PHYTOCHEMICAL AND BIOLOGICAL INVESTIGATION ON THE LEAVES OF *Saurauia roxburghii*

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTERS OF PHILOSOPHY (M. PHIL.) IN CHEMISTRY

SUBMTTED

BY

YUNUS AHMED

STUDENT NO. : 040803101F

REGISTRATION NO. : 040803101

SESSON: APRIL-2008

ORGANIC RESEARCH LABORATORY

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THESIS ACCEPTANCE LETTER

This thesis titled PHYTOCHEMICAL AND BIOLOGICAL INVESTIGATION ON THE LEAVES OF *Saurauia roxburghii* submitted by Yunus Ahmed, Roll No. 040803101F and Session-April 2008 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Masters of Philosophy (M.Phil) on 08 January 2012.

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Abbreviations and Symbols

CC	Column Chromatography
VLC	Vacuum Liquid Chromatography
TLC	Thin-Layer Chromatography
TMS	Tetra methyl silane
UV/VIS	Ultraviolet/visible Spectroscopy
IR	Infrared Spectroscopy
NMR	Nuclear Magnetic Resonance (Spectroscopy)
^{1}H NMR	Proton Nuclear Magnetic Resonance
^{13}C NMR	Carbon-13 Nuclear Magnetic Resonance
^{1}H-^{1}H COSY	Homonuclear Chemical Shift Correlation Spectroscopy
DEPT	Distortionless Enhancement by Polarization Transfer
NOE	Nuclear-Overhauser-Enhancement
HSQC	Heteronuclear Single Quantum Correlation
HMBC	Heteronuclear Multiple Bond Correlation
HPLC	High Performance Liquid Chromatography
ROESY	Rotating Frame Overhauser Enhancement Spectroscopy
MS	Mass Spectrometry
m/z	mass/charge
δ	Chemical shift
δ_H	Hydrogen NMR Chemical Shift
δ_C	Carbon NMR Chemical Shift
s	Single
d	Doublet
dd	double doublet
br t	Broad triplet
m	Multiplet
J	J-coupling (also called indirect dipole dipole coupling)
ppm	Parts per million
Hz	Hertz
MHz	Mega hertz

λmax	Maximal absorbance wavelength
R_f	Retardation factor/ Retention Factor
R_t	Retention Time
RP	Reversed Phase
Tab.	Table
IC_{50}	The half maximal inhibitory concentration
LC_{50}	The half maximal lethal concentration
CD_3OD	Methanol-d_4
$CHCl_3$	Chloroform
$CDCl_3$	Chloroform- d_4
MeOH/ CH_3OH	Methanol
HCl	Hydrochloric acid
H_2O	Water
ACN	Acetonitrile
CH_2Cl_2 / DCM	Dichloromethane
EA	Ethyl acetate
DMSO	Dimethyl sulphoxide
DCM	Dichloromethane
H_2SO_4	Sulphuric acid
NaCl	Sodium Chloride
KBr	Potassium bromide
VS	Vincristine Sulphate
µL	Micro Liter
µg	Micro gram
mL	Mili-Liter
mm	Mili-meter

ABSTRACT

The work in this thesis details the phytochemical and biological investigations of one species of *Actinidiaceae*, *Saurauia roxburghii*. Previously some works has been reported on this species, but chemical investigation on this species grown in Bangladesh has not been studied extensively. As the plant is being used in our country as a herbal medicine, it is necessary to have a knowledge of the constituents of the plant of our native species.The leaves of *S. roxburghii* was cold extracted with ethanol. The ethanol extract was partitioned into n-hexane, chloroform and ethylacetate and then subjected to column chromatography for fractionation over silica gel, successively with mixtures of n-hexane/dichloromethane, dichloromethane, mixtures of dichloromethane/ methanol and methanol respectively.

