52021) **(20)** has been advanced to phase III clinical trials by the Frence pharmaceutical company Ipsen-Beaufour for the treatment of septic shock in patients with severe sepsis caused by Gram-negative bacterial infections. Good results were also found in inflammatory and autoimmune disorders[22].

Gomisin A (**21**) is a lignan derivative isolated from the dry fruit *Schisandra chinensis*, a traditional Chinese medicine used for treatment of liver intoxication. Gomisin A was found to protect against hepatocarcinogenesis and liver damage in various animal models[22].

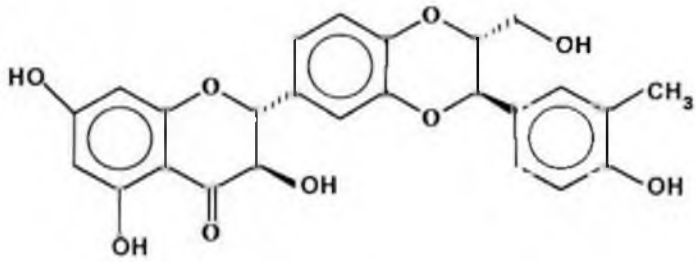



Ginkgolide B (**20**)



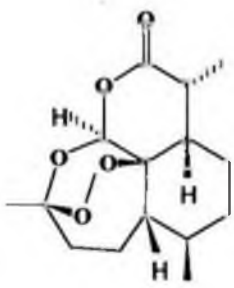

Gomisin A (**21**)

Another hepatoprotectant, Idb-1016, is also derived from the fruits of *Silybum marianum*, Idb-1016 (silipide) is a complex of the flavonolignan antioxidant, silybin **(22)**, and phosphatidylcholide. It is currently in phase III clinical development as an antihepatotoxic agent by Inverni Della Beffa (Synthelabo) in Italy[22].

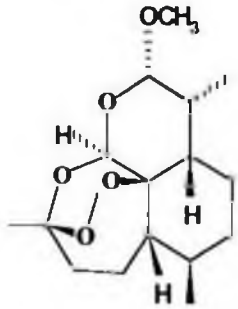



Silybin **(22)**

Malaria is still the most important tropical disease and the number of clinical cases is estimated to *ca.* 200 million annually. The most promising anti-malarial compound discovered so far is artemisinin (**23**) isolated in 1972 by Chinese scientists from the medicinal plant quighao (*Artemisia annus* L., Asteraceae). Artemisenin possesses a unique endoperoxide group that distinguishes it from the old generation of antimalarial drugs. It is effective in treating chloroquine-resistant cases and other severe cases without major toxicity. Artemether (**24**) a synthetic analogue of artemisinin, has been developed in the People's Republic of China. Two recent comparative clinical studies suggested that artemether is as effective as quinine in the treatment of severe malaria. Qinghao has been used for over 2000 years in China as febrifuge and in malaria therapy[29].

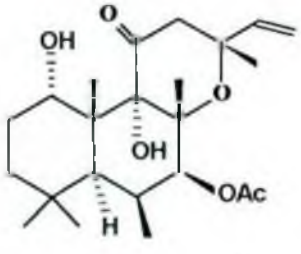



Artemisinin **(23)**

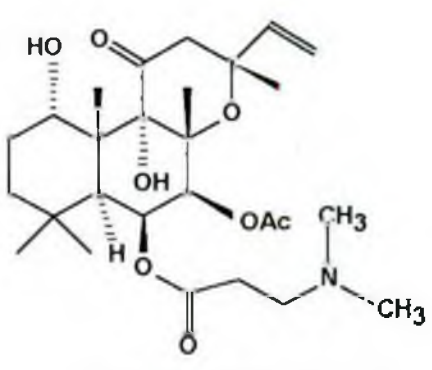



Artemether **(24)**

Forskolin (colforsin) (**25**), is a diterpene natural product isolated from the Indian plant *Coleus forskohlii* at Hoechst's research laboratories in India. The compound was first found to have blood pressure lowering and cardioactive properties. Later, forskolin was found as a potent adenylate cyclase activator[30]. Nippon Kayaku's semisynthetic efforts generated a water-soluble forskolin derivative, colforsin daproate (NHK-477) (**26**). The compound was shown to have reversible effects on the respiratory, circulatory, and autonomic nervous systems. Preliminary clinical trials of colforsin daproate demonstrated beneficial hemodynamic effects in heart failure patients. Colforsin daproate was then brought into phase II clinical trials in Japan for treatment of cardiac insufficiency and phase II trials for treatment of asthma[31].

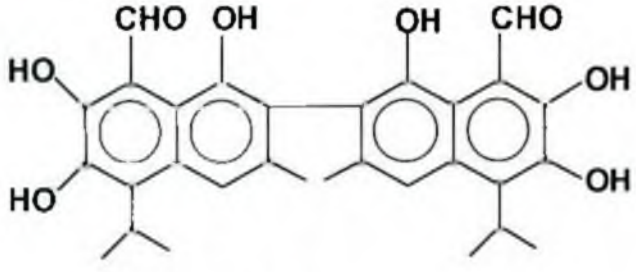



Forskolin (**25**)




Colforsin daproate (**26**)

The dimeric sesquiterpene gossypol (**27**), which occurs in the seeds of *Gossypium* species (Malvaceae), is one of the most interesting compounds for birth control activity[32,33].




Gossypol (**27**)

The data shown in the figures and tables listed below highlight[34] the invaluable role that natural products have played, and continue to play, in the drug discovery process related to all disease types (**Figure 1; Table 1**) and infectious diseases (**Figure 2; Table 2**).

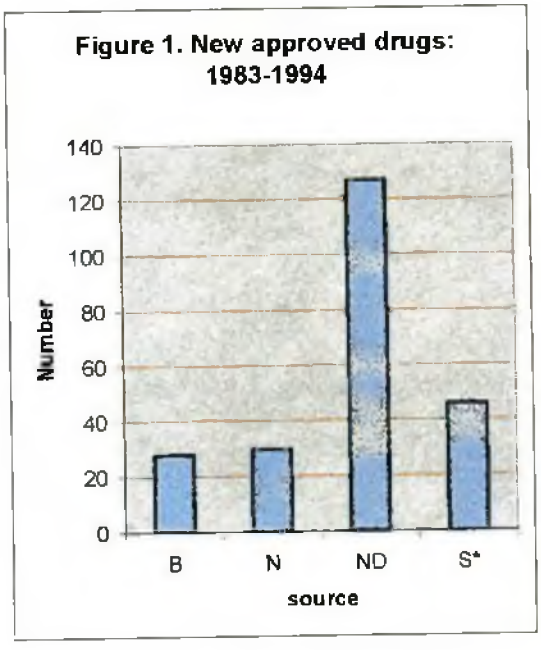



Figure 1. New approved drugs: 1983-1994[34].

The data have been analyzed in terms of number classified according to their origin as indicated below:

**B**: Biologics (vaccines, monoclonal, etc. derived from mammalian sources).

**N**: Derived from an unmodified natural product source.

**ND**: Derived from natural product source semisynthetics

**S**: From a synthetic source, but originally model on a natural product parent

**Table 1. New Approved Drugs: 1983-1994**[34]

| Diseases indication | Origin of drug | | | |
|---|---|---|---|---|
| | B | N | ND | S |
| Antiallergesic | | | 1 | |
| Antiarrhythmic | | | | 1 |
| Antibacterial | | 6 | 44 | |
| Anticancer and adjuvants | 6 | 4 | 11 | 4 |
| Antidiabetic | | 2 | | |
| Antiemetic | | | 1 | |
| Antifungal | | | 2 | 2 |
| Antiglaucoma | | | 2 | 2 |
| Antihistamine | | | | 25 |
| Anti-inflammatory | 1 | | 12 | |
| Antithrombotic | 2 | | 12 | |
| Antiulcer | | 1 | 9 | |
| Bronchodilator | | | 2 | |
| Calcium regulator | | | 1 | |
| Hormone | | | 4 | |
| Hypochlesterolemic | | 2 | 1 | |
| Hypolipidemic | | 1 | 1 | |
| Immunostimulant | 2 | 3 | 1 | |
| Immunosuppressant | 1 | 2 | 1 | 1 |
| Neoroleptic | | 1 | 1 | |
| Neotropic | | 1 | 1 | |
| Progestogen | | | 4 | |
| Miscellaneous | 16 | 8 | 26 | 2 |
| Total | 28 | 31 | 137 | 37 |

Of the new approved drugs reported between 1983 and 1994 (**Table 1**), drugs of natural origin (N and while 61% of the 31 anticancer drugs (excluding the six biologics) are naturally-derived (N and ND) or are modeled on a natural product parent (S).

**Table 2. New antiinfective Drugs, 1983-1994**[34]

| Generic name | Generic name |
|---|---|
| Compound source=N | |
| Drug use=antibacterail | |
| RV-11 | Miokamycin |
| Carumonam | Mupirocin |
| Isepamicin | Teicoplanin |
| Compound source=ND | |
| Drug use=antibacterail | |
| Arbekacin | Cefprozil |
| Aspoxicillin | Ceftazidime |
| Astromycin sulfate | Cefteram pivixil |
| Azithromycin | Ceftibuten |
| Aztreonam | Cefuroxime axetil |
| Cefbuperazone sodium | Cedfuzonam sodium |
| Cefdinir | Clarithromycin |
| Cefditoren pivoxil | Dirithromycin |
| Cefepime | Erythromycin acistrate |
| Cefetamet pivoxil HCl | Flomoxef sodium |
| Cefixime | Imipenem/cilastatin |
| Cefmenoxime HCl | Lenampicillin HCl |
| Cefminox sodium | Loracarbef |
| Cefodixime sodium | Merpenem |
| Ceforanide | Panipenem/betamipron |
| | Rifabutin |
| Cefotetan disodium | Rifapentine |
| Cefotiam hexetil HCl | Rifaximin |
| Cefpimizole | Rokitamycin |
| Cefotetan sodium | Roxithromycin |
| Cefpirome sulfate | Sultamycillin tosylate |
| Cefpodoxime proxetil | Temocillin disodium |
| Compound source=N | |
| Drug use=antimalarial | |
| Artemisinin | |
| Compound source=ND | |
| Drug use=antimalarial | |
| Mefloquine HCl | |
| Compound source=S | |
| Drug use=antiviral | |
| Didanosine | Stavudine |
| Famciclovir | Zalcitabine |
| Ganciclovir | Zidovudine |
| Sorivudine | |

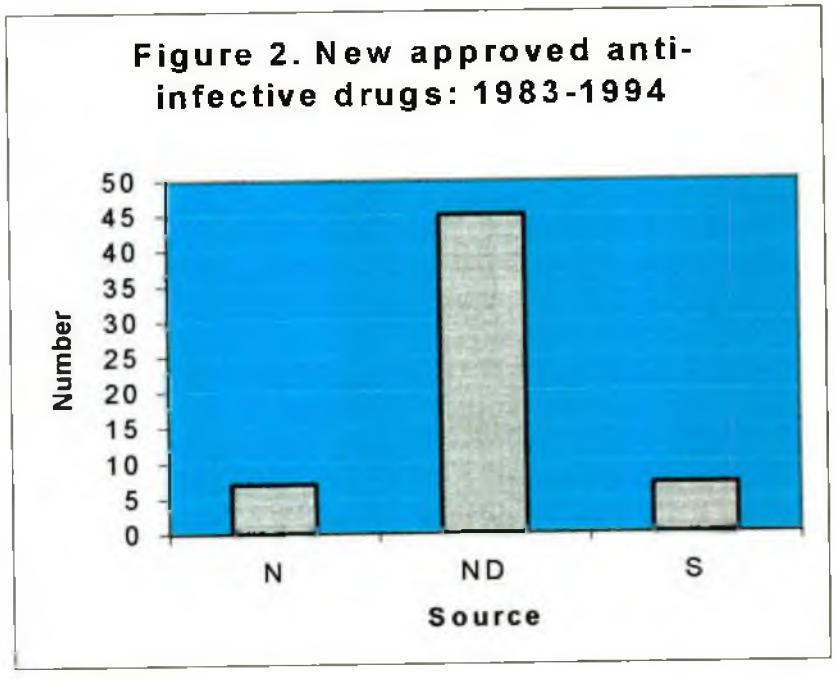



**Fig 2**: New approved antiinfective drugs: 1983-1994.

Of the 93 new approved antiinfective (**Table 2, Figure 2**), seven are actual natural products and 45 are semisynthetic derivatives, with seven based on natural product models (S) and no biological, giving a total of 63% of natural origin[34].

**1.3 Lipids, fats and fatty acids:**

Fatty acids are always present in varying amounts in all plant materials. These are important as membrane constituent in the chloroplasts and mitochondria. These substances also occur in considerable amount in the seeds or flowers of a number of plants and act as a storage form of energy to be used for germination. These fatty materials may influence the handling of the plant tissues as well as any chemical treatment done on it. Some fatty acids, which were isolated from the fungus, posses anti-biotic properties.

Lipids are important constituents of plant and animal tissue. They are comprised of different kinds of compounds. They are water-insoluble but very soluble in hydrocarbons and ethers.

Lipids are esters of long-chain carboxylic acids, which are usually unbranched. On the basis of the molecular structure, lipids can be divided into seven major classes[35]. These are:

i) Triacyl glycerol or triglyceride or fat
ii) Phosholipid
iii) Sphingolipid
iv) Glycolipid
v) Steroid
vi) Terpenoid
vii) Wax.

Fats are the most important members of this class, and are widely distributed throughout the animal and plant worlds.

Fats are esters of glycerol. These compounds are known as glyceryl esters. They are either oily liquids or waxy solids and contain the element carbon, hydrogen, and oxygen only. Individual fat is usually a mixture of esters of glycerol and two or three different fatty acids.

It has been established that fats are principally mixtures of mixed esters, that is, the general structural formula of a fat molecule is,

$CH_2OOC\text{-}R^1$
|
$CH\ OOC\text{-}R^2$
|
$CH_2OOC\text{-}R^3$

In which $R^1$, $R^2$, and $R^3$ may be same or different hydrocarbon radicals.

When fat is hydrolyzed, it gives a mixture of fatty acids. Fatty acids are long chain carboxylic acids. They are sparingly soluble in water. The fatty acids contain only one carboxylic group and thus monobasic. They fall roughly into three groups: saturated, unsaturated, and those, which contain branches or rings.

Palmitic and stearic acids are the most commonly encountered saturated fatty acids. Oleic acid, linoleic acid etc. are the common unsaturated fatty acids. The unsaturated acids can also exist in both *cis*-and *trans*-forms. The methylene groups bound to the double bonds in unsaturated oils are subject to chemical attack by oxygen.[36] A complex series of reaction results, which involve air-oxidation, hydroperoxide formation, polymerization, and cross-linking of polymer chains. The oxygen-initiated polymerization is responsible for use of multiply unsaturated fats as drying oils. Thus linseed oil is used as a binder and carrier of pigment in paints. Unsaturated acids are characterized by having one or more double bonds or ethylenic groups. These could be the following types: (i) monoenoic, (ii) dienoic, (iii) trienoic and (iv) polyenoic. Fats that process branched or cyclic fatty acid chains like hydnocarpic acid, gorlic acid, are relatively rare.[36]

Mammals and plants contain monoenoic and polyenoic fatty acids, whereas in bacteria, all the fatty acids containing double bonds are monoeoic. Plant and fish fats contain more multieoic fatty acids than animal fats.

Fatty acids required in the diet of mammals are called essential fatty acids. Linolic acid is the most abundant fatty acid in mammals, which makes up 10-20% of fatty acids for their

triacyl glycerols and phosphoglycerides. The most abundant fatty acids in natural glycerides are palmitic, stearic, oleic, and linolic acids. The plant glycerides contain relatively higher proportion of unsaturated acids.

Fatty acids are generally found[37] in ester linkage but are also found free and in loose molecular complexes in protein.

Fatty acids are weak electrolytes. They react with bases and alcohol, to give salts and esters, respectively.

### 1.4 Fatty acids in plant materials and their importance:

Although alkaloids, steroids, flavones and their glycosides, phenols and phenolic acids constitute a major portion of non-carbohydrate materials of a plant, fatty acids are always present in varying amounts in all plant materials.

The fatty acids that occur in nature usually have straight chains, and usually contain an even number of carbon atoms.

Fatty acids are insoluble in water and are extracted from plant tissues by organic solvents of low polarity like petrol, hexane, ether or chloroform. Although numerous fatty acids are now known in plants, the palmitic acid (C-16) is the major saturated acid in leaves lipids and also occur in varying quantities in some seeds oils. Stearic acid (C-18) is the major saturated acid in seed fats of a number of plant families. Unsaturated acids (mainly C-16 and C-18) are widespread in both leaf and seed oils. A number of rare fatty acids (e.g. erucic and sterculic acid) are found[38] in seed oils of a few plants. The plant glycerides have a relatively higher proportion of the more unsaturated acids.[39]

Fatty acids occur in plants primarily in bound form as fats or lipids. These lipids comprise up to 7%[38] of the dry weight in leaves in higher plants, about 1-5% in stems of green plants.[40] These are important as membrane constituent in the chloroplasts and mitochondria. These substances also occur in considerable amounts in the seeds or fruits

of a number of plants and act as a storage form of energy to be used during germination. These fatty materials may influence the handling of the plant tissues as well as any chemical treatment done on it.

A number of novel fatty acids that possess acetylenic and, occasionally, allenic linkages have been isolated from plant microorganisms. One of the most interesting of these is mycomycin, which is produced by the fungus Norcardia acidophilus. Another fatty acid, nemotinic acid, is isolated from a fungus, which is optically active and possesses antibiotic properties.[36]

Seed oils from plants such as olive, palm, coconut etc are exploited commercially and are used as edible oils, for soap manufacture and in the paint industry. Fats are sometimes converted into the methyl esters of carboxylic acids by the reaction with methanol in presence of a catalyst. These methyl esters can be catalytically reduced to straight chain primary alcohol of even carbon number, and from these a host of compounds can be synthesized. Therefore, the study of fatty acids, the major constituent of all fatty matters is important.

## 1.5 Analysis of fatty acids in different plant materials:

Analysis of fats and lipids are usually done by saponification of hexane/ dichloromethane extracts of plant materials and subsequent extraction with hexane after acidification. Fatty acids thus obtained in hexane are analyzed such as by High Performance Liquid Chromatography (HPLC) or esterified as methyl esters and analyzed by Gas Liquid Chromatography (GLC) or Gas Chromatography- Mass Spectroscopy (GC-MS).

Analysis of fatty of *E. heliosespia* seeds showed that it[42] contained 28% oil with lauric acid 2.85% myristic acid 5.49%, palmitic acid 9.88%, stearic acid 1.13% oleic acid 15.80% linoleic acid 22.14% and linolenic acid 42.71%.

Analysis of fatty acid contents of *Dillenia indica* fruit pulp showed that[43] the pulp contained lauric acid, palmitic acid, linoleic acid and behenic acid with the amount of 3, 28, 27, 19 and 23 mg/100 g of dry pulp, respectively.

It was found[44] that palmitic, oleic and linoleic acids were predominant and traces of capric, myristic, stearic and linolenic acids were present in kernel of rice.

The bark and stem of unretted jute plants[45] (*Corchorus capsularis* and *Corchorus olitorius*) were found to contain myristic, palmitic, stearic, oleic and linoleic acids.

It is found that safflower seed oil contained[46,47], 76% linoleic acid, 17% oleic acid and 2-5% saturated acids.

**1.6 Biological activity:**

The extracts of *Lagerstroemia speciosa* were tested by our research group for their anti-HIV and antidiabetic activities. The result showed that the extracts had significant anti-HIV and antidiabetic activities. The report of National Cancer Institute (NCI) showed that *Lagerstroemia speciosa* had high anti-HIV activity. According to *in vitro* Anti-HIV Drug Screening Results on primary screen, it was found that *ca.*58% and 55% viruses were inhibited described in **Fig. 4, 5**.

It is interesting that among the numerous medicinal plants *Lagerstroemia speciosa* has also tremendous antidiabetic activity. All parts of the plant have hypoglycemic activity. Different parts of *Lagerstroemia speciosa* plant have been used as medicine for the management of diabetes for a long time.

Literature survey showed[48,49] that all parts of the plant, *Lagerstroemia speciosa*, particularly methanol extracts of old leaves and bark have hypoglycemic activity ($p<0.01$) and ripe fruits contain hypoglycemic principles having activity equivalent to 6.0-7.7 units of insulin. In addition to that it has been used in the treatment of flow of urine, fever, diarrhea, ulcer etc.

## 1.7 A brief introduction to AIDS and diabetes:

As *Lagerstroemia speciosa* was selected for studying its possible anti-HIV and antidiabetic activities, before describing the plant and its medicinal importance, a brief introduction to AIDS and diabetes are given below.

### HIV and AIDS:

Infectious viral disease remains an important worldwide problem, because viruses resisted therapy longer than any other organism. The incidence of microbial infections are increasing alarmingly with the increase of sizable susceptible population of immunocompromised patients (ICP) including AIDS and AIDS-related Complex (ARC). AIDS (Acquired Immunodeficiency Syndrome) is a disease caused by a retrovirus, HIV (Human Immunodeficiency Virus), and characterized by failure of the immune system to protect against infections and certain cancers[50].

The World Health Organization (WHO) estimates[51] that by the year 2000, 90% of the AIDS cases will be notified from developing countries. In some countries AIDS became the 11$^{th}$ main cause of death (8$^{th}$ among male and 20$^{th}$ among female), when analyzed by age, AIDS was the second main cause of death, both sexes combined in the 20-49 age group[51]. Currently two kinds of therapeutic approaches are used for treatment of AIDS. One is to attack HIV reverse transcriptase, which is responsible for the viral genome transcription. The other is to inhibit HIV protease (HIV-1PR), which is essential for the processing of viral proteins and is regarded as one of the promising targets for development of anti-HIV agents[52]. Drug combinations based on these approaches can reduce the blood virus to an undetectable level. A small amount of virus, however, may still lurk inside the immune cells in a dormant state[53,54]. Another major obstacle of long term treatment of the disease is the remarkable tendency of HIV to mutate. Most, if not all, of the clinical chemotherapeutic agents for AIDS have shortcomings in terms of clinical resistance by HIV. Their high cost and severe side-effects further reduce the desirability of the currently available anti-HIV drugs. New anti-AIDS chemotypes should be of low toxicity and of potentially low cost.

**Diabetes mellitus:**

Diabetics is a Greek word, which means "Syphor". So diabetes is a disease characterized by polyurea. Latin word mellitus means "Honey". So diabetes mellitus literally means sweet (honeyed) polyurea.

Glucose is either absorbed in the intestine or produced by liver, the sugar travels in the blood to all the tissues of the body. There it serves as a source of energy and as a primordial precursor for other carbon-containing compounds.

When one eats carbohydrates, it is digested to glucose in the intestine, which it transfers to the blood stream. The resulting rise in the blood glucose stimulates the beta cells of the pancreas to release a hormone, insulin. Insulin sweeps out the glucose from the blood by either preventing the liver to release additional glucose or causing the muscle or fat cells to absorb more of the sugar. Muscle cells converts the glucose to glycogen, a polymerized carbohydrate that can be easily converted to glucose, when it is necessary. Fat cells convert glucose into droplets of fat for long-term storage. As blood glucose levels drop, the beta cells stop further secreting insulin, and the body's metabolism returns to the basal state[55].

When there is too little insulin or when muscle or fat cells resist its effect, blood glucose level rises all the time, producing hyperglycemia. The high concentration of sugar molecule creates an osmotic imbalance, so that the blood draws water from the cells and the kidneys excrete the water in the urine, along with an excess of salts. Dehydration and salt loss brought on by severe hyperglycemia can lead to coma and death. Milder hyperglycemia commonly called diabetes mellitus.

Diabetes mellitus is clinically defined as a syndrome characterized by hyperglycemia and polydypsia. Diabetes mellitus is a genetically determined disorder of the metabolism of carbohydrate, fat and protein associated with a relative of absolute insufficiency of insulin secretion and with varying degrees of insulin resistance. In its fully developed clinical expression it is characterized by fasting hypoglycemia and in the majority of

long-standing patients by microangiopathic vascular complications, especially in the eye and kidney, by an increased frequency of macrovascular disease such as coronary, heart and peripheral vascular disease and by neuropathy. The term diabetes does not denote a single disease entity but rather a heterogeneous group of disorders. Heterogeneity implies that there are differences among various groups of patients in terms of etiology and pathogenesis (genetic, environmental and immune factors), in natural history and in response to treatment. Diabetes therefore is not a single disease but a syndrome[56].

Hence, diabetes is termed as heterogeneous group of physical and metabolic disorders chemical evidences point to the conclusion that four types of diabetes generally occur in human body. They are as follows:

(i) Type 1: Insulin dependent diabetes mellitus (IDDM).
(ii) Type 2: Non insulin dependent diabetes mellitus (NIDDM).
(iii) Type 3: Malnutrition – related diabetes mellitus (MRDM).
(iv) Type 4: Impaired glucose tolerance (IGT).

NIDDM patients are two types (a) non-obese and (b) obese. Malnutrition – related diabetes mellitus (MRDM) is common to third world countries. IGT patients are also non-obese and obese. IGT is associated with certain conditions (e.g., drugs, pancreatic disease) and syndromes[56].

Diabetes mellitus is known to mankind in the dawn of the civilization. The problem of diabetes in the world is unbelievably serious and becoming worse because in spite of all efforts, diabetes are being diagnosed more often and living longer to suffer complications. Diabetes mellitus is one of the common chronic diseases of the present time. Diabetes mellitus is a leading cause of blindness, carries 2-3 times higher risk of heart attack, an even higher risk of stroke, and 5 times or greater risk of nephropathy.

The World Health Organization (WHO) estimates[57] that approximately 135 million people suffer from diabetes mellitus worldwide, and that this number will rise to almost 300 million by the year 2025.

Bangladesh is not an exception to the statistics. With change of life style in Bangladesh (rural as well as urban areas) diabetes is also increasing day by day. Recent statistics show that by the end of 2000, 7.5 million people in Bangladesh will be suffering from IGT and NIDDM and in 2005 it will be raised to more than 8.7 million[57].

Unfortunately, there is no reliable drug in respect of development of scientific knowledge so far we have. Keeping these facts in mind, we turned in searching of natural drugs those having hypoglycemic and anti-HIV activities. Isolation of pure compound(s) from plant extracts showing positive biological activities was also undertaken.

## 1.8 Traditional uses and pharmacology of plants in the treatment of diabetes Mellitus[58]:

From different databases it appears that more than 1200 species of organisms (including those from fungus and other microorganisms) have been used ethnopharmacologically to treat symptoms of diabetes mellitus. They include more than 725 genera in 183 families of plants. More than 200 pure compounds were isolated mainly from plants which have been claimed to possess hypoglycemic properties either *in vitro* or *in vivo*. Some of the chemical constituents of hypoglycemic plants are given in **Table 3**.

**Table 3: Chemical constituents of hypoglycemic plants are given below[58]:**

| **Plants with organic sulfur** | | |
|---|---|---|
| Plants | Active substance | Active part of plant |
| *Allium cepa* **L.**<br><br>*Allium sativum* **L.** | Allicin (allyl-propyl-disulphide), diallyl-disulphide-oxide etc. also kaempferol and quercetin-glycosides. | Bulb |
| *Brassica oleraceae* | Methyl- and ethyl-propyldisulphides and neoglucobrassicin (indol-myrosin glycoside (goitrigenous) | Leaves |
| **Plants with flavonosides** | | |
| *Ceiba pentandra* **(L.)**<br><br>**Geartin.** | Quercetin and kaempferol-glycosides, also methyl glucuronoxylan. | Juice, roots |
| *Centaurea perrotti*<br><br>*D. C.= C. calcitrapa =C. alexandrina* **L.** | Apegenin, naringenin quercetin and quercitin glycosides, also $\beta$ sitosterin and $\beta$ amyrin | Inflorescence |
| *Coccinia indica* W. and A. | Caffeic acid, quercetin, keampferol | Tuberous roots |
| *Phaseolous vulgaris* **L.** | Quercetin glycoside (querciturón), stigmesterin and $\gamma$ sitosterin, sulpher compounds. | Husks of beans |
| *Phyyllanthus niruri* **L.** | Quercetin, astralgin, leucodelphinidin glycosides, quercitrin, iso quercitrin | Leaves |
| *Scoparia dulcis* **L.** | Scoparoside (glycoside of 3-methyl-luteolin) | Plant |
| *Eucalyptus globulus* | Calyptoside (phenolic heteroside) | Leaves |

| | | |
|---|---|---|
| **Plants with anthocyanins, leuanthocyanins, catechols or tannins** | | |
| *Morus alba* **L.**<br><br>*Morus nigra* **L.** | Cyanidin delphinidin glucosides rutin, moracetin=quercetin triglycoside. | Leaves |
| *Musa paradisiaca*<br><br>*Var. sapientum Kuntze* | Leucodelphinidin- and leucocyanidin glycosides, 3-deoxyxanthincyanidin | Flowers |
| *Rhizophora mucronata* **Lam.** | Catechin and tannins | Bark and root |
| *Syzygium cumini* **L.** | Cyanidin 2-rhamnoglucoside galli- and elagi tannins | Fruit |
| *Vaccinium myrtillus* **L.** | Myrtillidin (7-methyl-delphidin) glycosides | |
| **Plants with phytosterin glycosides** | | |
| *Asterancantha longifolia* **Nees**= *Hygrophyla auriculata* | Steroid- and triterpine glycosides (lupeol-hygrosterol and phytosterols) | Root and seeds |
| *Ficus bengalensis* **L**<br><br>*F. glomerata* and spp. | $\beta$ sitosterin-D-glucoside | Root, bark |
| *Morus alba* **L**<br><br>*Morus nigra* **L** | $\beta$ sitosterin, sitosteryl carpate and palmitate | Leaves |
| *Momordica charantia* **L** | Charantin (phytosterin glycoside) | Fruit |
| *Momordica foetida*<br><br>Schum and Thonn. | Foetidin (sitosterol D-glycoside plus 5-25-stigmastadien glucoside) | Fruit |

| | | |
|---|---|---|
| *Maytenus senegalensis* **Lam** *Excell=Gymnosporia* (Roth) **Benth**. | $\beta$ sitosterin, triterpenes ($\beta$ amyrin and betulin) + hexacosane and hexacosanol | Leaves |
| *Polygonatum multiflorum* **L.** | Cardenolides` | Leaves |
| **Plants with hypoglycemic alkaloids** | | |
| *Catharanthus roseus* **G. Don.** | Leurosine (sulphate), vindoline (HCl), vindolinine (2HCl) | Roots |
| *Lupinus termis* | Sparteine, lupanine | Seeds |
| *Tecoma stans Juss* | Tecomine, tecostanine | Leaves |
| *Trigonella foenum graecum* **L.** | Trigonelline (betain) also coumarin and nicotinic acid | Seeds |

## 1.9 Aim of present research program:

Bangladesh possesses a rich heritage of traditional medicines with a large number of medicinal plants available throughout the country. Therefore, it is necessary to work on medicinal plants of Bangladesh. Our research group has been working for more than a decade with special interest on medicinal plant materials to find out different types of bioactive compound(s), for their possible therapeutic uses.

*Lagerstroemia speciosa* **L** locally known as Jarul is well known as ornamental tree. It is planted in different parts of the country including Dhaka university campus. In the campus area, a number of plants are available at the roadside for its beautiful lilac colored flowers. Besides the ornamental value, the plant has different types of medicinal value. The leaves, bark and flowers are being used by our traditional practitioners to treat various diseases. Pharmacological report showed that the bark and leaves extracts are being used for the management of diabetes.

A good number of plant extract have been investigated for hypoglycemic, anticancer and anti-HIV activities under a collaborative research program between the Department of Chemistry, University of Dhaka, Bangladesh Institute for Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorder (BIRDEM) and National Cancer Institute-Frederick Cancer Research and Development Center (NCI-FCRDC), Maryland, USA. Considering the medicinal value of the plant, it has been included by our research group in our on-going anticancer, anti-HIV and antidiabetic screening programs.

The present research work is a part of anti-HIV and antidiabetic research project. Leaves and bark extract of *Lagerstroemia speciosa* have been tested at NCI which showed significant protection against AIDS virus. The extracts of leaves and bark of the plant were also tested for hypoglycemic effects at BIRDEM. The extract showed significant hypoglycemic effects in streptozotocin induced diabetic model rats. Therefore, attempt has been under taken to isolate hypoglycemic and anti-HIV active compound(s) from the extracts of the plant.

The major part of the present research program involves chemical and biological studies of anti-diabetic and anti-HIV active extracts of the plant and identification and quantification of fatty acids. Anti-HIV results has been included in the work. Several species of *Lagerstroemia* genus are reported to have hypoglycemic activity but there was no report on hypoglycemic effect and identification and quantification of fatty acids of *Lagerstroemia speciosa* L. Therefore, *Lagerstroemia speciosa* (leaves, bark and flowers) was taken to isolate and characterize different compounds and identification and quantification of fatty acids.

## 1.10 Present study protocol:

The present study was designed to isolate pure compounds as well as to observe biological activities of the isolated compounds. This approach involved the following steps:

(a) Collection, proper botanical identification and drying of the plant materials;

(b) Preparation of appropriate extracts with different organic solvents (e.g., EtOH, MeOH, EtOAc, $CH_2Cl_2$ et.)

(c) Biological and pharmacological screening of crude extracts;

(d) Fractionation of the crude extract by using physico-chemical procedures, and monitored by biological tests, identification and separation of the active fractions;

(e) Several consecutive steps of chromatographic separation, where each fraction obtained has to be submitted to bioassay in order to follow the activity (activity guided fractionation);

(f) Isolation of the active constituents by chromatographic or other techniques and purification of the isolated compounds by repeated chromatography and crystallization;

(g) Establishment of the chemical structures of the pure compounds by $^{1}H$- and $^{13}C$-NMR, MS and 2D NMR spectroscopy as well as chemical analysis;

(h) Observation of biological activity by Brine Shrimp Lethality and determination of $LD_{50}$ for isolated pure compounds.

(i) Identifications and quantification of fatty acids by gas liquid chromatography (GLC) and gas chromatography-mass spectroscopy (GC-MS).

## 1.11 General description of Lythraceae family[59]:

*Lagerstroemia speciosa* (L) belongs to the family of Lythraceae. It comprises of 23 genera and 475 species and is widely distributed in most part of the world including Bangladesh, China, Thailand, Malaysia, tropical Australia and in the American tropics. Most of the tropical species are woody shrubs (trees in *Lagerstroemia* and *Lafoensia*). The Lythraceae is represented in the United States by one or more indigenous species of Lythrum (25-7, widespread), Ammannia (20-4, wide spread but more common to the south), Rotala (35-2, widespread), Didiplis (Mototypic, Minnesota to Texas and Florida), Decodon (motobtypic, eastern and central), cuphea (Parsonsia) (200-6, Kansas to Georgia and southward, 1 species to New England) and Heimia (2-1, Western Texas). The family is distinguished by the presence of the hypanthium, the superior ovary, the crumpled corolla, the often-unequal stamens of typically twice the number of sepals, and the seed without endosperm.

## 1.12 Medicinal importance of plant family Lythraceae:

The members of this family are claimed to have economic as well as medicinal importance. Some species are reported to be used as stimulant, hydragogue, drastic, purgative, febrifuge, narcotic[60]. Some species of this family show anti-inflammatory and diuretic properties.

## 1.13 Economic importance of the plant family Lythraceae:

Economically the family is important for members of ornamental value, notably the crape myrtle (*Lagerstroemia indica*) widely cultivated in warmer parts of the country, henna or mighonette tree (*Lawsonia inermis*) grown in California and much of the South, a dozen spices of Cuphea as garden annuals, and the spiked or purple loosestrife[59] (*Lythrum salicaria*).

Lythrum plants grow well in Bangladesh in flat lands. Most of them are planted in garden including Dhaka University Campus. According to Literature survey, the following species of Lythraceae family are available[61] which are given in **Table 4**.

**Table 4: Availability of various species of Lythraceae family in Bangladesh[61].**

| Genus | Species |
|---|---|
| 1. *Awsonia* **L** | (i) *Lawsonia alba* **Lamk**<br>*(ii) Lawsonia inermis* |
| 2. *Crypteronia* **Bl** | (i) *Crypteronia paniculatabi* |
| 3. *Lagerstroemia* **L** | (i) *Lagerstoremia parviflora* **Roxb**<br>(ii) *Lagerstoremia indica* **L**<br>(iii) *Lagerstroemia flos-reginae* **Retz.**<br>(iv) *Lagerstroemia macrocarpa* **Wall**<br>(v) *Lagerstroemia grandiflora.* |
| 4. *Duabanga* **Ham** | (i) *Duabanga sonneratioides* **Ham.** |
| 5. *Sonneratia* **L** | (i) *Sonneratia apetala* **Ham.**<br>(ii) *Sonneratia acida* **L** |
| 6. *Punica* **L** | (i) *Punica granatum* **L** |

## 1.14 General description of the genus *Lagerstroemia* L[60]

The *Lagerstroemia* **L** is a large plant generous including trees or shrubs. Its leaves are opposite, distichous, or the uppermost alternate, petiolate, oblong or ovate, often glaucous beneath. Its flowers are often large and showy, in axially and terminal lax, less often dense, 3-chotomous panicles; bracts 2 at apex of peduncles; bracteolate 2 on the pedicels. The number of sepals is 6, sometimes 7-9, connate in a funnel–shaped, smooth, grooved; angled or almost winged calyx–tube; lobes ovate, subacute, valvate. The number of petals is 6, sometimes 7-9, rarely 0, inserted at apex of calyx–tube, clawed more or less orbicular, wrinkled, the margin crisped, erose or fimbriate. Stamens numerous, inserted near base of calyx–tube; filaments much exserted. Carpels 3-6, Connate in a 3-6 celled ovary sessile at the base of the calyx–tube; style long, curved; stigma capitate. Fruit on ellipsoid, cariaceous or woody capsule, somewhat adnate below to the calyx, smooth, ellipsoid, with 3-6 celled and as many valves. Seeds numerous, rarely few, elongated, flat, erect, winged from their apex.

Fig. 3: Herbarium specimen of *Lagerstroemia speciosa* Linn.[63]

## 1.15 Description of the plant *Lagerstroemia speciosa* Linn.[60,61]

**Botanical name** : ***Lagerstroemia speciosa* Linn.**

**Family** : **Lythraceae**

English name : Queen Crape Myrtle

Local name : Jarul, Kantajarul, Paniajarul.

Other name : *Assam*: Ajhar; *Burma*: Eikmwe; *Hindi*: Arjuna; *Sanskrit*: Aujuna.

**Description:**

The plant *Lagerstroemia speciosa* Linn. is a large tree including 9-18 m high. The branches are widely spreading. The bark is pale, smooth, flaking off in irregular pieces. The leaves are 10-20 by 3.8-7.5 cm, oblong–lanceolate or elliptic, subacute, glabrous and finely reticulate on both surfaces, pale beneath, base acute or rounded; main nerves 10-13 pairs, prominent, curving upwards; petioles 6-10 mm long stout. Its flowers are 5.0-7.5 cm across, in large panicles sometimes reaching 30 cm long; pedicels stout, pubescent thickened upwards and articulated below the middle. The calyx is turbinate, 1.6 cm long covered with white or ferruginous tomentum ribbed with 12-14 prominent stout ridges, those opposite the calyx–teeth broader; teeth 6-7, triangular, acute, spreading, 6 mm long, thickened at the edges. The number of petals is 6-7, purple, 2.5-3.8 cm long, suborbicular or rotund–ovate, clawed, much undulate and crumpled, spreading. The stamens are all equal, shorter than the style. Capsules are ellipsoid or subglobose, 2.0-3.2 by 1.6-2.5 cm minutely apiculate. The seeds (including the wing) are 1.25-1.45 cm by 6.0-4.5 mm, glabrous, pale brown.

### 1.17 Chemical investigation of *Lagerstroemia species*:

Considering the importance of different species of *Lagerstroemia* as medicinal plants, extensive chemical works have been carried out on the leaves, bark, branches, seeds and many compounds have been isolated. Many of them are bioactive. The isolated compounds are cited below.

i) Abresoline; Di-Me ether (**28**);

ii) Abresoline; Di-Me ether, 5-epimer;

iii) Decinine; (-) –form (**29**);

iv) 3-*O*-Methylellagic acid (**30**);

v) Ellagic acid; 2,3–Di-Me ether, 8-*O*-$\beta$-D – glucopyranoside;

vi) Epoxydon; (+)- form, 4-Epimer (**31**);

vii) Flosin A (**32**);

viii) 21-Hopene–3,20-dione (**33**);

ix) 3-Hydroxycycloartan–24-one ; 3$\beta$-form (**34**);

x) 16-Hydroxy–8(17), 13-labdadien-15, 16-olide;16$\xi$-form, (**35**);

xi) Lagerine (**36**);

xii) Lagerine; Me ether;

xiii) Lagerstannin A (**37**);

xiv) Lagerstannin B (**38**);

xv) Lagerstannin C (**39**);

xvi) Lagerstroemin (**40**);

xvii) Lagerstroemin ; 1-Epimer;

xviii) Lagerstroemine (**41**);

xix) Lasubine I (**42**);

xx) Lasubine 1, 5–Epimer;

xxi) Luteolic acid; Diglucoside;

xxii) Lythrine (**43**);

xxiii) Reginin A (**44**);

xxiv) Reginin A; 1-Epimer;

xxv) Reginin C (**45**);

xxvi) Reginin D (**46**);

xxvii) Sinine (**47**);

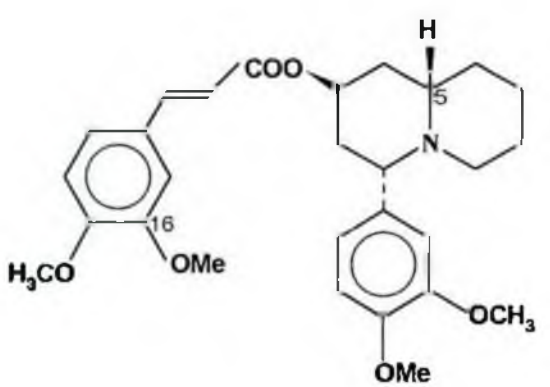
xxviii) Stigmast–4-ene-3,6-diol; (3β 6α, 24*R*)-form (**48**);

xxix) Verticillatine; 12,13-dihydro (**49**);

xxx) Vertine (**50**);

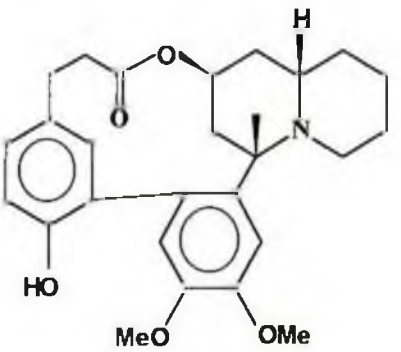
xxxi) Vertine; 12,13-Dihydro.

In 1978, Fuji, K *et al.*[65] isolated oil like alkaloid, di-methyl ether of abresoline (synonym: Subcosine II) (**28**) and di-methyl ether of abresoline, 5-epimer from the leaves of *Lagerstroemia subcostata.*




Abresoline (**28**)

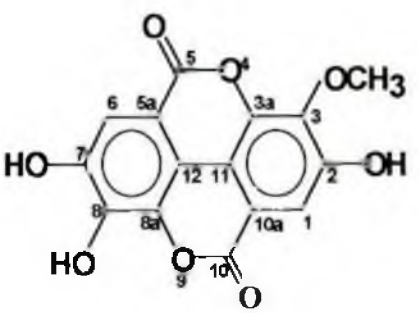
In 1962, Ferris, J P *et al.*[66] isolated an alkaloid decinine (**29**) from *Lagerstroemia indica.*




Decinine;(-)-form **(29)**

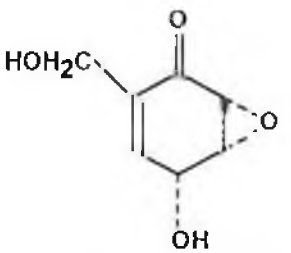
In 1976, Yazaki, Y *et al.*[67] isolated 3-*O*-methylellagic acid (**30**) from *Lagerstroemia subcostata*

In 1973, Pradhan, B P *et al.* [68] isolated Ellagic acid; 2,3–Di-Me ether, 8-*O*-*β*-D – glucopyranoside from the leaves of *Lagerstroemia speciosa.*



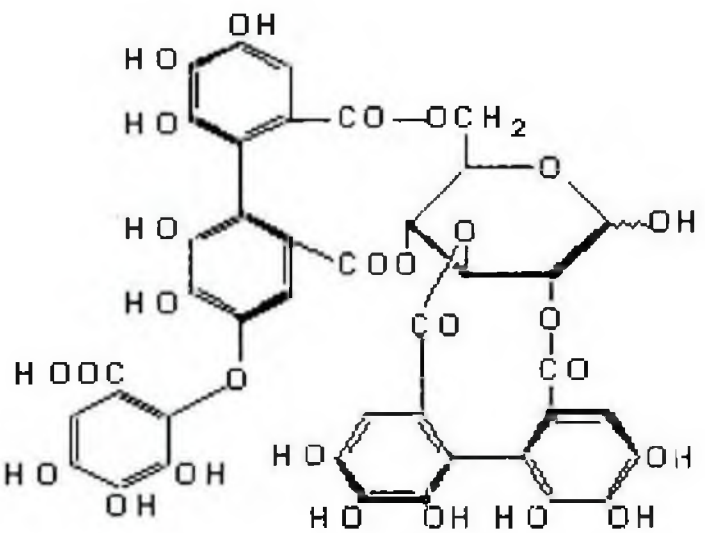

3-O-methyl ellagic acid **(30)**

In 1992 Nagata, T. *et al.*[69] isolated epiepoxydon (**31**) from *Lagerstroemia indica.*



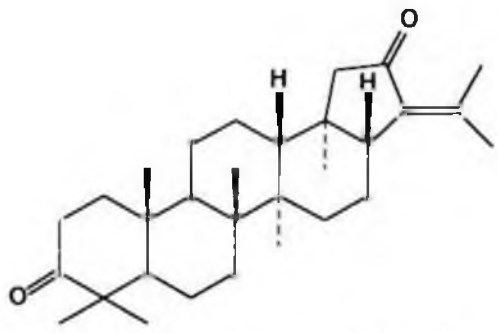
Epoxydon; (+)-form (**31**)

In 1991, Xu, Y. -M. *et al.*[70] isolated an off-white amorphous powder, flosin A (**32**) from the leaves of *Lagerstroemia flos-reginae* **Retz**.



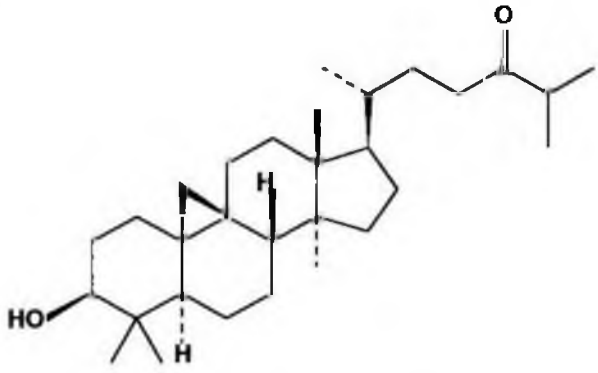
Flosin A (**32**)

In 1988, Barik, B R *et al.* [71] isolated needles like, 21-Hopene-3,20-dione (Synonym: Lageflorin) **(33)** from *Lagerstroemia parviflora.*



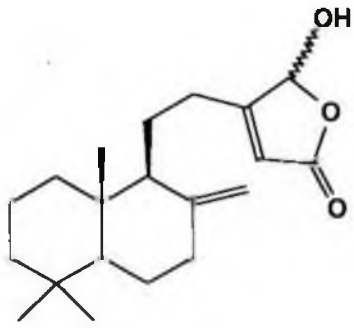

21-Hopene-3,20-dione (**33**)

In 1994, Della Greca, M. *et al.* [72] isolated crystalline 3-Hydroxycycloartan-24-one **(34)** ftom *Lagerstroemia lancasteri.*



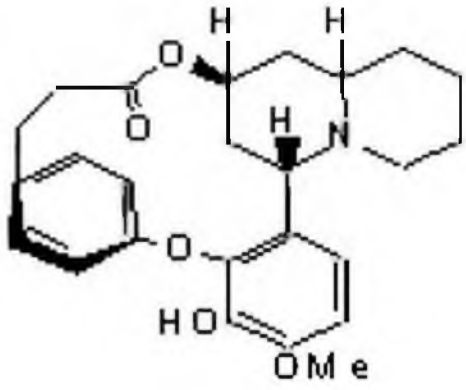

3-Hydroxycycloartan-24-one (**34**)

In 1992, Zdero, C. *et al.* [73] isolated 16-hydroxy-8(17), 13-labdadien-15, 16-olide (**35**) from *Lagerstroemia lancasteri*.



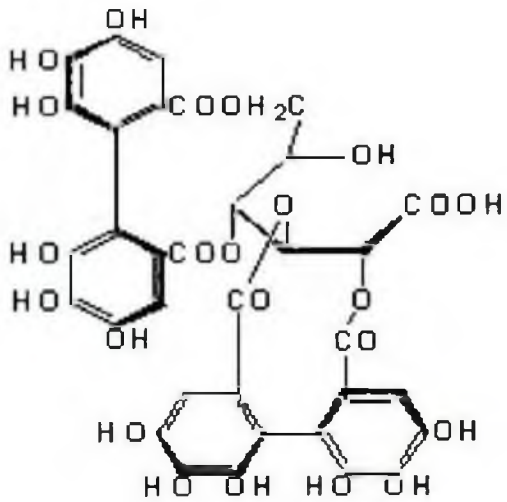

16-Hydroxy-8(17),13-labdadien-15,16-olide (**35**)

In 1971, Ferris, J. P. *et al.*[74] were isolated an alkaloid, lagerine (**36**) from *Lagerstroemia indica*.

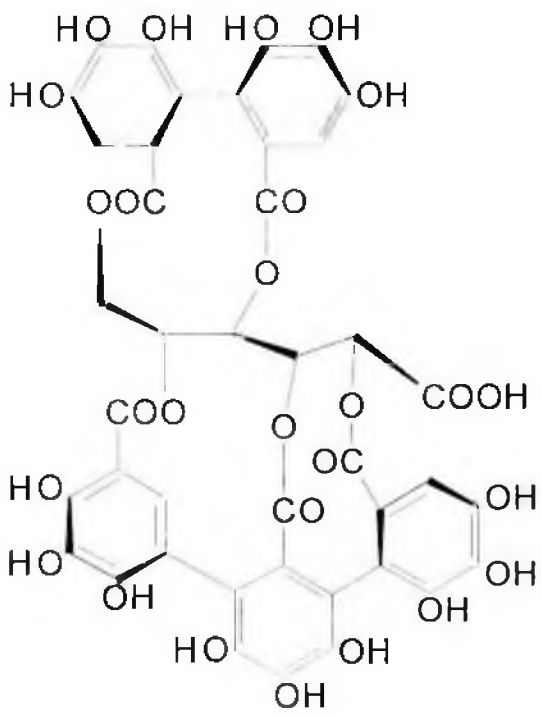



Lagerine (**36**)

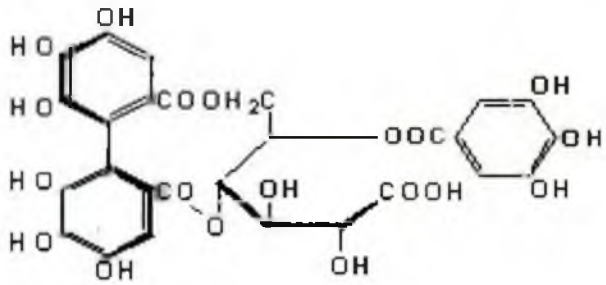
In 1992, Tanaka, T. *et al.*[75] isolated lagerstannin A (**37**), lagerstannin B (**38**) and lagerstannin C (**39**) from the fruits and leaves of *Lagerstroemia speciosa*.



Lagerstannin A (**37**)

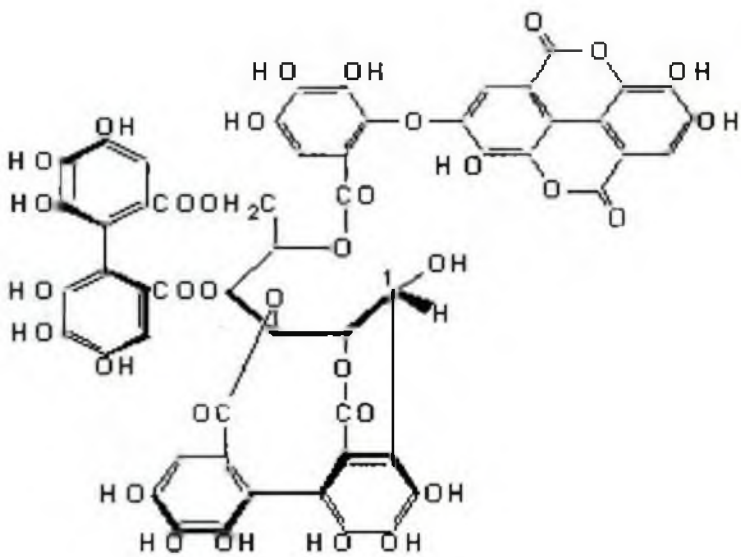


Lagerstannin B (**38**)



Lagerstannin C (**39**)

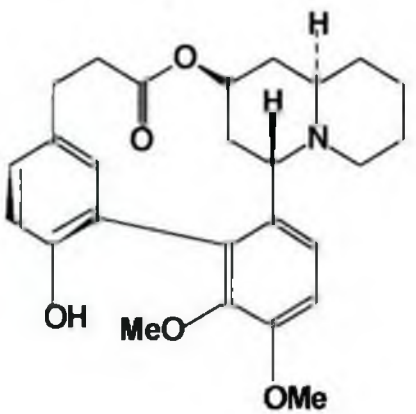
In 1991, Xu, Y. M. *et al.* [70] isolated lagerstroemin (**40**) were isolated from the leaves of *Lagerstroemia flos-reginae* **Retz**.



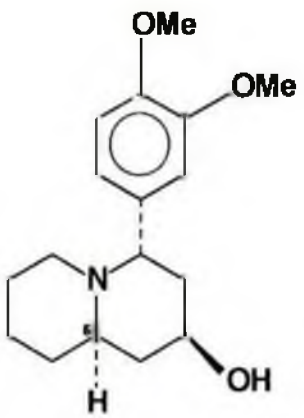
Lagerstroemin (**40**)

In 1971, Ferris, J. P. *et al.*[74] isolated lagerstroemine (**41**) from *Lagerstroemia indica.*

In 1978, Fuji, K. *et al.* [65] isolated an alkaloid, lasubine **I** (**42**) from *Lagerstroemia subcistata.*

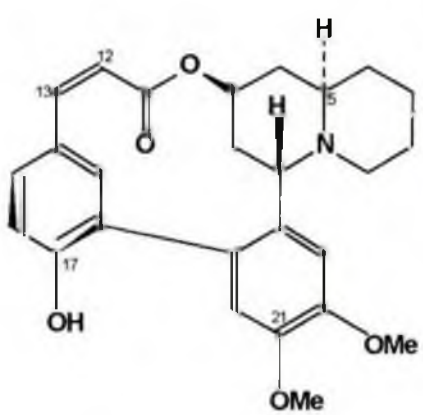



Lagerstroemine (**41**)



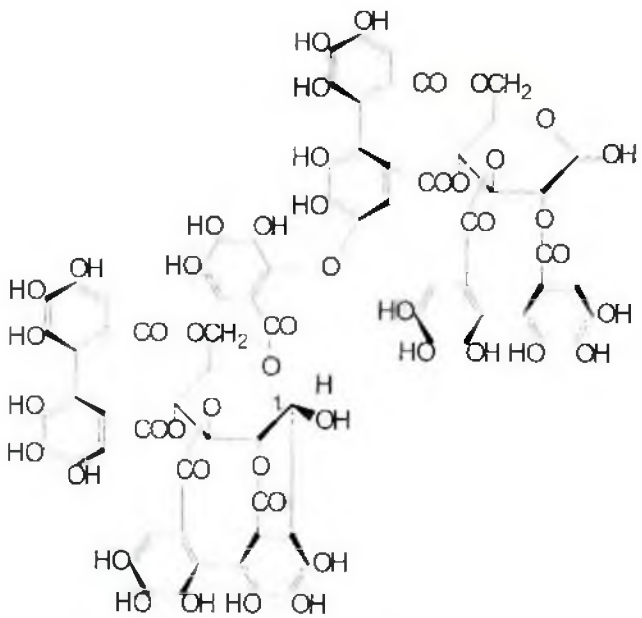

Lasubine I (**42**)

In 1964, Blomster, R. N. *et al.*[76] and Douglas, B. *et al.*[77], in 1975, Dominguez, X. A. *et al.*[78] and in 1978, Fuji, K. *et al.*[65] isolated lythrine (**43**) from *Lagerstroemia fauriei.*

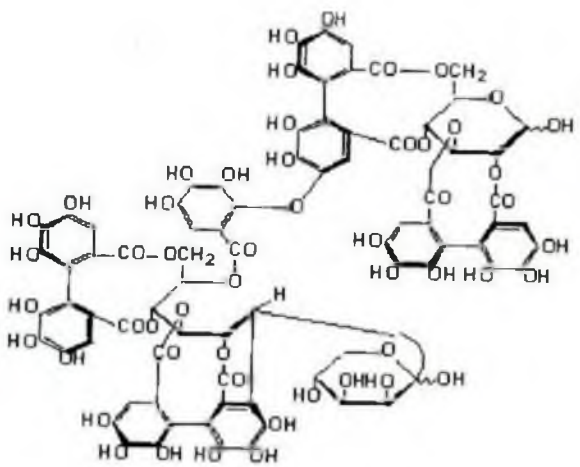



Lythrine (**43**)

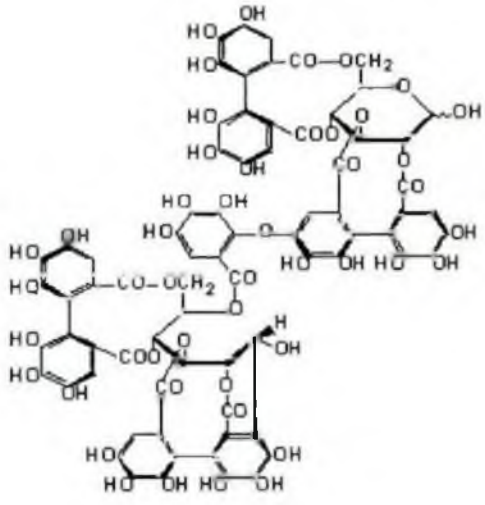
In 1991, Xu, Y. -M. *et al.*[70] isolated off-white ellgitannins, Reginin A (**44**), 1-Epimer Reginin B, Reginin C (**45**) and Reginin D (**46**) from the leaves of *Lagerstroemia flos-reginae* **Retz**.



Reginin A (**44**)



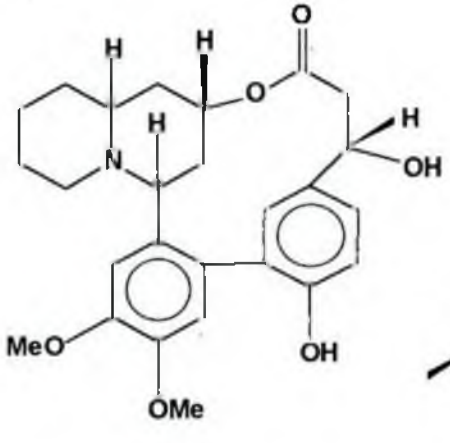
Reginin C (**45**)



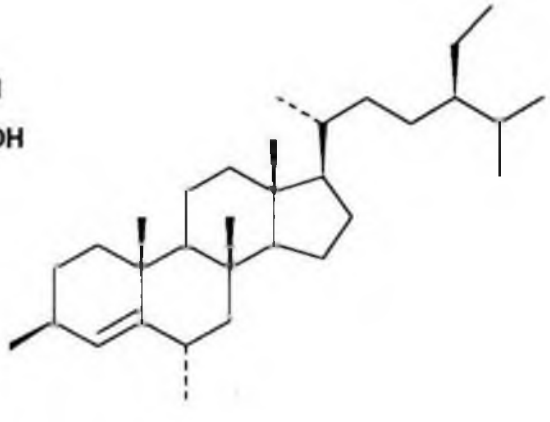
Reginin D (**46**)

In 1964, Blomster, R. N. *et al.*[76] isolated sinine (**47**) from *Lagerstroemia fauriei.*

In 1993, Gaspar, E. M. M. *et al.*[79] isolated stigmast-4-ene-3,6-diol (**48**) from *Lagerstroemia lancasteri.*

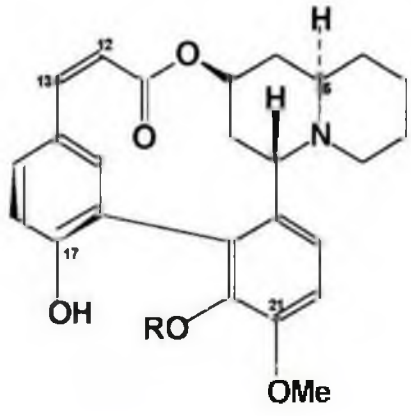


Sinine (**47**)



Stigmast-4-ene-3,6-diol (**48**)

In 1962, Ferris, J. P. *et al.*[74] isolated alkaloid, verticillatine (**49**) and Vertine (**50**) from *Lagerstroemia indica.*




Verticillatine, R=H (**49**)
Vertine , R=Me (**50**)

## 1.18 Chemical investigation and isolation of compounds from *Lagerstroemia speciosa* L

Literature survey showed that most of the compounds isolated from the leaves and bark of *Lagerstroemia speciosa* are tannins, ellagitannins and related compounds. Extract of leaves contains alanine, isoleucine, $\alpha$-aminobutyric acid and menthoeonine. But no alkaloids, glucosides, sterols, flavonoids are available in the plant *Lagerstroemia speciosa*[9].

Literature survey showed that the following compounds were isolated from *Lagerstroemia speciosa.* The name of the compounds are given below:

1) Colosolic acid (**51**);

2) Ellagic acid; 2,3–Di-Me ether, 8-*O*-$\beta$-D-Glucopyranoside of ellagic acid

3) Flosin A (**32**)

4) Lagerstannin A (**37**)

5) Lagerstannin B (**38**)

6) Lagerstannin C (**39**)

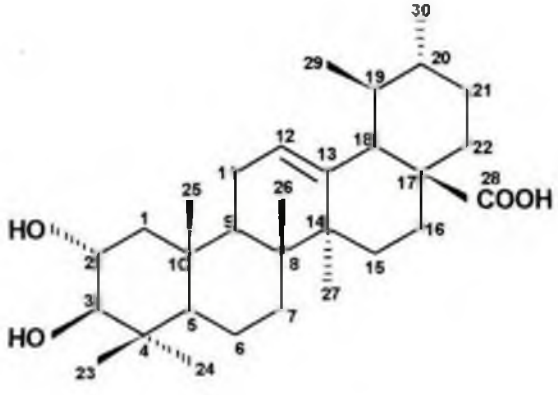
7) Lagerstroemin (**40**)

8) Reginin A (**44**)

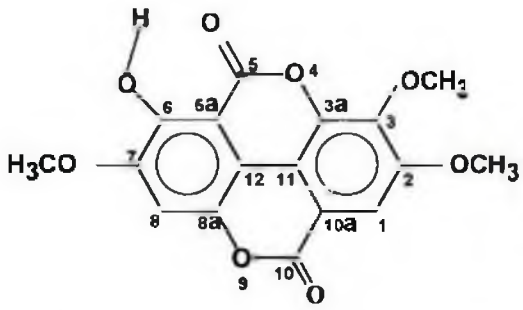
9) Reginin C (**45**)

10) Reginin D (**46**)

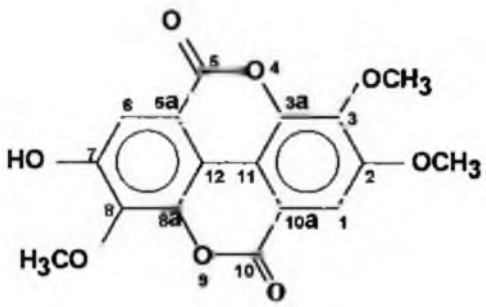
Two new compounds 2,3,7-Tri-*O*-methylellagic acid (**52**) and 2,3,8-Tri-*O*-methylellagic acid (**53**), *β*-sitosterol (**54**), sitosterol-3-*O*-*β*-D-glucoside (**55**), reported compound, colosolic acid (2α-hydroxyursolic acid)[48] (**51**), n-Tricosane (**56**), n-Heptacosane (**57**), n-Hentriacontane (**58**), together with more than sixteen different types of fatty acids were identified and quantified from leaves, bark and flowers of *Lagerstroemia speciosa* for the first time in this report. The structures of the compounds are given below:



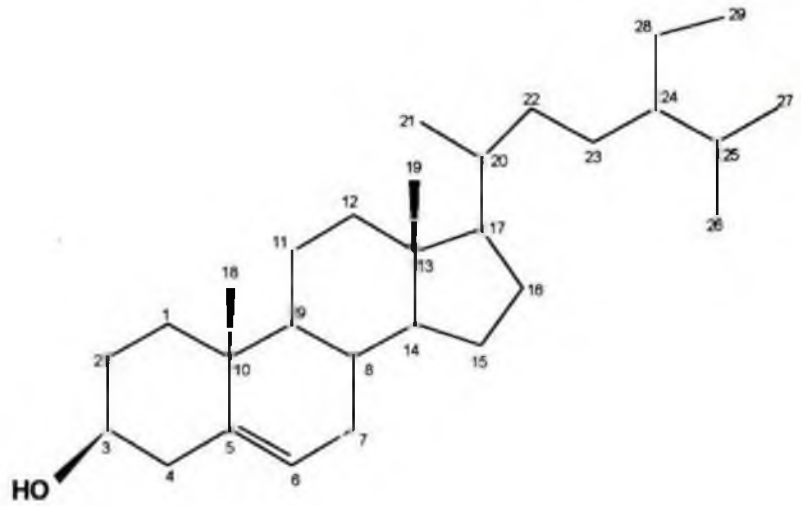

2,3-Dihydroxy-12-ursen-28-oic acid,
Colosolic Acid (LSM-4) (**51**)



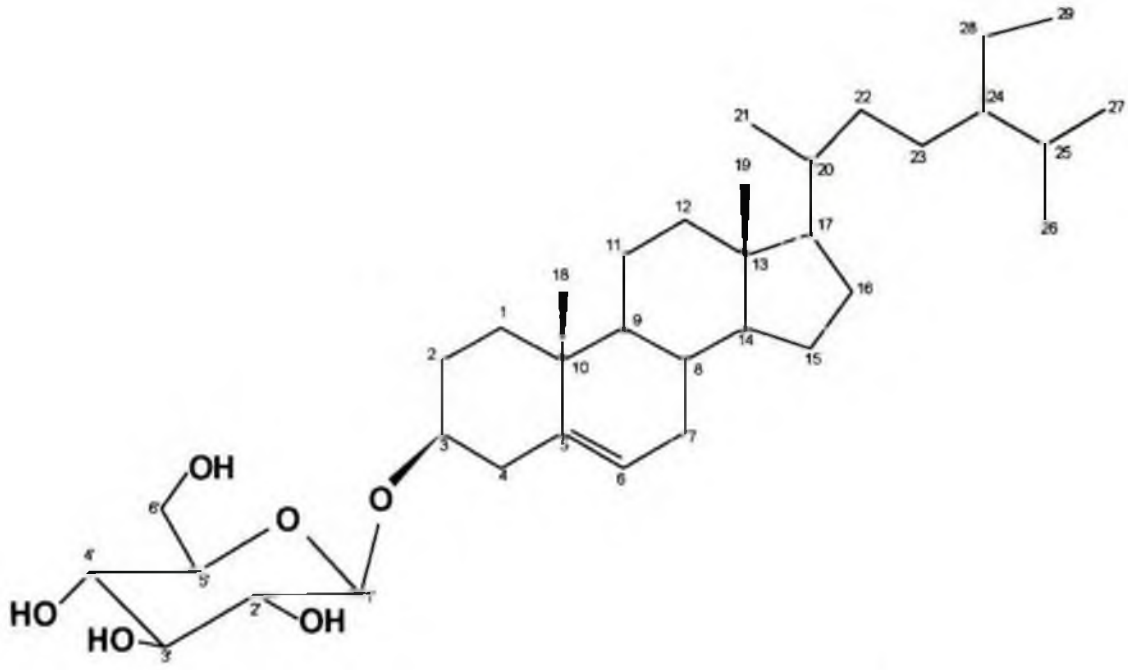

2,3,7-Tri-O-methylellagic acid (AR-7) (**52**)



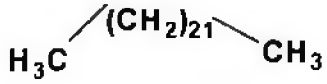

2,3,8-Tri-O-methylellagic acid (AR-2) (**53**)



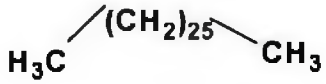

β–Sitosterol (AR-1) (**54**)



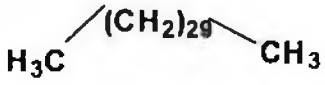

Sitosteryl-3-O-β–D-glucoside AR-3 (**55**)




n-Tricosane, JF-39 (**56**)

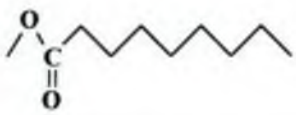



n-Heptacosane, JF-43 (**57**)

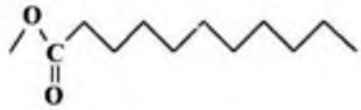



n-Hentriacontane, JF-53 (**58**)

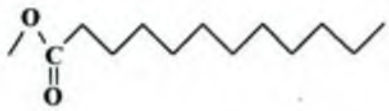
The structure of the identified and quantified fatty acid esters of *Lagerstroemia speciosa* **L** are given below:



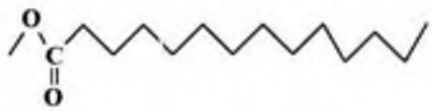
**Nonanoic acid, methyl ester**
**$C_{10}H_{20}O_2$**



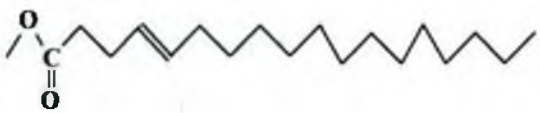
**Undecanoic acid, methyl ester**
**$C_{12}H_{24}O_2$**



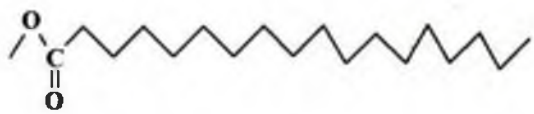
**Lauric acid, methyl ester**
**$C_{13}H_{28}O_2$**



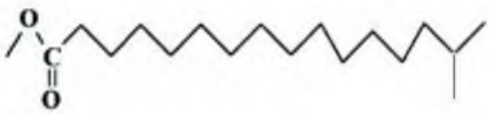
**Tetradecanoic acid, methyl ester**
**$C_{15}H_{30}O_2$**



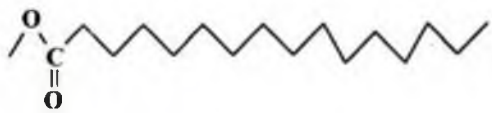
**Octadecenoic acid, methyl ester**
**$C_{19}H_{36}O_2$**



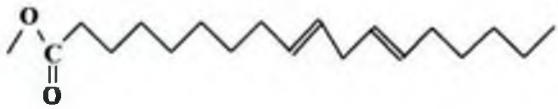
**Stearic acid, methyl ester**
**$C_{19}H_{38}O_2$**



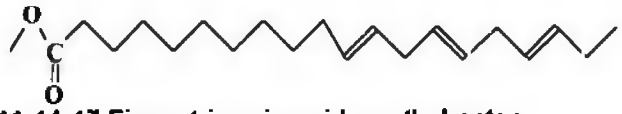
**Hexadecanoic acid, 14-methyl-, methyl ester**
**$C_{18}H_{36}O_2$**



**Palmitic acid, methyl ester**
**$C_{17}H_{34}O_2$**

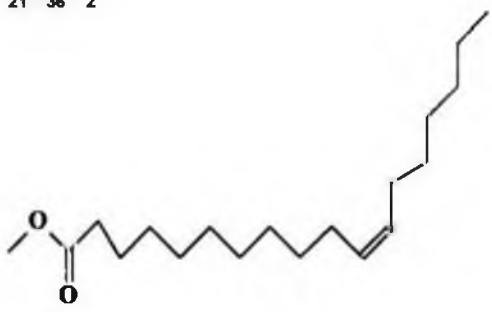


**9,12-Octadecadecadienoic acid (z, z)-, methyl ester**
**Linoleic acid, methyl ester**



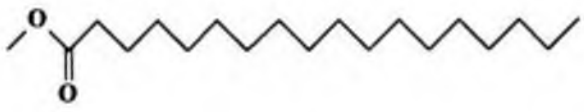
**11,14,17-Eicosatrienoic acid, methyl ester**

$C_{21}H_{36}O_2$



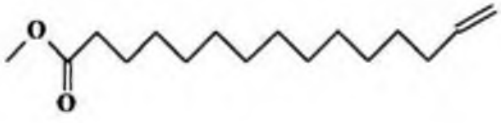
**11-Octadecenoic acid, methyl ester**

$C_{19}H_{36}O_2$



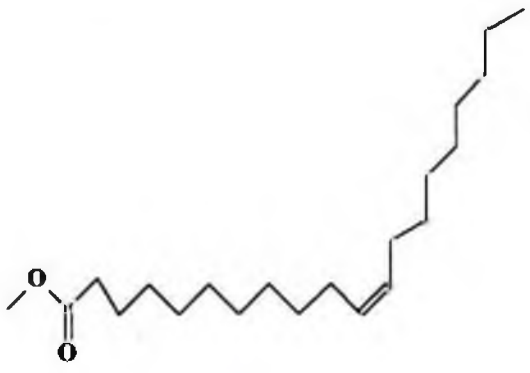
**Stearic acid, methyl ester**

$C_{19}H_{38}O_2$



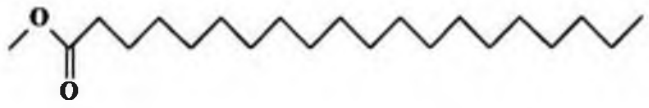
**14-Pentadecynoic acid, methyl ester**

$C_{16}H_{28}O_2$

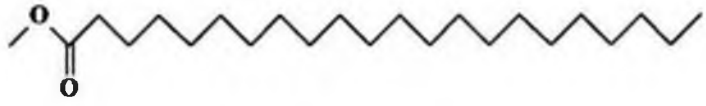


**Oleic acid, methyl ester**

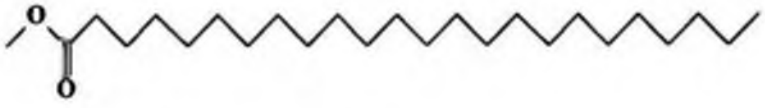
$C_{19}H_{36}O_2$



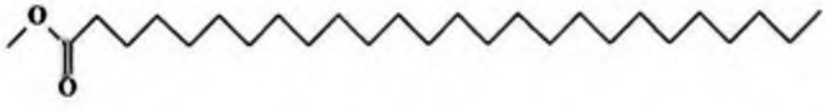
**Eicosanoic acid, methyl ester**
$C_{21}H_{42}O_2$



**Behenic acid, methyl ester**
$C_{23}H_{46}O_2$



**Tetracosanoic acid, methyl ester**
$C_{25}H_{50}O_2$



**Hexacosanoic acid, methyl ester**
$C_{27}H_{54}O_2$

# CHAPTER TWO: EXPERIMENTAL

# EXPERIMENTAL

## 2.1 General methods:

### 2.1.1 Solvents and chemicals:

Analytical or reagent grade (e.g., Sigma, E. Merck or BDH) solvents and chemicals were used in most of the experiments. Commercial grade of dichloromethane, ethyl acetate, methanol, ethanol, acetone etc. were distilled in glass distillation set before use.

### 2.1.2 Evaporation:

All evaporations were carried out under reduced pressure using a Buchi rotary evaporator at bath temperature not exceeding $40^0$C.

### 2.1.3 Freeze–drying:

Freeze-drying of aqueous extracts and fractions were carried out with a Varian 801 model LY-3-TT freeze-dryer. Aqueous extracts and fractions were first frozen in round-bottomed flasks by means of a methanol freezer (HETOFRIG, CB5, Denmark) at $-30^0$C to $-35^0$C and finally the materials were subjected to freeze-drying operation.

### 2.1.4 Chromatographic methods:

#### 2.1.4.1 Thin layer chromatography (TLC):

Two types of thin layer chromatography (TLC) were used (i) precoated TLC plates: 0.2 mm thin coating on glass or aluminum sheets; and (ii) manually prepared glass plates.

For thin layer chromatography some of the solvents used in this work are given below:
For the less polar fractions and compounds binary solvent systems were used e.g.,

(a) Hexane: dichloromethane (in different ratio)

(b) Hexane: ethyl acetate (in different ratio)

(c) Dichloromethane: methanol (in different ratio)

(d) Ethyl acetate: methanol (in different ratio)

and for more polar fractions and compounds trinary solvent systems were used, e.g.,

(e) Dichloromethane: methanol: water (in different ratio)

**2.1.4.2 Locations of spots:**

Irrigated plates were developed by one of the following methods to detect the positions of the spots.

(a) Examination under a UV light source with two different wave-lengths (254 nm and 350 nm).

(b) The plates were exposed to iodine vapor for five minutes.

(c) The plates were sprayed with 1% vanillin in concentrated sulphuric acid followed by heating in an oven at $120^0$C for 10 minutes.

**2.1.4.3 Column chromatography:**

**(a) Column:**

For this technique glass column of different size varying from large glass tubes (900 cm× 10 cm, i.d.) fitted with a rotaflow to small (30 cm× 1 cm, i.d.) burette like tube (30 cm× 1 cm), fitted with teflon flow control unit, were used.

**(b) Stationary phase:**

For stationary phase column grade silica gel (230–400 mesh; particle size 0.04-0.063 mm; ASTM, Art. 9385.5000, Merck) was used.

**(c) Preparation of column:**

The column was packed with silica gel slurry using lowest polar solvent. The packed column was equilibrated with two or three column volumes of mobile phase. Then the sample was introduced on the column bed and solvents with varying polarity (hexane to methanol) were used to elute the sample.

**2.1.4.4 Preparation of silica gel flash column:**

Glass columns of different sizes were used for chromatographic separations. Column grade silica gels of different mesh were used as stationary phase. To prepare a particular column the required amount of silica gel was swelled into a selected solvent (e.g., hexane, dichloromethane, ethyl acetate, methanol or a mixture of different solvents in different solvents in different ratio) for a while and then poured into the column with continuous flow of the solvent and occasional tapping of the column for homogeneous packing of the silica gel. The column was flashed with a fish aquarium air pump with three to four bed volumes of the equilibrating solvents in order to settle the column bed.

For flash column chromatography a fish aquarium air pump (EK 800) was used to apply pressure. Different fractions were collected in conical flasks (100 ml, 50 ml) manually.

**2.1.4.5 Preparation of Sephadex LH-20 gel column:**

The required amount of Sephadex LH-20 gel (particle size 25-100 μm, Pharmacia, Sweden) was suspended in water containing 0.1% 1-butanol (preservative) for two hours. The gel was degassed (30 minutes). The column was conditioned with three column volumes of water followed by methanol. The column was then equilibrated with three column volumes of water. The conditioned column was then ready for sample application.

### 2.1.5 Application of the sample into the column:

The crude extract or sub-fraction thereof was applied into the column either as a solution or in a ground form. To apply the sample in powder form the sample was dissolved in a particular solvent or a mixture of solvents and silica gel (sample: gel, 1: 2-3 w/w) was added to the sample solution and the mixture was evaporated to dryness. The dried material was ground thoroughly in a mortar to make fine powder and the powder was then applied into the column.

To apply the sample in the form of solution it was dissolved in a minimum volume of the column equilibrating solvent and was applied into the column. After application of the sample, some cotton or fresh silica gel was placed on the top of the silica gel bed so that the surface of the bed was not affected during solvent application.

### 2.1.6 Fractionation and monitoring procedure:

After application of the sample, the column was eluted with the equilibrating solvent and the polarity of the mobile phase was increased gradually by adding more polar solvent such as dichloromethane, ethyl acetate, and methanol. The eluted samples were collected in conical flasks or test tubes and the fractions were monitored by using TLC profiles. Fractions were combined on the basis of their $R_f$ values. The fractions were also combined by evaluating of their low-resolution $^1$H-NMR spectra where applicable.

### 2.1.7 Ultraviolet (UV) spectroscopy:

For ultraviolet spectrum a Shimadzu UV 160 A recording spectrometer was used. The samples were dissolved in solvents and the solutions were taken in 1 cm × 1 cm quartz cell for recording the spectrum.

### 2.1.8 Infrared (IR) spectroscopy:

A Shimadzu IR–470 spectrometer was used to obtain IR spectrum by making KBr pellets.

### 2.1.9 Nuclear magnetic resonance (NMR) spectroscopy:

NMR spectra of different extracts and fractions were recorded by using a 60 MHz NMR spectrometer (JEOL, JNM-PMX60SI). The $^{1}H$- and $^{13}C$-NMR spectra of pure compounds were recorded on a Varian 400 MHz spectrometer using $CDCl_3$ (for non-polar compounds) and $CD_3OD$, DMSO-$d_6$, Pyridine-$d_5$ (for polar compounds). In all cases tetramethyl silane (TMS) was used as the internal reference. Two-dimensional NMR (H-H COSY, NOESY) spectra were obtained using standard pulse sequences.

### 2.1.10 Gas-liquid chromatography (GLC):

Gas-liquid chromatography (GLC) was conducted with a PYE UNICAM PU 4500 gas chromatograph fitted with a flame ionization detector and Cp sil 88 or SE-54 quartz capillary column (25 m × 0.2 mm, i.d.). The separations were affected at 160-220$^0$C with 3$^0$C rise per minute. The quantitative evaluations of the peaks were obtained by using a LKB 2220 recording integrator.

### 2.1.11 Gas Chromatography-Mass Spectroscopy (GC-MS):

The separation and detection of components from a mixture of organic compounds is readily achievable by GC-MS. Gas chromatography (GC-17A model, Shimadzu Corporation) fitted with fused silica capillary column DB-1 (J & W), 0.25 μm film thickness was used. Flow rate of helium was 50 ml min$^{-1}$ at a pressure of 90 kPa. The GC-MS-QP 5050A mass spectrometer equipped with NIST 107 Library was used for comparison with published spectra. Minimum count for the peak integration was 18 × $10^6$. The GC was programmed at 80-240$^0$C with 5$^0$C rise per minute. MS was operating at 70 eV and the temperature of ion source was 240$^0$C.

### 2.1.12 Mass spectroscopy:

The mass spectra were recorded at 70 eV with a helium flow rate flow of 2.5 ml $min^{-1}$ with a Finnigan 4021 instrument. The fast atom bombardment mass spectrum ($FAB^{+}MS$) was recorded as a positive ion mode with m/z ranging 0.0020-1000.0000.

### 2.1.13 Preparation of vanillin– sulfuric acid reagent:

To prepare vanillin-sulfuric acid reagent, one gram of vanillin was dissolved in 100 ml of concentrated sulfuric acid. The prepared solution was then kept in a glass made sprayer. Irrigated TLC plates were sprayed and heated at $120^{0}$C for 10 minutes.

### 2.1.14 Melting point:

Melting point was recorded by Ogawa Seiki Co. Ltd. Melting point apparatus.

### 2.1.15 Test of carbohydrates:

#### (a) Phenol sulfuric acid test:

Aqueous phenol solution was prepared by mixing phenol (80 g) with water (20 ml). The mixture became homogeneous on shaking and it was stored in a stoppered bottle. Sample solution (500 µl) was taken in a test tube. Aqueous phenol (80%, 2 drops) was added and vortexed the sample solution. Concentrated sulfuric acid (3 ml) was added to the solution and it was vortexed again. A reddish brown color indicated the presence of carbohydrate in the sample.

**2.1.16 Test of steroids:**

**(a) Salkowski test:**

Small amount of sample was dissolved in a mixture of chloroform and methanol, then a few drops of concentrated sulfuric acid was added to the solution. A reddish color developed for steroids.

**(b) Liebermann-Burchard test:**

Small amount of sample was dissolved in a mixture of chloroform and methanol, then a few drops of concentrated sulfuric acid were added to it followed by 4-6 drops of acetic anhydride. A greenish color developed for steroids.

## 2.2 CHEMICAL STUDY ON THE DESIRED PLANT

### 2.2.1 Plant material:

*Lagerstroemia speciosa* **Linn.** (Family: Lythraceae) was selected for the present research work. Leaves, bark and flowers of the plant were studied in this investigation.

### 2.2.2 Collection of plant materials:

Fresh and matured leaves, bark, and flowers of *Lagerstroemia speciosa* **Linn.** were collected from the campus area of the University of Dhaka on January 07, 1999. Voucher specimen of the plant was submitted to the Bangladesh National Herbarium (BNH). Professor Salar Khan, Consultant of BNH, confirmed the identification of the plant. The plant materials were brought to our research laboratory. Associated dirt was removed by washing with water. The cleaned plant material was chopped and air-dried at room temperature followed by drying in an oven at $40^0$C. The dried plant materials were then ground (200 mesh) by a grinder.

### 2.2.3 Extraction of leaves, bark and flowers of *Lagerstroemia speciosa* Linn. with different solvents:

#### 2.2.3.1 Extraction of leaves with dichloromethane:

The powder of leaves (3.5 kg) of *L. speciosa* was introduced into a extraction tank and was submerged in dichloromethane for three days by changing the solvent every 24 h. After standing for 24 h at room temperature the solvent ($CH_2Cl_2$) was drained off and the process of extraction was repeated three times. The combined dichloromethane extract was evaporated to dryness. The dried extract was further dried in a freeze-dryer by a membrane pump in order to remove the residual solvent. The combined extract (184.0 g, LSLD) was kept at $-22^0$C in a freezer.

#### 2.2.3.2 Extraction of leaves with methanol:

The residue obtained after dichloromethane extraction (3.1 kg) was further extracted with methanol as described in **section 2.2.3.1**.The extract was stored in a reagent bottle at $-22^0$C in a freezer (122.0 g, LSLDM).

**Scheme 1: Extraction of leaves of *Lagerstroemia speciosa* Linn.**

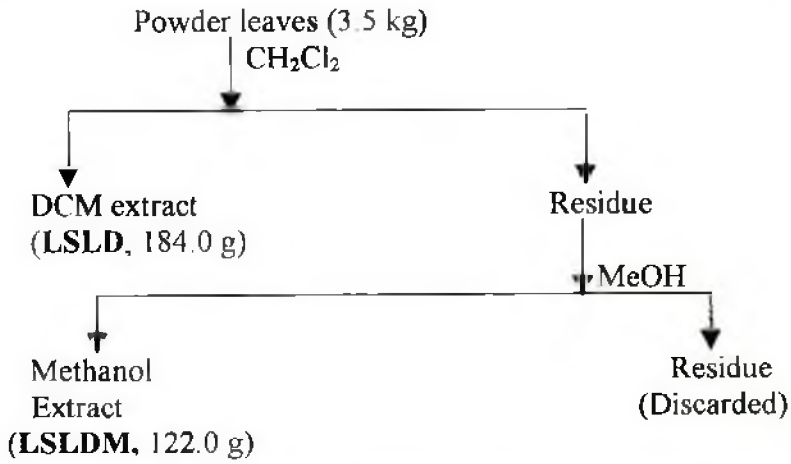



**Table 5: Percentage of different extractives of leaves of *Lagerstroemia speciosa* L.**

| Extractives | Amount ( g) | Percent of dry powder |
|---|---|---|
| DCM extract(LSLD) | 184.0 | 5.26 |
| Methanol extract (LSLDM) | 122.0 | 3.49 |

### 2.2.3.3 Extraction of bark with aqueous 80% ethanol:

Powdered bark of *Lagerstroemia speciosa* (3.0 kg) was extracted at room temperature with aqueous 80% ethanol (25 L) as described in **section 2.2.3.1**. The extract (LSBE, 87.02 g) was then stored in a reagent bottle at -22$^0$C in a freezer.

### 2.2.3.4 Partition of ethanol extract (LSBE) between dichloromethane and water:

Ethanol extract (LSBE, 87 g) was suspended in water (600 ml), ultrasonicated and the solution was transferred into a separating funnel. The aqueous solution was extracted with dichloromethane (6 × 200 ml). The dichloromethane soluble part was separated and evaporated to dryness (LSBED, 14.6 g).

The aqueous part was also evaporated to dryness. The dried part was further freeze-dried to remove the residual solvent (LSBEW, 65.3 g).

**Scheme 2: Extraction of bark of *Lagerstroemia speciosa* L**

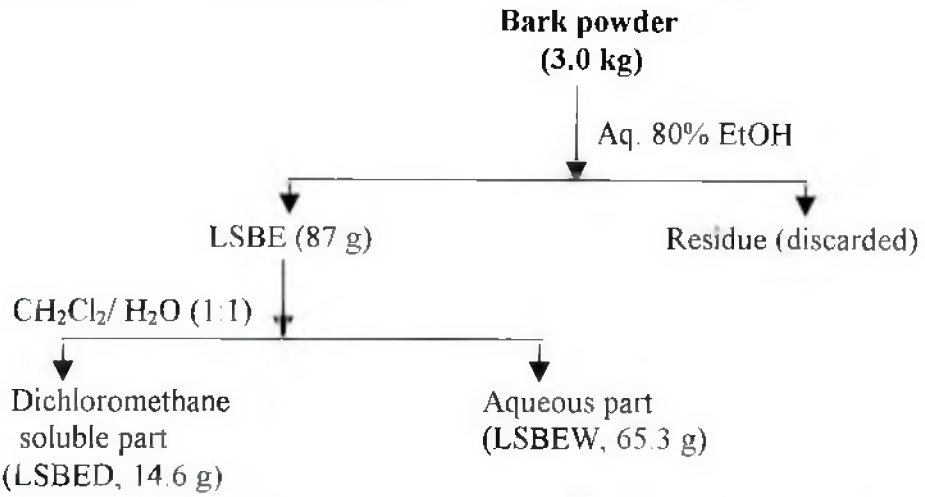



**Table 6: Percentage of different extractives of bark of *Lagerstroemia speciosa* L**

| Extractives | Amount (in g) | Percent (of dry powder) |
|---|---|---|
| Aqueous 80% ethanol extract (LSBE) | 87.0 | 2.9 |
| Dichloromethane soluble part (LSBED) | 14.6 | 0.49 |
| Water soluble part (LSBEW) | 65.3 | 2.18 |

### 2.2.3.4 Extraction of flowers with hexane:

The powder of flowers (300 g) was extracted with hexane as described in **sec. 2.2.3.1**. The amount of the extract was 2.4 g which was designed as LSFH.

Dichloromethane, methanol, ethanol and hexane extracts of leaves, bark and flowers of *Lagerstroemia speciosa* L were used for biological and chemical studies described in the respective sections.

## 2.3 BIOLOGICAL STUDIES

### 2.3.1 Brine Shrimp Lethality Assay:

Brine shrimp lethality assay[80, 81] of dichloromethane and methanol extracts of leaves, methanol and acetone fractions of bark and hexane extracts of flowers of *Lagerstroemia speciosa* were performed. The procedure is described below:

#### 2.3.1.1 Procedure:

**(a) Preparation of sea water:**

Sea salt (38 g, purchased from pet store in USA) was dissolved in distilled water (1 L) and then filter off.

**(b) Hatching of the brine shrimps eggs:**

Some seawater was kept in a small tray having partition (1: 4 ratio). Brine shrimp eggs were sprinkled on the large side of the tray covered with aluminum foil. The smaller compartment of the tray was kept open under a desk lamp. The hatched shrimps swam from the larger compartment into the smaller compartment within 24 hours. Living shrimps (age 48 h) were used to determine the toxicity of the extracts.

**(c) First round testing:**

Dichloromethane, methanol, ethanol and hexane extracts of leaves, bark and flowers (10 mg of each extract) were taken in 4 different vials (10 ml capacity) and seawater (5 ml) was added to each vial and ultrasonicated. From the solution of the above mentioned extracts (5 mg in 5 ml extracts) 500 µl, 250 µl, and 100 µl of each extract solution were taken in six different vials and sea water was added to make the volume 5ml. Afterwards 10 living shrimps drawn in Pasteur pipette were added to each vial and a few drops of yeast solution were added to the vials. For control, one vial with 5 ml sea water and 10 shrimps were taken. Number of mortality of the shrimps was counted after 24 h and 48 h. From the mortality using a NCI protocol, $LD_{50}$ of each extract was calculated and presented in **Table 7-11**.

**Table 7: Dichloromethane soluble part of leaves (LSLD) and mortality of the shrimps.**

| Amount of DCM extract (LSLD) (mg) | Mortality of shrimp out of 10 | | $LD_{50}$ at 24 h mg×$10^{-4}$/ml | $LD_{50}$ at 48 h mg×$10^{-4}$/ml |
|---|---|---|---|---|
| | After 24 h | After 48 h | | |
| 5.0 | 6 | 8 | 4.041 | 2.672 |
| 2.5 | 3 | 5 | | |
| 1.25 | 2 | 1 | | |
| 1.0 | 2 | 1 | | |
| Control | 0 | 0 | | |

**Table 8: Methanol soluble part of leaves (LSLDM) and mortality of the shrimps.**

| Amount of McOH Extract (LSLD) (mg) | Mortality of shrimp out of 10 | | $LD_{50}$ at 24 h mg×$10^{-4}$/ml | $LD_{50}$ at 48 h mg×$10^{-4}$/ml |
|---|---|---|---|---|
| | After 24 h | After 48 h | | |
| 5.0 | 6 | 8 | 2.950 | 1.670 |
| 2.5 | 5 | 7 | | |
| 1.25 | 4 | 4 | | |
| 1.0 | 1 | 3 | | |
| Control | 0 | 0 | | |

**Table 9: Methanol soluble part of bark (LSBEDM) and mortality of the shrimps.**

| Amount of MeOH Extract LSLD (mg) | Mortality of shrimp out of 10 | | $LD_{50}$ at 24 h mg×$10^{-4}$/ml | $LD_{50}$ at 48 h mg×$10^{-4}$/ml |
|---|---|---|---|---|
| | After 24 h | After 48 h | | |
| 5.0 | 6 | 8 | 4.016 | 2.247 |
| 2.5 | 3 | 6 | | |
| 1.25 | 1 | 2 | | |
| 1.0 | 1 | 2 | | |
| Control | 0 | 0 | | |

**Table 10: Acetone soluble part of bark (LSBEDA) and mortality of the shrimps.**

| Amount of Acetone Extract (LSBEDA) (mg) | Mortality of shrimp out of 10 | | $LD_{50}$ at 24 h mg×$10^{-4}$/ml | $LD_{50}$ at 48 h mg×$10^{-4}$/ml |
|---|---|---|---|---|
| | After 24 h | After 48 h | | |
| 5.0 | 6 | 8 | 3.431 | 2.247 |
| 2.5 | 4 | 6 | | |
| 1.25 | 3 | 2 | | |
| 1.0 | 2 | 2 | | |
| Control | 0 | 0 | | |

**Table 11: Hexane soluble part of flowers (LSFH) and mortality of the shrimps.**

| Amount of Hexane Extract LSFH (mg) | Mortality of shrimp out of 10 | | $LD_{50}$ at 24 h mg×$10^{-1}$/ml | $LD_{50}$ at 48 h mg×$10^{-4}$/ml |
|---|---|---|---|---|
| | After 24 h | After 48 h | | |
| 5.0 | 0 | 1 | The extract was not toxic $LD_{50} \cong 0$ | |
| 2.5 | 0 | 0 | | |
| 1.25 | 0 | 0 | | |
| 1.0 | 0 | 0 | | |
| Control | 0 | 0 | | |

### 2.4.1 Anti-HIV activity:

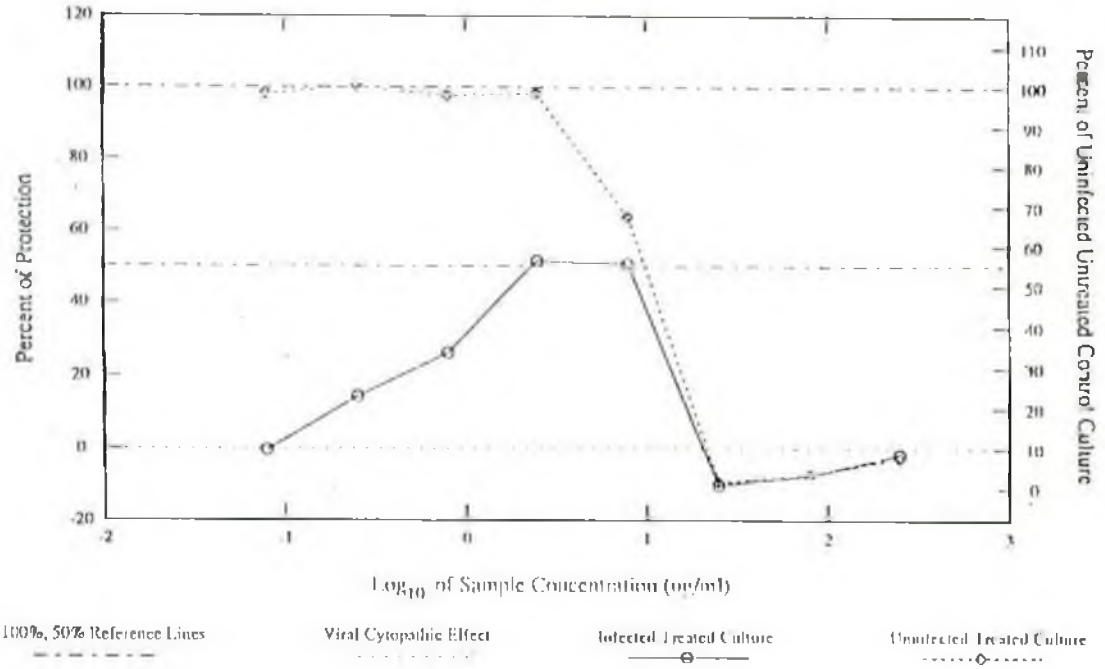
The dichloromethane and methanol extracts of bark of *Lagerstroemia speciosa* were tested at National Cancer Institute-Frederick Cancer Research and Development Center (NCI-FCRDC), Maryland, USA for anti-HIV activity.