The phytochemical investigation of n-hexane extract of *Saurauia roxburghii* led to the isolation of three compounds, compound-1 (**YSR-9**), compound-2 (**YSR-10**) and compound-3 (**YSR-2**) as well as the chloroform extract led to the isolation of other three compounds, compound-4 (**YSR-7**), compound-5 (**YSR-3**) and compound-6 (**YSR-5**) in pure form by repeated column chromatography over silica gel. All the compounds were identified by chemical methods and structure elucidation was done by spectroscopic analysis (UV, FT-IR, ^{1}H & ^{13}C NMR) and comparison of their spectral data with those published in the literature. Compound-1 and 2 were identified as steroids and compound-3 to 6 were identified as triterpenes. Compound-1 to 6 were named as Stigmasterol, *β*-sitosterol, 3*β*-hydroxy-urs-12-en-28-oic acid, 3*β*-hydroxy-olea-12-en, 3*β*-hydroxy-olea-12-en-28-oic acid and 2*α*,3*β*-dihydroxyolea-12-en-28-oic acid respectively. All of these compounds were isolated for the first time so far from this species *Saurauia roxburghii*.

From the brine shrimp lethality bioassay, it was found that the LC_{50} of crude ethanol, n-hexane, chloroform; column fractions H-13 of the crude hexane, C-19 of the crude extract of chloroform and column fractions E-13, E-18 and E-24 of the crude extract of ethanol were found to be 12.59, 14.79, 14.06, 11.75, 10.96,11.88, 4.37 and 6.92 µg/mL respectively. It was evident that all the test samples were very lethal to brine

shrimp nauplii. Column fractions E-18 and E-24 (4.37 and 6.92 µg/mL) of the crude extracts of ethanol showed quite potent activity in brine shrimp lethality bioassay.

For antibacterial activity, the crude ethanol and n-hexane, chloroform, ethyl-acetate extracts were screened against 14 test bacteria at a concentration of 500µg/disc. The extract showed good activity and other extracts showed moderate activity against most of the test Gram-positive and Gram-negative bacteria respectively but n-hexane, chloroform, ethyl-acetate extracts were found to be resistant to the *Bacillus megaterium, Salmonella typhi, Vibrio cholera* and *Bacillus polymyxa* and *Bacillus megaterium, Streptococcus pneumonia, Vibrio cholera* respectively.

The column fraction H-13 of the crude extract of n-hexane, C-15 and C-19 of the crude extract of chloroform and E-18 and E-24 of the crude extract of ethyl-acetate were screened against 8 test bacteria at a concentration of 400µg/disc.. These fractions showed low to mild antibacterial activity.

Fourteen (14) bacterial strains were taken to study the antibacterial activity of the pure compounds; compound-3 (**YSR-2**), compound-5 (**YSR-3**), compound-4 (**YSR-7**) and compound-1 (**YSR-9**) at a concentration of 300µg/disc.These compounds showed moderate activity against all the Gram-positive bacteria *Bacillus cereus, Bacillus megaterium, Bacillus subtilis, Staphylococcus aureus, Streptococcus pneumonia, Bacillus polymyxa, Mycobacterium tuberculosis* and Gram-negative bacteria *E.coli, Klebsiella sp., Proteus sp., Salmonella typhi, Shigella sonnei, Pseudomonas Aureus, Vibrio cholera* but compound-1(**YSR-9**) resistant to *Streptococcus pneumonia, Mycobacterium tuberculosis*; compound-3 (**YSR-2**) resistant to *Proteus sp., Shigella sonnei*; compound-4 (**YSR-7**) resistant to *Salmonella typhi, Pseudomonas Aureus* and compound-5 (**YSR-3**) resistant to *Salmonella typhi*.

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Author

YUNUS AHMED

CHAPTER-1

INTRODUCTION

1.1 General

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, in juries, wounds and fear of death had forced before time man to use any natural substance for relieving the pain and suffering caused by these abnormal conditions and for preserving health against diseases and death. Plants play a vital role for the existence of life in the universe. Primitive man started to distinguish nutritional and pharmacologically active plants for their survival. By their experience, this knowledge of herbal remedies was transferred from one generation to another at first orally and later in written from a papyri, backed clay tablets, parchments, manuscripts, pharmacopoeias and other works. Therefore, medicinal plants have been in use for the eradication and human suffering since ancient time.

As far as record goes, it appears that Babylonians (about 3000 years B.C.) were aware of a large number of medicinal plants and their properties. Some of them are still in use for the same purpose like henbane (*Hyoscyamus* Spp.), Opium (*papaver somniferum*), Castor oil (*Ricinus communis*), Aloe vera (*Aloe spp.*) etc [1].