The result is expressed in graph and presented in the next pages. From the graph it is found that DCM: MeOH extract of L. speciosa gave protection of HIV virus more than 56.5 and EC50 was 2.50 µg/ml **(Fig. 4, 5)**.

# National Cancer Institute
## Developmental Therapeutics Program
## *In Vitro* Testing Results

| | | | |
|---|---|---|---|
| NSC: N84339-0 / 1 | Plate: 6743 Lab: 9F SSPL: | | Assay: Primary Screen |
| Test Date: June 25, 1996 | Solubility Ind.: 1 | | COMI: |
| Report Date: February 20, 1997 | Cell Line: CEM-SS | | Solvent: DMSO 100% |

*In Vitro* Anti-HIV Drug Screening Results
Primary Screen



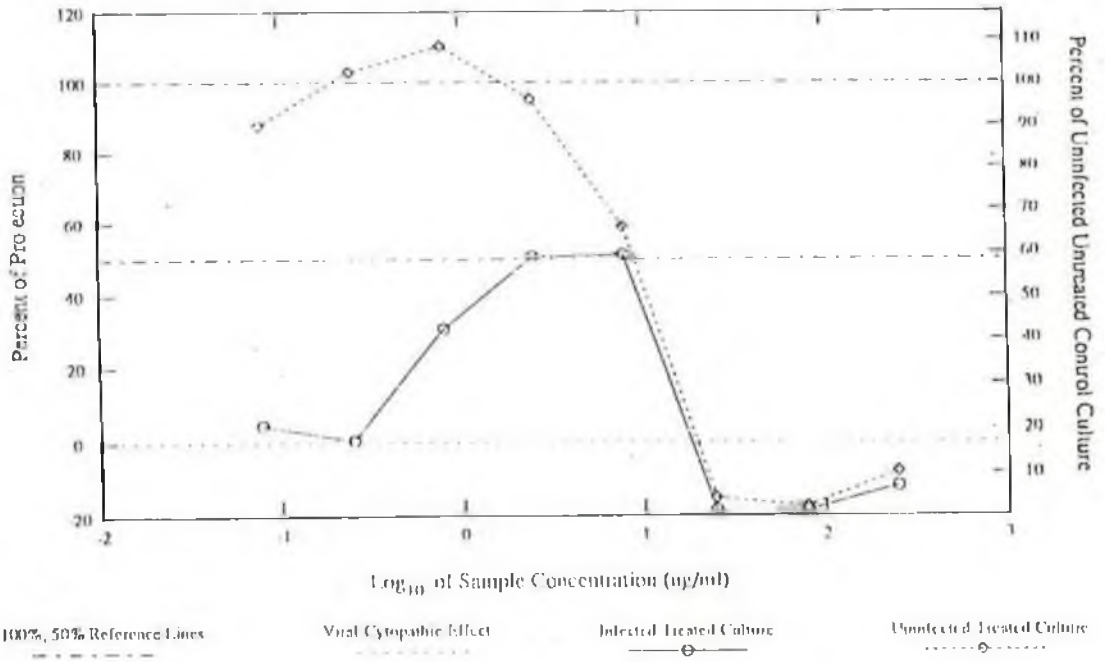

| Summary | | Dose (ug/ml) | Percent of Protection | Percent of Control | |
|---|---|---|---|---|---|
| Index | Concentration | | | Infected | Uninfected |
| IC50 (ug/ml) | $1.07 \times 10^{+1}$ | $7.94 \times 10^{-2}$ | -0.41 | 9.63 | 98.27 |
| EC50 (ug/ml) | $2.36 \times 10^{0}$ | $2.51 \times 10^{-1}$ | 14.40 | 22.96 | 100.17 |
| TI50 (IC/EC) | $4.56 \times 10^{0}$ | $7.93 \times 10^{-1}$ | 26.31 | 33.68 | 97.83 |
| Conclusion | | $2.50 \times 10^{0}$ | 51.48 | 56.24 | 98.27 |
| | | $7.92 \times 10^{0}$ | 50.60 | 55.54 | 67.44 |
| | | $2.50 \times 10^{+1}$ | -10.30 | 0.73 | 1.54 |
| | | $7.91 \times 10^{+1}$ | -7.54 | 3.22 | 3.30 |
| | | $2.50 \times 10^{+2}$ | -1.76 | 8.42 | 7.54 |

Fig 4: Anti-HIV screening results of DCM: MeOH (1: 1) extract

# National Cancer Institute
## Developmental Therapeutics Program
## *In Vitro* Testing Results

| | | | | |
|---|---|---|---|---|
| NSC: N84339-O/1 | Plate: 6730 | Lab: 9T | SSPL: | Assay: Primary Screen |
| Test Date: June 25, 1996 | Solubility Ind.: 1 | | | COMI: |
| Report Date: February 20, 1997 | Cell Line: CEM SS | | | Solvent: DMSO 100% |

*In Vitro* Anti-HIV Drug Screening Results
Primary Screen




| Summary | | Dose (ug/ml) | Percent of Protection | Percent of Control | |
|---|---|---|---|---|---|
| Index | Concentration | | | Infected | Uninfected |
| IC50 (ug/ml) | $1.06 \times 10^{+1}$ | $7.94 \times 10^{2}$ | 4.51 | 20.74 | 89.92 |
| EC50 (ug/ml) | $2.43 \times 10^{0}$ | $2.51 \times 10^{1}$ | 0.46 | 17.38 | 102.34 |
| TI50 (IC/EC) | $4.38 \times 10^{0}$ | $7.93 \times 10^{1}$ | 30.63 | 42.42 | 108.44 |
| Conclusion | | $2.50 \times 10^{0}$ | 50.58 | 58.98 | 96.02 |
| | | $7.92 \times 10^{0}$ | 51.01 | 59.34 | 65.78 |
| | | $2.50 \times 10^{-1}$ | -18.55 | 1.60 | 4.37 |
| | | $7.91 \times 10^{-1}$ | -18.65 | 1.52 | 2.50 |
| | | $2.50 \times 10^{-2}$ | -11.26 | 7.07 | 10.31 |

Fig. 5: Anti-HIV screening results of DCM: MeOH (1: 1) extract

## 2.5 CHEMICAL STUDIES

### 2.5.1 Fractionation of methanol soluble part (LSLDM, 120 g) of leaves:

Dried methanol extract was suspended in 10% methanol in ethyl acetate, ultrasonicated and stirred with magnetic stirrer for 3 h. The methanolic ethyl acetate soluble part was collected by centrifugation. The combined extract was evaporated to dryness. The 10% methanol soluble fraction was evaporated to dryness. The amount of 10% methanol soluble part was 16 g.

### 2.5.2 Fractionation of 10% methanol soluble part by Sephadex LH-20 gel column:

The 10% methanol soluble part (16 g) was dissolved in 20% methanol in water by sonication. The insoluble part was removed by centrifugation followed by cooling in a refrigerator and centrifuged. The clear solution was passed through the preconditioned Sephadex LH-20 gel column. The sample was eluted with 20% methanol in water, methanol and acetone successively and the eluents were collected in several conical flasks (500 ml). The colorless and green colored eluents were evaporated to dryness and two different parts, chlorophyll containing fraction (8.2 g) and chlorophyll free fraction (2.1 g) were obtained.

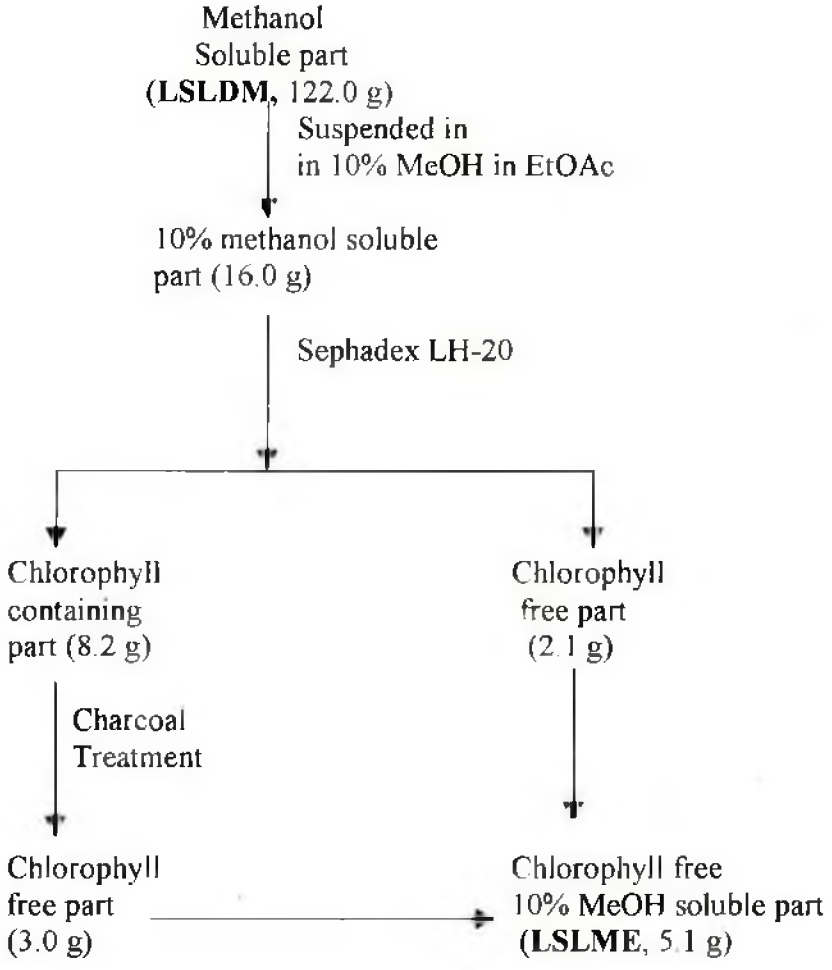
### 2.5.3 Removal of chlorophyll from the colored fraction (8.2 g):

Activated charcoal (25.0 g) was washed with water followed by methanol and acetone. The cleaned charcoal was dried at room temperature. The cleaned and conditioned charcoal was used to remove coloring materials from the sample.

The sample (8.2 g) was dissolved in methanol (250 ml). Then charcoal was added gradually with stirring and allowing to settle down. When methanol looked clear after adding ≈15 g of charcoal, the mixture was filtered and the filtrate was evaporate to dryness (3.0 g).

$^1$H-NMR spectra of chlorophyll free material from Sephadex LH-20 column and charcoal treated material were recorded. Both of the samples had similar $^1$H-NMR spectrum and these were combined. The amount of the combined fractions was 5.1 g (LSLME). The fractionation scheme is given below:

**Scheme 3: Fractionation of methanol soluble part of leaves**



**2.5.4 Fractionations of chlorophyll free fraction (LSLME) of leaves by column chromatography:**

TLC of LSLME (described in **section 2.1.4.1, 2.1.4.2**) performed in different solvent systems revealed that the extract was a mixture of several compounds.

**2.5.4.1 Preparation of column:**

A glass column (35 cm × 3 cm, i.d.) was packed with 70 g of column grade silica gel (230-400 mesh; particle size 0.04-0.063, ASTM, Art. 9385.5000, Merck) using dichloromethane as the equilibrating solvent. The column was flashed with two to three column volumes of dichloromethane and finally the column bed became 26.0 cm in length.

**2.5.4.2 Preparation of sample and application:**

Chlorophyll free methanol soluble part (LSLME, 5.1 g) of leaves was dissolved in a mixture of dichloromethane and methanol. Column grade silica gel (11.0 g) was added to the solution and the slurry was evaporated to dryness. The dried sample was ground with a mortar and pestle and then applied to the column (described in **section 2.1.5**)

**2.5.4.3 Fractionation of the applied sample:**

The applied sample (described in **section 2.5.4.2**) was eluted using dichloromethane and the polarity of the mobile phase was increased by adding ethyl acetate followed by methanol gradually. The eluted samples were collected in 100 ml conical flasks. The eluted samples were monitored by TLC and were combined on the basis of their $R_f$ values and finally five fractions ($LSLMEF_1$, $LSLMEF_2$, $LSLMEF_3$, $LSLMEF_4$ and $LSLMEF_5$) were obtained.

The amount of each fraction, the corresponding solvent polarity and TLC patterns are given in the **Table 12.**

**Scheme 4: Fractionation of chlorophyll free methanol soluble part of leaves**

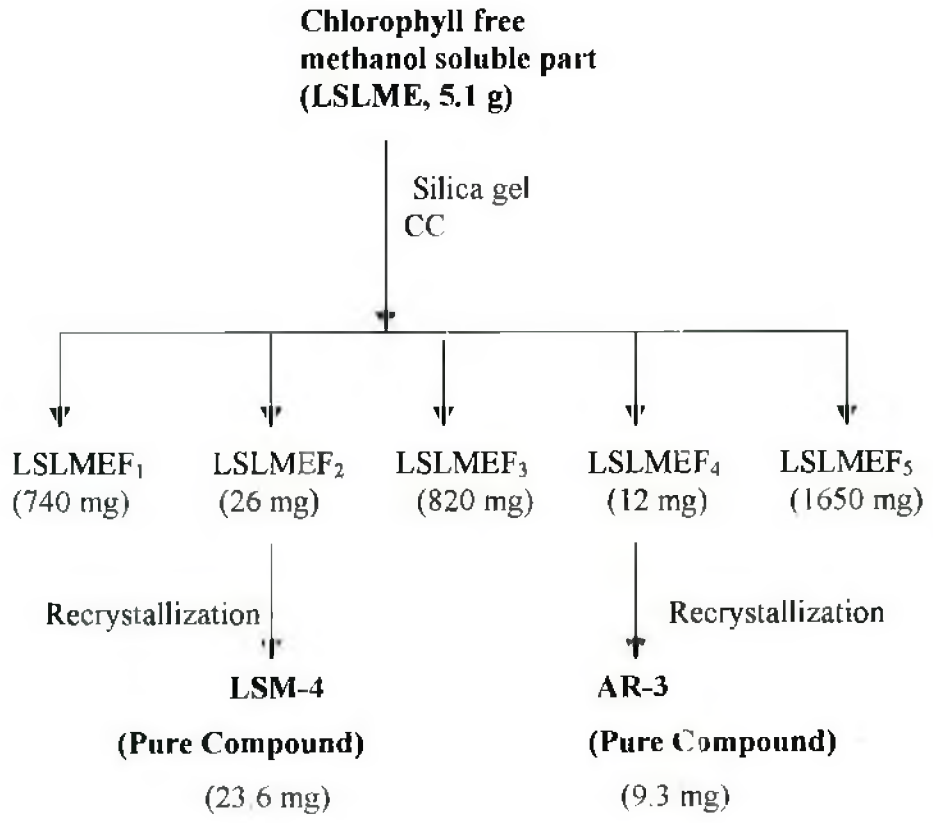



**Table 12: Different fractions of LSLME obtained by column chromatography.**

| Fraction No. | Solvent polarity (%) | | | Amount (mg) | TLC pattern |
|---|---|---|---|---|---|
| | $H_2O$ | MeOH | $CHCl_3$ | | |
| $LSLMEF_1$ | 1 | 4 | 95 | 740 | Elongated three spots |
| $LSLMEF_2$ | 2 | 6 | 92 | 26 | Single spot with tailing |
| $LSLMEF_3$ | 2 | 7 | 91 | 820 | Elongated two spots |
| $LSLMEF_4$ | 2 | 8 | 90 | 12 | Single spot with tailing |
| $LSLMEF_5$ | 4 | 12 | 86 | 1650 | Elongated three spots from base line |

### 2.5.5 Purification of fraction $LSLMEF_2$:

The fraction $LSLMEF_2$ gave a single spot with tailing in TLC. The fraction was washed repeatedly with hexane and dichloromethane to remove associated slight impurities. After washing, a white residue was obtained. The white residue was crystallized from mixture of hexane: dichloromethane (5:1) and a pure compound **LSM-4** (23.6 mg) was obtained.

### 2.5.6 Characterization of the compound LSM-4

#### 2.5.6.1 Physical characteristics of the compound LSM-4:

The compound LSM-4 was a white crystalline solid having **melting point 243-245$^0$C**. It was soluble in a mixture of dichloromethane and methanol. It gave violet color with vanillin-sulfuric acid reagent in TLC with **$R_f$ value 0.6** (2% MeOH in $CH_2Cl_2$ v/v) which indicated that LSM-4 might be a triterpene.

#### 2.5.6.2 Structure elucidation of the compound LSM-4 by spectroscopic methods:

**(a) Infrared (IR) spectroscopy:**

The **IR spectrum** of the compound LSM-4 (**Fig 6**) had absorption frequencies at 3410, 2910, 2850, 1680, 1442, 1365, 1280, 1105, 1050, 995, 980, 660 $cm^{-1}$.

**(b) $^1$H-NMR ($CDCl_3$+$CD_3OH$, 300 MHz) spectroscopy:**

In the **$^1$H-NMR spectrum** of the compound LSM-4 (**Fig 7**) had signals at 5.05 (1H,br *s*, H-12), 3.45 (1H, *d*, *J*=9.0 Hz, H-3α), 2.75 (1H, *d*, *J*=9.6 Hz, H-18α), 0.88, 0.81, 0.78, 0.61, .60 (each 3H, *s*, 5 × $CH_3$), 0.73 (3H, *d*, *J*=6.4Hz, $CH_3$), 0.65 (3H, *d*, *J*=5.9Hz) ppm.

**(c) Mass spectroscopy:**

**Electron impact mass spectrum (EIMS)** of the compound LSM-4 (**Fig 8**) had the molecular ion peak at *m/z* 472.7, base peak at *m/z* 247.9 and other intense peaks at *m/z* 441.7, 427.7, 407.7, 254.8, 202.9, 132.9 and 54.9.

### 2.5.7 Purification of fraction $LSLMEF_4$:

The fraction $LSLMEF_4$ gave a single spot with tailing in TLC. The fraction was washed with hexane and dichloromethane to remove associated slight impurities. After washing, a white residue was obtained. The white residue was crystallized from the mixture of dichloromethane: methanol (9:1 v/v) and gave a pure compound **AR-3** (9.3 mg) was obtained.

### 2.5.8 Characterization of the compound AR-3

#### 2.5.8.1 Physical characteristic of the compound AR-3:

The compound AR-3 was a white solid. It gave characteristic violet color of steroid with vanillin-sulfuric acid reagent. It was soluble in a mixture of methanol and chloroform and also soluble in pyridine. It gave positive test of steroid (Salkowski test and Leibermann-Burchard test described in **section 2.1.16**). It also gave positive test of sugar (phenol-sulfuric acid test described in **section 2.1.15**). The result indicated that the compound AR-3 might be a steroidal glycoside. **Melting point** of compound AR-3 was **283-285$^0$C**.

#### 2.5.8.2 Structural elucidation of the compound AR-3 by spectroscopic methods:

**(a) $^1$H-NMR (Pyridine, 400 MHz) spectroscopy:**

In the **$^1$H-NMR spectrum** of the compound AR-3 are shown in **(Fig 9, 9a, 9b)** and the spectral data are given in **Table 13**.

**Table 13: $^{1}$H-NMR spectral data of the compound AR-3 (Pyridine, 400 MHz)**

| Chemical shift (ppm) | Splitting pattern | Integration | Assignment |
|---|---|---|---|
| 5.34 | Distorted doublet | 1H | Olefmic proton |
| 5.05 | Doublet | 1H | Anomeric proton of sugar moiety |
| 3.92-4.57 | Multiplet | | Oxygenated methine protons of sugar moiety |
| 0.66 | Singlet | 3H | $CH_3$ group |
| 0.89 | Doublet | 3H | $CH_3$ group |
| 0.87 | Doublet | 6H | 2 $CH_3$ groups |
| 0.93 | Singlet | 3H | $CH_3$ group |
| 0.85 | Singlet | 3H | $CH_3$ group |

**(b) $^{13}$C-NMR spectroscopy:**

The **$^{13}$C-NMR spectrum** of the compound AR-3 **(Fig 10, 10a, 10b, 10c)** had 35 signals indicating that the compound contained 35 carbons. The spectral data are given in **Table 14**.

**Table 14: $^{13}$C-NMR data of the compound AR-3 (Pyridine, 400 MHz).**

| Position of carbons | Chemical shift (ppm) of AR-3 | Position of Carbons | Chemical shift (ppm) of AR-3 |
|---|---|---|---|
| C-1 | 37.51 | C-19 | 19.44 |
| C-2 | 30.28 | C-20 | 36.41 |
| C-3 | 78.13 | C-21 | 19.24 |
| C-4 | 39.37 | C-22 | 34.24 |
| C-5 | 140.94 | C-23 | 26.44 |
| C-6 | 121.94 | C-24 | 46.08 |
| C-7 | 32.09 | C-25 | 29.50 |
| C-8 | 32.20 | C-26 | 19.03 |
| C-9 | 50.38 | C-27 | 19.99 |
| C-10 | 36.95 | C-28 | 23.42 |
| C-11 | 21.31 | C-29 | 12.18 |
| C-12 | 39.98 | C-1′ | 102.61 |
| C-13 | 42.51 | C-2′ | 75.37 |
| C-14 | 56.86 | C-3′ | 78.50 |
| C-15 | 24.53 | C-4′ | 78.64 |
| C-16 | 28.56 | C-5′ | 71.74 |
| C-17 | 56.28 | C-6′ | 62.88 |
| C-18 | 12.00 | | |

**(c) Fast atom bombardment mass spectroscopy (FAB$^+$MS):**

The molecular mass of AR-3 was found to be $MH^+$ at *m/z* at 547 in **FAB$^+$MS (Fig 12, 12a)** ran with glycerol as the matrix. The major peaks appeared at *m/z* 396 [$MH^+$-Glu-$H_2O$], 213, 255, 145 and 213.

**2.5.9 Fractionation of dichloromethane extract of leaves (LSLD) by column chromatography:**

**2.5.9.1 Preparation of column:**

A glass column (35 cm× 12 cm, i.d.) was packed with 450 g of column grade silica gel using hexane as the equilibrating solvent. The column was flashed with three column volumes of hexane and finally the column bed became 23 cm.

**2.5.9.2 Preparation of sample and application:**

Dichloromethane extract (LSLD, 120.0 g) of leaves was dissolved in a mixture of dichloromethane and methanol and 240 g of column grade silica gel was added to the solution and the slurry was evaporated to dryness. The dried sample was ground to powder with a mortar and pestle and then applied to the column (described in **section 2.1.5**).

**2.5.9.3 Fractionation of the applied sample:**

The applied sample (described in **section 2.5.9.2**) was eluted using hexane and the polarity of the mobile phase was increased by adding dichloromethane followed by ethyl acetate gradually. The eluted samples were collected in 57 conical flasks. The eluted samples were monitored by TLC and were combined on the basis of their $R_f$ values and finally six fractions were obtained.

The amount of each fraction, the corresponding solvent polarity and TLC patterns are given in **Table 15.**

**Table 15: Different fractions of dichloromethane extract of leaves obtained by column chromatography.**

| Fraction No. | Solvent polarity (%) | | | Amount (g) | TLC pattern |
|---|---|---|---|---|---|
| | Hexane | $CH_2Cl_2$ | EtOAc | | |
| $LSLDF_1$ | 100 | 0 | 0 | 4.6 | Single spot |
| $LSLDF_2$ | 20 | 80 | 0 | 10.3 | Four spots |
| $LSLDF_3$ | 0 | 100 | 0 | 9.0 | Separated more than five spots |
| $LSLDF_4$ | 0 | 50 | 50 | 19.0 | Chlorophyll containing five spots |
| $LSLDF_5$ | 0 | 20 | 80 | 15.0 | Two spots with tailing |
| $LSLDF_6$ | 0 | 0 | 100 | 31.1 | Two spots with tailing from the base |

### 2.5.10 Fractionation of $LSLDF_3$ by column chromatography:

A glass column (26 cm× 4.5 cm; i.d.) was packed with 260 g of column grade silica gel using hexane as the equilibrating solvent. The column was flashed with three column volumes of hexane and finally the column bed became 18 cm.

The sample was ($LSLDF_3$, 9 g) was applied to the column in powdered form as described in section 2.1.5 The sample was eluted using hexane and the polarity of the mobile phase was increased by adding dichloromethane followed by ethyl acetate gradually. The eluted fractions were collected in 60 conical flasks. Five different fractions were obtained by pooling different fractions on the basis of their $R_f$ values.

The amount of each fraction, the corresponding solvent polarity and TLC patterns are given in the **Table 16.**

**Table 16: Different fractions $LSLDF_3$ obtained by column chromatography.**

| Fraction No. | Solvent polarity (%) | | | Amount (g) | TLC pattern |
|---|---|---|---|---|---|
| | Hexane | $CH_2Cl_2$ | EtOAc | | |
| $LSLDC_2F_1$ | 100 | 0 | 0 | 0.24 | Two spots with tailing |
| $LSLDC_2F_2$ | 90 | 10 | 0 | 0.32 | Elongated two spots |
| $LSLDC_2F_3$ | 60 | 40 | 0 | 4.00 | Four spots with tailing |
| $LSLDC_2F_4$ | 0 | 100 | 0 | 0.36 | Elongated three spots |
| $LSLDC_2F_5$ | 0 | 80 | 20 | 0.53 | No separated spots |
| $LSLDC_2F_6$ | 0 | 20 | 80 | 0.28 | Tailing |
| $LSLDC_2F_7$ | 0 | 0 | 80 | 0.62 | Tailing |

### 2.5.11 Fractionation of $LSLDC_2F_3$ by column chromatography:

A glass column (26 cm× 3 cm; i.d.) was packed with 110 g of column grade silica gel using hexane as the equilibrating solvent. The column was flashed with three column volumes of hexane and finally the column bed became 16 cm.

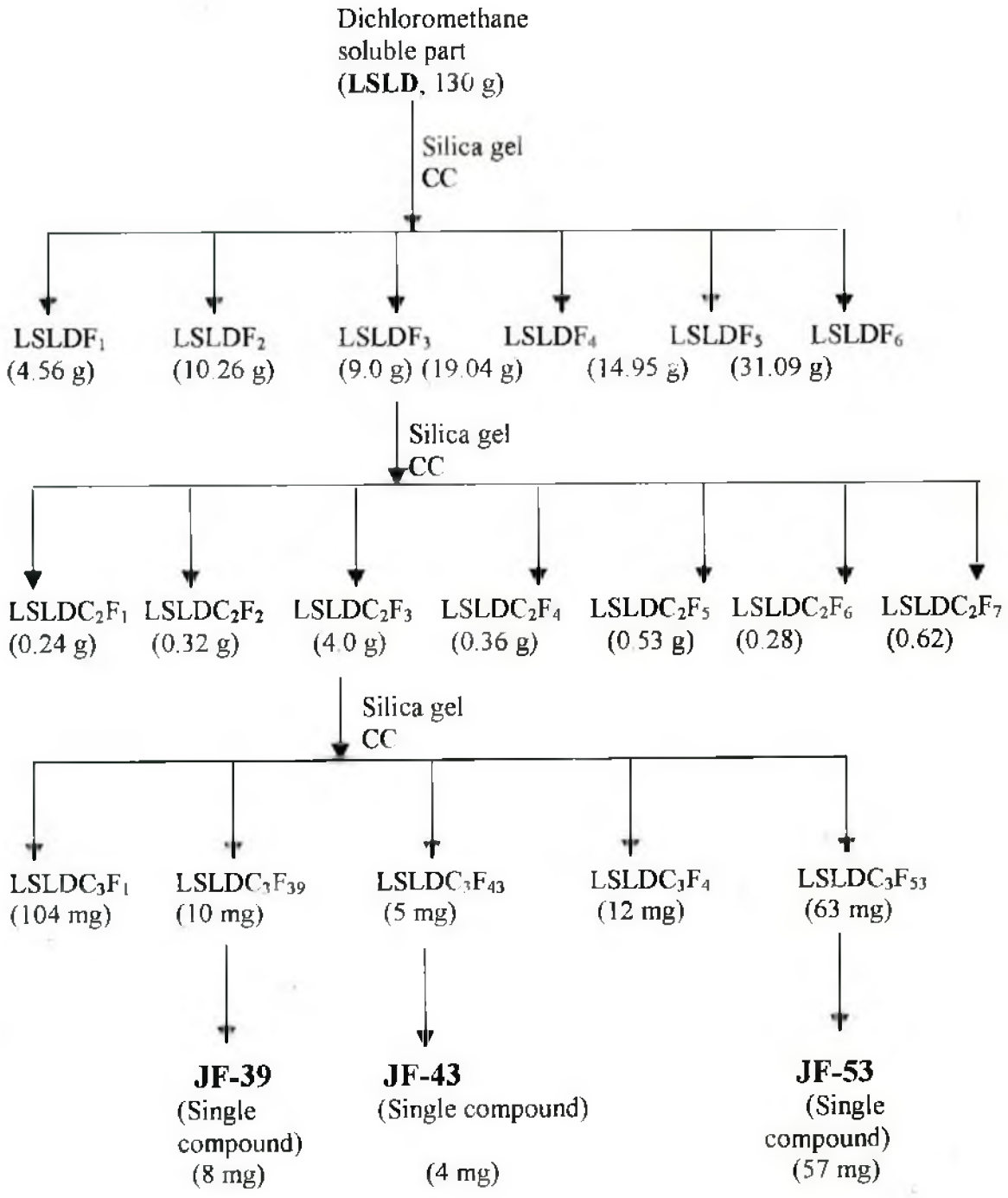
The sample was ($LSLDC_2F_3$, 4.0 g) was applied to the column in powdered form as described in section 2.1.6. The sample was eluted using hexane and the polarity of the mobile phase was increased by adding dichloromethane followed by ethyl acetate gradually. The eluted fractions were collected in 54 conical flasks. Five different fractions were obtained by pooling different fractions on the basis of their $R_f$ values.

The amount of each fraction, the corresponding solvent polarity and TLC patterns are given in the **Table 17.**

**Table 17: Different fractions of $LSLDC_2F_3$ obtained by column chromatography.**

| Fraction No. | Solvent polarity (%) | | | Amount (mg) | TLC pattern |
|---|---|---|---|---|---|
| | Hexane | $CH_2Cl_2$ | EtOAc | | |
| $LSLDC_3F_1$ | 80 | 20 | 0 | 104 | Two spots with tailing |
| $LSLDC_3F_2$ | 70 | 30 | 0 | 8 | One spot with tailing |
| $LSLDC_3F_3$ | 60 | 40 | 0 | 4 | One spots with tailing |
| $LSLDC_3F_4$ | 0 | 100 | 0 | 12 | No separated spots |
| $LSLDC_3F_5$ | 0 | 80 | 20 | 630 | One spots with tailing |

**Scheme 5: Fractionation of dichloromethane soluble part (LSLD) by column chromatography:**



### 2.5.12 Purification of fraction $LSLDC_3F_{39}$:

The fraction $LSLDC_3F_{39}$ gave a single spot with tailing in TLC. The fraction was repeatedly washed with methanol to remove associated impurities. After washing, a white solid was obtained JF-39 (8 mg).

### 2.5.13 Characterization of compound JF-39:

The compound JF-39 was a white solid. It gave violet color with vanillin-sulfuric acid reagent. It was readily soluble in hexane and dichloromethane. Melting point of compound JF-39 was $67^0$C.

### 2.5.13.1 Structure elucidation of the compound JF-39 by spectroscopic methods:

#### (a) Infrared (IR) spectroscopy:

In **infrared spectrum** of the compound JF-39 **(Fig 13)** there were absorption frequencies at 2905, 2830, 1455, 1360, 730 and 720 $cm^{-1}$.

#### (b) Gas Chromatography-Mass spectroscopy (GC-MS):

The gas chromatograph of the compound JF-39 **(Fig 14)** showed a single peak with retention time 21.08. The mass fragmentation pattern **(Fig 15)** of the corresponding chromatograph showed peaks at *m/z* 267, 236, 209, 195, 181, 167, 153, 139, 125, 111, 97, 83, 69, 57 and 43.

### 2.5.14 Purification of fraction $LSLDC_3F_{43}$:

The fraction $LSLDC_3F_{43}$ gave a single spot with tailing in TLC. The fraction was repeatedly washed with methanol to remove associated impurities. After washing, a white solid was obtained JF-43 (4 mg).

### 2.5.14.1 Structure elucidation of the compound JF-43 by Gas Chromatography-Mass spectroscopy (GC-MS):

The gas chromatograph of the compound JF-43 showed a single peak with retention time 57.93. The corresponding gas chromatograph gave a mass pattern (**Fig 16**) showed the molecular peak at *m/z* 380 and the other intense peaks were at *m/z* 239, 225, 197, 183, 169, 155, 141, 127, 113, 99, 85, 71, 57 and 43.

### 2.5.15 Purification of fraction $LSLDC_3F_{53}$:

The fraction $LSLDC_3F_{53}$ gave a single spot with tailing in TLC. The fraction was washed with methanol repeatedly to remove associated impurities. After washing, a white solid was obtained and assigned as JF-53 (630 mg).

### 2.5.15.1 Structural elucidation of the compound JF-53 by Gas Chromatography-Mass spectroscopy (GC-MS):

The gas chromatograph showed a single peak with retention time 86.68. The corresponding gas chromatograph gave a mass fragmentation (**Fig 17**) in MS peaks at *m/z* 436 and the other intense peaks were at *m/z* 239, 225, 210, 183, 169, 155, 141, 127, 113, 99, 85, 71, 57 and 43.

### 2.5.16 Fractionations of dichloromethane extract of bark (LSBED) with Diion HP-20.

#### 2.5.16.1 HP-20 Diion:

HP–20 is a polyaromatic, hydrophobic compound. The Diion HP-20 was poured through a sintered funnel. Then the eluents were treated with silver nitrate for the test of $Cl^-$. Until it was free from chloride ion, the Diion was washed with methanol followed by acetone.

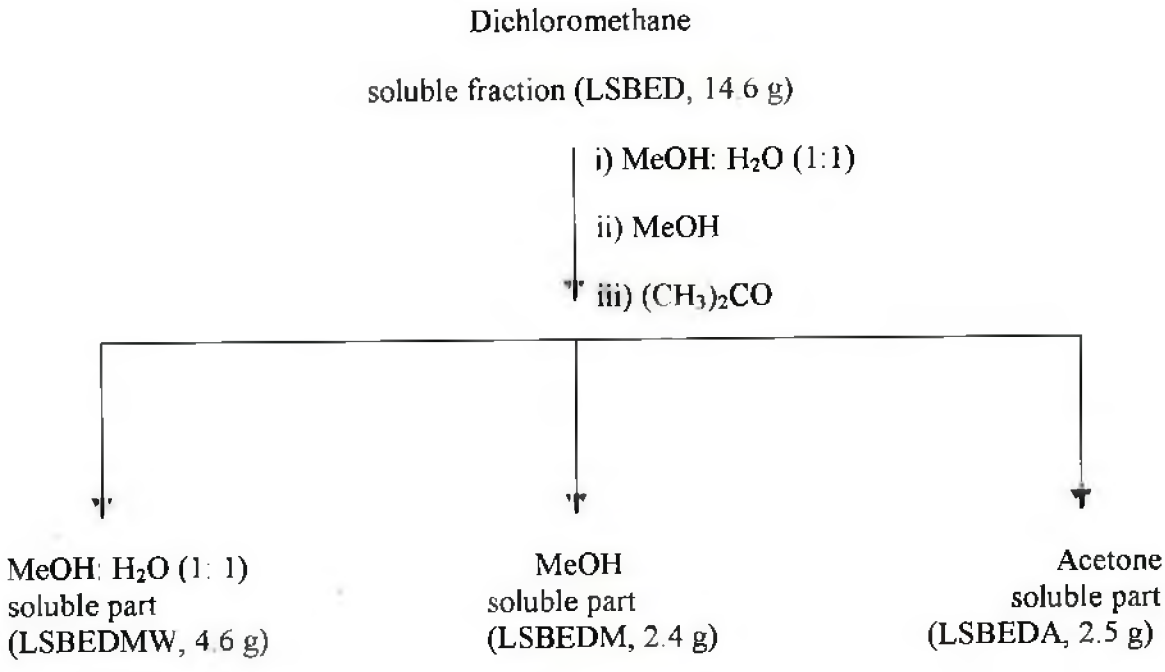
#### 2.5.16.2 Preparation of HP–20 column:

A glass column (55.6 cm × 8.0 cm; i.d.) was packed with preconditioned HP–20 Diion (35 g) using methanol and water mixture (1: 1) as the mobile phase. The column was equilibrated by passing two column volumes of mobile phase.

#### 2.5.16.3 Fractionations of dichloromethane extract of bark (LSBED) with Diion HP-20 gel column chromatography.

The dichloromethane soluble part (LSBED, 14.6 g) was suspended in the mixture of methanol and water (1: 1). Then the suspension was passed through the prepacked HP–20 column. The sample was eluted with methanol and water (1: 1) followed by methanol, and acetone. The elutes were collected in three conical flasks and the elutes were evaporated to dryness gave (LSBEDMW, 4.6 g); (LSBEDM, 2.4 g) and (LSBEDA, 2.5 g). The scheme of fractionation and the amount of each fractions are given below.

**Scheme 6: Fractionation of dichloromethane soluble part (LSBED) by diion HP-20 gel column**




**2.5.17 Fractionation of methanol soluble part (LSBEDM) by column chromatography:**

A glass column (18 cm × 2.5 cm; i.d.) was packed with column grade silica gel (60 g) using dichloromethane as the equilibrating solvent. The column was flashed with three column volumes of dichloromethane and finally the column bed became 15 cm.

The sample was (LSBEDM, 2.4 g) was applied to the column in the powder form as described in the **section 2.1.5**. The sample was eluted using dichloromethane and adding ethyl acetate followed by methanol gradually increased the polarity of solvent. The eluted fractions were collected in 59 conical flasks. Six different fractions were obtained by pooling different fractions on the basis of their $R_f$ values.

The amount of each fraction, the corresponding solvent polarity and TLC patterns are given in the **Table 18**.

**Scheme 7: Fractionation of methanol soluble part (LSBEDM) by column chromatography**

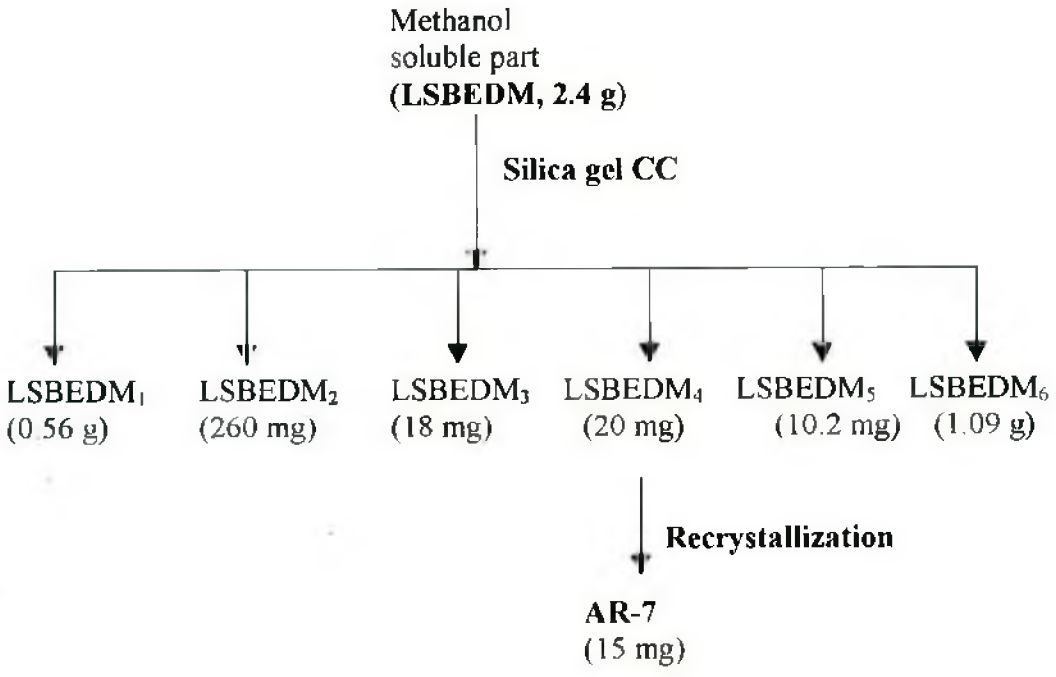



**Table 18: Different fractions of LSBEDM obtained by column chromatography.**

| Fraction No. | Solvent polarity (%) | | | Amount (mg) | TLC pattern |
|---|---|---|---|---|---|
| | $CHCl_3$ | EtOAc | MeOH | | |
| $LSBEDM_1$ | 80 | 20 | 0 | 560 | Elongated four spots |
| $LSBEDM_2$ | 50 | 50 | 0 | 260 | Elongated four spots |
| $LSBEDM_3$ | 0 | 100 | 0 | 18 | Elongated two spots |
| $LSBEDM_4$ | 0 | 95 | 5 | 20 | Single spot with tailing |
| $LSBEDM_5$ | 0 | 80 | 20 | 10.2 | Elongated three spots |
| $LSBEDM_6$ | 0 | 70 | 30 | 1090 | Elongated four spots |

### 2.5.18 Purification of fraction $LSBEDM_4$:

The fraction $LSBEDM_4$ gave a single spot with tailing in TLC. The fraction was repeatedly washed with hexane and dichloromethane to remove associated impurities. After washing, a white solid was obtained. The white residue was crystallized from the mixture of solvent dichloromethane: methanol (4:1 v/v). The crystalline solid was named as AR-7 (15 mg).

### 2.5.19 Characterization of the compound AR-7

#### 2.5.19.1 Physical characterization of the compound:

The compound AR-7 was isolated as a light yellow amorphous solid. It was sparingly soluble in methanol but completely soluble in the mixture of chloroform and methanol and also in pyridine. It had a melting point 296-298$^0$C. It gave pink colour with vanillin-sulfuric acid reagent on TLC with $R_f$ value 0.68 using dichloromethane and methanol (4: 1) as mobile phase.

#### 2.5.19.2 Structure elucidation of the compound AR-7 by spectroscopic methods:

**(a) $^1$H-NMR (DMSO, 400 MHz) spectroscopy:**

In the $^1$H-NMR spectrum of the compound AR-7 (**Fig 18, 18a, 18b**) had signals at 8.21 (*s*, 1H, -OH at C-6) for hydroxyl proton and 7.59 (*s*, 2H, C-1 & C-8) ppm for two aromatic protons. The three sharp signals were at 4.11 (*s*, 3H, -$OCH_3$ at C-7), 4.04 (*s*, 3H, -$OCH_3$ at C-3) and 3.99 (*s*, 3H, -$OCH_3$ at C-2) ppm. The spectral data are given in the **Table 19.**

**Table 19: $^1$H-NMR data and assignment of the compound AR-7 (Pyridine)**

| Chemical shift In ppm | Splitting Pattern | Integration | Assignment |
|---|---|---|---|
| 8.21 | Singlet | 1H | One –OH proton in aromatic ring |
| 7.59 | Singlet | 2H | Two aromatic protons |
| 4.11 | Singlet | 3H | Three -$OCH_3$ protons |
| 4.04 | Singlet | 3H | Three -$OCH_3$ protons |
| 3.99 | Singlet | 3H | Three -$OCH_3$ protons |

**(b) $^{13}$C-NMR spectroscopy:**

The **$^{13}$C-NMR spectrum** of the compound AR-7 **(Fig. 19, 19a)** had 17 signals indicating that the compound contained 17 carbons. The two down field signals at 158.39 and 158.28 ppm were assigned to two carbonyl carbons. The signal at 154.35 ppm was due to carbon which contain a hydroxyl group. The two signals appeared at 117.67 and 107.46 in DEPT were due to methylene carbons. Three most upfield signals at 61.47, 61.31 and 56.73 were due to three oxymethyl groups. The other signals at 114.08, 112.87, 112.73 and 111.43 were quaternary aromatic carbons.

**(c) Mass spectroscopy:**

The fast atom bombardment mass spectrum **($FAB^+MS$) (Fig. 21, 21a)** showed the pseudo molecular ion peak at *m/z* 367 for $[M+H+Na]^+$, $[344+1+23]^+$, thus establishing a molecular formula $C_{17}H_{12}O_8$ and other intense peaks were at *m/z* 309, 307, 275, 155, 152, 119, 85, 57, 47, 23.

**2.5.20 Fractionation of acetone soluble part of bark (LSBEDA) by column chromatography:**

A glass column (26 cm x 3 cm; i.d.) was packed with column grade silica gel (70 g) using 80% hexane in dichloromethane as the equilibrating solvent. The column was flashed with three column volumes of 80% hexane in dichloromethane and finally the column bed became 15 cm.

The sample was (LSBEDA, 2.5 g) was applied to the column in the powder form as described in **section 2.1.5**. The sample was eluted with 80% hexane in dichloromethane and the polarity of the solvent was gradually increased with ethyl acetate followed by methanol. The eluted fractions were collected in 31 conical flasks. Five different fractions were obtained by pooling different fractions on the basis of their $R_f$ values.