The Chinese have an effective and unique system of medicine. The earliest known Chinese pharmacopoeia, the Pen Tsao, described over 300 medicinal plants and their uses. Although various of several ancient pharmacopoeia still exist today, the main surviving text book was on herbalism written in the 16th century by the physician Li Shih-Cheu describing almost 200 herbs and 10,000 herbal remedies. Today Chinese herbalism is very much an orthodox from of therapy and preventive treatment in China and it is increasingly practiced in the west. Chinese herbalism can be used for a wide range of ailments, including asthma, skin diseases, menstrual problems, digestive disturbances, migraine and is effective when used on its own or in conjunction with another therapy such as acupuncture.

The material media of the great Greek physician Hippocrates (460-370 B.C.) consists of some 300 to 400 medicinal plants which included opium, mint rosemary, sage and verena[1]. In the middle age, the great Greek pharmacist-physician Galen (131-200 A.D.) used a large number of medicinal plants in preparing his recipes.

The Arabian Muslim physicians like Al-Razi and Ibne Sina (9^{th} to 14^{th} century A.D.) brought about a revolution in the medicine by bringing new drugs of plant and mineral origin into general use. Enriching the original Greek system of medicine by introducing these new materials and knowledge they laid down the foundation stone of modern western medicine.

The medicinal use of plants in the Indian subcontinent is the Rig Veda (4500 – 1600 BC), which noted that Indo-Aryans used the Soma plant (*Amanita muscaria)* as a medicinal agent. The Vedas made many references to healing plants including sarpagondha (*Rauvolfia serpentine*), while a comprehensive Indian Herbal, the Charaka Samhita, cities more than 500 medicinal plants [2].

Since disease, decay & death have always co-existed with life, the study of diseases & their treatment must also have been contemporaneous with the dawn of the human intellect. 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 & 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 & it has been estimated that only 5-15% of the approximately 2,50,000-5,00,000 species of higher plants of which more than 80,000 are medicinal has been investigated pharmacologically [3]. Thus there are considerable chances of finding new natural compounds with pharmacological activities, useful for the development of new drugs.

Scientists are now working together to find out new drug for incurable diseases. Taxonomist, Chemist, Biochemist, Pharmacologist & Pharmacist are working under collaborative program for making a plant product(s) into a commercial drug.

1.2 Medicinal importance of plant materials

Plants not only provide man with food, shelter, and medicine, but also the sustaining oxygen. From ancient time to modern age the human has been successfully used plants and plant products as effective therapeutic tools for fighting against diseases and various health hazards.

Although with the advent of synthetic drugs the use and procurement of plant derived drugs have declined to a large extent, a large number of drugs of modern medicine are obtained from plant sources. According to some generous estimates, almost 80% of the present day medicines are directly or indirectly obtained from plants [4].

As therapeutic use of plants continued with the progress of civilization and development of human knowledge, scientists endeavored to isolate different chemical constituents from plants, put them to biological and pharmacological tests and thus have been able to identify and isolate therapeutically active compounds, which have been used to prepare modern medicines. In course of time their synthetic analogues have also been prepared. In this way, the discovery of vincristine was done from *Catharanthus roseus*, which is, used in the treatment of cancer[5].

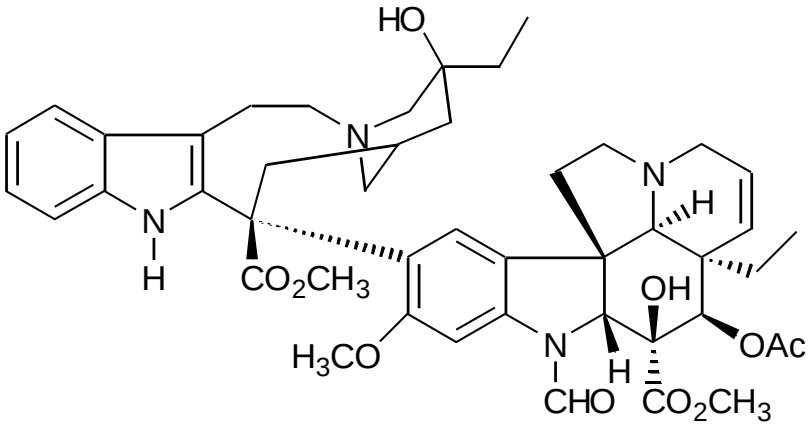


Fig: 1.1 Vincristine

Calanolide-A is a reverse-transcriptase inhibitor isolated from the Malaysian rainforest tree, *Calophyllum Langerum* by the US NCI. It has exhibited synergistic anti-HIV activity in combination with nucleoside reverse-transcriptase inhibitor, including AZT, ddI and ddC [6]. Medichem pharmaceuticals and the state of Sarawak, Malaysia have begun clinical development of Calanolide A as a potential treatment for AIDS and HIV infections.

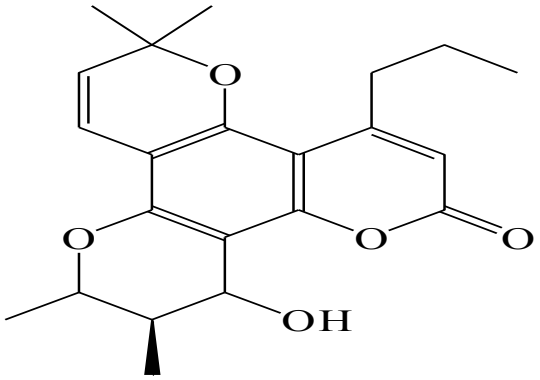


Fig: 1.2 Calanolide-A

Ancient Chinese medical texts written 2000 years ago describe the herb *Artemisia annua* as a medicine for malaria. From this indication, in the late 1960s Chinese researchers initiated evaluation of various extracts of this herb. Bioassay guided isolation yielded the new anti-malarial compound artemisinin (3) which is effective in treating chloroquine resistant cases and other severe cases without major toxicity.

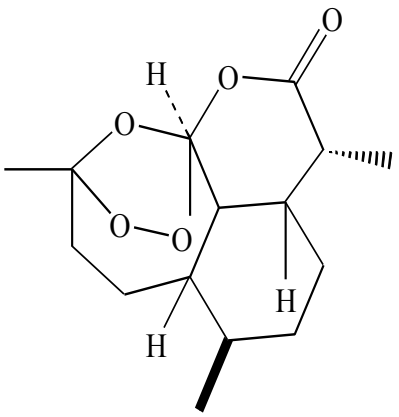


Fig: 1.3 Artemisinin

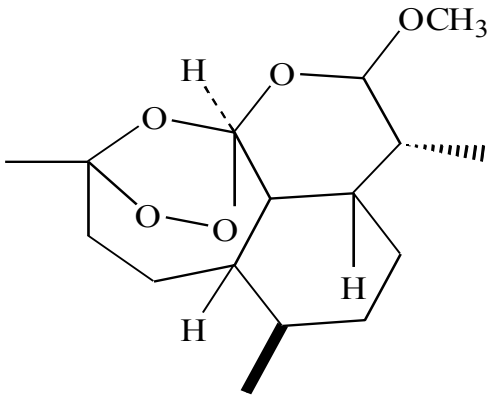


Fig: 1.4 Artem ether

Artemether, a synthetic analogue of artemisinin has been developed in the People"s Republic of China. Two recent clinical studies suggested that artemether is as effective as quinine in the treatment of severe malaria [7].

For several centuries, elderly people in some parts of Mainland China have brewed tea from the leaves of the club moss (*Huperzia serrata*) for improvement of their memory. In the early 1980s, Chinese scientist isolated huperzine-A from the plant Club moss as a potent, reversible and selective inhibitor of acetycholinesterase. A total synthesis has been developed due to very low levels in nature, and the product is found to be a promising candidate for the treatment of cholinergic related neurodegenerative disorders such as Alzheimer"s disease

(AD). In a prospective, multicenter, double-blind trial with 103 patients, huperzine A was found to be safe and superior to placebo and induced improvement in memory cognition and behavior in about 58% of patients with AD[8].