The amount of each fraction, the corresponding solvent polarity and TLC patterns are given in the **Table 20.**

**Scheme 8: Fractionation of Acetone soluble part (LSBEDA) by chromatographic techniques which are shown below:**

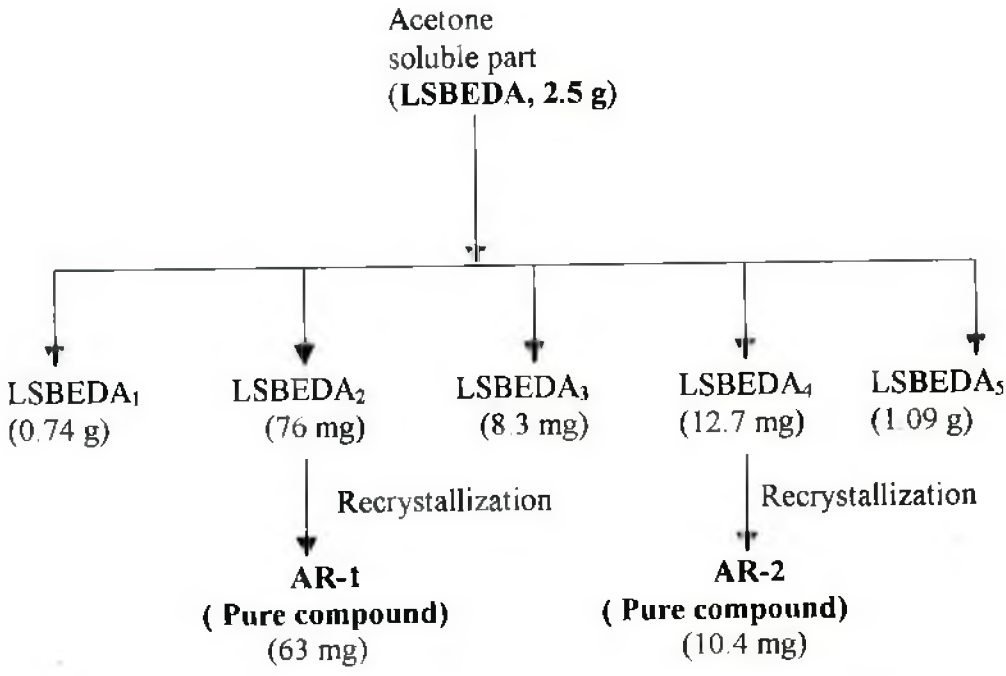



**Table 20: Different fractions of acetone soluble part (LSBEDA) obtained by column chromatography.**

| Fraction No. | Solvent polarity (%) | | | | Amount (mg) | TLC pattern |
|---|---|---|---|---|---|---|
| | Hexane | $CHCl_3$ | EtOAc | MeOH | | |
| $LSBEDA_1$ | 80 | 20 | 0 | 0 | 740 | Elongated four spots |
| $LSBEDA_2$ | 0 | 100 | 0 | 0 | 76 | Single spot with tailing |
| $LSBEDA_3$ | 0 | 98 | 2 | 0 | 8.3 | Elongated two spots |
| $LSBEDA_4$ | 0 | 95 | 5 | 0 | 12.7 | Single spot with tailing |
| $LSBEDA_5$ | 0 | 0 | 100 | 20 | 1090 | Elongated three spots |

**2.5.21 Purification of the fraction $LSBEDA_2$:**

The fraction $LSBEDA_2$ gave a single spot with tailing in TLC. The fraction was repeatedly washed with hexane to remove associated impurities. After washing, a white residue was obtained. The white residue was crystallized from the mixture of hexane: dichloromethane (8:2 v/v) and very nice needle shaped crystals were obtained. The crystalline solid was named as AR-1 (63 mg).

### 2.5.22 Characterization of the compound AR-1:

#### 2.5.22.1 Physical characteristics and chemical tests:

Compound AR-1 was a colorless crystalline solid having melting point $118^0$-$119^0$C. It was readily soluble in chloroform. It gave a single spot on TLC with $R_f$ value of 0.60 using chloroform as mobile phase. It gave positive tests for steroids. Thus, the compound AR-1 might be a steroid.

#### 2.5.22.2 Structure elucidation of compound AR-1 by spectroscopic methods:

**(a) Infrared (IR) spectroscopy:**

The IR spectrum of the compound AR-1 **(Fig 22)** had absorption frequencies at 3415, 2920, 2850, 1640, 1455, 1370, 1055, 980 and 960 $cm^{-1}$.

**(b) $^1$H-NMR spectroscopy:**

The $^1$H-NMR spectrum of the compound AR-1 is shown in **(Fig 23)** and the spectral data are given in the **Table 21**.

**Table 21: $^1$H-NMR spectral data of the compound AR-1 (Pyridine, 400 MHz)**

| Chemical shift (ppm) of AR-1 | Splitting pattern | Integration | Assignment |
|---|---|---|---|
| 0.99 | Singlet | 3H | $-CH_3$ protons |
| 0.66 | Singlet | 3H | $-CH_3$ protons |
| 0.90 | Doublet | 6H | Two $-CH_3$ protons |
| 0.66 | Singlet | 3H | $-CH_3$ protons |
| 0.80 | Triplet | 3H | $-CH_3$ protons |
| 5.35 | Broad doublet | 1H | Olefinic proton |
| 3.50 | Septet | 1H | Oxymethelene proton $-CH_2$-CHOH-$CH_2$- |
| 1.03-2.29 | | | Different methylene ($-CH_2-$) and methyne (>CH-) protons |

**(c) $^{13}$C-NMR spectroscopy:**

The $^{13}$C-NMR spectrum of the compound AR-1 **(Fig 24, 24a)** had 29 signals indicating that the compound contained 29 carbons and the chemical shift of carbons (δC in ppm; C1to C29) are 37.26, 31.66, 71.80, 42.30, 140.76, 121.71, 31.91, 31.91, 50.14, 36.50, 21.08, 39.78, 42.32, 56.77, 24.30, 28.24, 56.06, 11.85, 19.39, 36.14, 18.77, 33.95, 26.09, 45.84, 29.16, 19.80, 19.03, 23.07, 11.97.

**(d) Mass spectroscopy:**

The mass spectrum of the compound AR-1 **(Fig 26)** had peaks at 397 $[M-H_2O+H]^+$, 414-18+1, 55, 69, 81, 119, 145, 159, 213, 273, 381, and 395.

**2.5.23 Purification of fraction LSBEDA$_4$:**

The fraction LSBEDA$_4$ gave a single spot with tailing in TLC. The fraction was repeatedly washed with hexane and dichloromethane to remove associated impurities. After washing, a white residue was obtained. The white residue was crystallized from the mixture of dichloromethane: methanol (4:1 v/v). The crystalline solid was assigned as **AR-2** (10.4 mg).

**2.5.24 Characterization of the compound AR-2**

**2.5.24.1 Physical characteristics of the compound AR-2:**

The compound AR-2 was a white crystalline solid having melting point 294-297$^0$C. It was soluble in dimethylsulfoxide (DMSO). It gave a single spot on TLC with $R_f$ value 0.65 using methanol in dichloromethane (1: 4 v/v). It gave violet color with vanillin-sulfuric acid reagent.

### 2.5.24.2 Structure elucidation of the compound AR-2 by spectroscopic methods:

#### (a) $^1$H-NMR (DMSO, 400 MHz) spectroscopy:

In the $^1$H-NMR spectrum of the compound AR-2 (**Fig 27, 27a**) had signals at 7.60 (*s*, 1H, at C-6) 7.49 (*s*, 1H, C-1) ppm for two aromatic protons. The three sharp signals were at 4.50 (*s*, 3H, -$OCH_3$ at C-8), 4.45 (*s*, 3H, -$OCH_3$ at C-3) and 4.00 (*s*, 3H, -$OCH_3$ at C-2) ppm indicated the presence of three oxymethyl groups in the compound AR-2. The $^1$H-NMR data are given in **Table 22**.

**Table 22: $^1$H-NMR data of the compound AR-2 (Pyridine, 400 MHz)**

| Chemical shift (ppm) | Splitting pattern | Integration | Assignment |
|---|---|---|---|
| 7.5 | Singlet | 1H | One aromatic proton at C-1 |
| 7.6 | Singlet | 1H | One aromatic proton at C-6 |
| 4.05 | Singlet | 3H | Three -$OCH_3$ protons at C-8 |
| 4.03 | Singlet | 3H | Three -$OCH_3$ protons at C-3 |
| 3.99 | Singlet | 3H | Three -$OCH_3$ protons at C-2 |

#### (b) Mass spectroscopy:

The fast atom bombardment mass spectrum (**FAB$^+$MS**) (**Fig 28, 28a, 28b**) showed the pseudo molecular ion peak at *m/z* 345 for $[M+H]^+$, thus establishing a molecular formula $C_{17}H_{12}O_8$ and other intense peaks at *m/z* 306, 235, 157, 79, 63, 47.

**2.5.25 Analysis of fatty acids:**

**2.5.25.1 Identification and quantification of fatty acid as methyl esters:**

The hexane extract (150 mg) of *Lagerstroemia speciosa* **L** (flowers) was taken in a pear-shaped flask. Methanolic sodium hydroxide (0.5 M, 5 ml) was added to it and shaked well. The mixture was refluxed for 30 minute in a boiling water bath. Then the mixture was evaporated to dryness by means of a rotary evaporator.

Boron-trifluoride methanol complex ($BF_3$-MeOH, 5 ml) was added to the dried material. The mixture was heated on a boiling water bath for 6-10 minute. The mixture was then evaporated to dryness and extracted with hexane (3×15 ml). The hexane soluble part containing the methylesters of fatty acids was analyzed by GLC/ GC-MS **(Scheme 9)**.

Fatty acid esters of leaves and bark were also identified following same procedure as described above.

Methylesters of caprylic, nonanoic, undecanoic, lauric, myristeric, palmitic, oleic, stearic, arachidic and behenic acids were used as reference fatty acid esters in the GLC method. These authentic methylesters of fatty acids were obtained from the Sigma Chemical Co., USA.

The percentage of each fatty acid was calculated by comparing its peak area with the total peak area. The results are presented in the **Table 23-25**.

The individual fatty acid esters were also identified by using gas chromatography (GC) and mass spectrometry (MS) and the components were compared with published spectra of NIST 107 Library (Shimadzu Corporation).

It was found that palmitic, oleic, stearic acids were the major quantity of fatty acids found in the extract of flowers. On the other hand, Oleic, palmotic, stearic acids and oleic, palmitic, linoleic acids were found as major acids in leaves and bark, respectively.

**Scheme 9: Extraction and preparation of methyl esters of fatty acids of *Lagerstroemia speciosa* L:**

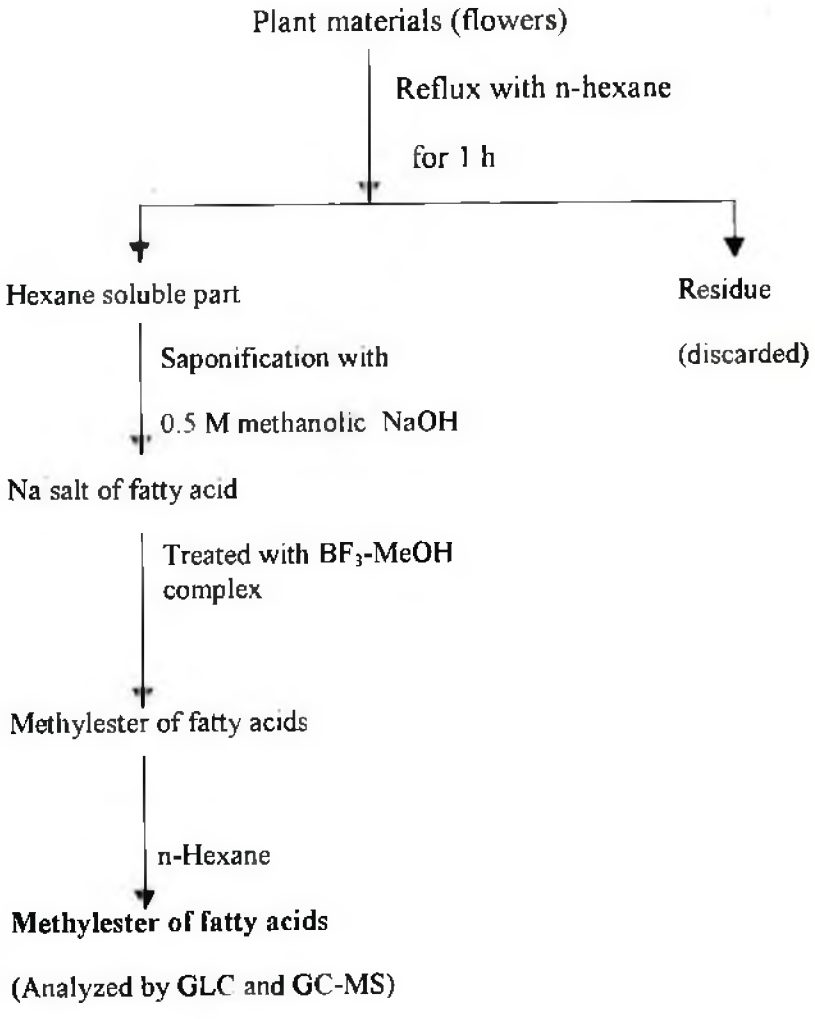


**Table 23: Analysis of fatty acids esters of *Lagerstroemia speciosa* L (Flower)**

| Retention time | Name of the fatty acids (as methyl esters) | Molecular formula | Relative % of fatty acids |
|---|---|---|---|
| 8.12 | Nonanoic acid | $C_{10}H_{20}O_2$ | 0.27 |
| 11.39 | Undecanoic acid | $C_{12}H_{24}O_2$ | 0.28 |
| 18.87 | Lauric acid | $C_{13}H_{26}O_2$ | 0.28 |
| 26.40 | Myristic acid | $C_{15}H_{30}O_2$ | 1.75 |
| 29.31 | 4-Octadecenoic acid | $C_{19}H_{36}O_2$ | 0.80 |
| 33.50 | Palmitic acid | $C_{17}H_{34}O_2$ | 24.25 |
| 36.78 | 15-methyl hexadecanoic acid | $C_{18}H_{36}O_2$ | 0.39 |
| 38.70 | Linoleic acid | $C_{19}H_{34}O_2$ | 15.69 |
| 38.79 | 11, 14, 17-Ecosatrienoic acid | $C_{21}H_{36}O_2$ | 5.85 |
| 39.00 | 11-Octadecenoic acid | $C_{19}H_{36}O_2$ | 0.76 |
| 39.16 | Stearic acid | $C_{19}H_{38}O_2$ | 5.84 |
| 45.25 | Oleic acid | $C_{19}H_{36}O_2$ | 0.34 |
| 45.95 | Ecosanoic acid | $C_{21}H_{42}O_2$ | 4.83 |
| 50.93 | Behenic acid | $C_{23}H_{46}O_2$ | 0.26 |
| 58.47 | Tetracosanoic acid | $C_{25}H_{50}O_2$ | 1.17 |
| 69.65 | Hexacosanoic acid | $C_{27}H_{54}O_2$ | 0.73 |

**Table 24: Analysis of fatty acids esters of *Lagerstroemia speciosa* L (leaves)**

| Retention time | Name of the fatty acids (as methyl esters) | Molecular formula | Relative % of fatty acids |
|---|---|---|---|
| 11.99 | Lauric acid | $C_{13}H_{26}O_2$ | 4.72 |
| 17.59 | Myristic acid | $C_{15}H_{30}O_2$ | 4.08 |
| 22.40 | Palmotelic acid | $C_{15}H_{30}O_2$ | 1.93 |
| 22.97 | Palmitic acid | $C_{17}H_{34}O_2$ | 16.09 |
| 27.16 | Linoleic acid | $C_{19}H_{34}O_2$ | 7.50 |
| 27.32 | Oleic acid | $C_{19}H_{36}O_2$ | 16.19 |
| 27.97 | Stearic acid | $C_{19}H_{38}O_2$ | 6.57 |
| 32.65 | Arachidic acid | $C_{19}H_{38}O_2$ | 4.84 |
| 50.93 | Behenic acid | $C_{23}H_{46}O_2$ | Trace amount |

**Table 25: Analysis of fatty acids esters of *Lagerstroemia speciosa* L (Bark)**

| Retention time | Name of the fatty acid (as methyl esters) | Molecular formula | Relative % of fatty acids |
|---|---|---|---|
| 17.35 | Myristic acid | $C_{15}H_{30}O_2$ | 2.32 |
| 22.15 | Palmotelic acid | $C_{15}H_{30}O_2$ | 1.18 |
| 22.73 | Palmitic acid | $C_{17}H_{34}O_2$ | 23.43 |
| 26.92 | Linoleic acid | $C_{19}H_{34}O_2$ | 11.14 |
| 27.09 | Oleic acid | $C_{19}H_{36}O_2$ | 27.14 |
| 27.97 | Stearic acid | $C_{19}H_{38}O_2$ | 6.57 |
| 32.36 | Arachidic acid | $C_{19}H_{38}O_2$ | 3.83 |
| 38.24 | Behenic acid | $C_{23}H_{46}O_2$ | 0.75 |

# CHAPTER THREE: RESULTS & DISCUSSION

## 3. RESULTS AND DISCUSSION

### 3.1 Collection of plant materials:

Fresh and matured leaves, bark and flowers of *Lagerstroemia speciosa* **Linn.** were collected from the campus area of the University of Dhaka. The leaves, bark and flowers were brought to our Research Laboratory and associated dirt was removed by washing with water. The collected leaves, bark and flowers were cleaned, chopped and dried in an oven at $40^0$C and ground to powder for extraction.

### 3.2 Extractions of leaves, bark and flowers of *Lagerstroemia speciosa* Linn. with different solvents:

#### 3.2.1 Extraction of leaves with dichloromethane and methanol:

The powder of leaves (3.5 kg) of *L. speciosa* was extracted with dichloromethane as described in **section 2.2.3.1**. The extract was evaporated to dryness (184.0 g, 5.76% of leaves powder, LSLD) and kept at $-22^0$C in a freezer.

The residue obtained after extraction with dichloromethane (3.1 kg) was further extracted with methanol as described in **section 2.2.3.2**. The extract (122.0 g, 3.49% of leaves powder, LSLDM) was stored in a reagent bottle at $-22^0$C in a freezer.

#### 3.2.2 Extraction of bark with aqueous 80% ethanol:

The bark powder (3.0 kg) was extracted with aqueous 80% ethanol (6 × 4 L) at room temperature as described in **section 3.2.1**. The extract (87.0 g, 2.9% of bark powder, LSBE) was then stored in a reagent bottle at $-22^0$C in a freezer. The scheme of extraction and the amount and percentage of different extractives of *L. speciosa* are given in **section 2.2.3.3** and **Table 6.**

### 3.2.3 Partition of ethanol extract (LSBE) between dichloromethane and water:

Ethanol extract (LSBE, 87 g) was suspended in water, ultrasonicated and the solution was transferred into a separating funnel. The aqueous solution was partitioned with dichloromethane. The dichloromethane soluble part was separated and evaporated to dryness (14.6 g, 0.49% of bark powder, LSBED).

The aqueous part was also evaporated to dryness. The dried part was further freeze-dried to remove the residual solvent (65.3 g, 2.18% of bark powder, LSBEW). The ethanol extract (LSBE, 87 g) was partitioned between dichloromethane and water to divide the extract into lipophilic and hydrophilic parts. The lipophilic partitioned fraction was much lower than hydrophilic fraction.

### 3.2.4 Fractionation of dichloromethane soluble part (LSBED) of bark by Diion HP-20 gel column:

The dichloromethane soluble part (14.6 g) was suspended in the mixture of methanol and water in the ration 1:1. The suspension was fractionated by Diion HP-20 gel column to divide dichloromethane soluble part into acetone, methanol and methanol-water (1: 1) soluble fractions. The eluents were evaporated to dryness and 2.5 g of acetone (LSBEDA), 2.4 g of methanol (LSBEDM) and 4.6 g of methanol-water (1:1) (LSBEDMW), respectively soluble parts were obtained. The dried three fractions were stored in reagent bottles at - $22^0$C in a freezer.

### 3.3 Extraction of flowers with hexane:

The powder of flowers (300 g) was extracted with hexane as described in **section 2.2.3.5**. The amount of the extract was 2.4 g and designed as LSFH.

Dichloromethane, methanol, ethanol and hexane extracts of leaves, bark and flowers of *Lagerstroemia speciosa* **L** were used for biological and chemical studies described in the respective sections.

### 3.4 Fractionation of methanol soluble part (LSLDM, 120 g) of leaves:

The dried methanol extract was suspended in 10% methanol in ethyl acetate and extracted three times. The 10% methanol soluble fraction was evaporated to dryness. The amount of 10% methanol soluble part was 16.0 g.

### 3.5 Fractionation of 10% methanol soluble part by Sephadex LH-20 gel column:

The 10% methanol soluble part (16 g) was fractionated by Sephadex LH-20 gel column. Then several solvents such as 20% methanol in water, methanol and acetone were passed successively through the column and the eluents were collected in several conical flasks (500 ml). The eluents were evaporated to dryness and two different parts of chlorophyll containing fraction (8.2 g) and chlorophyll free fraction (2.1 g) were obtained. The chlorophyll containing part was treated with charcoal to remove chlorophyll. The chlorophyll was completely absorbed by charcoal and it was decolorized. The amount of the decolorized sample was 3.0 g. Both the fractions of chlorophyll free parts were combined by evaluating by $^1$H-NMR (60 MHz) spectra. The amount of the combined fractions was 5.1 g that was designed as LSLME.

### 3.6 Fractionation of chlorophyll free methanol soluble part of leaves (LSLME) by column chromatography:

The chlorophyll free methanol soluble part (LSLME, 5.1 g, described in **section 2.5.4**) was fractionated by silica gel column chromatography using silica gel as adsorbent and eluted with dichloromethane and methanol with increasing polarity. The eluents were collected in conical flasks and monitored by TLC (described in **section 2.5.4.3**). The eluted samples were combined and five fractions (e.g., $LSLMEF_1$, $LSLMEF_2$, $LSLMEF_3$, $LSLMEF_4$ and $LSLMEF_5$) were obtained.

### 3.7 Purification of the fraction $LSLMEF_2$:

The fraction $LSLMEF_2$ gave a single spot in TLC and was purified by washing repeatedly with hexane. The residue was crystallized from hexane and dichloromethane mixture (5: 1; v/v) and a pure compound LSM-4 was obtained (23.6 mg).

### 3.8 Characterization of the compound LSM-4:

#### 3.8.1 Physical characteristics of the compound LSM-4:

The compound LSM-4 was a white crystalline solid having **melting point 243-245$^0$C**. It was soluble in a mixture of dichloromethane and methanol. It gave violet color with vanillin-sulfuric acid reagent in TLC with **$R_f$ value 0.6** (2% MeOH in $CH_2Cl_2$ v/ v) indicating its terpenoidal nature.

#### 3.8.2 Structure elucidation of the compound LSM-4 by spectroscopic methods:

#### (a) Infrared (IR) spectroscopy:

The **IR spectrum** of the compound LSM-4 (**Fig. 6**) had absorption frequencies at 3410 for the stretching of –OH group. The absorption at 2910 $cm^{-1}$ and 2850 $cm^{-1}$ for symmetric and symmetric stretching of –C-H bonds. The absorption at 1680 $cm^{-1}$ indicated the unconjugated double bond (>C=C<) stretching vibration. Absorption at 1442 and 1365 $cm^{-1}$were due to methyl groups (–$CH_3$) asymmetric and symmetric bending (scissoring), respectively. The absorption at 1050 $cm^{-1}$was due to C-O stretching vibration.

**(b) $^1$H-NMR ($CDCl_3$+$CD_3OH$, 300 MHz) spectroscopy:**

In the **$^1$H-NMR spectrum** of the compound LSM-4 (**Fig.** 7) had signal at 5.05 ppm (1H,br *s*, H-12) for olefmic proton. The doublet at 3.45 ppm for the α-proton at C-3 (1H, *d*, *J*=9.0 Hz, H-3α). The doublet at 2.75 ppm for the proton at C-18 (1H, *d*, *J*=9.6 Hz, H-18α). The signals at 0.88, 0.81, 0.78, 0.61, 0.60 for five methyl groups (each 3H, *s*, 5 × $CH_3$) attached to quaternary carbons. The doublet at 0.73 ppm (3H, *d*, *J*=6.4Hz, $CH_3$) and 0.65 (3H, *d*, *J*=5.9Hz, $CH_3$) for two methyl groups attached to -CH< carbons.

**(c) Mass spectroscopy:**

**Electron impact mass spectrum (EIMS)** of the compound LSM-4 **(Fig. 8)** had the molecular ion peak at *m/z* 472.7 corresponding to the molecular formula $C_{30}H_{48}O_4$ and the base peak at *m/z* 247.9 and other intense peaks at *m/z* 441.7, 427.7, 407.7, 254.8, 202.9, 132.9 and 54.9.

Mass fragmentation pattern was compared with triterpenoid library search and the data matched with the data of 2,3-dihydroxy-12-oleanen-28-oic acid (colosolic acid).

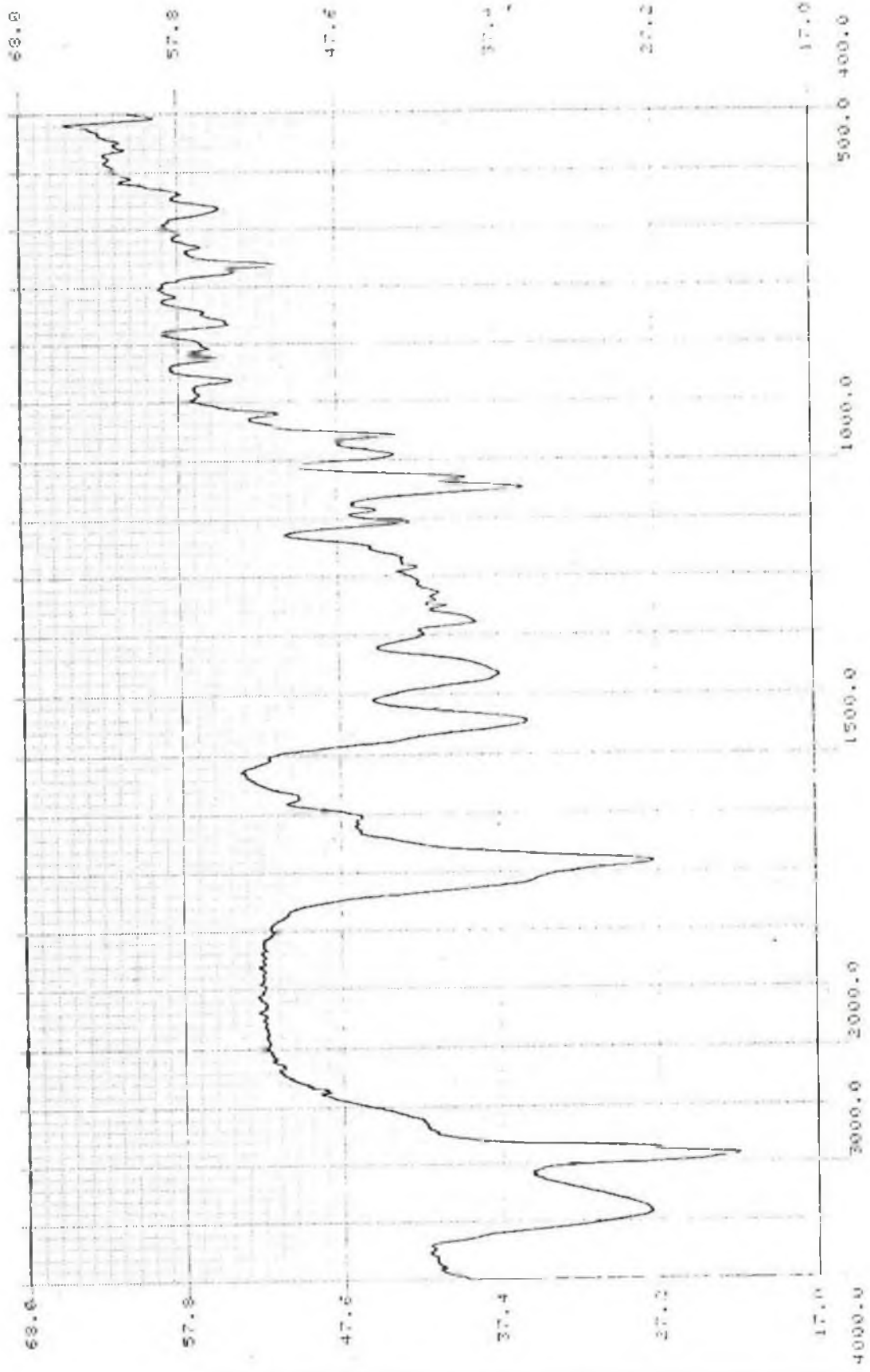



Fig 6: Infrared (IR) spectrum of the compound LSM-4

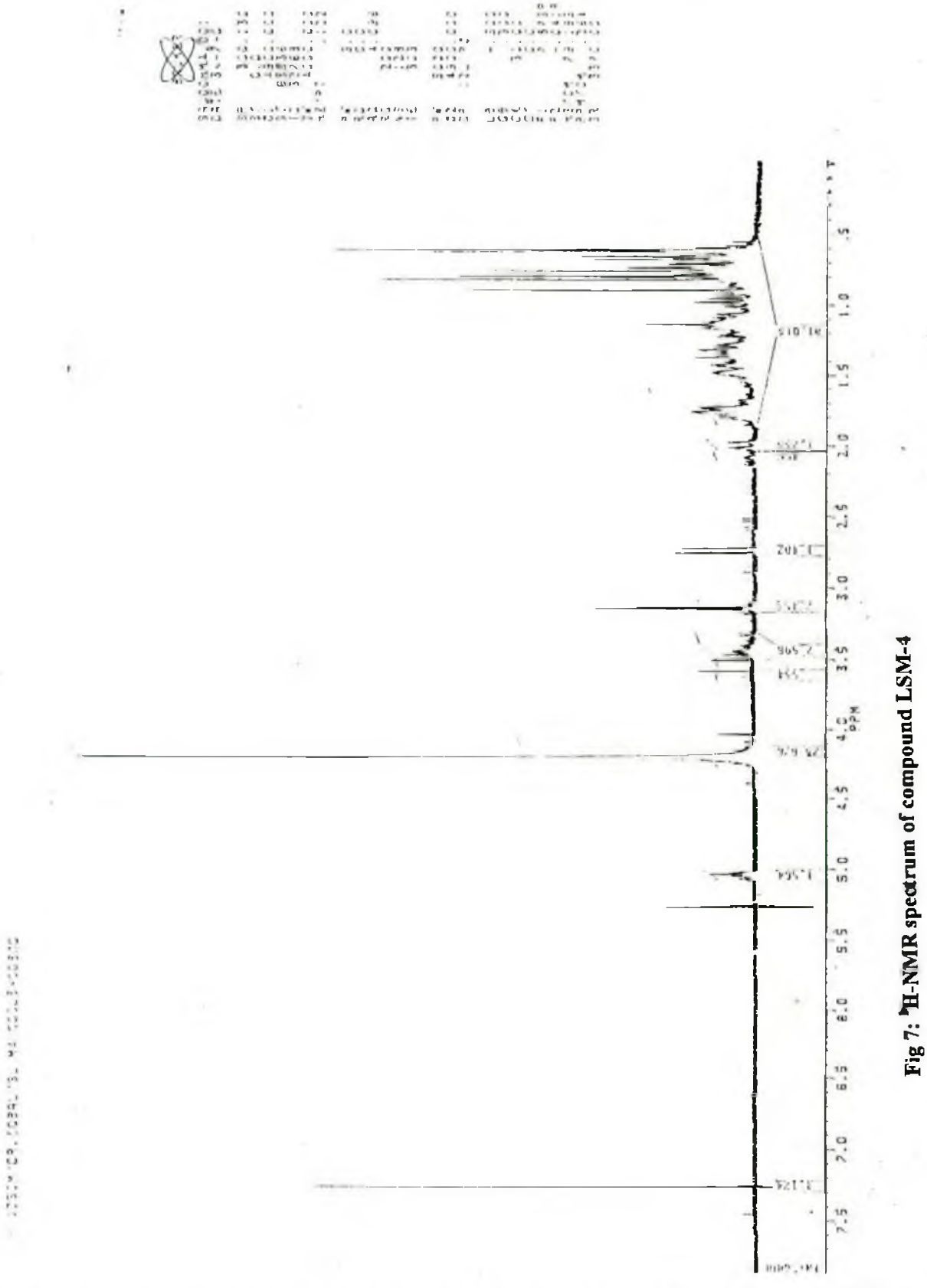


Fig 7: $^1$H-NMR spectrum of compound LSM-4

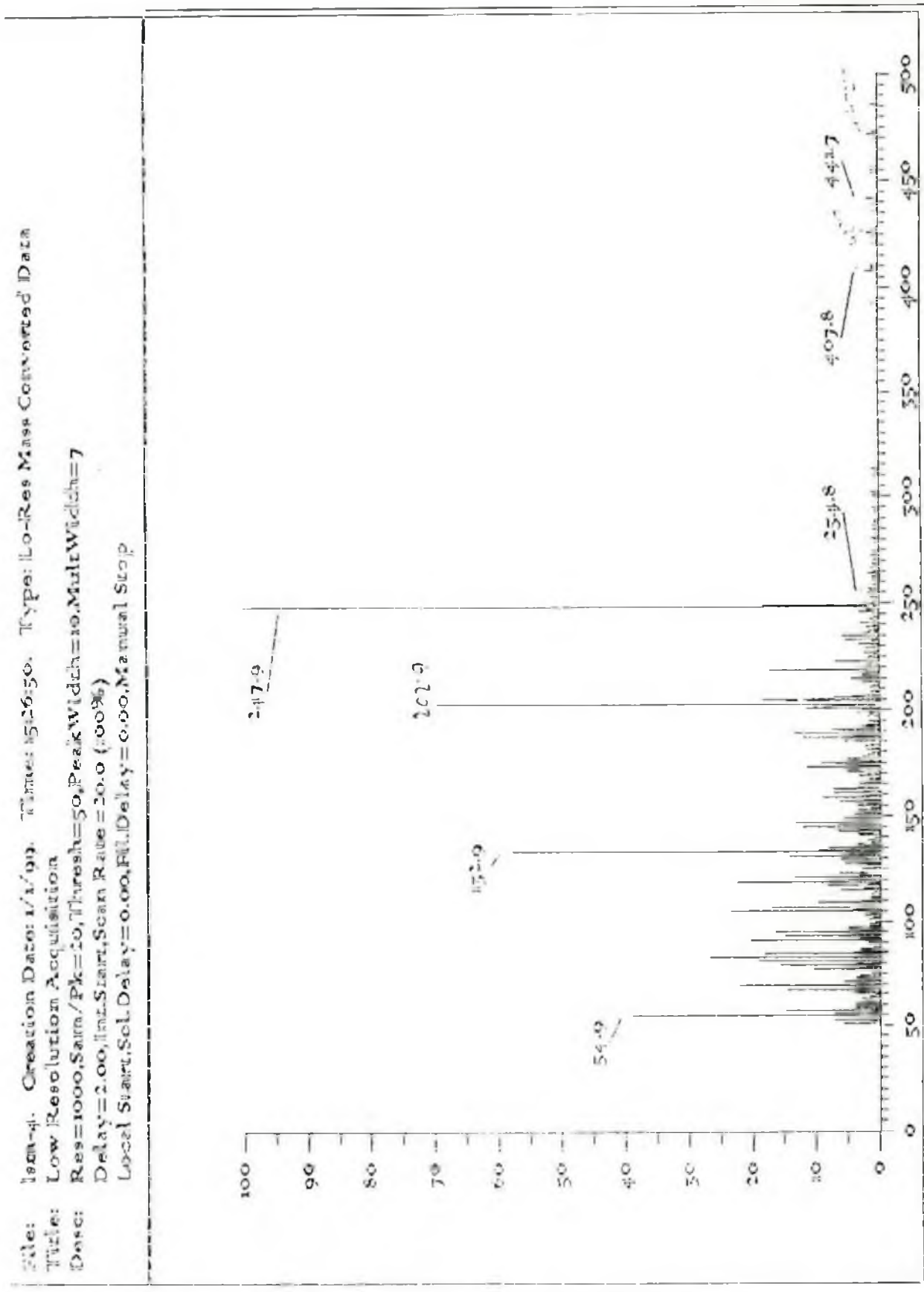
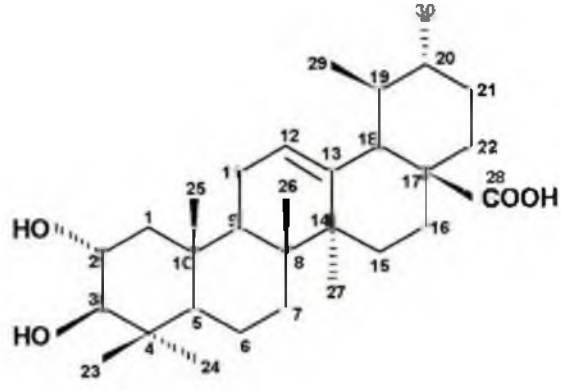



Fig. 8: Electron Impact Mass Spectrum (EMS) of the compound LSM-4

### 3.8.2.1 Structure of the compound LSM-4:

On the basis of physical characteristics and spectroscopic data it was found that the compounds LSM-4 was a pentacyclic triterpenoid. The $^1$H-NMR spectral data were compared with the reported[48] $^1$H-NMR spectral data of terpenoids. From the spectroscopic analysis and comparison it was confirmed that the isolated pure compound LEM-4 was 2,3-dihydroxy-12-oleanen-28-oic acid (Colosolic acid) that has the following structure.




2,3-Dihydroxy-12-ursen-28-oic acid,
Colosolic Acid (LSM-4) (**51**)

Colosolic acid has been previously reported[48] from the leaves of *Lagerstroemia speciosa* **Linn.**

### 3.8.3 Purification of the fraction $LSLMEF_4$:

The fraction $LSLMEF_4$ gave a single spot in TLC and was purified by washing repeatedly with hexane and methanol to remove associated impurities. After washing the material was purified by recrystallization. The TLC pattern showed a single spot with $R_F$ value 0.30 by using methanol and dichloromethane mobile phase (1: 9 v/v). The solution was kept for crystallization at room temperature in the above solvent system. White amorphous powder was formed. The powder was collected from the solution and dried at room temperature and finally dried by a freeze drier and kept in desicator. The amount of the compound (AR-3) was 9.3 mg.

### 3.8.4 Characterization of the compound AR-3:

#### 3.8.4.1 Physical characteristic of the compound AR-3:

The compound AR-3 was a white solid. It gave characteristic violet color of steroid with vanillin-sulfuric acid reagent. It was soluble in a mixture of methanol and chloroform and also in pyridine. It gave positive test of steroid (Salkowski test and Leibermann-Burchard test, described in **section 2.1.16**). It also gave positive test of sugar (phenol-sulfuric acid test, described in **section 2.1.15**). The result indicated that the compound AR-3 might be a steroidal glycoside. **Melting point** of compound AR-3 was **283-285$^0$C.**

#### 3.8.4.2 Structure elucidation of the compound AR-3 by spectroscopic methods:

#### (a) $^1$H-NMR (Pyridine, 400 MHz) spectroscopy:

In the **$^1$H-NMR spectrum** of the compound AR-3 **(Fig. 9, 9a, 9b)** had distorted doublet at 5.34 ppm (1 H, $J_{6,7}$=3.6 Hz), was supposed by a single olefinic proton at C-6. The doublet at δ 5.05 ppm (1H, $J_{1,2}$=6.0 Hz) was for anomeric proton of a sugar moiety. Upfield chemical shift and large coupling constant indicated *β*-glycoside linkage of the sugar residue to the aglycone part. In the upfield region of the $^1$H-NMR spectrum, there were signals of six methyl groups. Two signals at 0.66 (3H, *s*) and 0.93 (3H, *s*) were due to two methyl groups attached to methyne carbon (shown by doublet) resonated at 0.89 (3H, *t*) and 0.87 (6H, *t*) and one was due to methylene proton at 0.85. A complicated

multiplet in the region of 3.92-4.57 ppm was for the oxygenated methine protons of the sugar moiety.

**Table 26: $^{1}$H-NMR spectral data of the compound AR-3**

| Chemical shift (δ in ppm) | Splitting pattern | Integration | Assignment |
|---|---|---|---|
| 5.34 | Distorted doublet | 1H | Olefmic proton |
| 5.05 | Doublet | 1H | Anomeric proton of sugar moiety |
| 3.92-4.57 | Multiplet | | Oxygenated methine protons in sugar moiety |
| 0.93 | Singlet | 3H | -$CH_3$ group |
| 0.98 | Doublet | 6H | Two –$CH_3$ group |
| 0.89 | Triplet | 3H | -$CH_3$ group |
| 0.87 | Triplet | 3H | -$CH_3$ group |
| 0.66 | Singlet | 3H | -$CH_3$ group |

**(b) $^{13}$C-NMR spectroscopy:**

The **$^{13}$C-NMR spectrum** of the compound AR-3 **(Fig. 10, 10a, 10b, 10c)** had 35 signals indicating that the compound contained 35 carbons and 29 signals for steroid skeleton and 6 signals for sugar residue. The most down shielded signals at 140.94 and 121.94 ppm were assigned to the two olefinic carbons C-5 and C-6, respectively. To distinguish between methyl, methylene, methine and quaternary carbons present in the compound, DEPT-135$^{0}$ and DEPT-90$^{0}$ were carried out. The signals at 12.00 (C-18), 19.44 (C-19), 19.24 (C-21), 19.03 (C-26), 19.99 (C-27), 12.18 (29) ppm were assigned to six methyl groups attached to quaternary carbons. Eleven signals for methylene carbons at 37.51 (C-1), 30.28 (C-2), 39.37 (C-4), 32.09 (C-7), 21.31 (C-11), 39.98 (C-12), 24.53 (C-15), 28.56 (C-16), 34.24 (C-22), 26.44 (C-23), 23.42 (C-28) and nine signals for methyne carbons at 78.13 (C-3), 121.94 (C-6), 32.20 (C-8), 50.38 (C-9), 56.86 (C-14), 56.28 (C-17), 36.41 (C-20), 46.08 (C-24), 29.50 (C-25). The signals at 140.94 (C-5), 36.95 (C-10)

and 42.51 (C-13) ppm for three quaternary carbons. The signals at 102.61, 75.37, 78.50, 78.64, 71.74, 62.88 for the carbons of glucoside units e.g. for carbons C-1′ (anomeric carbon), C-2′, C-3′, C-4′, C-5′, and C-6′. The $^{13}$C-NMR data corresponded to the $^{13}$C-NMR data glucose moiety. The $^{13}$C-NMR data of AR-3 and that of the reported data are given in **Table 27.**

**Table 27: $^{13}$C-NMR data of AR-3 (Pyridine, 400 MHz) and that of reported[83] data of sitosteryl-3-*O*-*β*-D-glucoside**

| Position of carbons | Chemical shift (in ppm) of AR-3 | Sitosteryl-3-*O*-*β*-D-glucoside[83] | Position of Carbons of AR-3 | Chemical shift (in ppm) | Sitosteryl-3-*O*-*β*-D-glucoside[83] |
|---|---|---|---|---|---|
| C-1 | 37.51 | 37.5 | C-19 | 19.44 | 19.4 |
| C-2 | 30.28 | 30.2 | C-20 | 36.41 | 36.5 |
| C-3 | 78.13 | 78.5 | C-21 | 19.24 | 19.1 |
| C-4 | 39.37 | 40.0 | C-22 | 34.24 | 34.3 |
| C-5 | 140.94 | 140.9 | C-23 | 26.44 | 26.5 |
| C-6 | 121.94 | 121.6 | C-24 | 46.08 | 46.1 |
| C-7 | 32.09 | 32.1 | C-25 | 29.50 | 29.5 |
| C-8 | 32.20 | 32.1 | C-26 | 19.03 | 19.0 |
| C-9 | 50.38 | 50.3 | C-27 | 19.99 | 20.0 |
| C-10 | 36.95 | 36.9 | C-28 | 23.42 | 23.4 |
| C-11 | 21.31 | 21.3 | C-29 | 12.18 | 12.2 |
| C-12 | 39.98 | 39.2 | C-1′ | 102.61 | 102.5 |
| C-13 | 42.51 | 42.4 | C-2′ | 75.37 | 75.2 |
| C-14 | 56.86 | 57.0 | C-3′ | 78.50 | 78.2 |
| C-15 | 24.53 | 24.5 | C-4′ | 78.64 | 78.2 |
| C-16 | 28.56 | 28.6 | C-5′ | 71.74 | 71.7 |
| C-17 | 56.28 | 56.2 | C-6′ | 62.88 | 62.8 |
| C-18 | 12.00 | 12.0 | | | |

**(c) Mass spectroscopy:**

The molecular mass is supposed to be $MH^+$ at ***m/z*** 547 in **FAB$^+$MS (Fig 12, 12a)** ran with glycerol as the matrix. The peak appeared at *m/z* 396 [$MH^+$-Glu-$H_2O$], which indicated the loss of the glucose unit. The other remaining signals corresponded to ***m/z*** 213 side chain [$C_{10}H_{21}$], 255 [$MH^+$-side chain-Glu], 145 [$MH^+$-Glu-C ring frag], 213 [$MH^+$-Glu-D ring frag]. The mass fragmentation pattern was similar to the sitosteryl-3-*O*-*β*-D-glucoside.