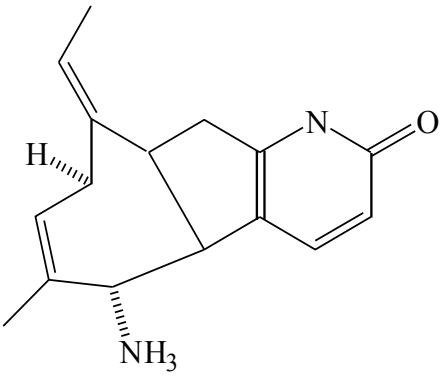


Fig: 1.5 Huperzine

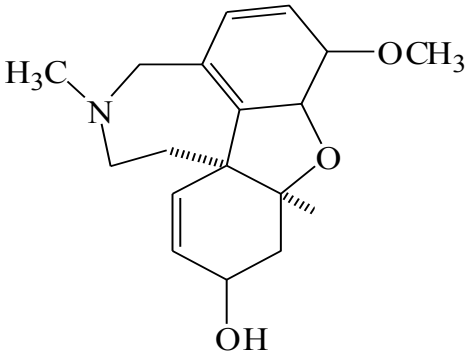


Fig: 1.6 Galathmine

Galanthamine is a long acting, centrally active competitive cholinesterase inhibitor; a natural product isolated from *Galanthus nivalis* in the 1950s. Galanthamine under the name of Nivalein is marketed in Austria for AD and in Germany for other indication such as facial neuralgia [9].

In the antidiabetes area, the past decade has witnessed the market introduction of several α-glucosidase inhibitors derived from natural products. Acarbose, a complex oligosaccharide one of them, was isolated from *Actinoplanes* sp. At Bayer from a search for α-glucosidase enzyme inhibitors. By inhibiting α-glucosidase, acarbose decreases the release of glucose from ingested carbohydrate and slows the increase of food-induced blood glucose levels. Acarbose is now approved in Germany, Japan, the US and other countries and has been used as adjuvant therapy in diabetes [10, 11].

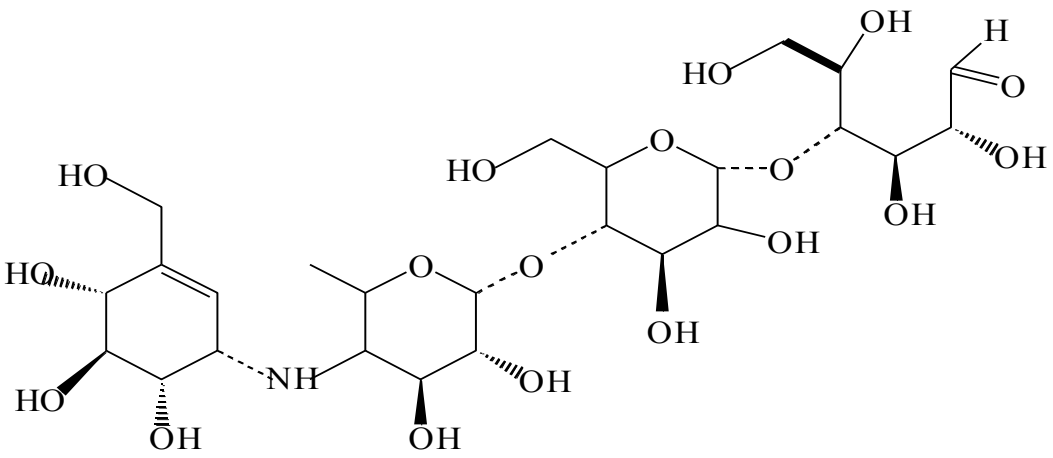


Fig: 1.7 Acarbose

Forskolin (Colforsin) is a diterpene natural product isolated from the Indian plant *Coleus forskohlii* at Hoechsts research labs in India and have blood pressure lowering and cardioactive properties. Later Forskolin was found as a potent adenylate cyclase activator [12]. Colforsin daproate (8) is a semisynthetic product of forskolin derivative and was then brought into phase III clinical trials in Japan for treatment of cardiac insufficiency and phase II trials for treatment of asthma [13].

Triptolide is an active component isolated from the Chinese plant *Tripterygium wilfordii*, a plant traditionally used for the treatment of rheumatoid arthritis. A variety of formulation was developed in Mainland and China are shown to be effective in the treatment of inflammatory and autoimmune diseases. Triptolide was demonstrated to significantly inhibit arthritis in animal modal and have potent cytotoxicity [14].

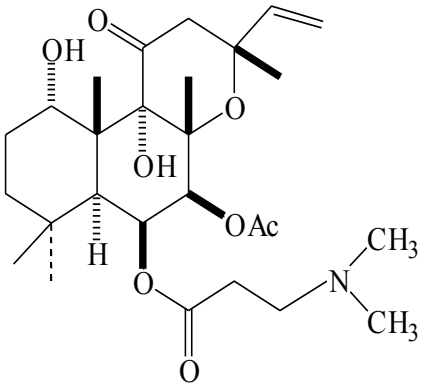


Fig: 1.8 Colforsin daproate

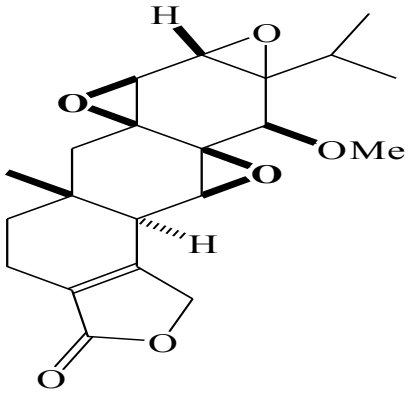


Fig: 1.9 Triptolide

Gomisin A is a lignin derivative isolated from the dry fruit of *Schisondra chinensis*, a traditional Chinese medicine used for the treatment of liver intoxication. Gomisin A was found to be hepatoprotectant and protect liver damage in various animal models

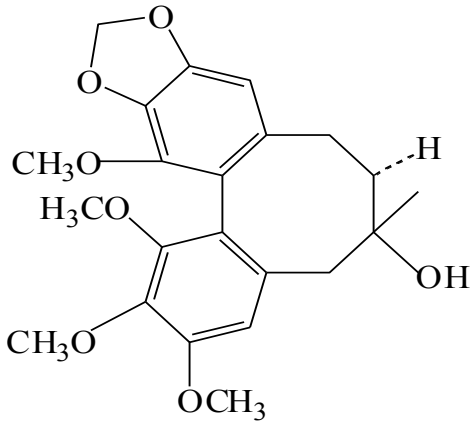


Fig: 1.10 Ginkgolide B

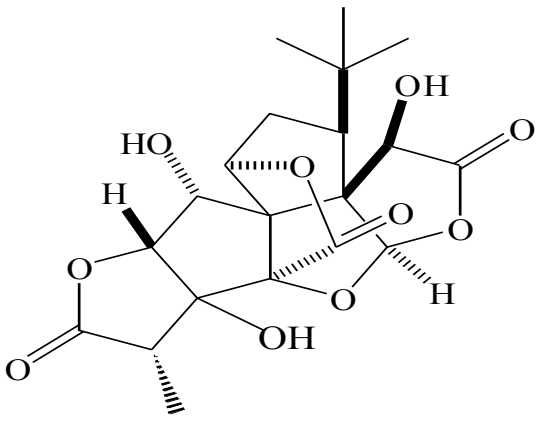


Fig: 1.11 Gomisin A

The Chinese tree *Ginkgo biloba* has been used therapeutically for thousands of years. More recently, extracts of the leaves have become available in many European countries as over-the counter products for the treatment of cerebral vascular insufficiency and tinnitus. Ginkgolides, a class of unique diterpene cage like molecules were isolated from the leaves of *Ginkgo biloba* and represent a group of highly selective platelet activity factor (PAF) receptor antagonists. Among them, Ginkgolide B has been advanced to phase III clinical trials for the treatment of septic shock in patients with severe sepsis caused by Gram positive bacterial infections and also good result in inflammatory and autoimmune disorders [16].

Morphine was first isolated by Serturner in 1806 followed by Codeine in 1832 by Robiquet and then the non-morphine alkaloid papaverine by Merck in 1848 from the seeds of *Poppy*. Dextromethorphan is a semisythentic product of morphine used in most cough syrup today.

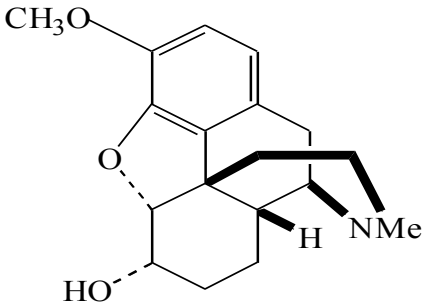


Fig: 1.12 Dextromethorphan

Two active anti-tumor agents etoposide and teniposide was isolated from the root of various species of the genus *Podophyllum*. These plants possess a long history of medicinal use by early American and Asian cultures, including the treatment of skin cancer and wart [17].