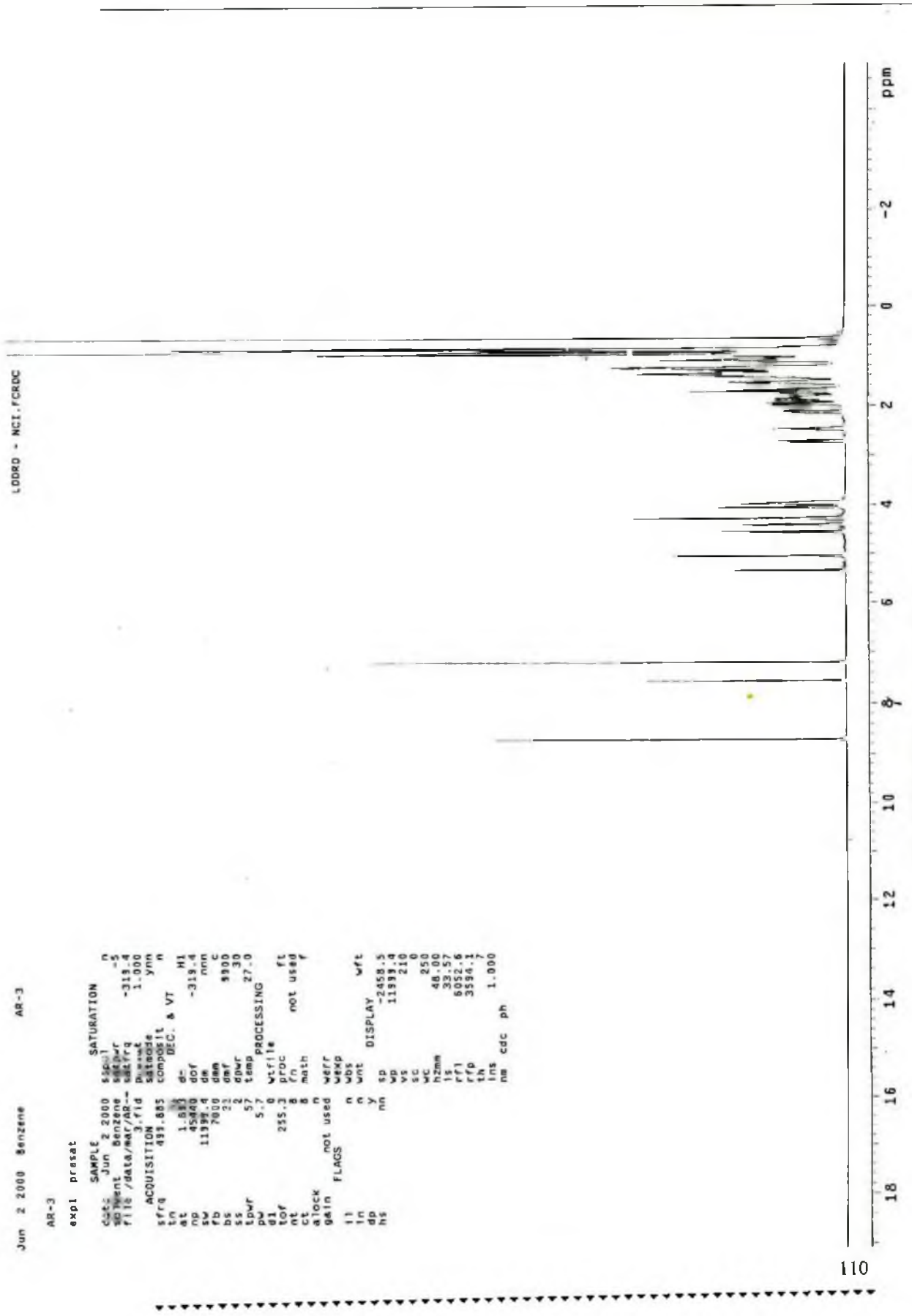



Fig. 9: $^{1}$H-NMR spectrum of the compound AR-3

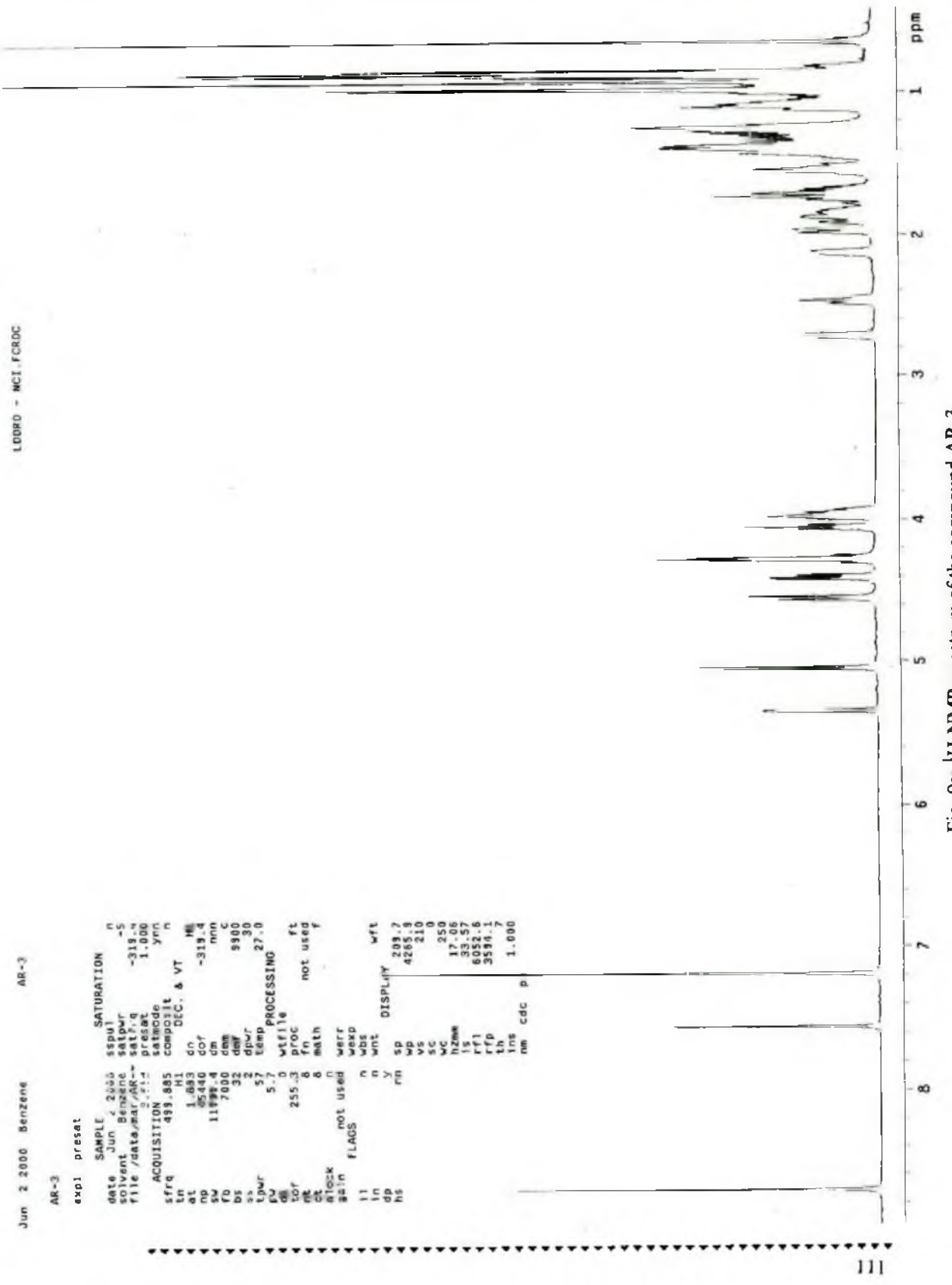



Fig. 9a. $^{1}$H-NMR spectrum of the compound AR-3

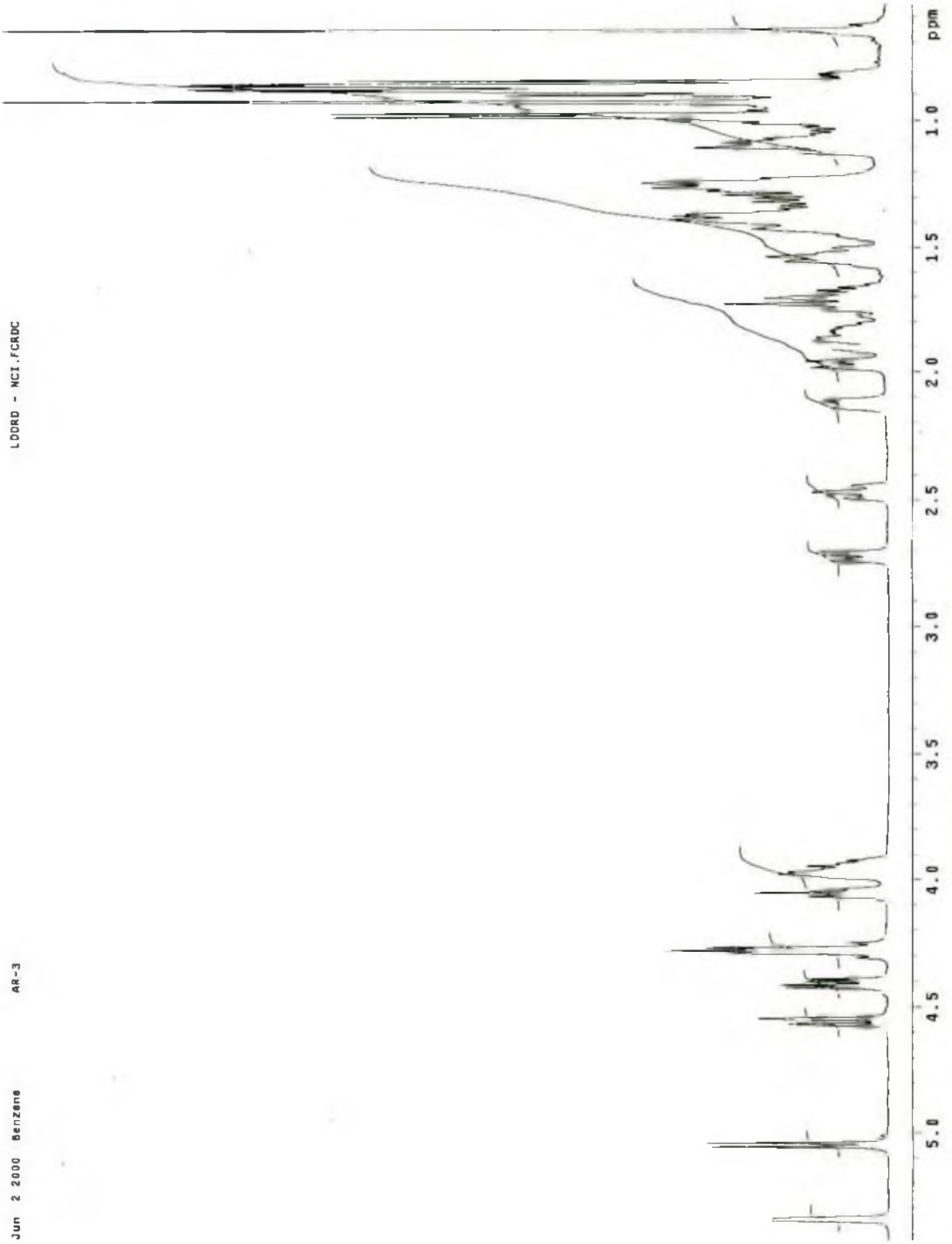



Fig. 9b: $^{1}$H-NMR spectrum of the compound AR-3

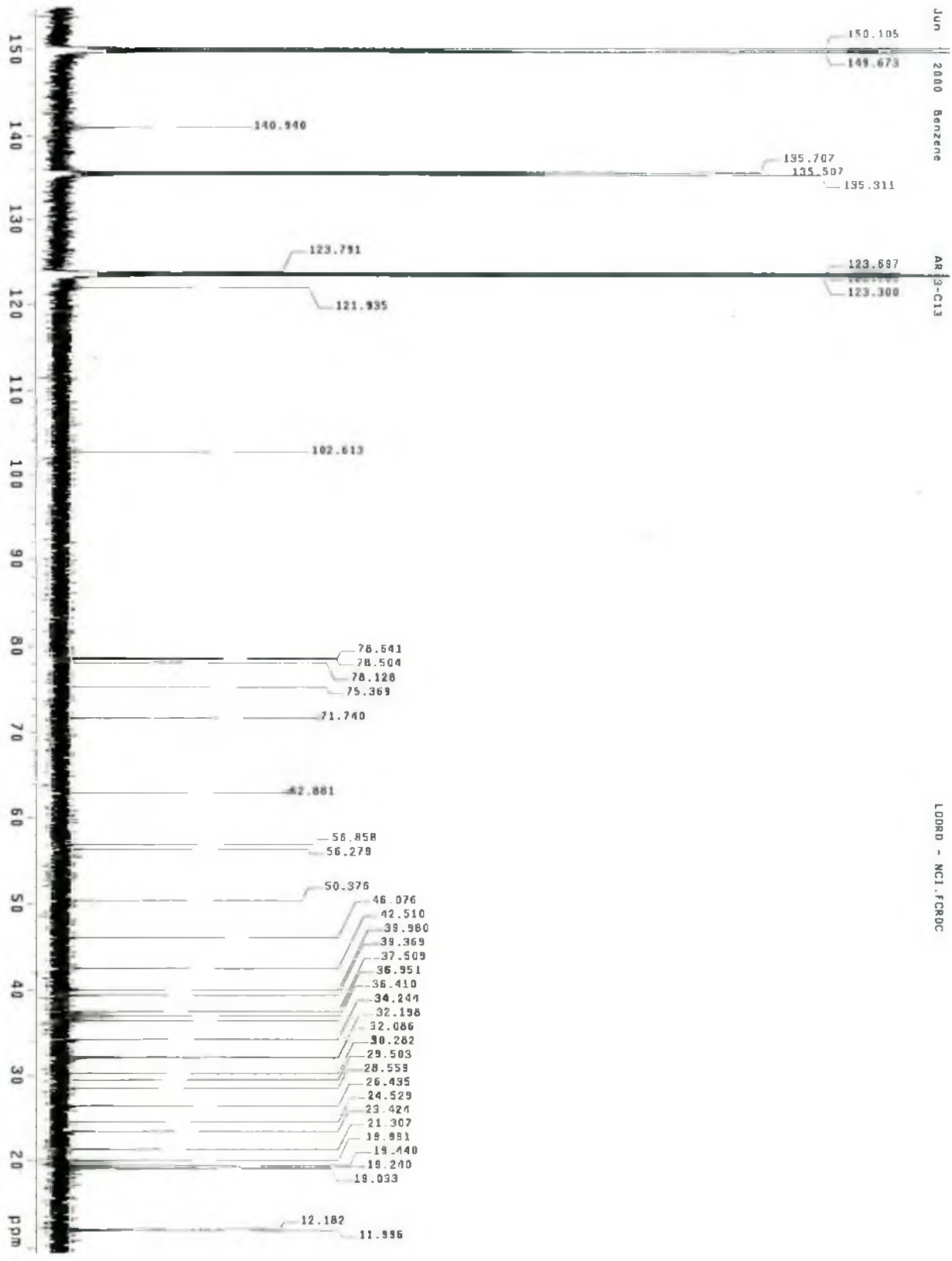



Fig. 10: $^{13}$C-NMR spectrum of the compound AR-3

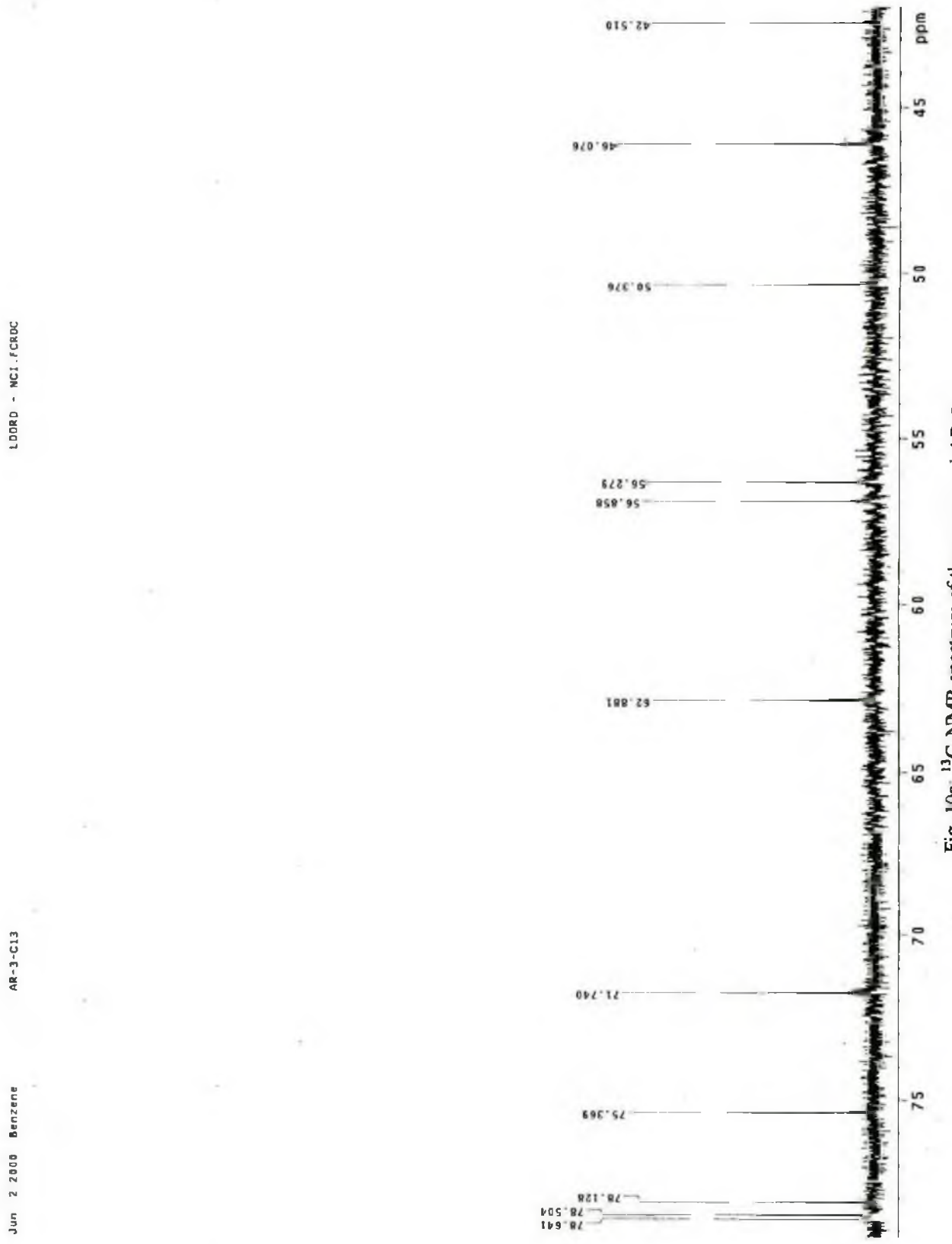



Fig. 10a $^{13}$C-NMR spectrum of the compound AR-3

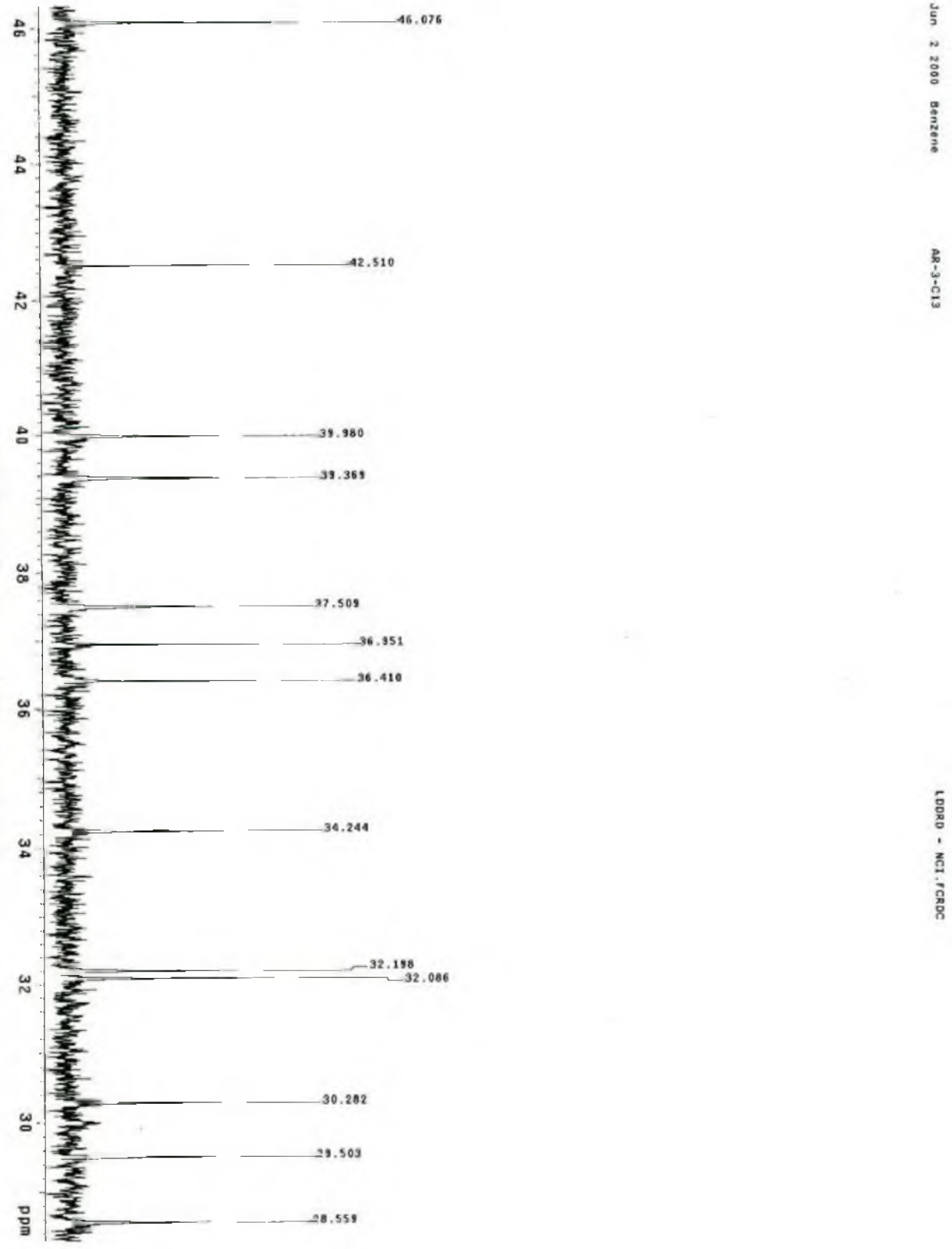



Fig. 10b: $^{13}$C-NMR spectrum of the compound AR-3

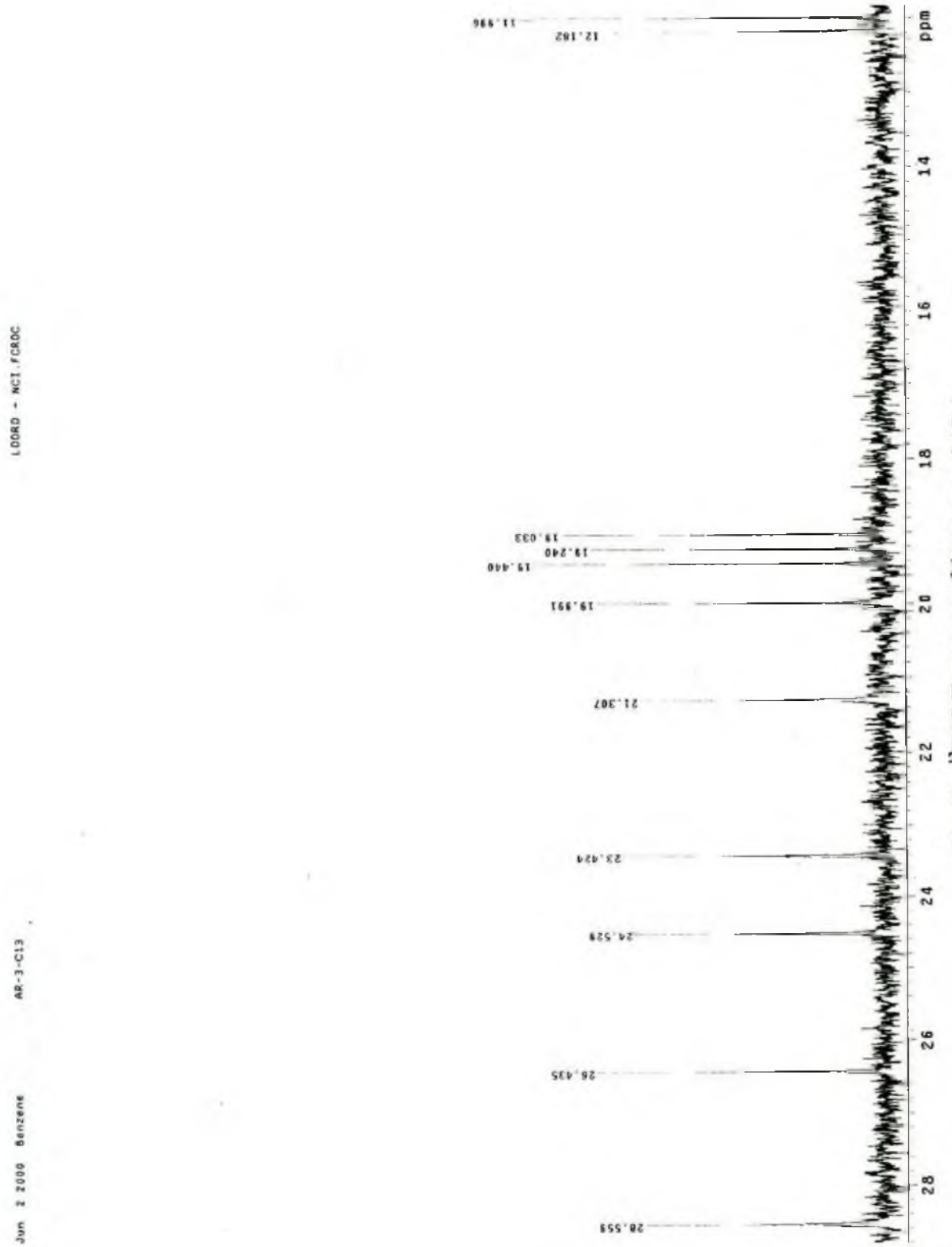



Fig. 10c: $^{13}$C-NMR spectrum of the compound AR-3

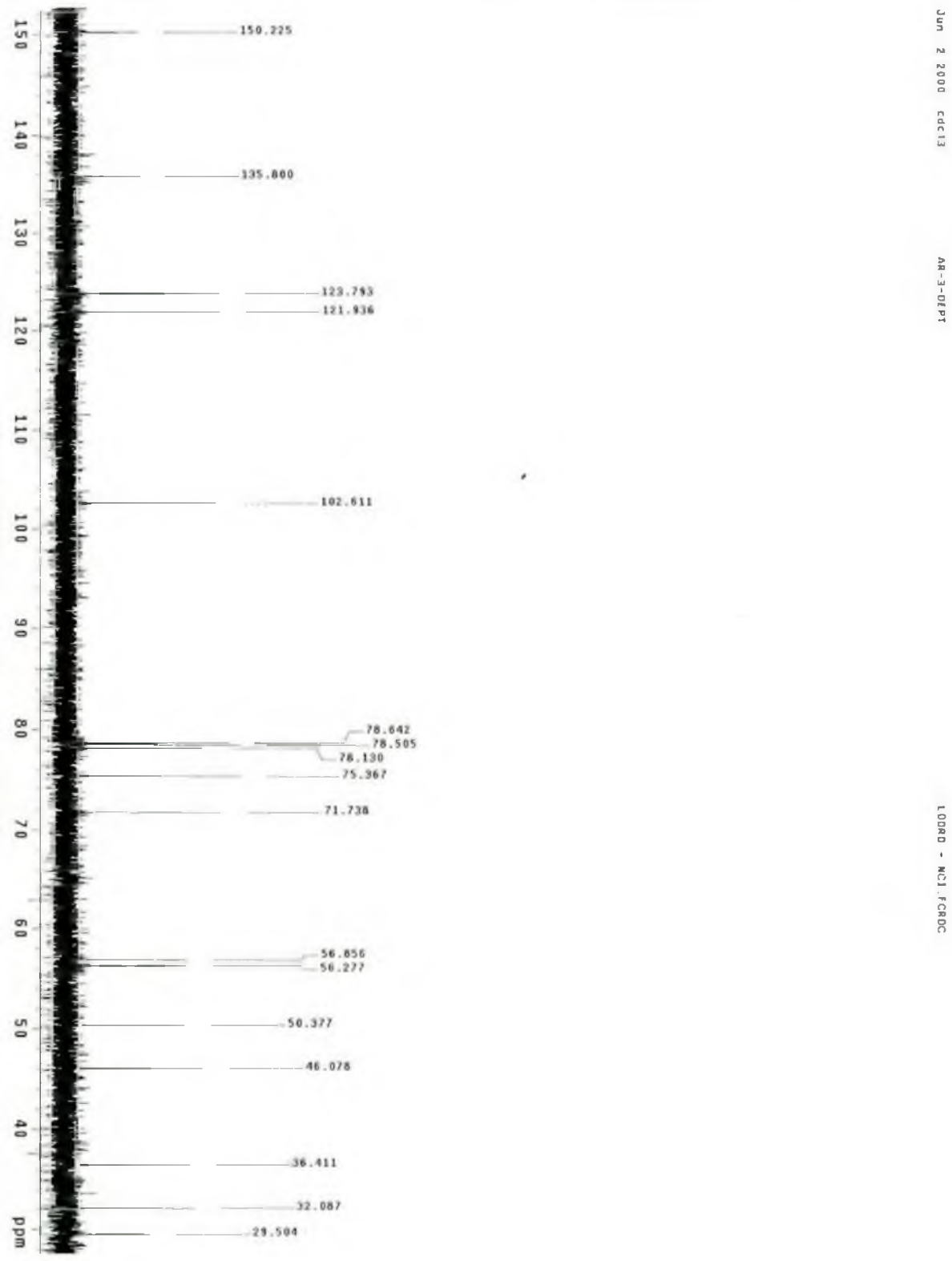



Fig. 11: DEPT spectrum of the compound AR-3

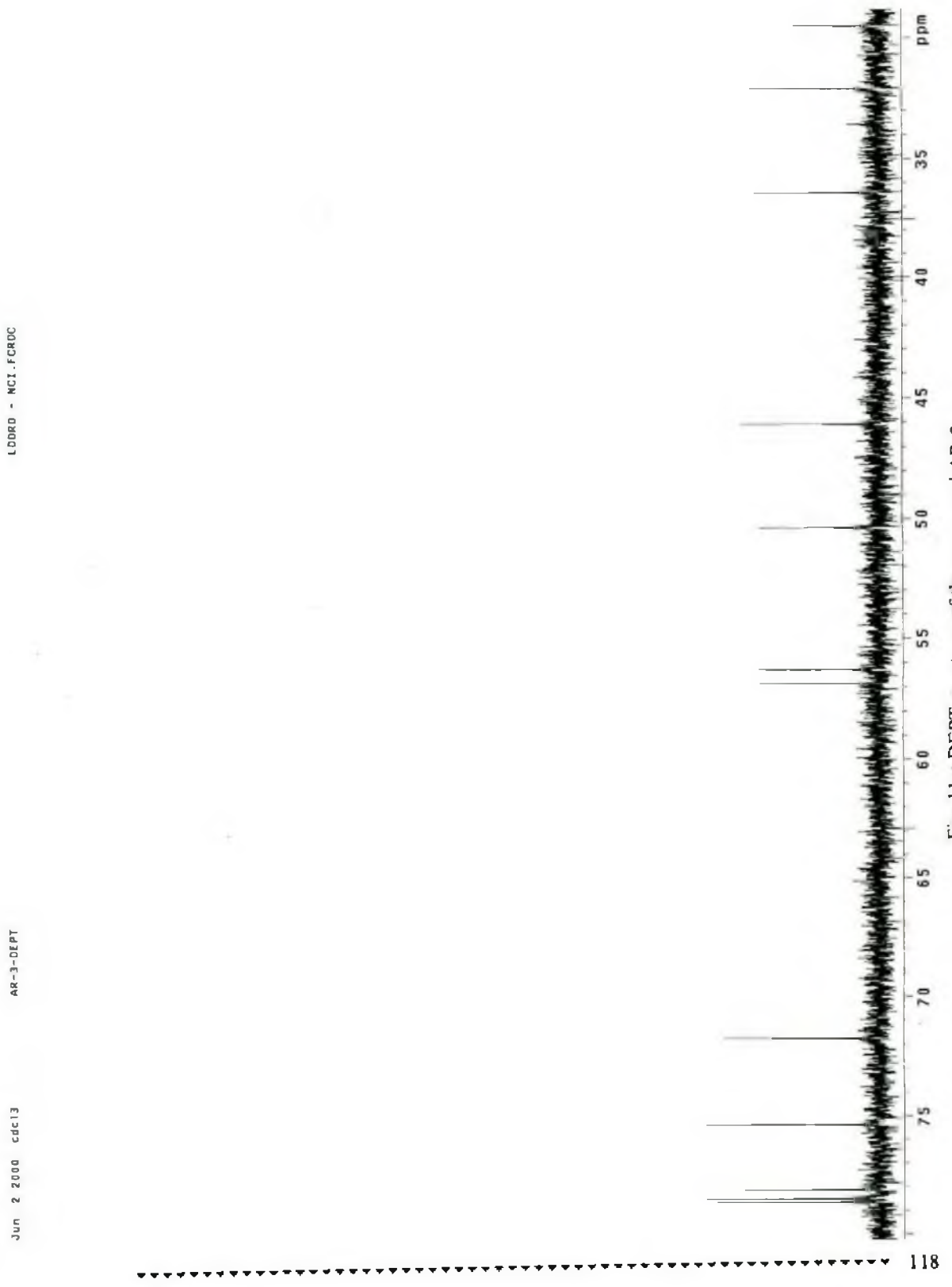



Fig 11a: DEPT spectrum of the compound AR-3

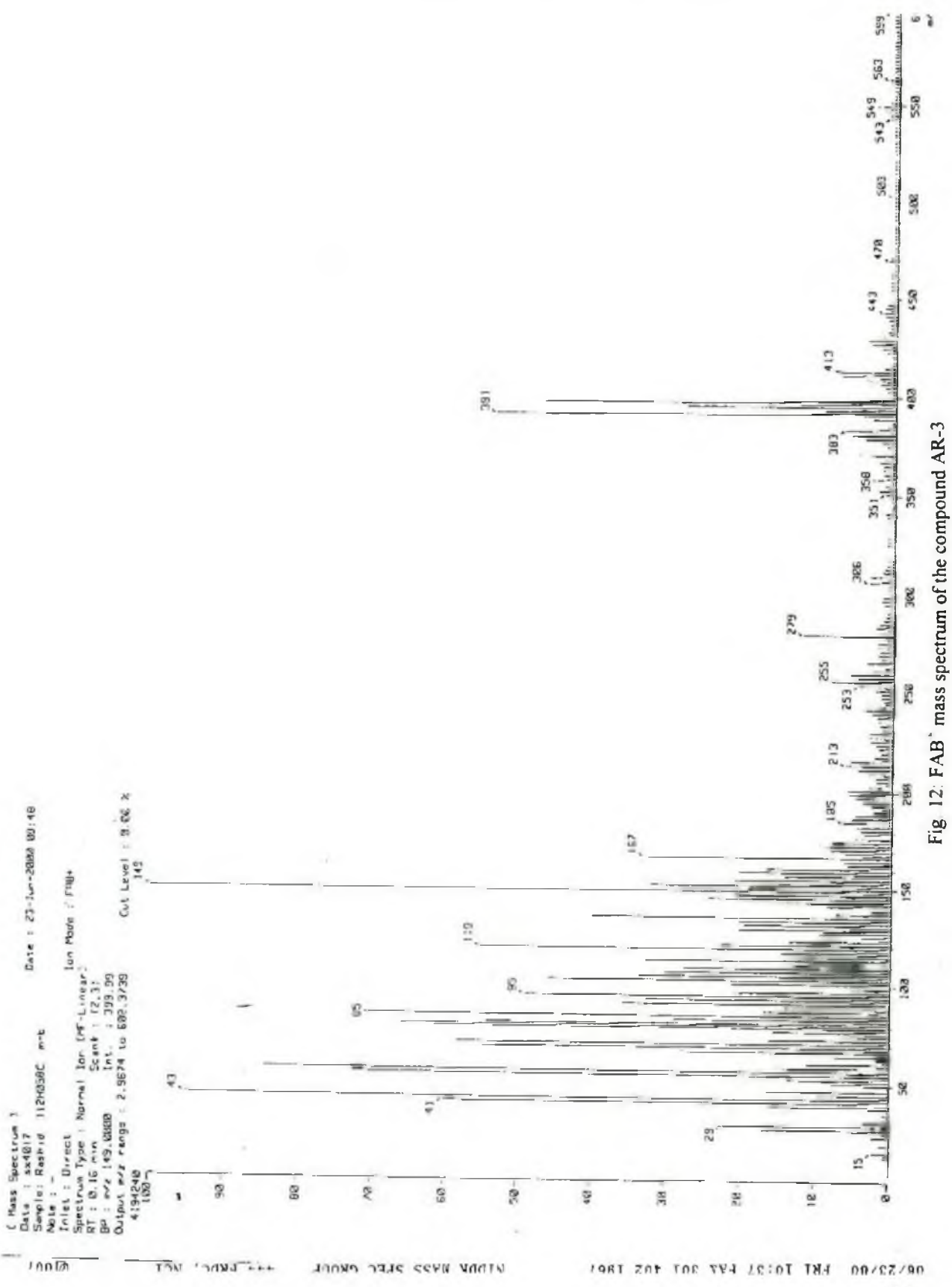


Fig 12: FAB mass spectrum of the compound AR-3

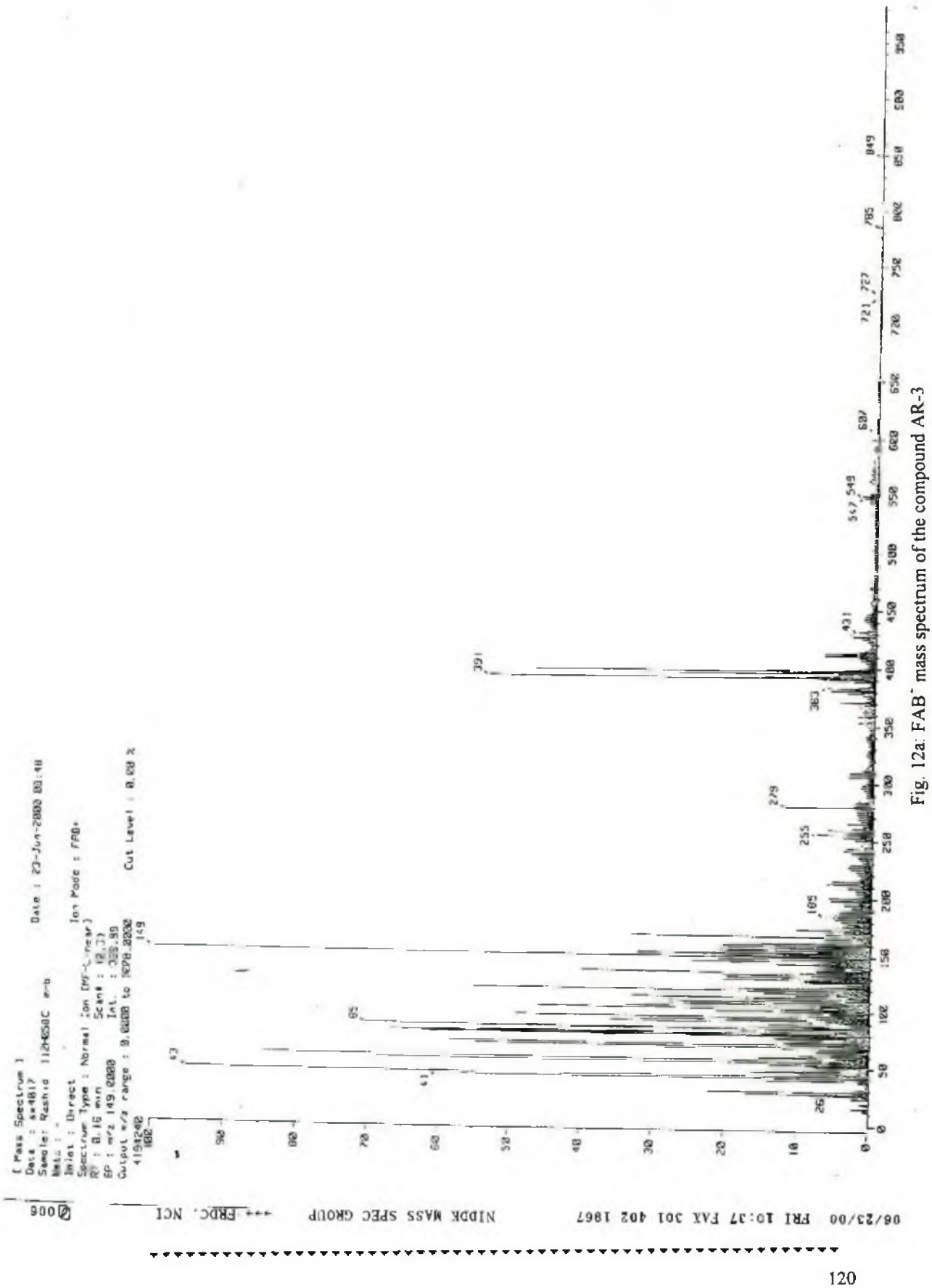
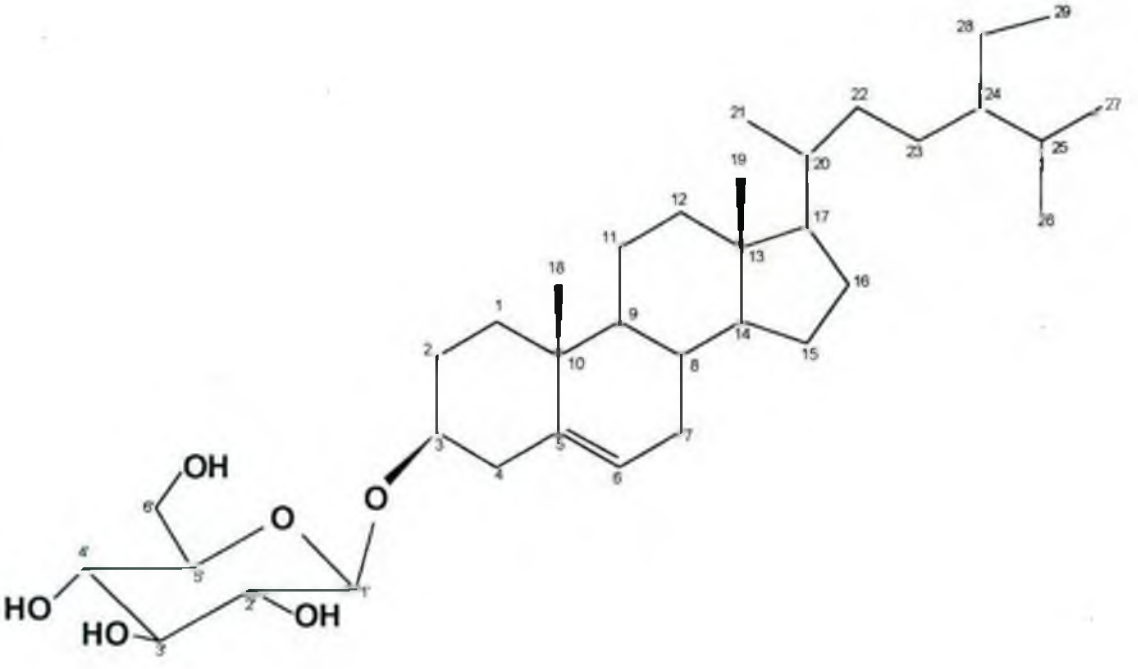


Fig. 12a: FAB$^+$ mass spectrum of the compound AR-3

### 3.8.4.3 Structure of the compound AR-3:

On the basis of physical characteristics and spectroscopic data it was found that the compound AR-3 was a steroidal glucoside. The $^{1}$H-, $^{13}$C-NMR spectral data were compared with the reported[82] $^{1}$H-, $^{13}$C-NMR spectral data of steroidal and their glucosides. From the spectroscopic analysis and comparison with known compound it was found that the isolated compound AR-3 was sitosteryl-3-*O*-*β*-D-glucoside.

The structure of sitosteryl-3-*O*-*β*-D-glucoside (AR-3) is given below:




Sitosteryl-3-*O*-β-D-glucoside AR-3 (**55**)

Computer literature survey NAtural PRoduct ALART (NAPRALART search in December 1998) and Dictionary of Natural Product on CD-ROM (Chapman & Hall, release 6:2) of *Lagerstroemia speciosa* L confirmed that sitosterol-3-*O*-*β*-D-glucoside although a known compound but it has not been reported earlier from this plant.

### 3.9 Fractionation of dichloromethane extract of leaves (LSLD) by column chromatography:

Dichloromethane extract (LSLD, 120 g) of leaves was fractionated by silica gel column chromatography by using hexane, dichloromethane and ethylacetate. The eluted samples were monitored by TLC and were combined on the basis of their $R_f$ values and finally six fractions were obtained (described in **Table 15**) and the fraction three was designed as $LSLDF_3$.

### 3.10 Fractionation of $LSLDF_3$ by column chromatography:

The sample was ($LSLDF_3$, 9 g) was fractionated by column chromatography and finally five different fractions were obtained by pooling different fractions on the basis of their $R_f$ values.

The amount of each fraction and the corresponding solvent TLC patterns are given in **Table 16** (described in **section 2.5.10**).

### 3.11 Fractionation of $LSLDC_2F_3$ by column chromatography:

The sample $LSLDC_2F_3$ (4 g) was further fractionated by column chromatography and finally five different fractions were obtained by pooling different fractions on the basis of their $R_f$ values.

The amount of each fraction, the corresponding solvent polarity and TLC patterns are given in **Table 17** (described in **section 2.5.11**).

### 3.11.1 Purification of fraction $LSLDC_3F_{39}$:

The fraction $LSLDC_3F_{39}$ gave a single spot with tailing in TLC. The fraction was washed repeatedly with methanol to remove associated impurities. After washing, a white solid was obtained and assigned as JF-39.

### 3.11.2 Characterization of compound JF-39:

The compound JF-39 was a white solid. It gave characteristic violet color with vanillin-sulfuric acid reagent with $R_f$ value 0.4 in 10% dichloromethane in hexane. It was readily soluble in hexane and dichloromethane. Melting point of the compound JF-39 was $67^0$C.

### 3.11.3 Structure elucidation of the compound JF-39 by spectroscopic methods:

#### (a) Infrared (IR) spectroscopy:

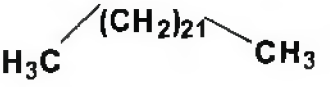
The **infrared spectrum** of the compound JF-39 **(Fig. 13)** there were absorption frequencies at 2905 and 2830 $cm^{-1}$ for symmetric and asymmetric stretching. The absorption frequencies at 1455 and 1360 $cm^{-1}$ for scissoring and 730 and 720 $cm^{-1}$ for bending.

#### (b) Gas Chromatography-Mass spectroscopy (GC-MS):

The gas chromatograph of the compound **(Fig. 14)** showed a single peak with retention time 21.08. The corresponding GC-MS showed the peak at m/z 267, 236, 209, 195, 181, 167, 153, 139, 125, 111, 97, 83, 69, 57 and 43.

From spectroscopic data the compound JF-39 was confirmed as n-Tricosane, which is a known hydrocarbon but isolated for the first time from *Lagerstroemia speciosa*.