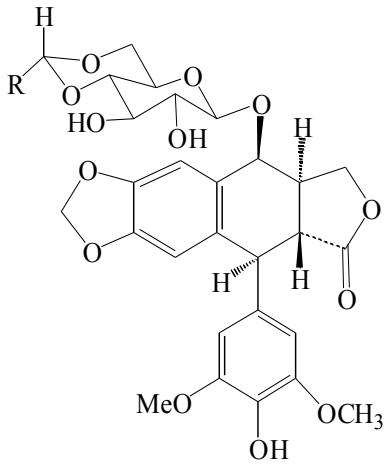


Fig: 1.13 Etoposide (13) R = Me;

Fig: 1.14 Teniposide (14) R = 

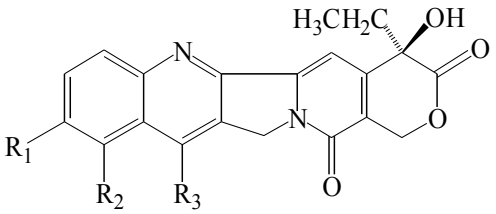


Fig: 1.15 Camptothecin (15) $R_1 = R_2 = R_3 = H$;

Fig: 1.16 Toptican (16) R_1 =OH, R_2=$CH_2N(CH_3)_2$, R_3= H;

Camptothectin was isolated from the Chinese ornamental tree *Camptotheca acuminata* by Wani and Wall[18]. It was advanced to clinical trial by NCI in the 1970s but was dropped because of severe bladder toxicity. But toptican is a modified camptothecin was approved for use in the USA in 1996. The discovery of quinine was done from *Cinchona* bark, which is, used in the treatment of malaria by French scientist Caventon and Pelletier[19].

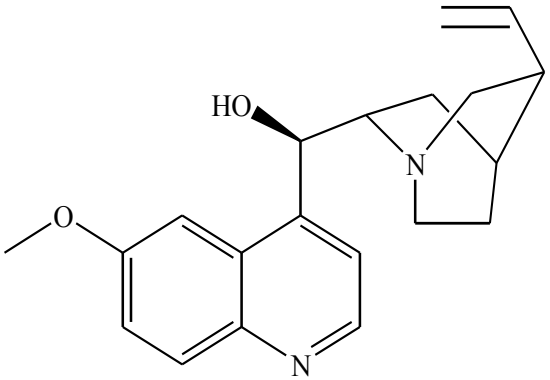


Fig: 1.17 Quinine

Uses of folk medicine represent the way of shortcut discovery of modern medicine. An inventory of medicinal plants compiled by WHO on the basis of literature from 91 countries including the classical text on Ayurvedic and Unani medicine list 21000 species of "medicinal plants".According to WHO, around 80% of the world"s 5.76 billion populations in the developing world rely on herbal remedies for their basic health care need (Medicinal plants, India). The use of medicinal plants as a source for relief from illness can be traced back over five millennia from written documents of the early civilizations in China, India and near east but it is doubtless an art as old as mankind. Even today, plants are the almost exclusive source of drugs for the majority of the world"s population. In industrialized countries, medicinal plant research has had its ups and downs during the last decades.

Plants will continue to be extremely important as source of new drugs as evidenced by recent approvals in the United States of several new plant derived drugs based on the secondary metabolites of plants. For example, in the treatment of refractory ovarian cancer, new drug has recently been approved in the United States from taxol, an anti cancer taxane diterpenoid derived from the relativity scarce pacific western Yew tree, *Taxus brevifolia* Nutt. A relatively new semi-synthetic antineoplastic agent based on podophylotoxin is etoposide a constituent of the Mayapple *Podophyllum petatum*, which is useful in the chemotherapeutic treatment of refractory testicular carcinomas, small cell lung carcinomas, non-Hodgkin"s lymphoma and non lymphocytic leukemia.

1.3 Status of medicinal plants in Bangladesh

The number of medicinal herbs included in the *Materia Medica* of traditional medicine in this subcontinent at present stands at about 2000. About 450 to 500 of such medicinal herbs have so far been enlisted as growing or available in Bangladesh. Almost 80% of rural population is dependent on medicinal plants for their primary health care. Herbal medicine is widely used in Bangladesh in traditional healthcare system such as Ayurvedic, Unani, Hekimi and other form of folk treatments[2].

1.4 The plant family: Actinidiaceae

On the basis of morphology and habitat, the Actinidiaceae is a very homogeneous plant family. They are trees, shrubs or herbs often with milky juice. Leaves are alternate or opposite, rarely divided or compound; stipules usually small, caduceus or persistent, rarely connate in a bud protecting sheath; glands sometimes are at the apex of the petiole or at the base of the leaf-blade [20, 21].