The structure of the compound JF-39 is given below:




n-Tricosane, JF-39 (**56**)

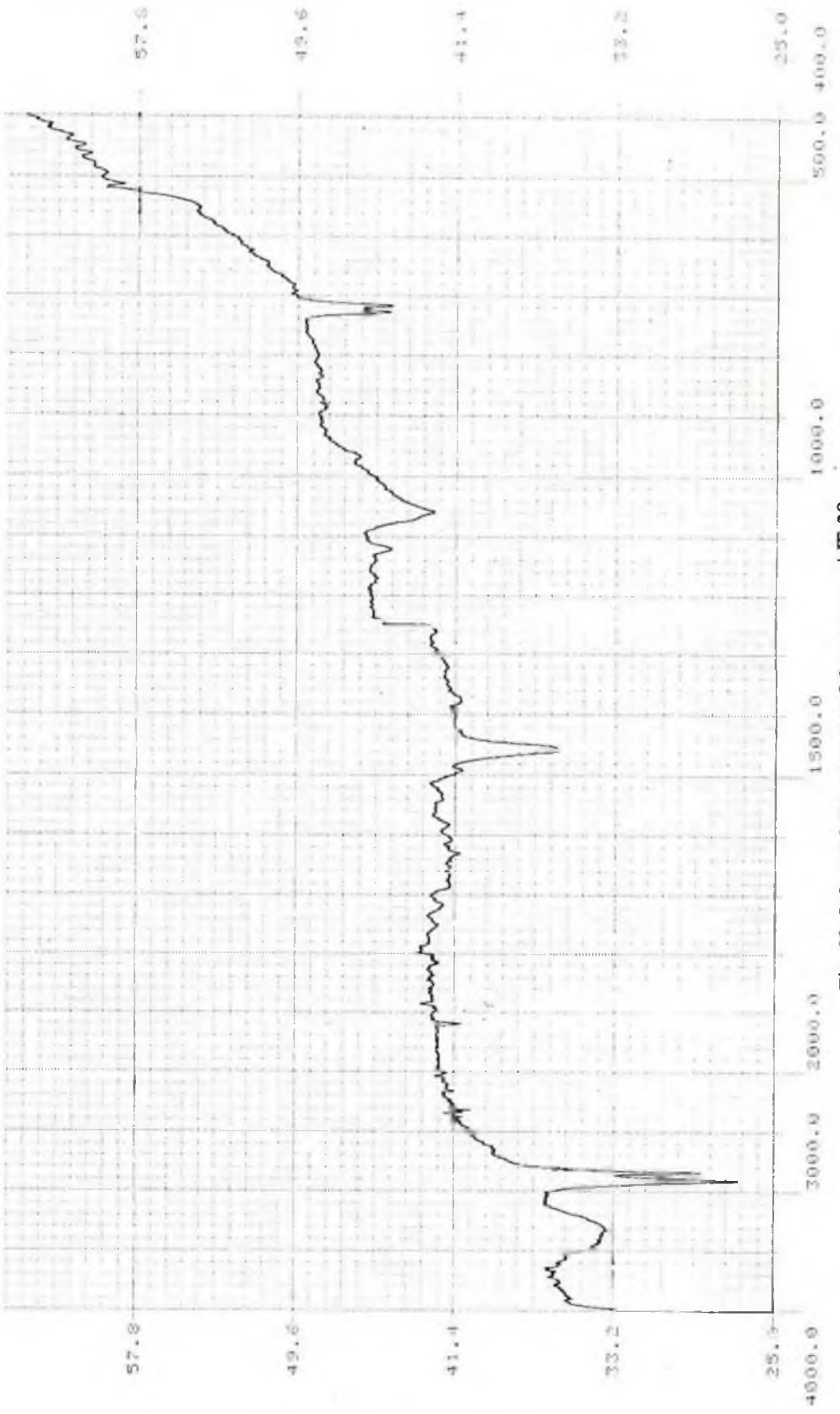



Fig 13: Infrared (IR) spectrum of the compound JF-39

ANALYTICAL RESEARCH DIVISION

*** CLASS-5000 *** Report No. = 1 Data : N.D70 00/04/24 14:17:28
Sample : AO-1
ID : 1ppm
Sample Amount : 1
Dilution Factor : 1
Type : Unknown
Operator : Dr.Mozaffar
Method File Name : N.MET

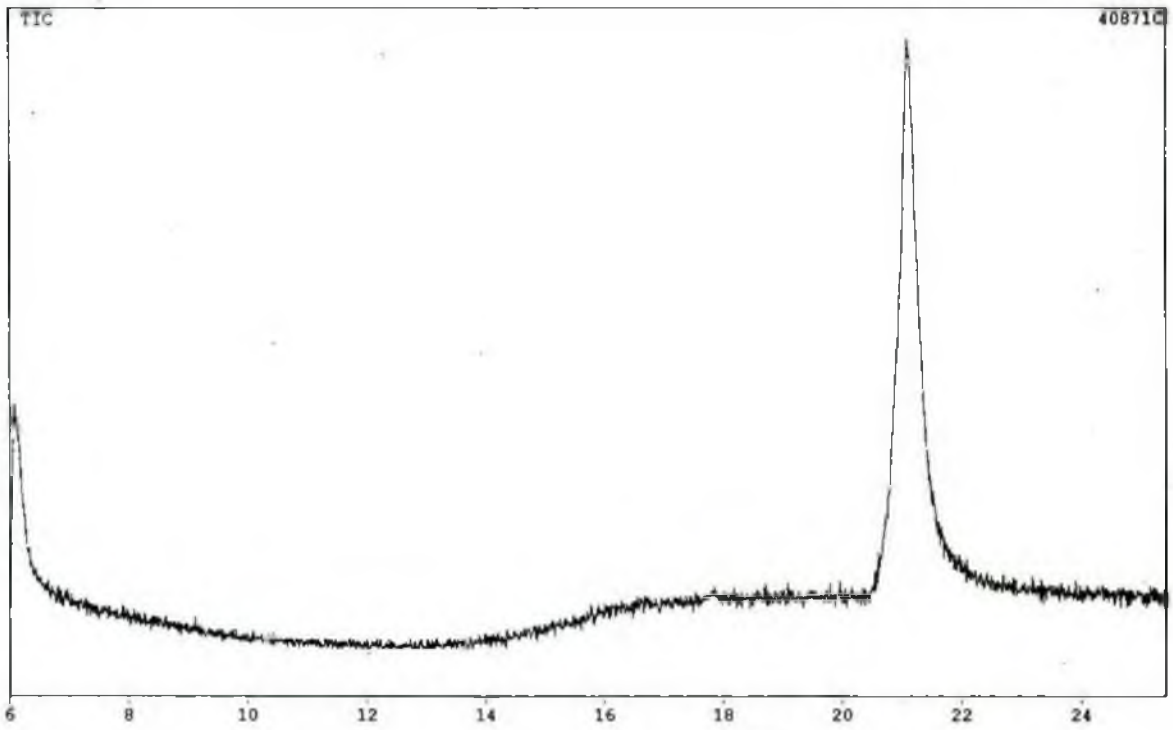



Fig. 14: Gas Chromatograph of the compound JF-39

Scan # : 1811 B.G. Scan # : ( 1863 - 1881 )
Mass Peak # : 62 Ret. Time : 21.083
Base Peak : 57.15 ( 26345)

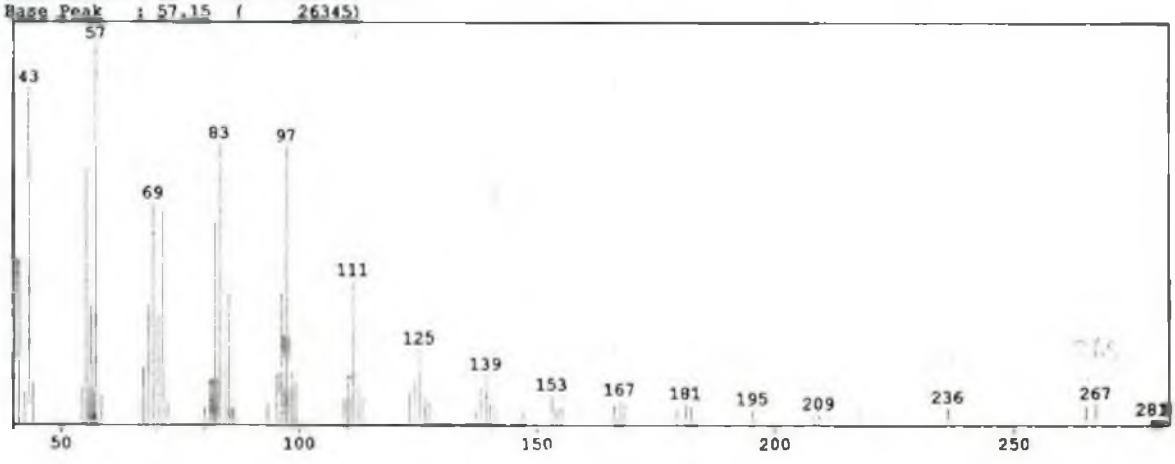



Fig. 15: Mass fragmentation of the compound JF-39

### 3.11.4 Purification of fraction $LSLDC_3F_{43}$:

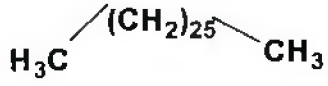
The fraction $LSLDC_3F_{43}$ gave a single spot with tailing in TLC. The fraction was washed repeatedly with methanol to remove associated impurities. After washing, a white solid was obtained and the solid was assigned as JF-43 (4 mg).

### 3.11.4.1 Structure elucidation of the compound JF-43 by Gas Chromatography and Mass spectroscopy (GC-MS):

The gas chromatograph of the compound JF-43 showed a single peak with retention time 57.93. The corresponding gas chromatograph gave a mass fragmentation pattern (**Fig. 16**) showed the molecular ion peak at *m/z* 380 corresponding to the molecular formula $C_{27}H_{56}$. The other intense peaks were at *m/z* 239, 225, 197, 183, 169, 155, 141, 127, 113, 99, 85, 71, 57 and 43.

The mass fragmentation was compared with known hydrocarbon in the library of mass spectrometer. By mass matching it was suggested that the compound JF-43 was a long chain aliphatic hydrocarbon n-Heptacosane that also isolated for the first time from this plant.

The structure of the compound JF-43 is given below:




n-Heptacosane, JF-43 (**57**)

ANALYTICAL RESEARCH DIVISION

*** CLASS-5000 *** Report No. = 1 Data : H.D43 00/07/25 14:18:23
Sample : LSHF1
ID : 1ppm
Sample Amount : 1
Dilution Factor : 1
Type : Unknown
Operator : Dr.Mozaffar
Method File Name : JOSIM.MET

Scan # : 6472 B.G. Scan # : ( 6480 - 6491 )
Mass Peak # : 42 Ret. Time : 57.925
Base Peak : 57.15 ( 34730)

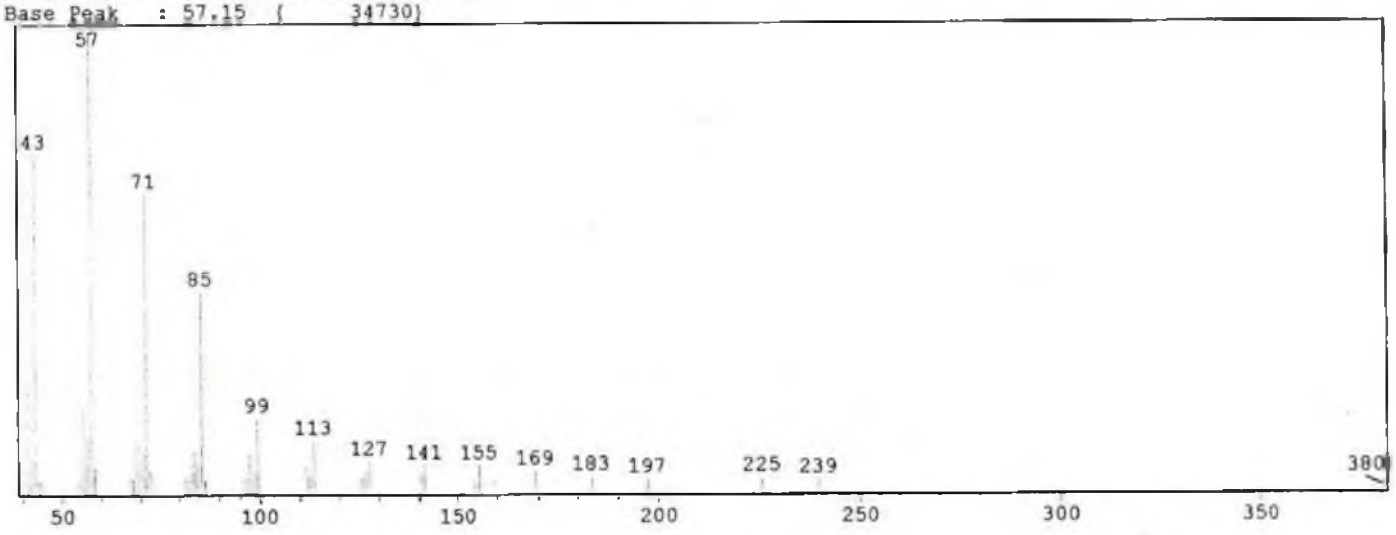



Fig. 16: Mass fragmentation of the compound JF-43

### 3.11.5 Purification of fraction $LSLDC_3F_{53}$:

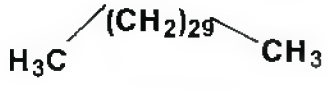
The fraction $LSLDC_3F_{53}$ gave a single spot with tailing in TLC. The fraction was washed repeatedly with methanol to remove associated impurities. After washing, a white solid was obtained and it was designed as JF-53.

### 3.11.5.1 Structure elucidation of the compound JF-53 by Gas Chromatography and Mass spectroscopy (GC-MS):

The gas chromatograph of the compound JF-53 showed a single peak with retention time 86.68. The corresponding gas chromatograph gave a mass fragmentation pattern **(Fig. 17)** showed the molecular peak at *m/z* 436 corresponding to the molecular formula $C_{31}H_{64}$. The other intense peaks were at *m/z* 239, 225, 210, 183, 169, 155, 141, 127, 113, 99, 85, 71, 57 and 43.

The mass fragmentation was compared with known hydrocarbon in the library of mass spectrometer. By mass matching it was suggested that the compound JF-43 was a long chain aliphatic hydrocarbon n-Hentriacontane that also isolated for the first time from this plant.

The structure of the compound JF-53 is given below.




n-Hentriacontane, JF-53 (**58**)

ANALYTICAL RESEARCH DIVISION

*** CLASS-5000 *** Report No. = 1 Data : H.D43 00/07/25 14:18:23
Sample : LSHF1
ID : 1ppm
Sample Amount : 1
Dilution Factor : 1
Type : Unknown
Operator : Dr.Mozaffar
Method File Name : JOSIM.MET

Scan # : 9922 B.G. Scan # : ( 9959 - 9973 )
Mass Peak # : 42 Ret. Time : 86.675
Base Peak : 57.15 ( 37546)

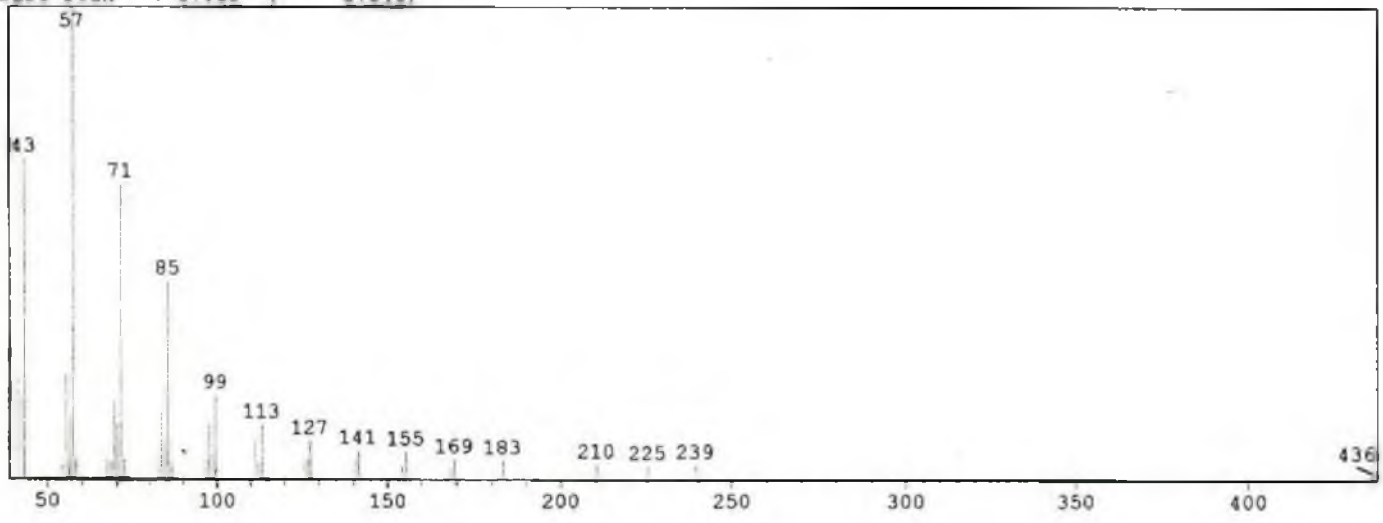



Fig. 17: Mass fragmentation of the compound JF-53

### 3.12 Fractionation of methanol soluble part of bark (LSBEDM, 2.4 g) by column chromatography:

Methanol soluble part was fractionated by silica gel column chromatography and eluted with chloroform followed by ethyl acetate and methanol with increasing polarity. According to the TLC pattern six different fractions (e.g., $LSBEDM_1$, $LSBEDM_2$, $LSBEDM_3$, $LSBEDM_4$, $LSBEDM_5$ and $LSBEDM_6$) were obtained.

### 3.13 Purification of fraction $LSBEDM_3$:

The fraction $LSBEDM_3$ gave a single spot with tailing in TLC. The fraction was washed repeatedly with hexane and dichloromethane to remove associated impurities. After washing, a pale yellow amorphous solid was obtained and designed as AR-7.

### 3.14 Characterization of the compound AR-7:

#### 3.14.1 Physical characteristics:

The compound AR-7 was sparingly soluble in methanol but completely soluble in pyridine and the mixture of chloroform and methanol. It had a **melting point 296-298$^0$C**.

#### 3.14.2 Structure elucidation of AR -7 by spectroscopic methods:

#### (a) $^1$H-NMR (DMSO, 400 MHz) spectroscopy:

In the **$^1$H-NMR spectrum** of the compound AR-7 **(Fig. 18, 18a, 18b)** there were signals at 8.21 ppm for aromatic hydroxyl proton (1H, *s*, hydroxyl proton in aromatic ring), 7.59 ppm for two aromatic protons (2H, *s*, Two aromatic protons) and three signals at 4.11 ppm (3H, *s*, $-OCH_3$), 4.04 ppm (3H, *s*, $-OCH_3$) and 3.99 ppm (3H, *s*, $-OCH_3$) for the presence of three oxymethyl protons in the compound AR-7. The $^1$H-NMR data and assignment of the compound AR-7 are given in the **Table 28**.

**Table 28: $^{1}$H-NMR data of the compound AR-7 (Pyridine. 400 MHz)**

| Chemical shift (ppm) | Splitting Pattern | Integration | Assignment |
|---|---|---|---|
| 8.21 | Singlet | 1H | One -OH proton in aromatic ring |
| 7.60 | Singlet | 2H | Two aromatic protons |
| 4.11 | Singlet | 3H | Three -$OCH_3$ protons |
| 4.04 | Singlet | 3H | Three -$OCH_3$ protons |
| 3.99 | Singlet | 3H | Three -$OCH_3$ protons |

**(b) $^{13}$C-NMR spectroscopy:**

The **$^{13}$C-NMR spectrum** of the compound AR-7 (**Fig. 19, 19a**) had 17 signals indicating that the compound contained 17 carbons. The $^{13}$C-NMR data indicated that the compound might be a derivative of ellagic acid. The two down field signals at 158.39 and 158.28 ppm were assigned to two carbonyl carbons at C-5 and C-10 positions. The signal at 154.35 ppm was due to carbon C-6 which contain a hydroxyl group. The two signals at 117.67 and 107.46 were due to methylene C-6 and C-1 carbons. Three most upfield signals at 61.47, 61.31 and 56.73 were due to three oxymethyl groups attached to C-7, C-2 and C-3 carbons. The other signals at 114.08, 112.87, 112.73 and 111.43 were quaternary aromatic carbons.

From $^{1}$H- and $^{13}$C-NMR data the structure of the compound AR-7 was found to be 2,3,7-Tri-*O*-methylellagic acid.

**(c) $FAB^+$Mass spectroscopy:**

The fast atom bombardment mass spectrum (**$FAB^+$MS**) of the compound AR-7 (**Fig. 21, 21a**) had peak at *m/z* 344+23+1 [base peak; M+Na+H], thus suggesting an empirical formula $C_{17}H_{12}O_8$. The other intense peaks were at *m/z* 327.8, 300.9, 285.8, 171.9, 115.0 102.9 and 52.9.

$FAB^+$ Mass spectrum also confirmed that the compound AR-7 was 2,3,7-Tri-*O*-methyl ellagic acid.

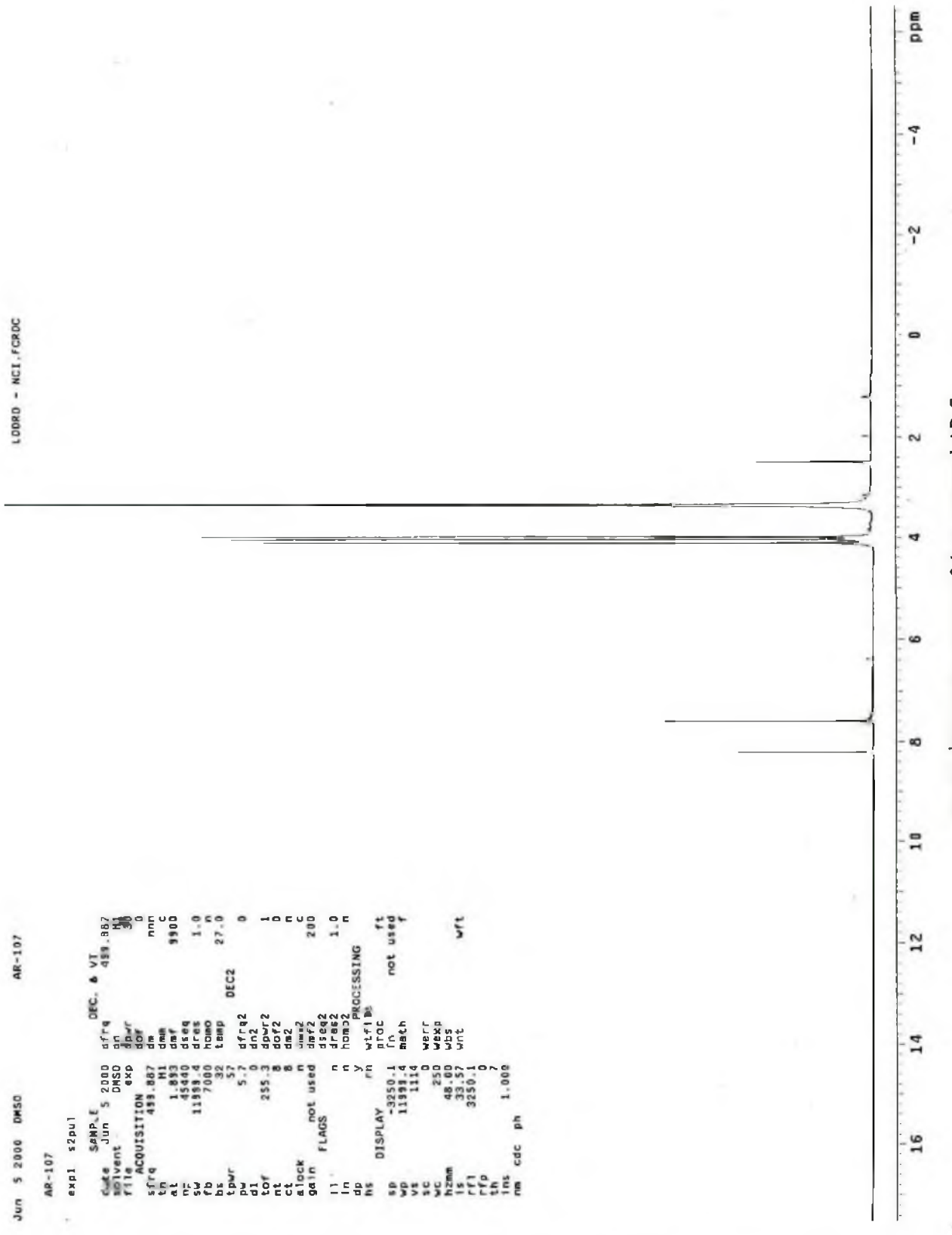



Fig. 18: $^{1}$H-NMR spectrum of the compound AR-7

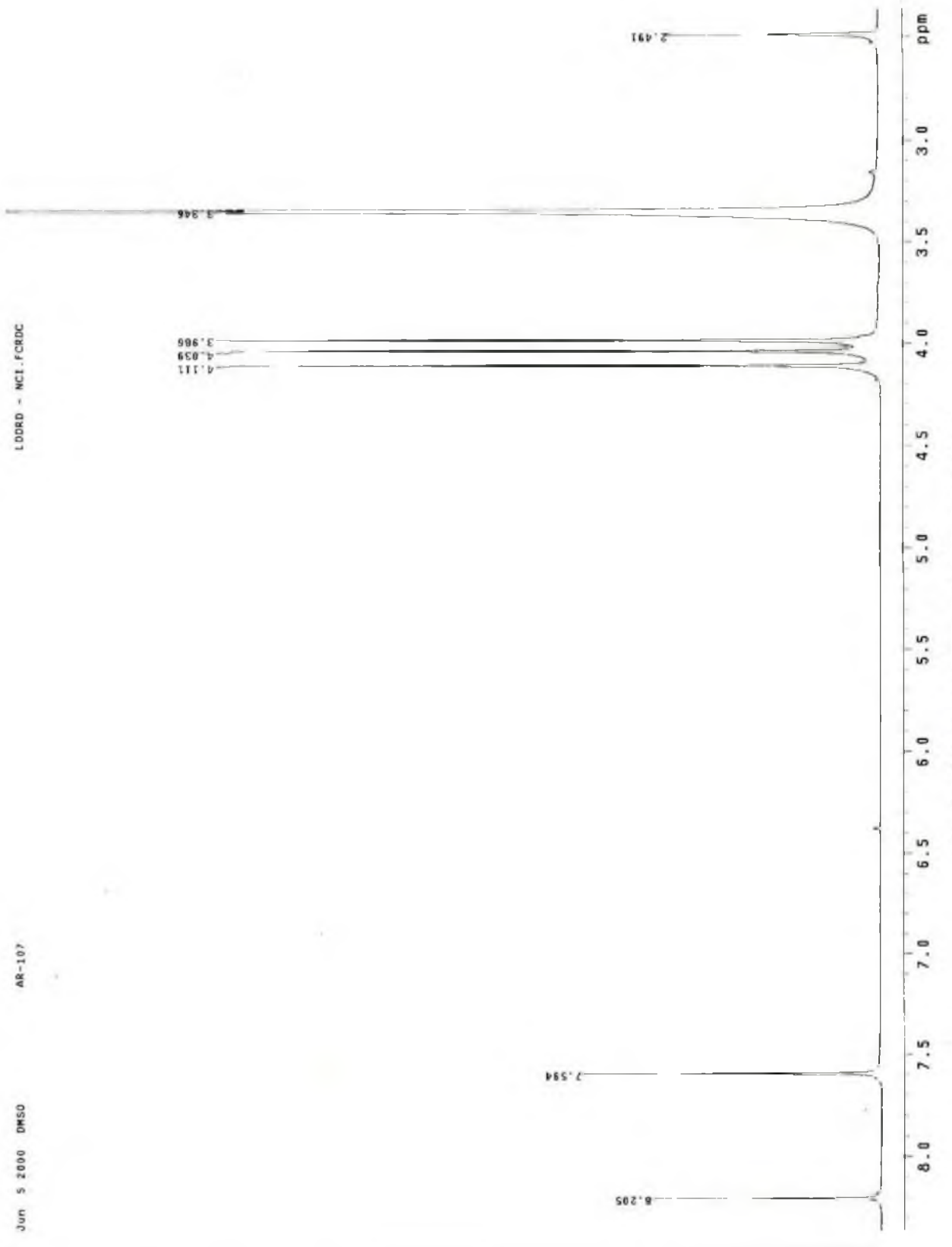



Fig 18a: $^1$H-NMR spectrum of the compound AR-7

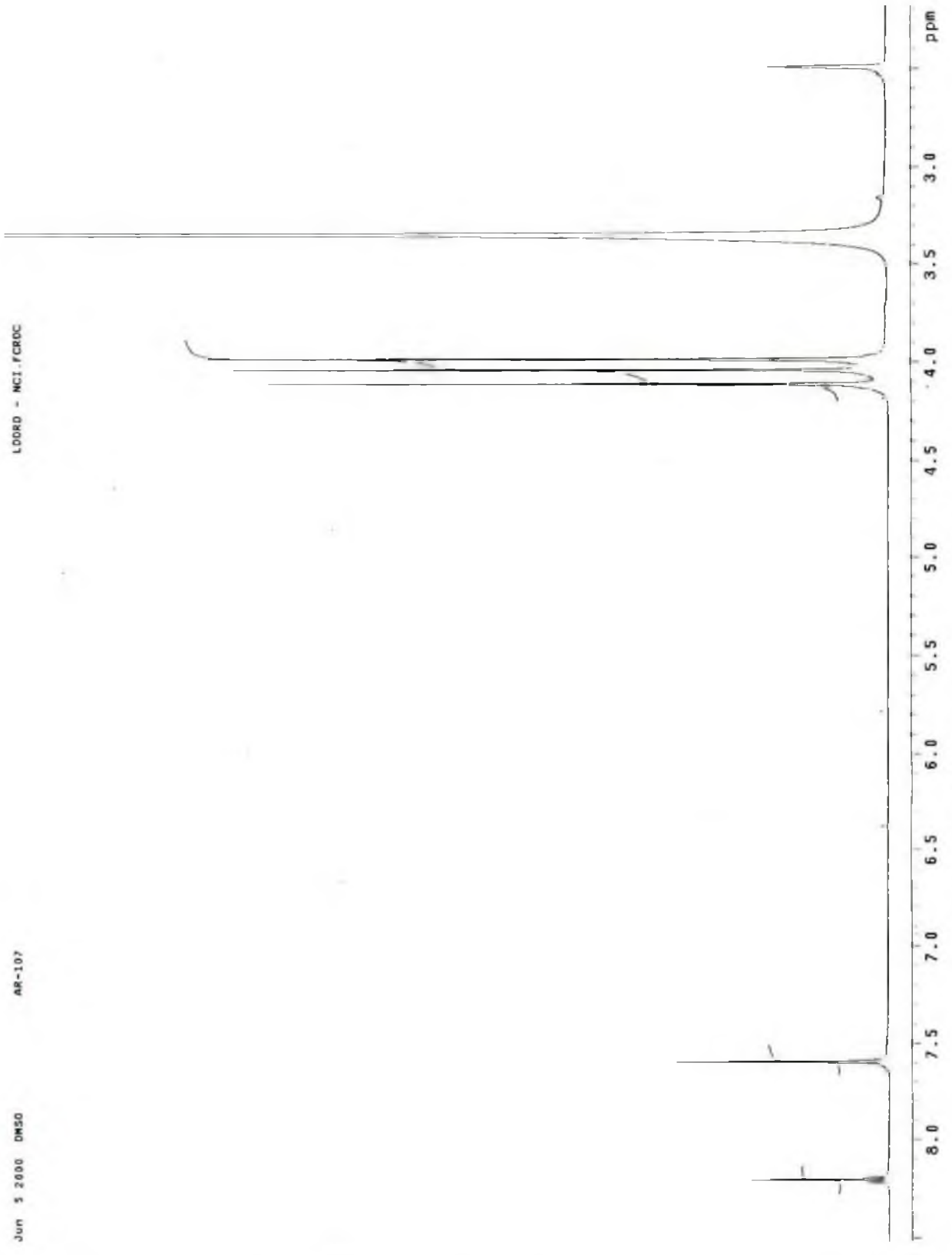



Fig. 18b: $^1$H-NMR spectrum of the compound AR-7

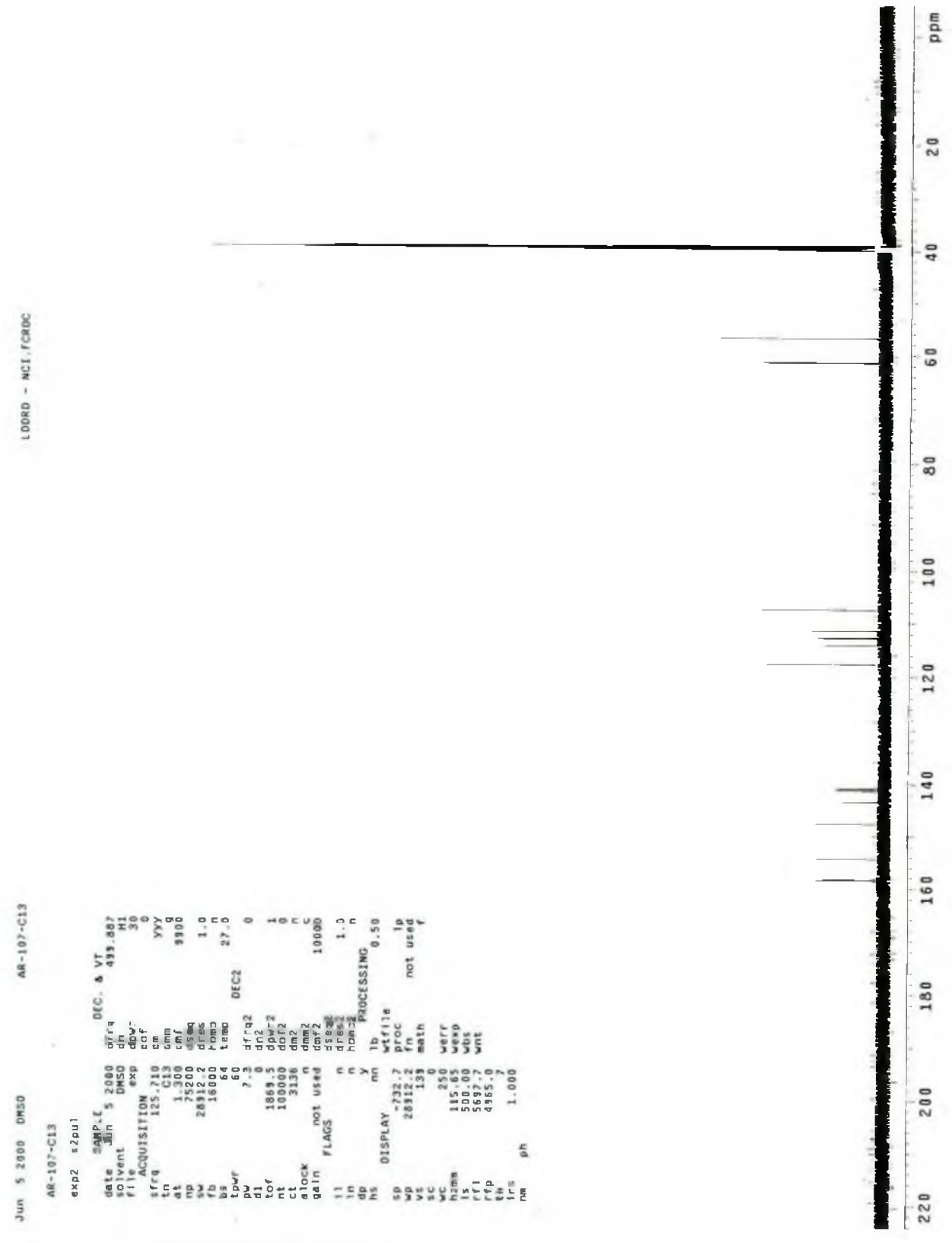



Fig. 19: $^{13}$C-NMR spectrum of the compound AR-7

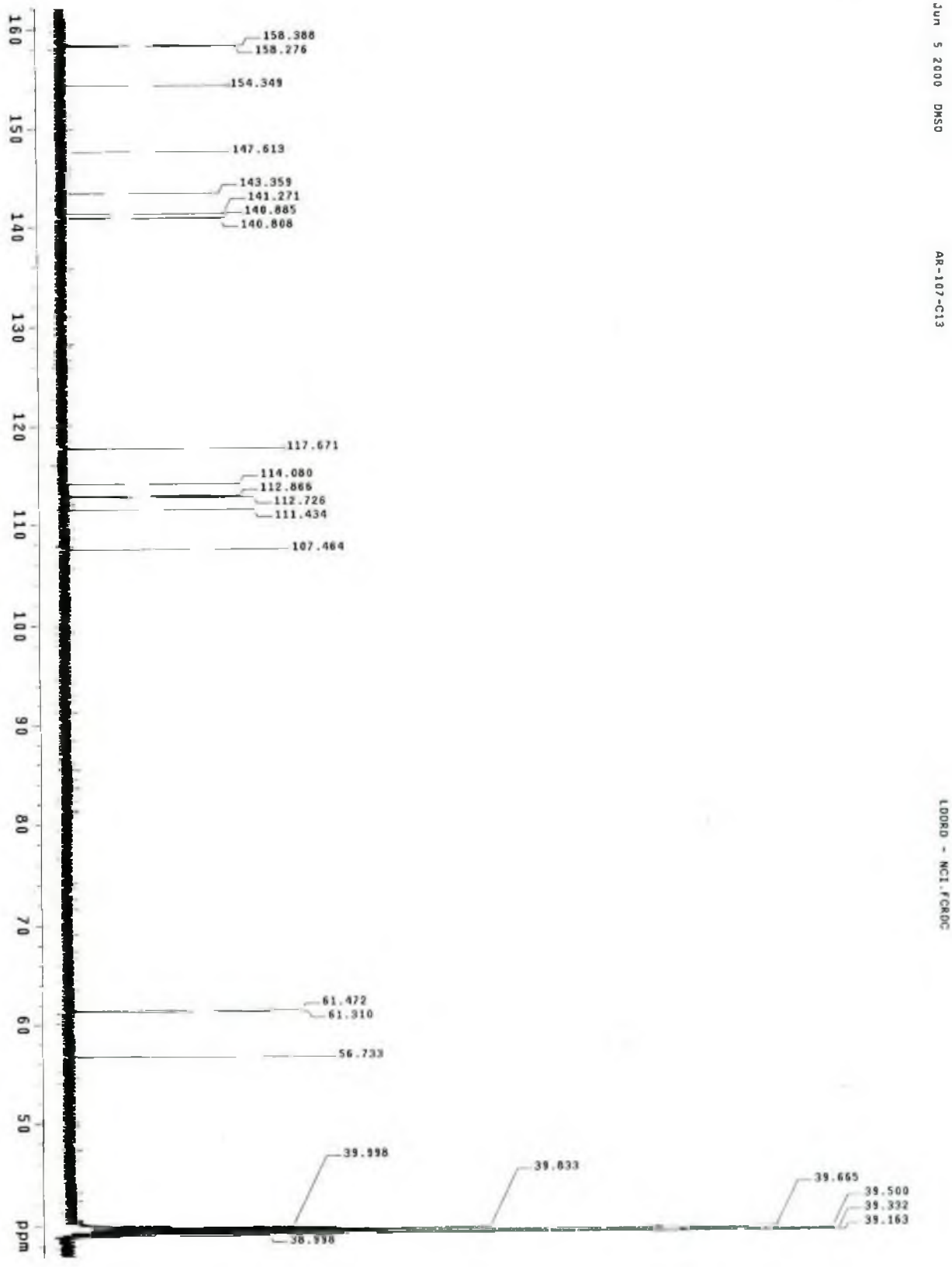



Fig. 19a. $^{13}$C-NMR spectrum of the compound AR-7

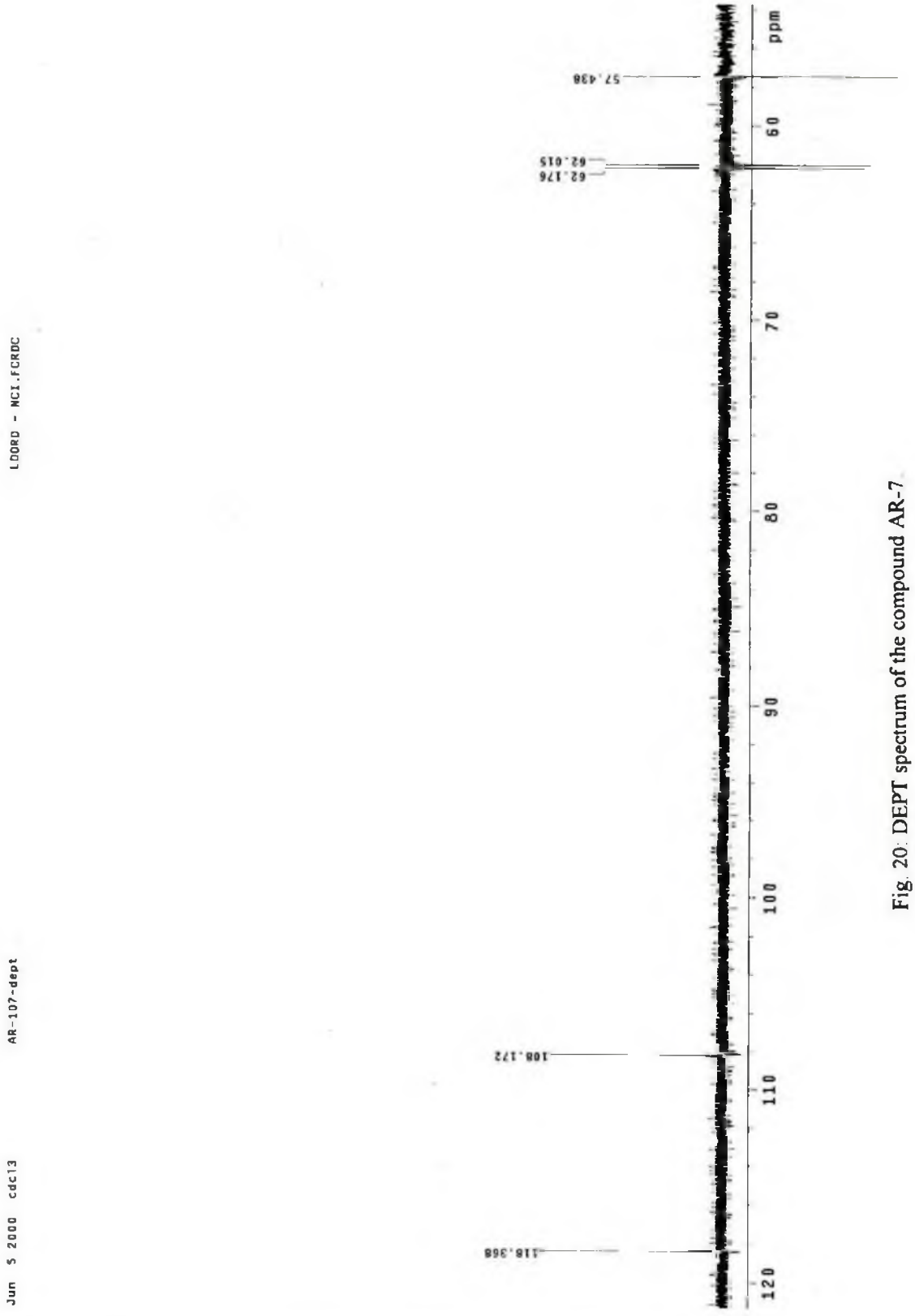



Fig. 20: DEPT spectrum of the compound AR-7

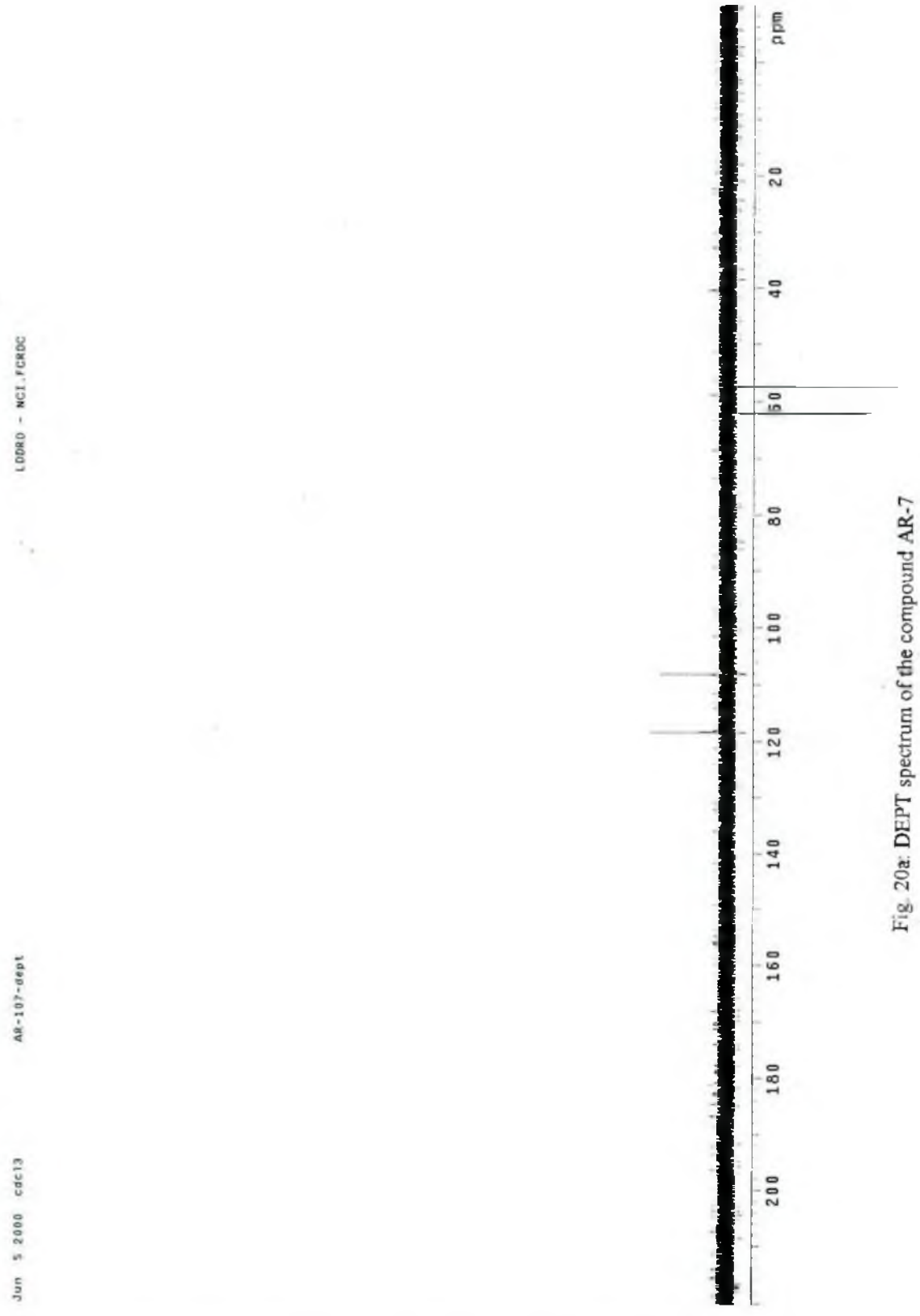



Fig. 20a: DEPT spectrum of the compound AR-7

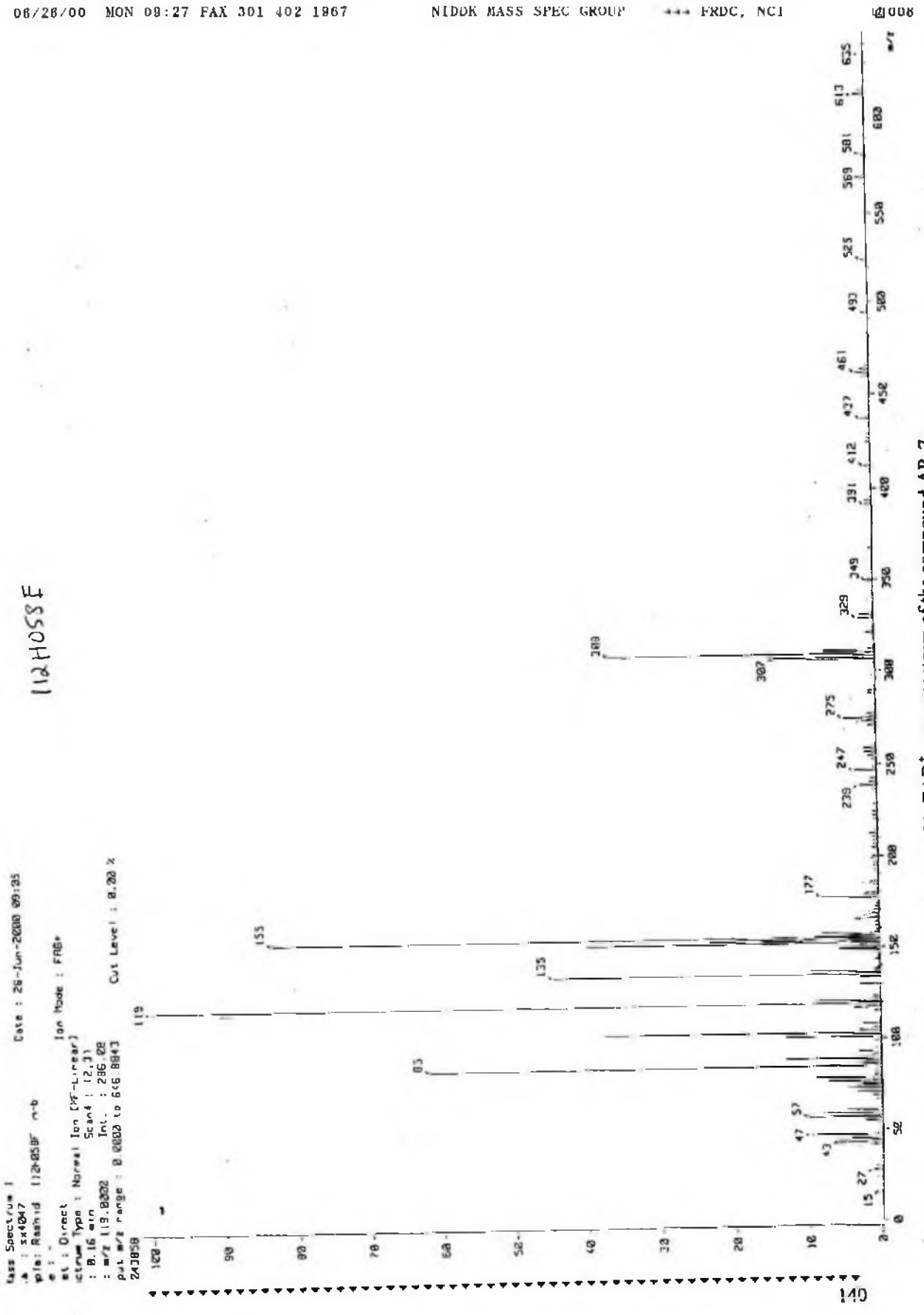



Fig 21: FAB$^+$ mass spectrum of the compound AR-7

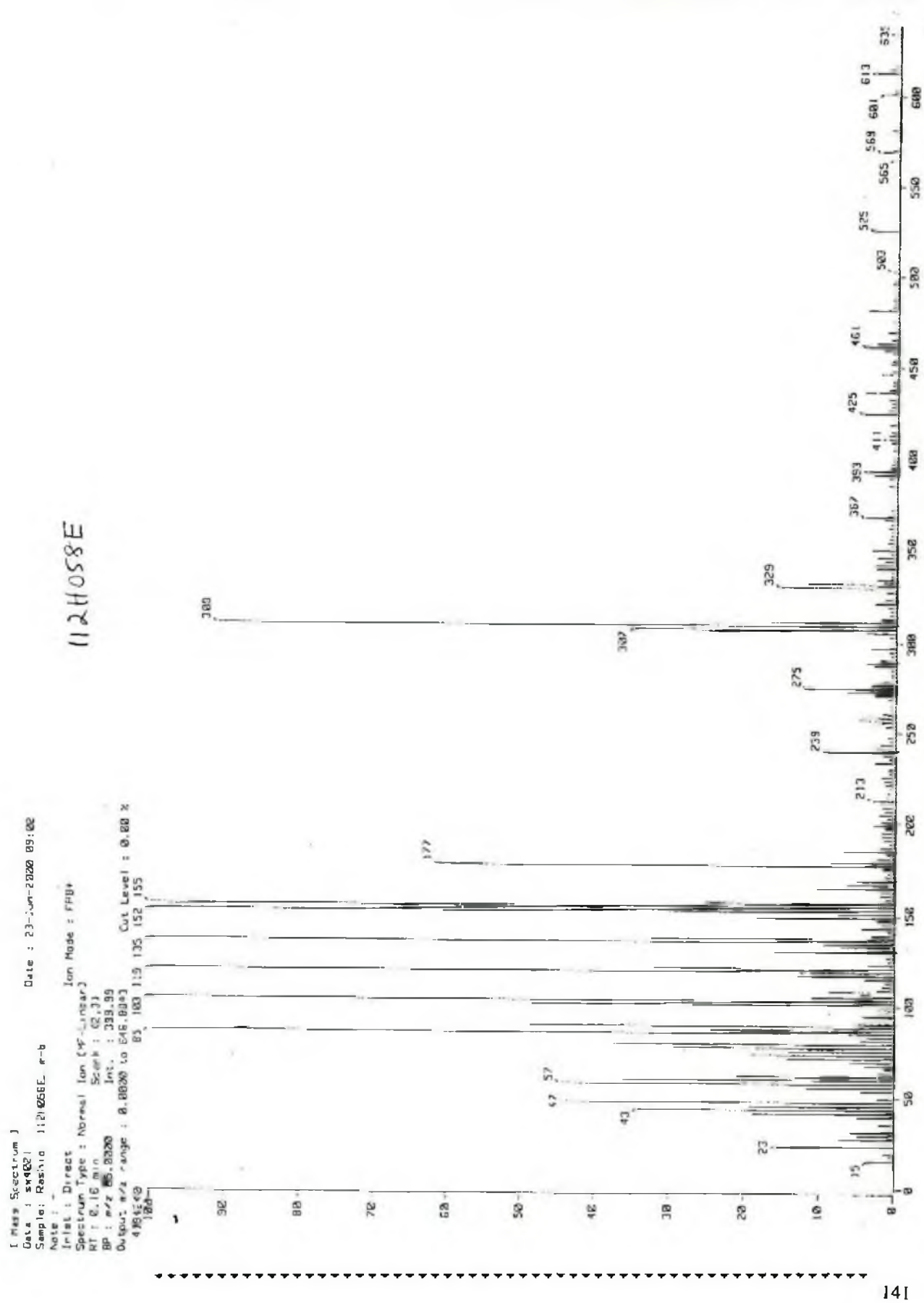
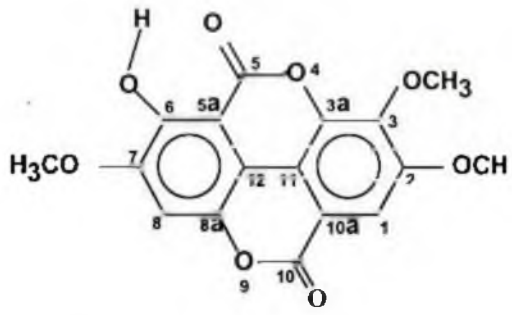



Fig. 21a: FAB+ mass spectrum of the compound AR-7

### 3.14.3 Structure of the compound AR-7:

On the basis of physical characteristics and spectroscopic data it was concluded that the compound AR-7 was 2,3,7-Tri-*O*-methylellagic acid.

The structure of 2,3,7-Tri-*O*-methylellagic acid (AR-7) is given below:




2,3,7-Tri-*O*-methylellagic acid (AR-7) (**52**)

From computer literature survey NAtural PRoduct ALART (NAPRALART search in December 1998) and Dictionary of Natural Product on CD-ROM (Chapman & Hall, release 6:2) of *Lagerstroemia speciosa* **L.** it was found that 2,3-Di-*O*-methyl, 2,7-*O*-methylellagic acids were reported earlier from plant sources but not from *Lagerstroemia* genus and 2,3,7-Tri-*O*-methylellagic acid has not been reported earlier from this plant.

### 3.15 Fractionation of acetone soluble part (LSBEDA) of bark by column chromatography:

The dried extract of acetone soluble part (LSBEDA; 2.31 g) of *Lagerstroemia speciosa* gave three spots on TLC. The fraction was further fractionated by column chromatography using silica gel as adsorbent and eluted with dichloromethane and methanol and five different fractions (e.g., $LSBEDA_1$, $LSBEDA_2$, $LSBEDA_3$, $LSBEDA_4$ and $LSBEDA_5$) were obtained.

### 3.16 Purification of fraction $LSBEDA_2$:

The fraction $LSBEDA_2$ gave a single spot with tailing in TLC. The fraction was washed repeatedly with hexane and dichloromethane to remove associated impurities. After washing, a white residue was obtained. The white residue was crystallized from the mixture of dichloromethane: hexane (1: 5 v/v). The crystalline solid was assigned as AR-1 (63 mg).

### 3.17 Characterization of the compound AR-1

#### 3.17.1 Physical characteristics and chemical tests:

Compound AR-1 was a colorless crystalline solid having **melting point $138^0$-$139^0$C**. It was readily soluble in chloroform. It gave positive test for steroid.

#### 3.17.2 Structure elucidation of compound AR-1 by spectroscopic methods:

#### (a) Infrared (IR) spectroscopy:

The **IR spectrum** of the compound AR-1 **(Fig 22)** had absorption at 3415 $cm^{-1}$ which was due to –OH stretching vibration. Sharp absorption at 2920 and 2850 $cm^{-1}$ were due to –C-H asymmetric and symmetric stretching respectively. Weak absorption at 1640 $cm^{-1}$ indicated the presence of isolated double bond (>C=C<) stretching. Absorption at 1455 $cm^{-1}$ was due to $-CH_2-$ bending vibration (scissoring). It was the characteristic absorption of methylene ($-CH_2-$) group. The absorption at 1370 $cm^{-1}$ was due to geminal dimethyl

group. This was the characteristic peak of isopropyl group. The absorption frequency at 1055 $cm^{-1}$ was due to C-O stretching vibration at 980 and 960 $cm^{-1}$ were the characteristic peaks of steroid.

**(b) $^1$H-NMR spectroscopy:**

The **$^1$H-NMR spectrum (Fig. 23)** of the compound AR-1 had two sharp two singlets at 0.66 (3H, *s*) and 0.99 (3H, *s*) which indicated the presence of two angular methyl protons attached with quaternary carbons (>C<).

The two doublets at 0.91 and 0.89 (6H, *d*, *J*=5.2 Hz) and 0.80 (3H, *d*, *J*=3.4 Hz) indicated that the presence of three methyl groups attached with methine carbons (>CH-). One distorted triplet at 0.82 (3H, t) indicated the presence of one methyl group attached with methylene carbon. The triplet was distorted due to long range coupling with neighboring other protons.

A distorted doublet at 5.38 (H, *d*, *J*=4.0) indicated the presence of double bond in between olefinic quaternary carbon (>C<) and olefinic methine carbon (>CH-). Low coupling constant of *J*=4.0 Hz indicated that the double bond was endocyclic.

The characteristic septet at 3.49 was due to a proton attached to the carbon having adjacent –OH group and two methylene groups *e.g.*, $-CH_2-CHOH-CH_2-$. The approximate coupling constant of 11.4 Hz indicated that the hydroxyl group was in $\beta$–orientation.

The other signals (multiplets, triplets, doublets, singlets) between 1.01-2.29 ppm were due to different methylene ($-CH_2-$) and methine (>CH-) protons presents in the compound AR-1.

The $^1$H-NMR spectral data of the compound AR-1 are given in the **Table 29**.

**Table 29: $^{1}$H-NMR spectral data of the compound AR-1 (Pyridine, 400 MHz)**

| Chemical shift (ppm) | Splitting pattern | Integration | Assignment |
|---|---|---|---|
| 5.34 | Distorted doublet | 1H | Olefinic proton |
| 5.045 | Doublet | 1H | Anomeric proton of sugar moiety |
| 3.92-4.57 | Multiplet | | Oxygenated methine protons sugar moiety |
| 0.66 | Singlet | 3H | -$CH_3$ group |
| 0.87 | Doublet | 3H | -$CH_3$ group |
| 0.86 | Doublet | 6H | Two –$CH_3$ groups |
| 0.85 | Singlet | 3H | -$CH_3$ group |

**(c) $^{13}$C-NMR spectroscopy:**

The **$^{3}$C-NMR spectrum (Fig. 24, 24a** and **Table 30**) of the compound AR-1 had 29 signals indicating that the compound contained 29 carbons. The signal at 71.80 ppm was assigned to oxymethine carbon C-3 and two most deshielded signals at 140.76 and 121.71 ppm were due to two quaternary olefmic carbons C-5 and C-6, respectively. To distinguish between methyl, methylene, methine and quaternary carbons present in the compound, DEPT-135$^{0}$ and DEPT-90$^{0}$ were carried out. Chemical shifts in the $^{13}$C NMR spectra were compared with the reported data of steroidal compounds and the assignments were made accordingly. Chemical shifts at 37.26, 31.66, 42.30, 31.91, 21.08, 39.78, 24.30, 28.24, 33.95, 26.09 and 23.07 ppm were assigned to C-1, C-2, C-4, C-7, C-11, C-12, C-15, C-16, C-22, C-23 and C-28, respectively. Eight signals at 71.79, 31.88, 50.10, 56.74, 56.02, 36.13, 45.80 and 29.11 ppm were assigned to eight methine carbons C-18, C-19, C-21, C-26, C-27 and C-29, respectively. Six signals at 11.85, 19.39, 18.77, 19.80, 19.03 and 11.97 ppm were assigned to six methyl carbons C-18, C-19, C-21, C-26, C-27 and C-29, respectively. Two other deshielded signals at 36.50, 42.32 ppm were assigned to two quaternary carbons C-10 and C-13, respectively.

**Table 30. $^{13}$C-NMR data of AR-1 and that of reported[83] data of $\beta$-sitosterol**

| Position of Carbons | Types of Carbons | Compound AR-1 | $\beta$-sitosterol[83] |
|---|---|---|---|
| C-1 | $-CH_2-$ | 37.26 | 37.31 |
| C-2 | $-CH_2-$ | 31.66 | 31.57 |
| C-3 | -CH-OH | 71.80 | 71.69 |
| C-4 | $-CH_2-$ | 42.30 | 42.25 |
| C-5 | >C< | 140.76 | 140.76 |
| C-6 | >CH- | 121.71 | 121.59 |
| C-7 | $-CH_2-$ | 31.91 | 31.92 |
| C-8 | >CH- | 31.91 | 31.92 |
| C-9 | >CH- | 50.14 | 50.17 |
| C-10 | >C< | 36.50 | 36.51 |
| C-11 | $-CH_2-$ | 21.08 | 21.11 |
| C-12 | $-CH_2-$ | 39.78 | 39.81 |
| C-13 | >C< | 42.32 | 42.33 |
| C-14 | >CH- | 56.77 | 56.79 |
| C-15 | $-CH_2-$ | 24.30 | 24.32 |
| C-16 | >CH- | 28.24 | 28.26 |
| C-17 | >CH- | 56.06 | 56.11 |
| C-18 | $-CH_3$ | 11.85 | 11.87 |
| C-19 | $-CH_3$ | 19.39 | 19.40 |
| C-20 | >CH- | 36.14 | 36.17 |

| | | | |
|---|---|---|---|
| C-21 | $-CH_3$ | 18.77 | 18.82 |
| C-22 | $-CH_2-$ | 33.95 | 33.95 |
| C-23 | $-CH_2-$ | 26.09 | 26.13 |
| C-24 | >CH- | 45.84 | 45.55 |
| C-25 | >CH- | 29.16 | 29.18 |
| C-26 | $-CH_3$ | 19.80 | 19.84 |
| C-27 | $-CH_3$ | 19.03 | 19.07 |
| C-28 | $-CH_2-$ | 23.07 | 23.09 |
| C-29 | $-CH_3$ | 11.97 | 12.32 |

**(d) $FAB^+$ Mass spectroscopy:**

The **fast atom bombardment mass spectrum ($FAB^+MS$)** of the compound AR-1 **(Fig. 26)** had the molecular ion peak at *m/z* 397 $[M-H_2O+H]^+$, 414-18+1 indicating that 414 was the molecular mass corresponding the molecular formula $C_{29}H_{50}O$.

The base peak at 43 and other intense peaks were at *m/z* 55, 69, 81, 119, 145, 159, 213, 273, 381, and 395. The fragmentation pattern was very similar to those observed for $C_{29}$ steroids.

On the basis of chemical results and spectroscopic data (UV, IR, $^1$H-NMR, $^{13}$C-NMR spectral data of AR-1 were compared with the reported $^{13}$C-NMR, mass spectroscopy) it was observed that the compound AR-1 is very similar to the known steroid $\beta$-sitosterol. The physical characteristics and $^{13}$C-NMR spectral data of AR-1 were compared with the reported $^{13}$C-NMR data of steroids. It was found that the data fitted very well with the $^{13}$C-NMR spectral data of $\beta$-sitosterol, which is a very common steroid present in almost all kinds of plants. But it was isolated from the bark of *Lagerstroemia speciosa* **Linn.** for the first time.

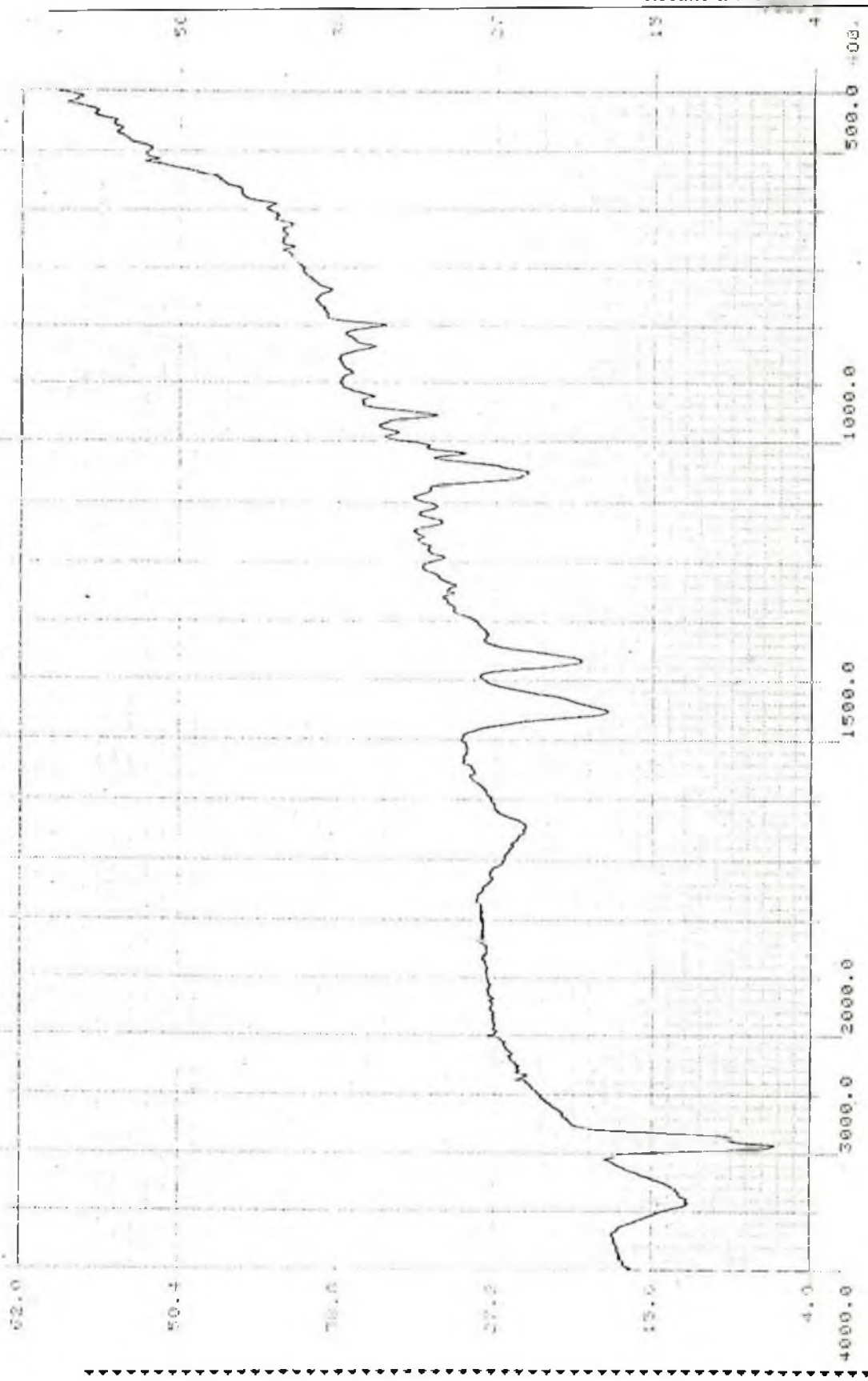



Fig. 22: Infrared (IR) spectrum of the compound AR-1

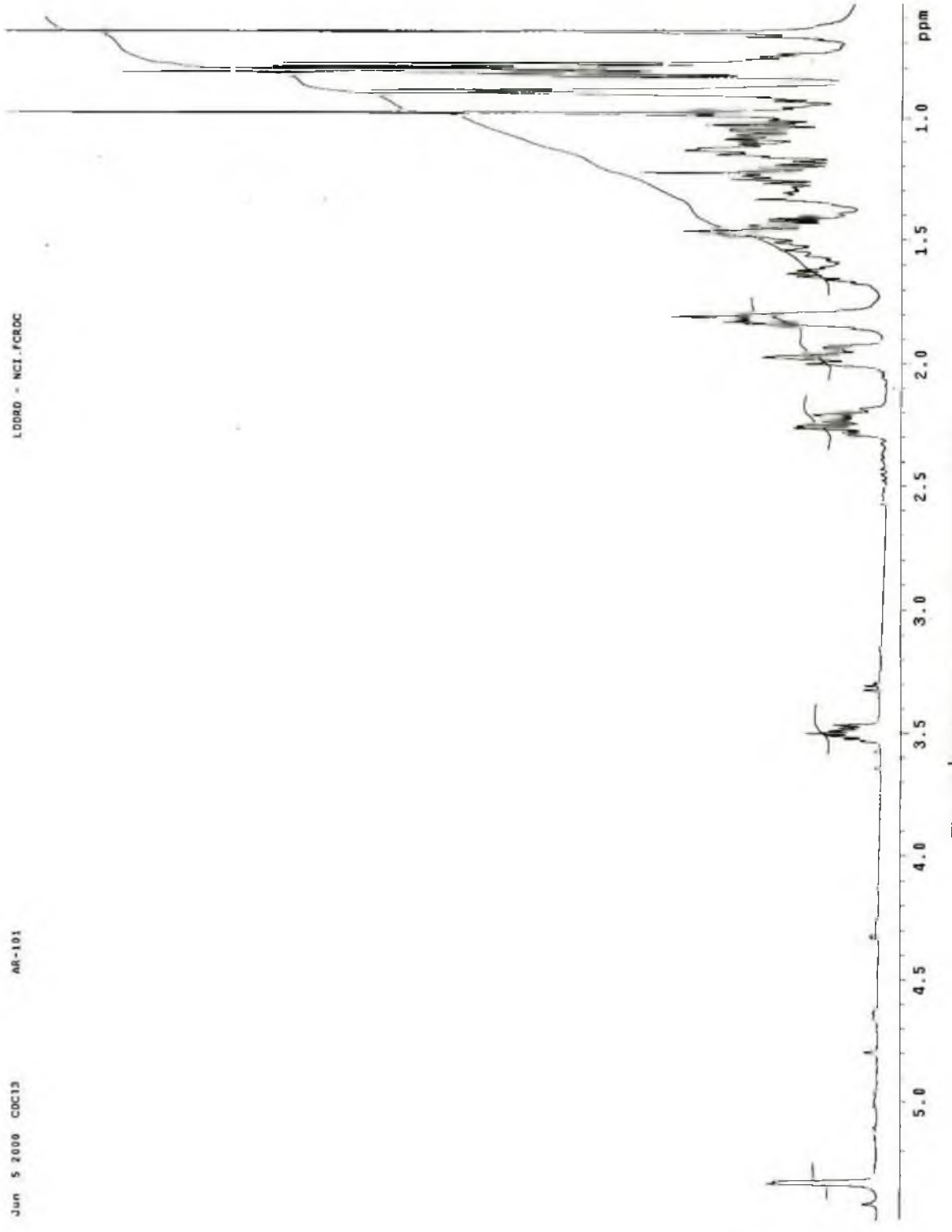



Fig. 23: $^1$H-NMR spectrum of the compound AR-1

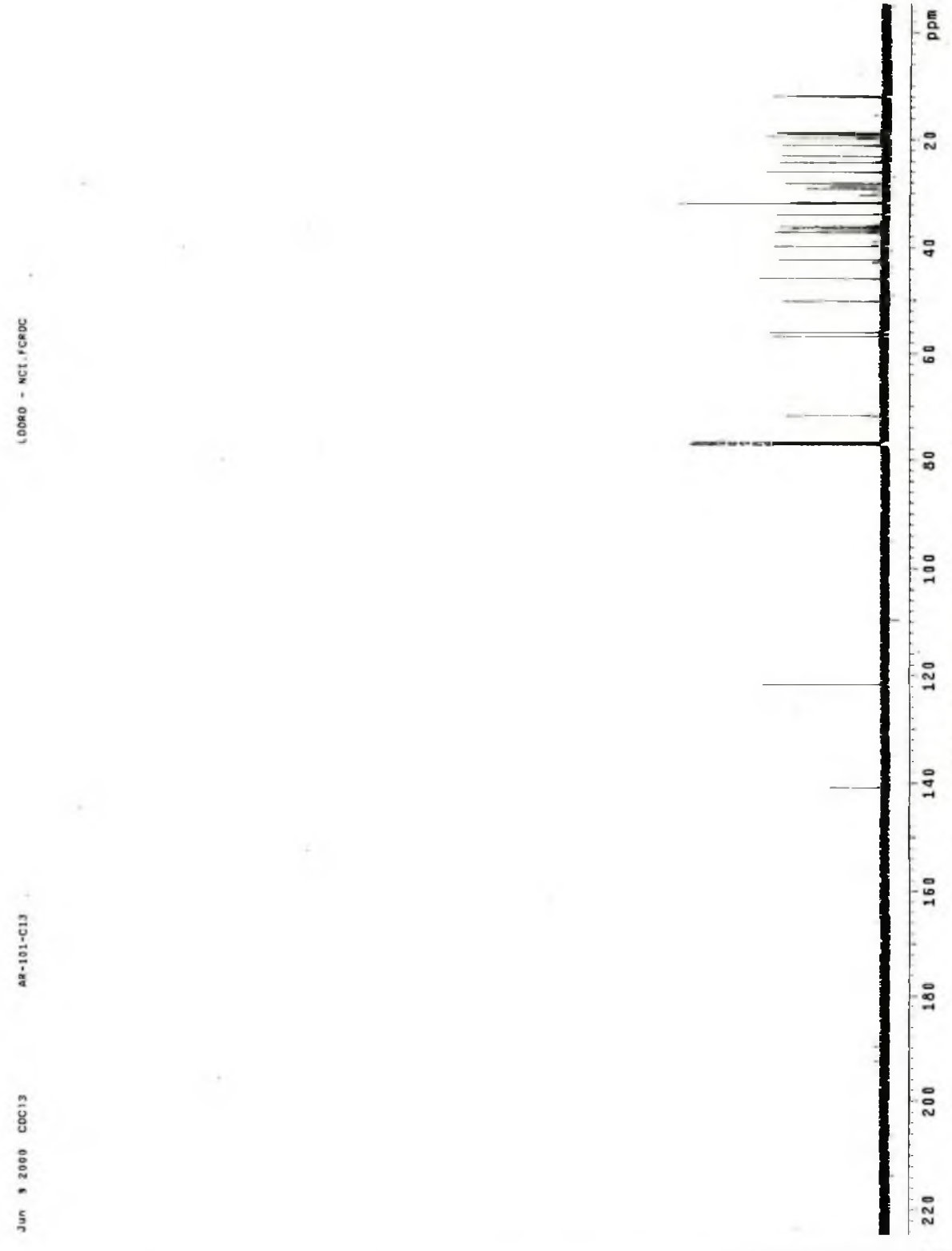



Fig 24. $^{13}$C-NMR spectrum of the compound AR-1

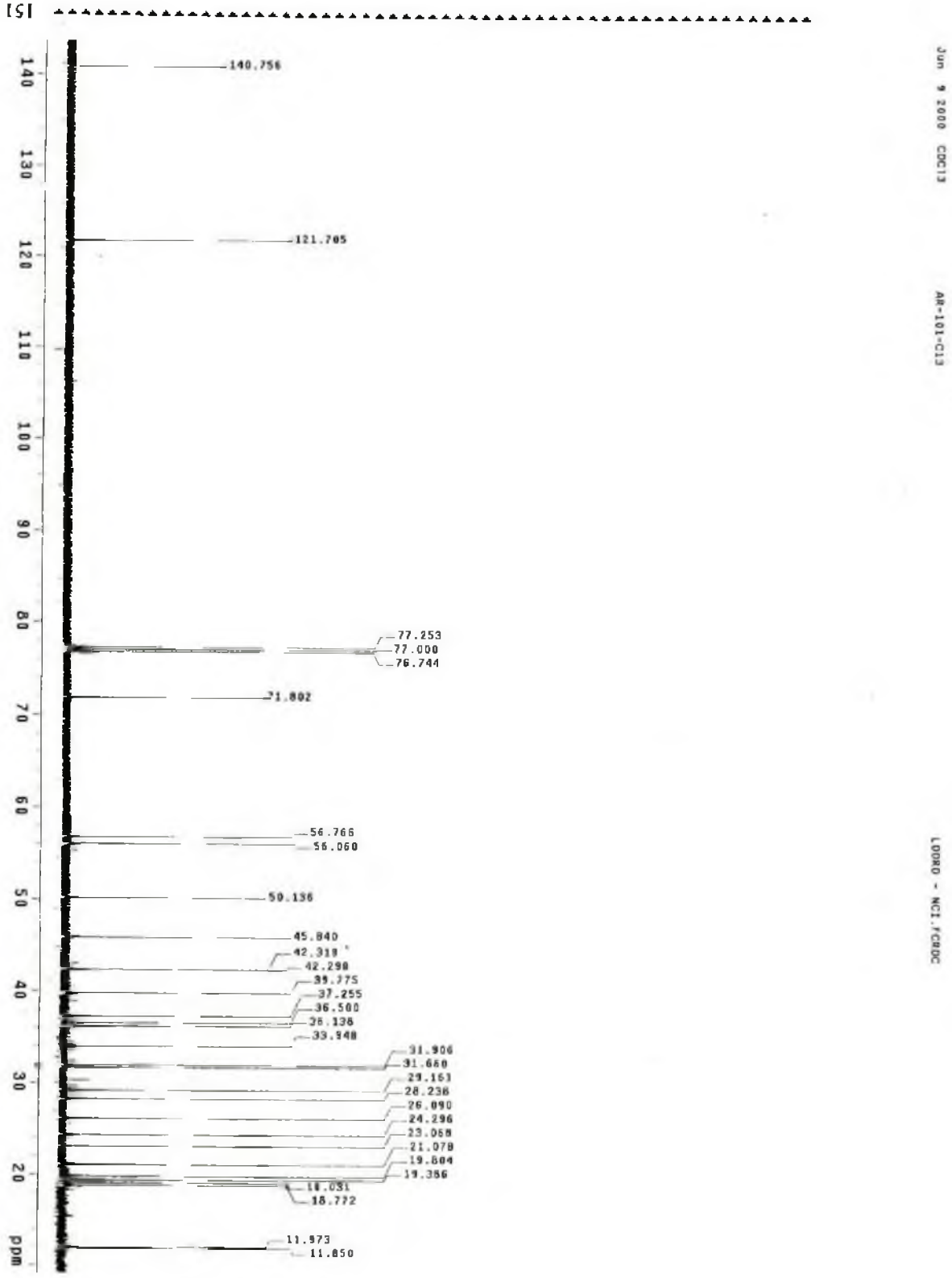



Fig 24a: $^{13}$C-NMR spectrum of the compound AR-1

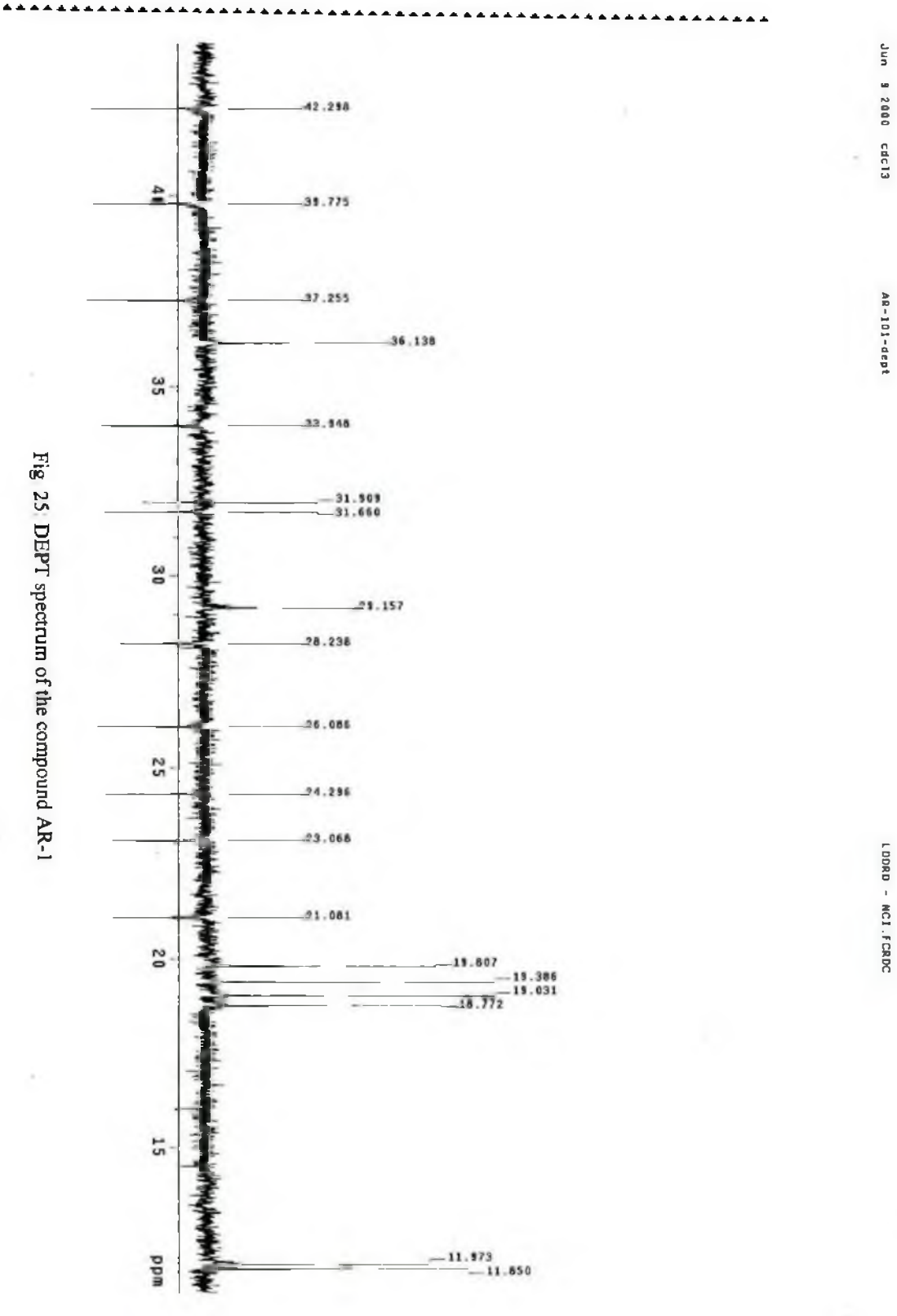



Fig 25 DEPT spectrum of the compound AR-1

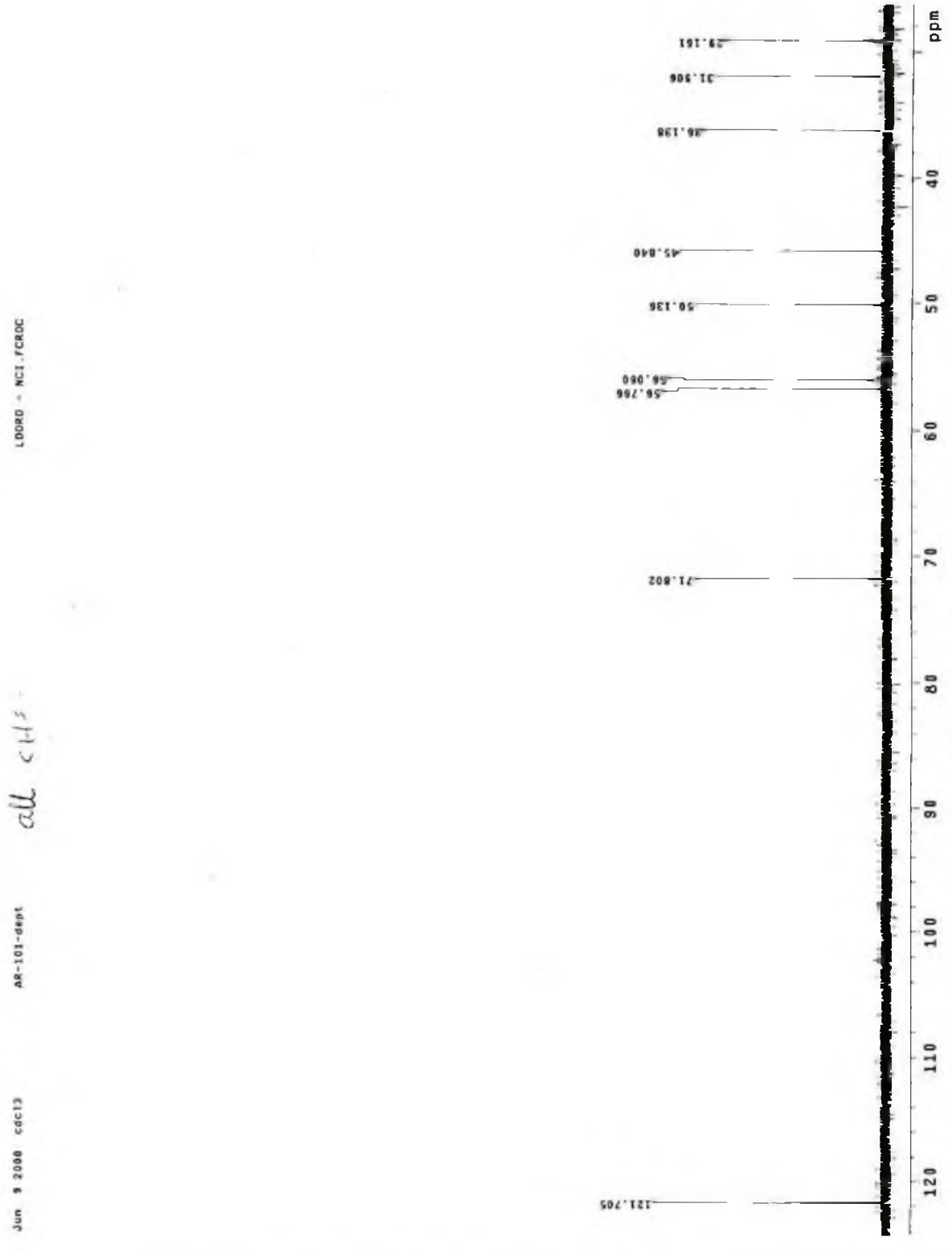



Fig. 25a: DEPT spectrum of the compound AR-1

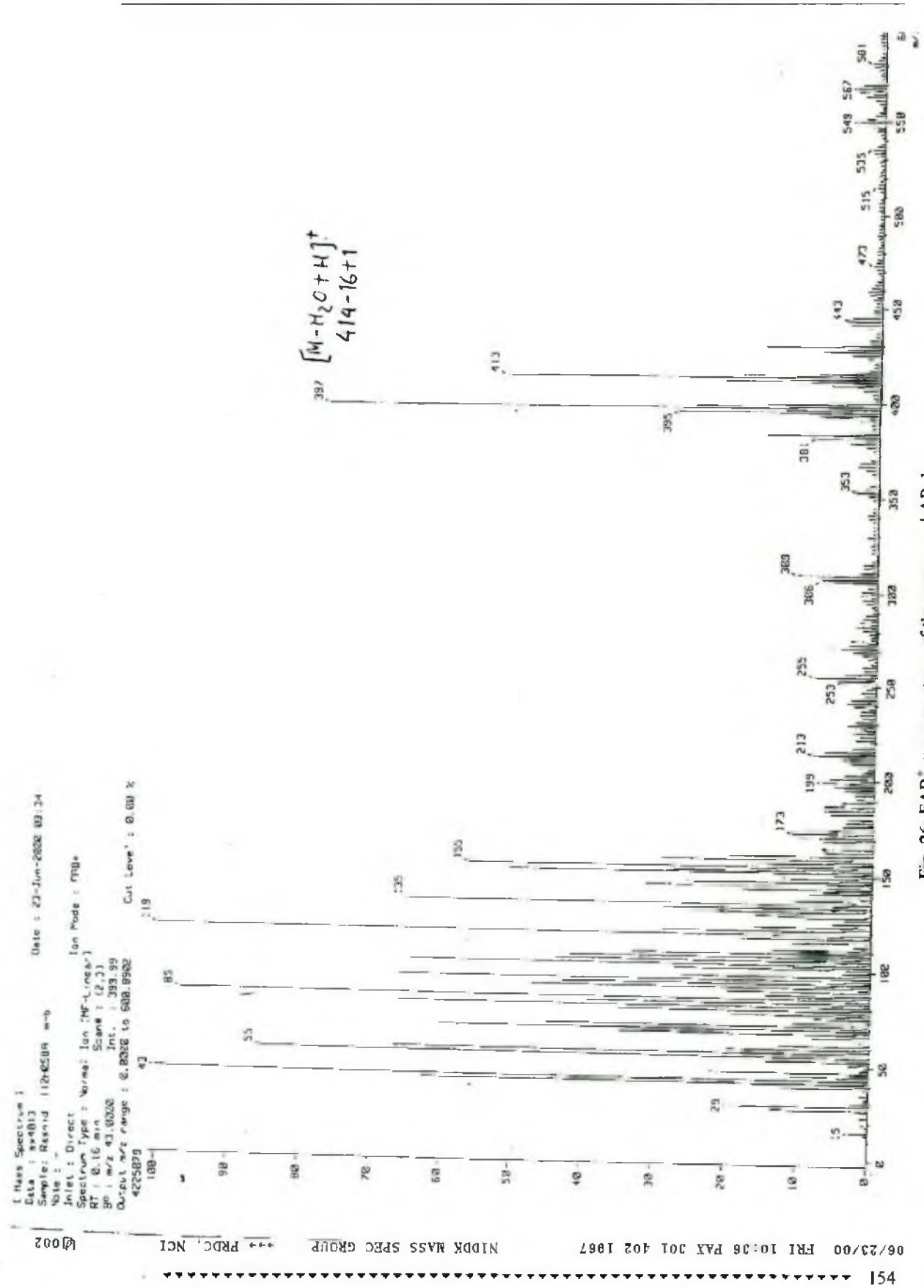
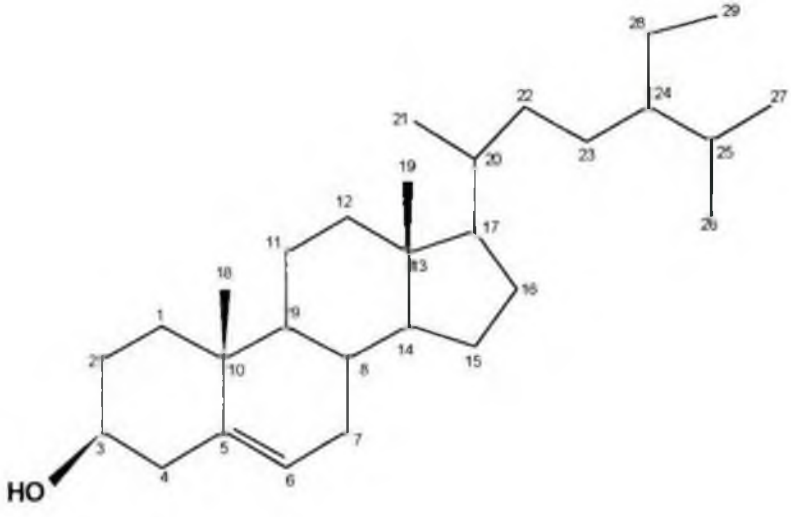



Fig. 26 FAB$^+$ mass spectrum of the compound AR-1

### 3.17.3 Structure of compound AR-1:

On the basis of physical characteristics and spectroscopic data it was found that the compounds AR-1 was a steroid. The $^{1}$H-, $^{13}$C-NMR spectral data were compared with the reported[83] $^{1}$H-, $^{13}$C-NMR spectral data of steroid. From the spectroscopic analysis and comparison it was found that the isolated compound AR-1 was confirmed as $\beta$-sitosterol.

The structure of $\beta$-sitosterol (AR-1) is given below.




$\beta$-Sitosterol (AR-1) (**54**)

Computer literature survey NAtural PRoduct ALART (NAPRALART search in December 1998) and Dictionary of Natural Product on CD-ROM (Chapman & Hall, release 6:2) of *Lagerstroemia speciosa* **Linn.** confirmed that $\beta$-sitosterol has not been reported earlier from this plant.

### 3.18 Purification of fraction $LSBEDA_4$:

The fraction $LSBEDA_4$ gave a single spot with tailing in TLC. The fraction was washed repeatedly with hexane and dichloromethane to remove associated impurities. After washing, a white residue was obtained. The white residue was crystallized from the mixture of solvent dichloromethane: methanol (4:1 v/v). The crystalline solid was assigned as **AR-2** (10.4 mg).

### 3.19 Characterization of the compound AR-2

#### 3.19.1 Physical characteristics of the compound AR-2:

The compound AR-2 was a white crystalline compound having **melting point 296-297$^0$C**. It was soluble in dimethylsulfoxide (DMSO). It gave a single spot on TLC with **$R_f$ value 0.65** using a mixture of methanol in dichloromethane (1: 4 v/v).

#### 3.19.2 Structure elucidation of the compound AR-2 by spectroscopic methods:

#### (a) $^1$H-NMR (DMSO, 400 MHz) spectroscopy:

In the **$^1$H-NMR spectrum** of the compound AR-2 **(Fig. 27, 27a)** had signal at 7.5 ppm was assigned to the proton attached to C-1 carbon (*s*, 1H, aromatic proton at C-1) and the other signal at 7.6 ppm was assigned to proton attached at C-6 carbon (*s*, aromatic proton at C-6). There was no signal for hydroxyl proton attached to C-7. The three sharp signals were at 4.05 (*s*, 3H, -$OCH_3$ at C-8), 4.03 (*s*, 3H, -$OCH_3$ at C-3) and 3.99 (*s*, 3H, -$OCH_3$ at C-2) indicated the presence of three oxymethyl groups in the compound AR-2. The $^1$H-NMR data are given in **Table 31**.

**Table 31: $^1$H-NMR data of the compound AR-2 (Pyridine, 400 MHz)**

| Chemical shift (ppm) | Splitting pattern | Integration | Assignment |
|---|---|---|---|
| 7.5 | Singlet | 1H | One aromatic proton at C-1 |
| 7.6 | Singlet | 1H | One aromatic proton at C-6 |
| 4.05 | Singlet | 3H | Three -$OCH_3$ protons at C-8 |
| 4.03 | Singlet | 3H | Three -$OCH_3$ protons at C-3 |
| 3.99 | Singlet | 3H | Three -$OCH_3$ protons at C-2 |

**(b) FAB$^+$ Mass spectroscopy:**

The **fast atom bombardment mass spectrum (FAB$^+$MS) (Fig. 28, 28a, 28b)** showed the pseudo molecular ion peak at *m/z* 345 for $[M+H]^+$, thus establishing a molecular formula $C_{17}H_{12}O_8$ and other intense peaks at *m/z* 306, 235, 157, 79, 63, 47.

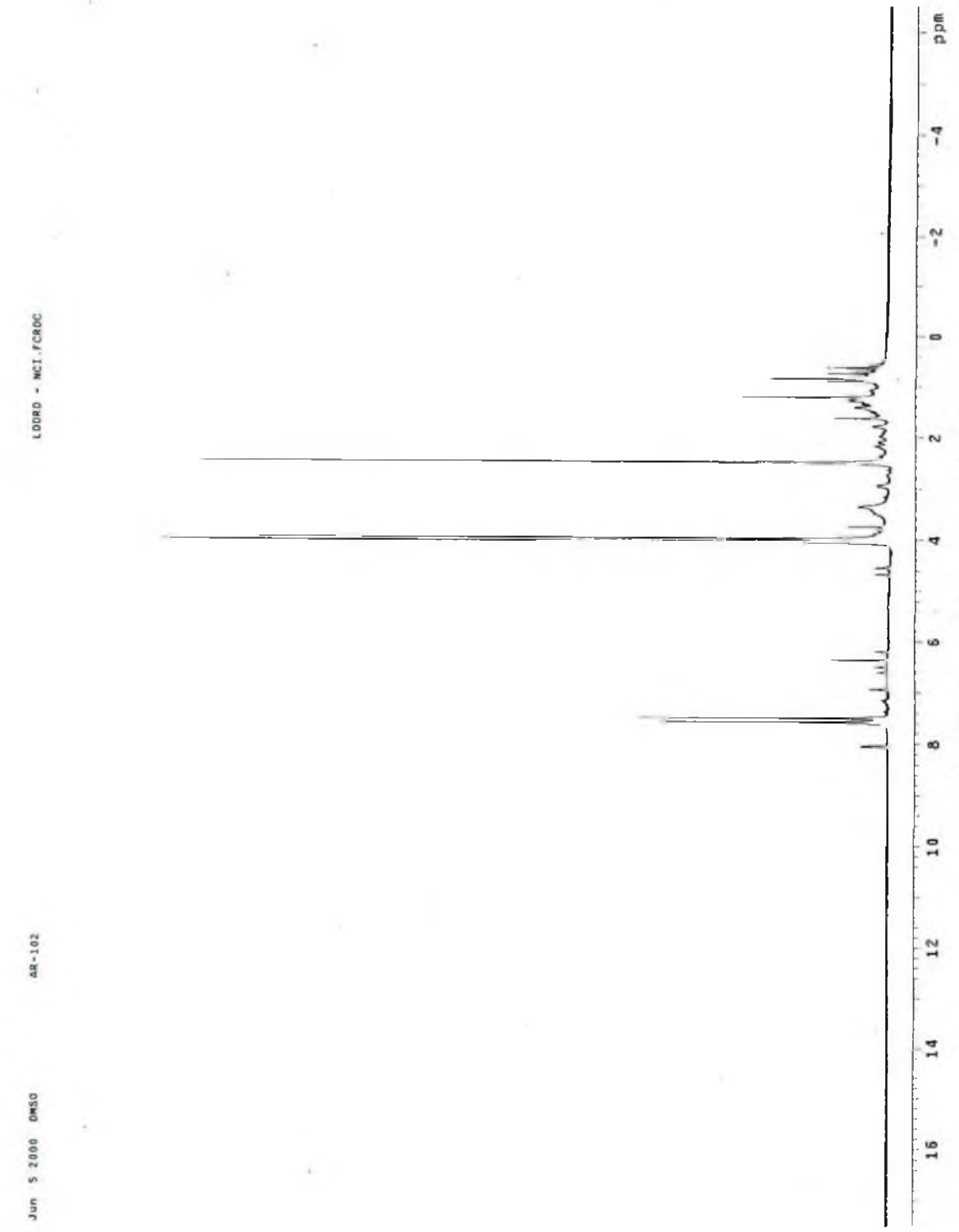



Fig. 27: $^1$H-NMR spectrum of the compound AR-2

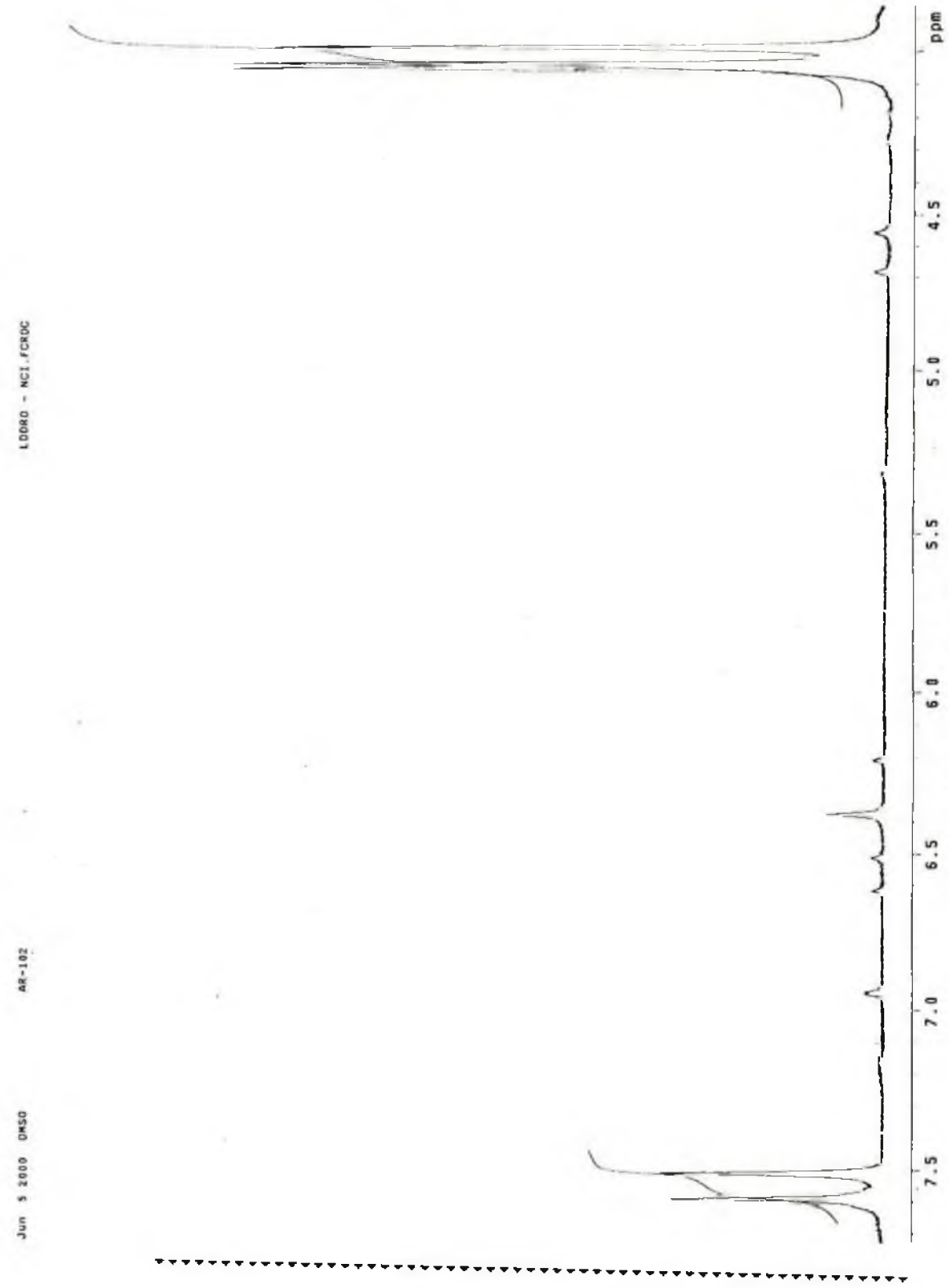



Fig. 27a: $^{1}$H-NMR spectrum of the compound AR-2

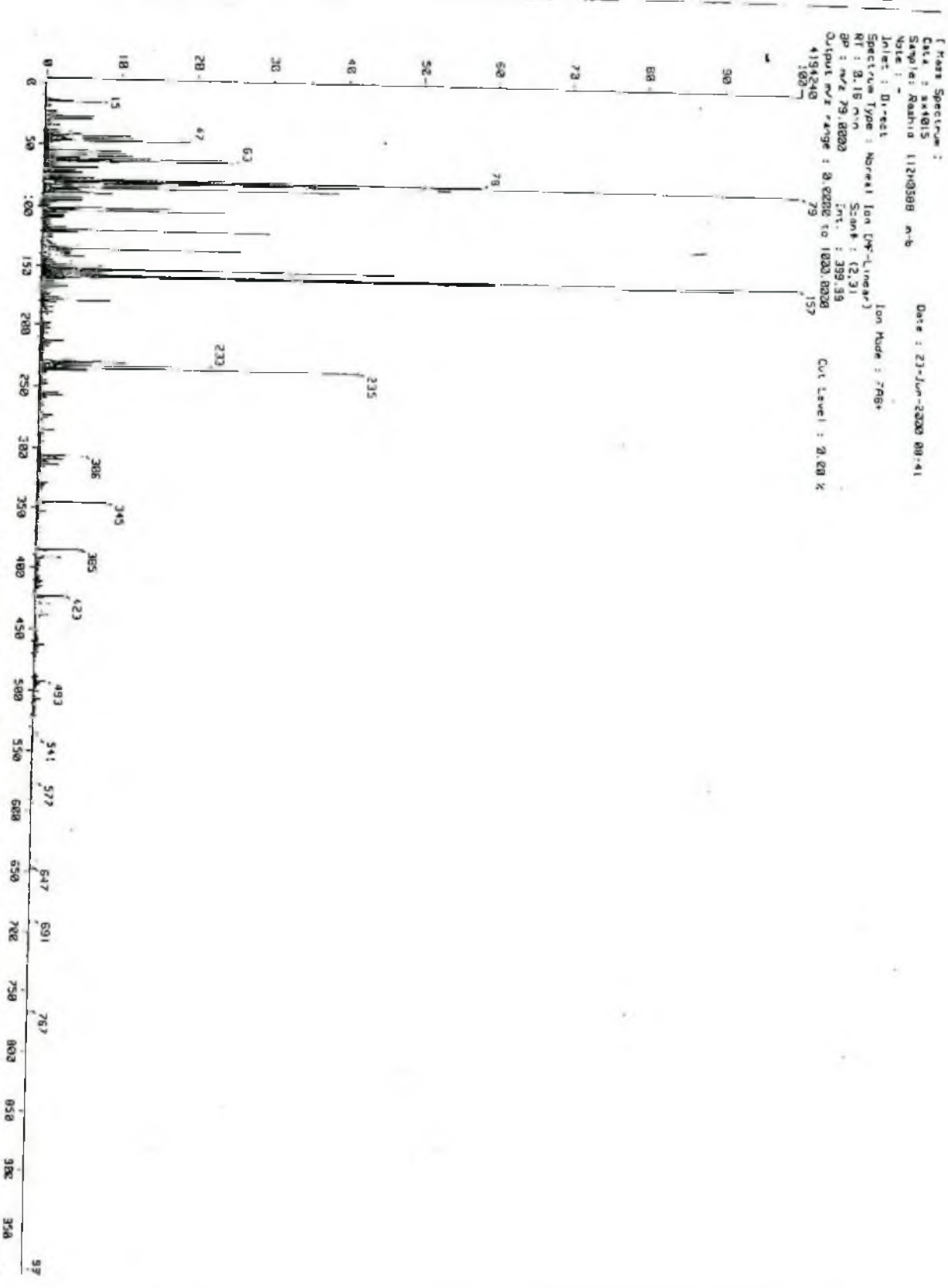



Fig. 28: FAB$^+$ mass spectrum of the compound AR-2

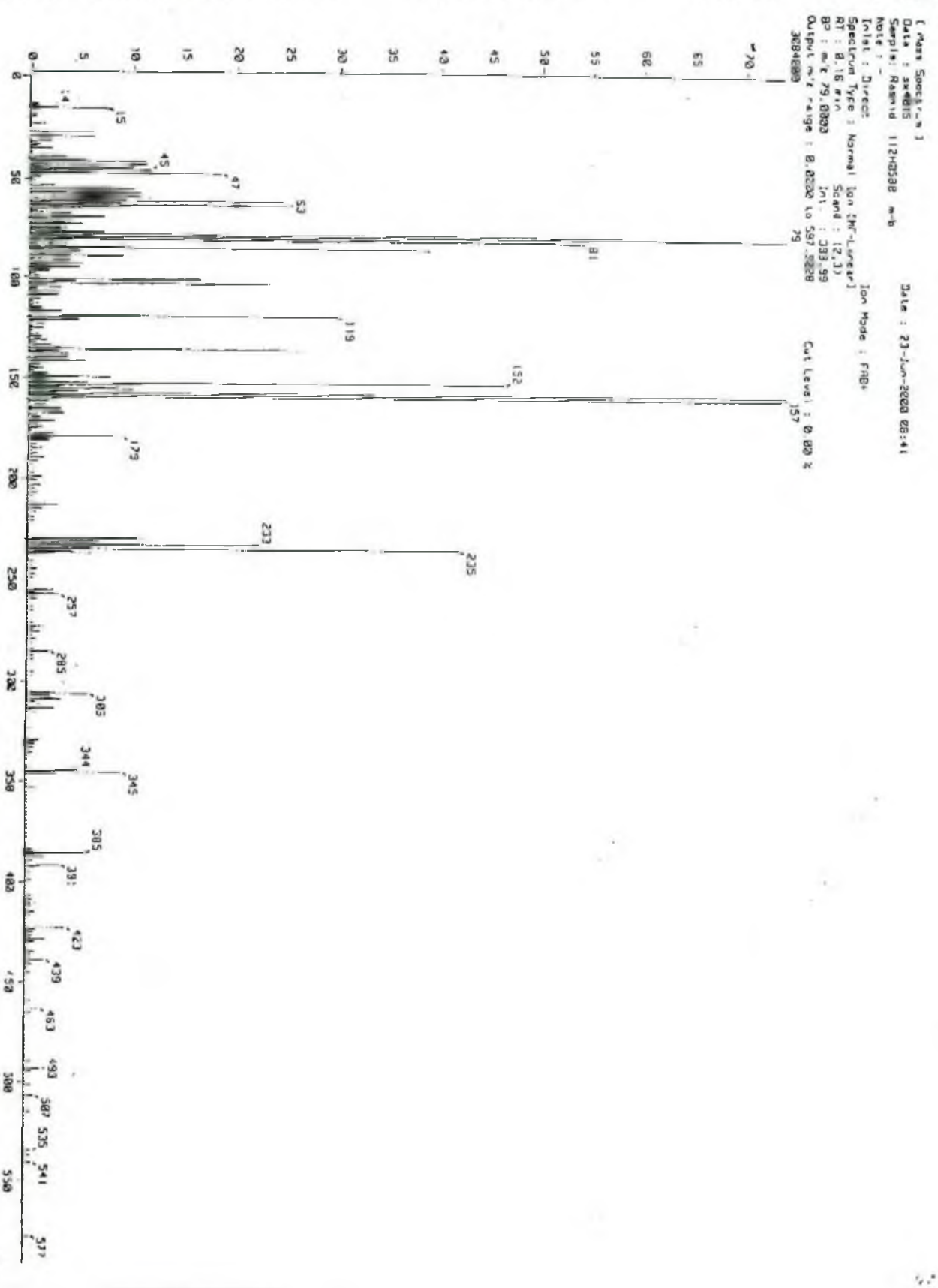
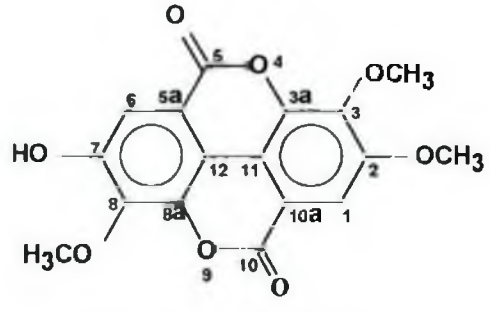



Fig. 28a: FAB$^+$ mass spectrum of the compound AR-2

### 3.19.3 Structure of the compound AR-2:

On the basis of physical characteristics and spectroscopic data it was revealed that the compound AR-2 was an ellagic acid derivative. The $^{1}$H- and $^{13}$C-NMR data were compared with reported $^{1}$H- and $^{13}$C-NMR data of ellagic acid derivatives. From the spectroscopic analysis and comparison it was confirmed that the isolated pure compound AR-2 was 2,3,8-Tri-*O*-methylellagic acid.

The structure of 2,3,8-Tri-*O*-methylellagic acid (AR-2) is given below:




2,3,8-Tri-*O*-methylellagic acid (AR-2) (**53**)

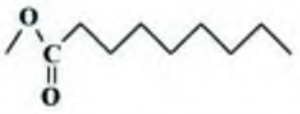
Computer literature survey NAtural PRoduct ALART (NAPRALART search in December 1998) and Dictionary of Natural Product on CD-ROM (Chapman & Hall, release 6:2) of *Lagerstroemia speciosa* **Linn.** confirmed that 2,3,8-Tri-*O*-methylellagic acid is a new compound but it was not been reported earlier from this plant.

### 3.20 Identification and quantification of fatty acids:

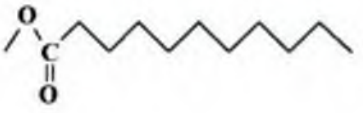
Sixteen fatty acids from flowers, ten fatty acids from leaves and nine fatty acids from bark were identified and quantified from the hexane and dichloromethane extracts of *Lagerstroemia speciosa* **L** (described in **section 2.5.25)**. At first, the hexane extract of flowers was saponified with methanolic sodium hydroxide to isolate the fatty acids. The fatty acids were then, esterified by treating with borontrifluoride-methanol complex (described in **section 2.5.25.2**). The individual fatty acid esters were identified by using gas chromatography (GC) and Mass spectrometry. The components were also compared with published spectra of NIST 107 Library (Shimadzu Corporation). Sixteen fatty acids namely nonanoic acid, undecanoic acid, lauric acid, myristic acid, 4-octadecenoic acid, palmitic acid, 15-methyl hexadecanoic acid, linoleic acid, 11, 14, 17-ecosatrienoic acid, 11-octadecenoic acid, stearic acid, oleic acid, ecosanoic acid, behenic acid, tetracosanoic acid, hexacosanoic acids as methylesters from flowers (described in **Table 23)**. Similarly, nine fatty acids namely lauric acid, myristic acid, palmotelic acid, palmitic acid, linoleic acid, stearic acid, oleic acid, arachidic acid, behenic acids as methylesters from leaves (described in **Table 24)** and nine fatty acids namely myristic acid, palmotelic acid, palmitic acid, linoleic acid, stearic acid, oleic acid, arachidic acid, behenic acids as methylesters from bark (described in **Table 25**) were identified and quantified.

The identification and quantification of fatty acids from this plant is reported here for the first time.

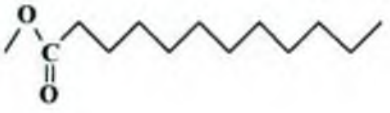
The structure of the identified and quantified fatty acid esters of *Lagerstroemia speciosa* **Linn.** are given below:



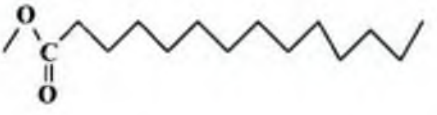
**Nonanoic acid, methyl ester**
$C_{10}H_{20}O_2$



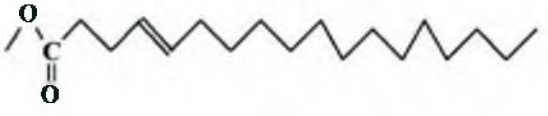
**Undecanoic acid, methyl ester**
$C_{12}H_{24}O_2$



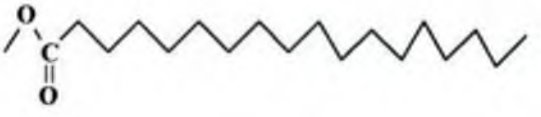
**Lauric acid, methyl ester**
$C_{13}H_{28}O_2$



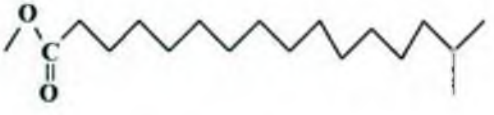
**Tetradecanoic acid, methyl ester**
$C_{15}H_{30}O_2$



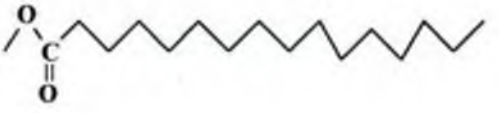
**Octadecenoic acid, methyl ester**
$C_{19}H_{36}O_2$



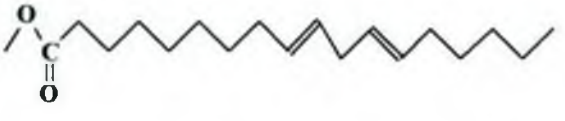
**Stearic acid, methyl ester**
$C_{19}H_{38}O_2$



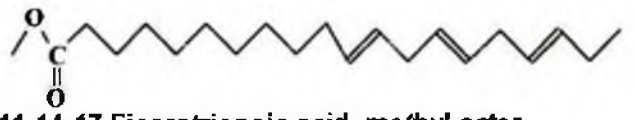
**Hexadecanoic acid, 14-methyl-, methyl ester**
$C_{18}H_{36}O_2$



**Palmitic acid, methyl ester**
$C_{17}H_{34}O_2$

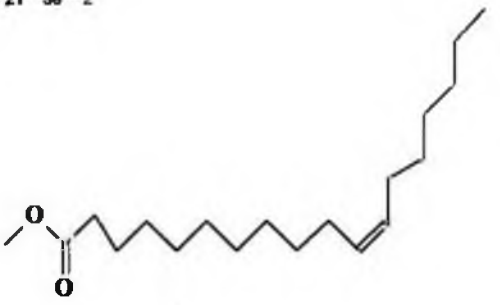


**9,12-Octadecadecadienoic acid (z, z)-, methyl ester**
**Linoleic acid, methyl ester**



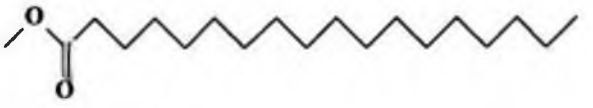
11,14,17-Eicosatrienoic acid, methyl ester

$C_{21}H_{36}O_2$



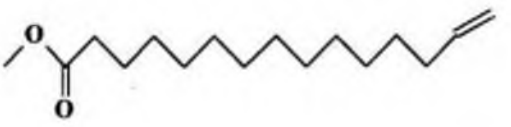
11-Octadecenoic acid, methyl ester

$C_{19}H_{36}O_2$



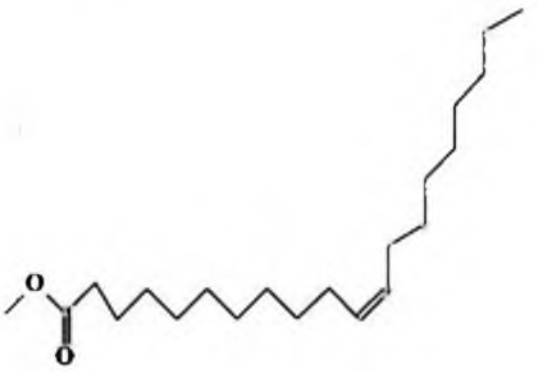
Stearic acid, methyl ester

$C_{19}H_{38}O_2$



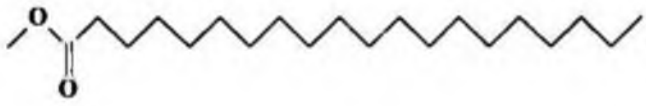
14-Pentadecynoic acid, methyl ester

$C_{16}H_{28}O_2$

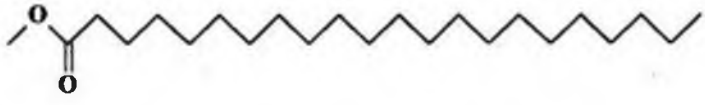


Oleic acid, methyl ester

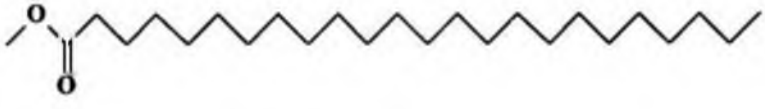
$C_{19}H_{36}O_2$



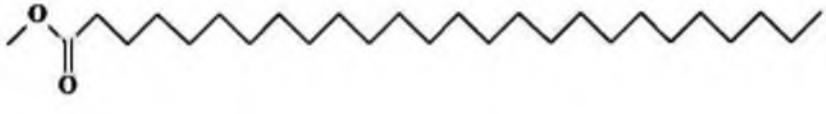
**Eicosanoic acid, methyl ester**
$C_{21}H_{42}O_2$



**Behenic acid, methyl ester**
$C_{23}H_{46}O_2$



**Tetracosanoic acid, methyl ester**
$C_{25}H_{50}O_2$



**Hexacosanoic acid, methyi ester**
$C_{27}H_{54}O_2$

## 3.21 BIOLOGICAL STUDIES

### 3.21.1 Brine shrimp lethality assay of dichloromethane, methanol soluble parts of leaves, methanol, acetone soluble parts of bark and hexane soluble part of flowers and pure compounds LSM-4, AR-2, and AR-7:

Brine shrimp lethality bioassay is a recent development[80, 81] to assay the bioactivity of extracts and compounds. Bioactive compounds are almost always toxic at higher dose. This bioassay has several advantages and superior to other cytotoxicity studying procedures such as it is rapid in process, inexpensive and simple. Brine Shrimp Lethality Assay of dichloromethane, methanol extracts of leaves, methanol, and acetone soluble parts of bark and hexane extracts of flowers of *Lagerstroemia speciosa* were performed by following the National Cancer Institute-Frederick Cancer Research and Development Center (NCI-FCRDC) protocol. Young shrimps (24-48 h) mortality by plant extracts are considered a measure of toxicity of the extracts. Our research group following the same protocol as used by NCI has also adopted tabletop assay for brine shrimp mortality.

Dichloromethane, methanol extracts of leaves, methanol, acetone soluble parts of bark, hexane extracts of flowers and pure compounds LSM-4, AR-2 and AR-7 isolated from *Lagerstroemia speciosa* were tested for brine shrimp mortality at top dose of 1 mg/ml with 10 shrimps. Shrimps were found dead after 24 h and 48 h. More than 5 shrimps dead after 24 h are considered for determination of $LD_{50}$ of the extract by making serial dilution of the extract followed by mortality tests (statistical calculation by NCI soft-ware calculation available at organic research laboratory, DU). The dichloromethane, methanol soluble parts of leaves, methanol, acetone soluble parts of bark were toxic at the top dose and maximum exposure (48 h, **Table 7, Table 8, Table 9, Table 10**) and hexane extract of flowers was nontoxic even at top dose and maximum exposure (48 h, **Table 11**). Therefore, serial dilution experiment was not performed.

The mortality of the shrimps and $\mathbf{LD_{50}}$ of isolated three pure compounds are given in **Tables 32-34**.

**Table 32: Brine shrimp mortality of pure compound LSM-4 (Colosolic acid)**

| Amount of LSME-4 (in mg) | Mortality of shrimp out of 10 | | $LD_{50}$ at 24 h (mg/ml)$\times 10^{-4}$ | $LD_{50}$ at 48 h (mg/ml) $\times 10^{-4}$ |
|---|---|---|---|---|
| | After 24 h | After 48 h | | |
| 1.25 | 3 | 6 | 7.2 | 2.2 |
| 0.5 | 2 | 5 | | |
| 0.25 | 0 | 2 | | |
| Control | 0 | 0 | | |

**Table 33: Brine shrimp mortality of pure compound AR-2 (2,3,8-Tri-*O*-methylellagic acid)**

| Amount of AR-2 (in mg) | Mortality of shrimp out of 10 | | $LD_{50}$ at 24 h (mg/ml) $\times 10^{-4}$ | $LD_{50}$ at 48 h (mg/ml) $\times 10^{-4}$ |
|---|---|---|---|---|
| | After 24 h | After 48 h | | |
| 1.25 | 4 | 7 | 7.5 | 1.9 |
| 0.5 | 2 | 3 | | |
| 0.25 | 1 | 2 | | |
| Control | 0 | 0 | | |

**Table 34: Brine shrimp mortality of pure compound AR-7 (2,3,7-Tri-*O*-methyl ellagic acid)**

| Amount of AR-7 (in mg) | Mortality of shrimp out of 10 | | $LD_{50}$ at 24 h (mg/ml) $\times 10^{-4}$ | $LD_{50}$ at 48 h (mg/ml) $\times 10^{-4}$ |
|---|---|---|---|---|
| | After 24 h | After 48 h | | |
| 1.25 | 5 | 6 | 9.3 | 1.2 |
| 0.5 | 3 | 3 | | |
| 0.25 | 1 | 1 | | |
| Control | 0 | 0 | | |

# CHAPTER FOUR: SUMMARY

## Chapter Four

## SUMMARY

*Lagerstroemia speciosa* is one of the important medicinal plants of Lythraceae family and was selected for present research work. Its local name is Jarul. Different parts of the plant are used for the treatment of various diseases such as stimulant, hydragogue, drastic, purgative, febrifuge, diabetes management, small ulcer, astringent, narcotic, anti-inflammatory and diuretic etc. Leaves, bark and flowers of *Lagerstroemia speciosa* **Linn.** were collected from the campus area of the University of Dhaka. Voucher specimens of the plant were submitted to the Bangladesh National Herbarium (BNH). The collected bark materials were cleaned, chopped and dried in an oven at $40^0$C and ground to powder for extraction.

The extracts of *Lagerstroemia speciosa* **Linn.** were tested by our research group for their anti-HIV and antidiabetic activities. The results showed that the extracts had significant anti-HIV (56.5% protection, $EC_{50}$ was 2.50 μg/ml) (**Fig 4, 5**) and antidiabetic activities.

For chemical work, the powdered of leaves (3.5 Kg) of *L. speciosa* **Linn.** were first extracted with dichloromethane and the residue was extracted with methanol and 184 g (5.26%) of dichloromethane (LSLD) and 122 g (3.49%) of methanol extracts (LSLDM) were obtained.

The bark ground (3.0 kg) of *L. speciosa* **Linn.** was extracted with aqueous 80% ethanol (6 × 4 L) and 87.0 g (2.9%) of ethanol extract (LSBE) was obtained. The ethanol extract was partitioned between dichloromethane and water. After partition, 16.3 g (0.49%) of dichloromethane (LSBED) and 72.0 g (2.18%) of water (LSBEW) soluble parts were obtained.

Dichloromethane, methanol extracts of leaves and methanol, acetone soluble parts of bark and hexane extract of *L. speciosa* **Linn.** were tested for brine shrimp lethality assay. Brine shrimp lethality assay of the above-mentioned extracts and fractions of *L. speciosa* **Linn.** indicated that the extracts and fractions were toxic even at low dose *except* hexane extract of flowers.

The $LD_{50}$ of dichloromethane and methanol extracts of leaves were $4.0\times10^{-4}$ (at 24 h, in mg/ml), $2.7\times10^{-4}$ (at 48 h, in mg/ml) and $3\times10^{-4}$ (at 24 h, in mg/ml) and $1.7\times10^{-4}$ (at 48 h, in mg/ml), respectively and that of methanol and acetone extracts of bark were $4.0\times10^{-4}$ (at 24 h, in mg/ml), $2.2\times10^{-4}$ (at 48 h, in mg/ml) and $3.4\times10^{-4}$ (at 24 h, in mg/ml), $2.2\times10^{-4}$ (at 48 h, in mg/ml), respectively and that of hexane extract was zero.

The $LD_{50}$ of Colosolic acid (LSM-4), 2,3,8-Tri-O-methylellagic acid (AR-2), 2,3,7-Tri-O-methylellagic acid (AR-7) were $7.2\times10^{-4}$ (at 24 h, in mg/ml), $2.2\times10^{-4}$ (at 48 h, in mg/ml), $7.5\times10^{-4}$ (at 24 h, in mg/ml), $1.9\times10^{-4}$ (at 48 h, in mg/ml), $9.3\times10^{-4}$ (at 24 h, in mg/ml), $1.2\times10^{-4}$ (at 48 h, in mg/ml), respectively.

Anti-HIV test of the dichloromethane and methanol extracts of bark of *L. speciosa* **Linn.** were performed at National Cancer Institute-Frederick Cancer Research and Development Center (NCI-FCRDC), Maryland, USA. Both the fractions showed significant protection (>50%) of the HIV infected cells in comparison with normal or unaffected cells.

The methanol extract of bark contained more polar compounds, before performing chromatographic separation the extract was resolubilized with 10% methanol in ethyl acetate to achieve partial fractionation of the methanol extract. The 10% methanol in ethyl acetate soluble fraction was further fractionated by Sephadex LH-20 gel column chromatography. Two fractions were obtained such as (i) chlorophyll containing fraction and (ii) chlorophyll free fraction. Charcoal treatment was carried out to remove chlorophyll and ran $^1$H-NMR (60 MHz) of both the fractions. Both the fractions were combined after evaluating $^1$H-NMR spectra.

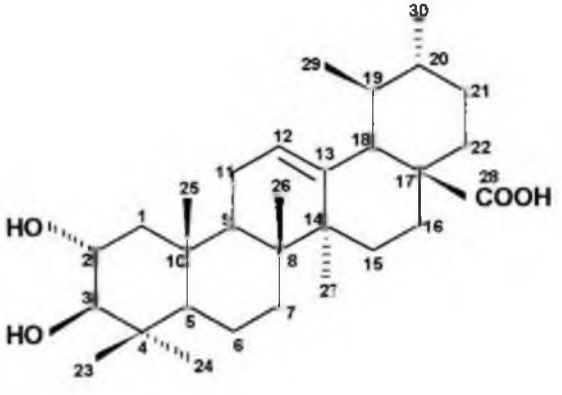
The chlorophyll free 10% methanol in ethyl acetate soluble fraction (LSLME, 5.1 g) was fractionated by silica gel column chromatography and five fractions were collected on the basis of their $R_f$ values.

The fraction $LSLMEF_2$ appeared as a single spot in TLC with $R_f$ value 0.6 in 2% methanol in dichloromethane as mobile phase. The fraction was purified by washing repeatedly with hexane and dichloromethane. After washing, the white residue was obtained and assigned as LSM-4 (23.6 mg). The compound LSM-4 was a crystalline solid having a melting point 243-245$^0$C.

To characterize each of the compounds, physical and chemical properties as well as spectral data were fully studies. Physical form, color solubility and $R_f$ were observed among various physical properties. Among spectroscopic technique, $^1$H-NMR, $^{13}$C-NMR, DEPT, mass analysis were carried out for structural elucidation.

The structure of the compound LSM-4 was elucidated from its physical characteristics as well as various spectroscopic methods. It was found that the compound LSM-4 had one olefinic double bond, two hydroxyl groups, one carboxylic group, and seven methyl groups.

On the basis of physical characteristics and spectroscopic data it was found that the compounds LSM-4 was a terpenoid. The $^1$H-NMR spectral data were compared with the reported[48] $^1$H-NMR spectral data of terpenoids. From the spectroscopic analysis and comparison it was confirmed that the isolated pure compound LSM-4 was 2,3-dihydroxy-12-oleanen-28-oic acid (Colosolic acid) that has the following structure.



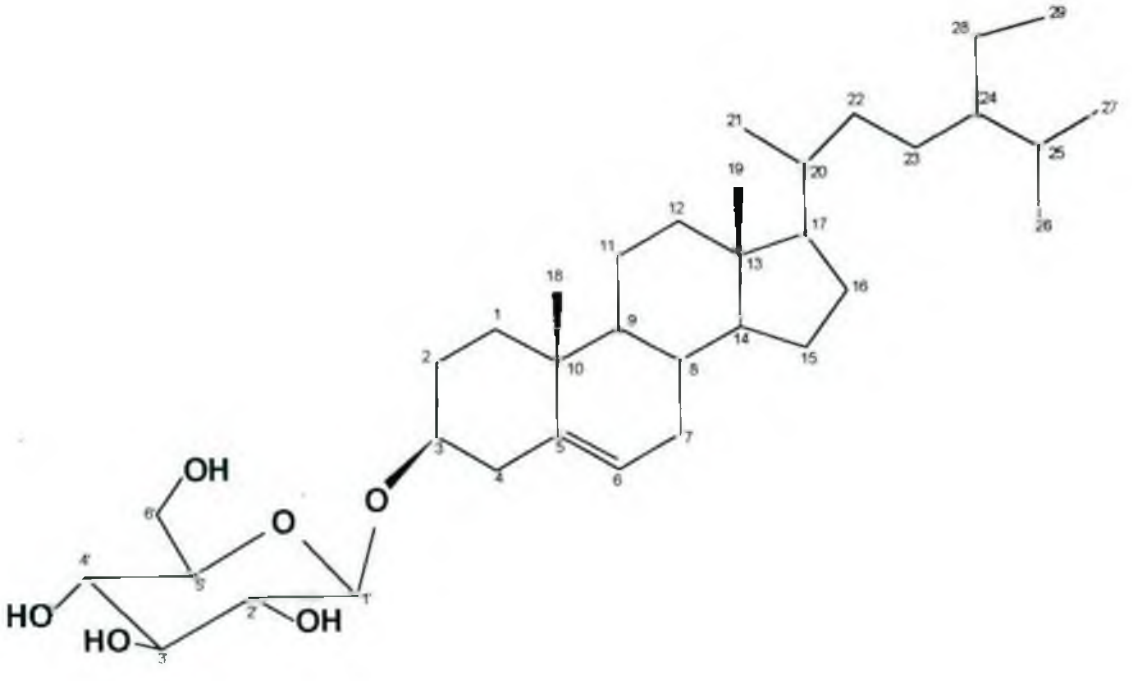

2,3-Dihydroxy-12-ursen-28-oic acid,
Colosolic Acid (LSM-4) (**51**)

The fraction $LSLMEF_4$ appeared as a single spot in TLC with $R_f$ value 0.3 in 10% methanol in dichloromethane (1: 10 v/v) using as mobile phase. The fraction was purified by washing repeatedly with hexane and dichloromethane. After washing, the white residue was obtained and assigned as AR-3 (9.3 mg). The compound AR-3 was a crystalline solid having melting point at 283-285$^0$C.

The structure of the compound AR-3 was elucidated from its physical characteristics as well as various spectroscopic methods. It was found that the compound AR-3 had one olefmic double bond, six methyl groups and a glucose unit.

On the basis of physical characteristics and spectroscopic data it was found that the compound AR-3 was a steroid glucoside. The $^1$H-, $^{13}$C-NMR spectral data were compared with the reported $^1$H-, $^{13}$C-NMR spectral data of steroid glucoside. From the spectroscopic analysis and comparison it was found that the isolated compound AR-3 was sitosteryl-3-*O*-*β*-D-glucoside.

The structure of sitosteryl-3-*O*-*β*-D-glucoside (AR-3) is given below:




Sitosteryl-3-O-β-D-glucoside AR-3 (**55**)

Computer literature survey NAtural Product ALART (NAPALART search in December 1998) and Dictionary of Natural Product on CD-ROM (Chapman & Hall, release 6:2) of *Lagerstroemia speciosa* L confirmed that sitosteryl-3-*O*-*β*-D-glucoside although a known compound but it was not been reported earlier from the plant.

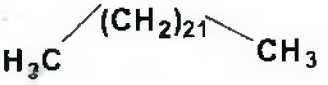
The dichloromethane soluble part (LSLD, 130.0 g) of leaves was fractionated by column chromatography and six fractions were obtained. The fraction number three was designed as $LSLDF_3$.

The fraction was ($LSLDF_3$, 9 g) was further fractionated by silica gel column chromatography and seven fractions were obtained. The fraction number three was designed as $LSLDC_2F_3$.

The subfraction ($LSLDC_2F_3$, 4.0 g) was further fractionated by silica gel column chromatography and five different fractions were obtained by pooling different fractions on the basis of their $R_f$ values.

The fraction $LSLDC_3F_{39}$ gave a single spot with tailing in TLC. The fraction was washed with methanol repeatedly to remove associated impurities. After washing, a white solid was obtained and designed as JF-39 (8 mg). The melting point of the compound JF-39 was $67^0$C.

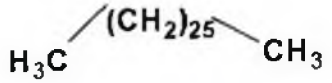
The structure of the compound JF-39 was given by evaluating the IR, mass spectrum and retention time of the gas chromatograph as n-Tricosane, which was isolated for the first time from this plant.




n-Tricosane, JF-39 (**56**)

The fraction $LSLDC_3F_{43}$ gave a single spot with tailing in TLC. The fraction was washed with methanol repeatedly to remove associated impurities. After washing, a white solid was obtained and designed as JF-43 (4 mg).

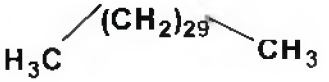
The structure of the compound JF-43 was given as n-Heptacosane by evaluating the mass spectrum and retention time of the gas chromatograph, which was isolated for the first time from this plant.



n-Heptacosane, JF-43 (**57**)

The fraction $LSLDC_3F_{53}$ gave a single spot with tailing in TLC. The fraction was washed with methanol repeatedly to remove associated impurities. After washing, a white solid was obtained and designed as JF-53 (57 mg).

The structure of the compound JF-53 was given by evaluating mass spectrum and retention time of the gas chromatograph as n-Hentriacontane, which was isolated for the first time from this plant.



n-Hentriacontane, JF-53 (**58**)

Dichloromethane extract of *L. speciosa* of bark (LSBD, described in section 2.2.3.4) which showed significant anti-HIV activity (described in section 2.4.1) was taken for isolation of pure bioactive compound(s).

The dichloromethane soluble part of bark (LSBED, 16.3 g) of *L. speciosa* was fractionated by a Diion HP-20 gel column and eluted with acetone, methanol and methanol: water (1:1). The eluents were evaporated to dryness and three fractions were

obtained such as (i) 2.5 g of acetone soluble part (LSBEDA), (ii) 2.4 g of methanol soluble part (LSBEDM) and (iii) 4.6 g of methanol-water soluble part (LSBEDMW).

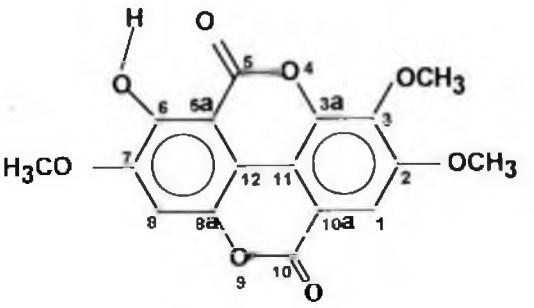
The dried extract of methanol soluble part (LSBEDM, 2.4 g) was fractionated by column chromatography by using silica gel as adsorbent and eluted with dichloromethane, ethyl acetate and methanol with increasing polarity and six fractions were obtained. After washing the fraction $LSBEDM_4$ with hexane a pure crystalline compound AR-7 was obtained (15 mg).

The compound AR-7 gave a round single spot in TLC with $R_f$ value 0.68 using methanol in dichloromethane (1: 4; v/v) as mobile phase and melting point was 296-298$^0$C.

The structure of the compound AR-7 was elucidated from its physical characteristic as well as spectroscopic methods. It was found that the compound had three oxymethyl groups, one hydroxyl group, two carbonyl groups two aromatic protons.

On the basis of physical characteristics and spectroscopic data it was concluded that the compound AR-7 was 2,3,7-Tri-*O*-methylellagic acid.

The structure of 2,3,7-Tri-*O*-methylellagic acid (AR-7) is given below.




2,3,7-Tri-O-methylellagic acid (AR-7) **(52)**

Computer literature survey NAtural PRoduct ALART (NAPRALART search in December 1998) and Dictionary of Natural Product on CD-ROM (Chapman & Hall, release 6:2) of *Lagerstroemia speciosa* L confirmed that 2,3,7-Tri-*O*-methylellagic acid is a new compound but it has not been reported earlier from the plant sources.

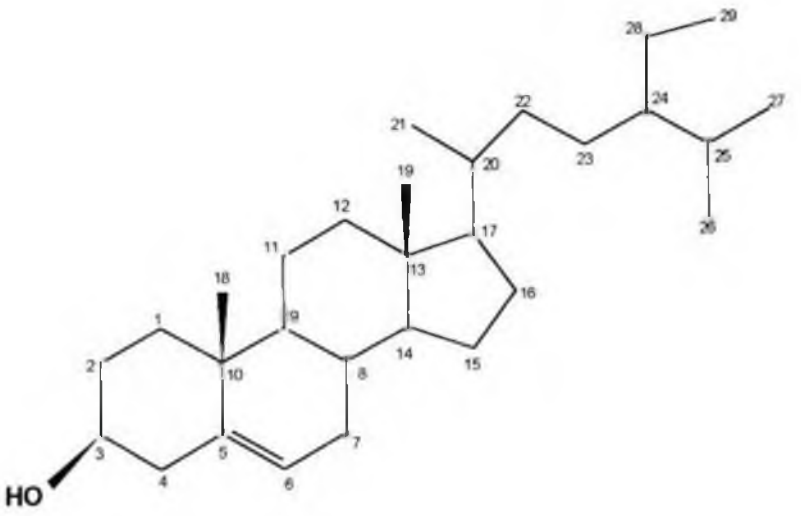
The dried extract of acetone soluble part (LSBEDA, 2 5 g) was fractionated by column chromatography by using silica gel as adsorbent and eluted with dichloromethane, ethyl acetate and methanol with increasing polarity.

The fraction $LSBEDA_2$ appeared as a single spot in TLC with $R_f$ value 0.60 in dichloromethane using as mobile phase. The fraction was purified by washing repeatedly with hexane. After washing, the white residue was recrystallized from a mixture of hexane and dichloromethane (4: 1). Very nice needle like crystals were obtained and assigned as AR-1 (63 mg). The compound AR-1 was a needle like crystal having melting point at 138-139$^0$C. It gave a positive test of steroid. Thus the compound AR-1 was suspected to be a steroid.

The structure of the compound AR-1 was elucidated from its physical characteristics as well as various spectroscopic methods. It was found that the compound AR-1 had one olefinic double bond, two hydroxy groups, six methyl groups.

On the basis of physical characteristics and spectroscopic data it was found that the compounds AR-1 was a steroid. The $^1$H-, $^{13}$C-NMR spectral data were compared with the reported $^1$H-, $^{13}$C-NMR spectral data of steroid. From the spectroscopic analysis and comparison with the reported value it was confirmed that the isolated compound AR-1 was $\beta$-sitosterol.

The structure of $\beta$-sitosterol (AR-1) is given below:




β–Sitosterol (AR-1) (**54**)

Computer literature survey NAtural PRoduct ALART (NAPRALART search in December 1998) and Dictionary of Natural Product on CD-ROM (Chapman & Hall, release 6:2) of *Lagerstroemia speciosa* **Linn.** confirmed that $\beta$-sitosterol has not been reported earlier from this plant.

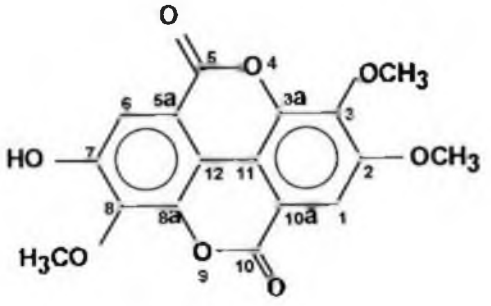
The fraction $LSBEDA_4$ appeared as a single spot in TLC with $R_f$ value 0.65 in methanol in dichloromethane (1: 4 v/v) using as mobile phase. The fraction was purified by repeatedly washing with hexane. After washing, the white solid was crystallized was obtained and named as AR-2 (10.4 mg).

The purified compound AR-2 gave a single spot on TLC spraying with vanillin-sulphuric acid reagent. It was sparingly soluble in methanol but completely soluble in the mixture of chloroform and methanol. It had a melting point of 296-297$^0$C.

The structure of the compound AR-2 was elucidated from its physical characteristic as well as spectroscopic methods. It was found that the compound had three oxymethyl groups, two carbonyl groups two aromatic protons.

On the basis of physical characteristics and spectroscopic data it was revealed that the compound AR-2 was 2,3,8-Tri-*O*-methylellagic acid.

The structure of 2,3,8-Tri-*O*-methylellagic acid (AR-2) is given below:




2,3,8-Tri-*O*-methylellagic acid (AR-2) (**53**)

Computer literature survey NAtural PRoduct ALART (NAPRALART search in December 1998) and Dictionary of Natural Product on CD-ROM (Chapman & Hall, release 6:2) of *Lagerstroemia speciosa* **Linn.** confirmed that 2,3,8-Tri-*O*-methylellagic acid is a new compound but it has not been reported earlier from the plant sources.

More than sixteen fatty acids were isolated from plant materials of *Lagerstroemia speciosa* **Linn.** (flowers, leaves and bark). The individual fatty acids were identified and quantified as their methyl esters by GLC/ GC-MS.

Sixteen fatty acids from flowers, ten fatty acids from leaves and nine fatty acids from bark were identified and quantified from the hexane and dichloromethane extracts of *Lagerstroemia speciosa* **Linn.** (described in **section 2.5.25**). At first, the hexane extract of flowers was saponified with methanolic sodium hydroxide to isolate the fatty acids. The fatty acids were then, esterified by treating with borontrifluoride-methanol complex (described in **section 2.5.25.2**). The individual fatty acid esters were identified by using gas chromatography (GC) and Mass spectrometry. The components were also compared with published spectra of NIST 107 Library (Shimadzu Corporation). Sixteen fatty acids namely nonanoic acid, undecanoic acid, lauric acid, myristic acid, 4-octadecenoic acid, palmitic acid, 15-methyl hexadecanoic acid, linoleic acid, 11, 14, 17-ecosatrienoic acid, 11-octadecenoic acid, stearic acid, oleic acid, ecosanoic acid, behenic acid, tetracosanoic acid, hexacosanoic acids as methylesters from flowers (described in **Table 23)**. Similarly, nini fatty acids namely lauric acid, myristic acid, palmotelic acid, palmitic acid, linoleic acid, stearic acid, oleic acid, arachidic acid, behenic acids as methylesters from leaves (described in **Table 24)** and eight fatty acids namely myristic acid, palmotelic acid, palmitic acid, linoleic acid, stearic acid, oleic acid, arachidic acid, behenic acids as methylesters from bark (described in **Table 25**) were identified and quantified.

It was found that palmitic, linolic, stearic, ecosanoic acids were the major quantity of fatty acids found in the extract of flowers. On the other hand, Oleic, palmitic, stearic, arachidic acids of the extract of leaves and palmotic, linoleic, stearic acids were found as the major quantity.

# CHAPTER FIVE: REFERENCES

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## REFERENCES


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