Flowers: usually small or minute, always 1-sexual; inflorescence various, usually compound, sometimes (Euphorbia) of single naked 1- staminate florels in a perianth-like involucres surrounding a solitary pistil, more commonly of main inflorescence centripetal, auxiliary or racemose, the subdivisions cymose sometimes wholly cymose in terminal dichotomous panicles, reduced to simple clusters or solitary florets. Perianth often small, sometimes obsolete, often dissimilar in the 2- sexes, usually simple, calycine with vaivate or imbricate segments, sometimes calycine and 2-seriate imbricate, with segments all similar or occasionally dissimilar rarely double, the inner then of 4-5 small scale-like, or very rarely conspicuous petals.

Male flowers: Torus sometimes forming an intra staminal disk or with disk-glands or lobes alternate with the stamens of the outer series, stamens various, sometimes solitary or flower than, sometimes as many as the sepals or petals, sometimes indefinite (rarely very numerous); filaments free or connate; anthers 2-celled; often didymous with longitudinal, transverse or porous dehiscence. Rudimentary ovary present[20, 21]. **Female flowers:** Sepals usually larger and less connate than in the male. Petals sometimes smaller and less often present than in the male. Disk hypogynous or of discrete glands or 0. Ovary superior, sessile or stipitate, usually of 3 (rarely more or 2) carpel more or less united; ovules 1-2 in each carpel, pendulous from inner angle of the cell, the funicle often thickened; styles as many as the carpel, free or united or entire divided; stigmatic surface usually on the inner surface of the styles or style-arms.

Fruit: is usually a capsule of three 2-valved 1-2 seeded cocci separating from a persistent axis or a drupe with 1-3 cells or of one or more combined nuts. Seeds laterally attached at or above the middle of the cells, with or without an aril or caruncle at the hilum; albumen fleshy embryo straight, enclosed in the albumen, cotyledons flat, leafy and radicle superior; rarely albumen and cotyledons fleshy[20, 21].

Three genera (*Actinidia*, *Clematoclethra* and *Saurauia*) and and 250 species [22]distributed in the tropics and subtropics of Asia, and South and Central America[23]. However three genera (one endemic) and 66 species (52 endemic) in China[20]. Familiy synonyms: Saurauiaceae [21].

1.4.1 Medicinal Importance of Actinidiaceae

Table 1.1 Medicinal importance and uses of *Actinidiaceae* plants are listed below

Genus	Plant parts	Medicinal or other uses
1. Actinidia	Root	Roots known as „ipicacuanha" in the West Indies are powerful emetic. It is also applied to skin for leucoderma and for scorpion and centipede bites.
	Bark	Root, along with the bark of *Moringa olifera*, is used to induce permanent sterility in women.
2. Clematoclethra	Aerial parts	Ethanolic extract of aerial parts is hypotensive. Hypotensive, CNS depressant
	Extract of fruits	Used against malaria.
	Root and stem	In headache, fever, spleen trouble madness, epilepsy, convulsions, icterus, scabies, syphilis, ulcer, hydrocele, foul breathe, cholera etc.
	Seed	In asthma.
3. Saurauia	Stems and leaves	used as herbal medicines against a large number of severe diseases like asthma, bronchitis, and central nerves system (CNS) depression.
	Fruits	Extracts of fruits is used for hepatitis B, ulcers, eczema

1.5 Taxonamy of Genus *Saurauia* [25]

Trees or shrubs. Branchlets usually with unguiculate hairs or subulate scales. Leaves petiolate; petiole scaly or not, rarely long setose; leaf blade tomentose or not abaxially, veins with scales or stiff hairs, numerous lateral veins diverging parallel to midvein, margin serrate. Inflorescences thyrsoid, composed of terminal cymes, solitary or fasciculate, usually scaly, tomentose or glabrous. Pedicels 2-bracteate. Flowers hermaphroditic or plants functionally dioecious. Sepals 5, strongly imbricate. Petals 5, imbri-cate, usually connate at base. Stamens 15–130; filaments adnate to base of petals; anthers obtrigonal, dorsifixed, dehiscing longitu-dinally or poricidally. Ovary 3–5-loculed, with many ovules per locule; styles 3–5, connate below middle, rarely free; stigma simple to discoid. Fruit baccate, white to pale green, rarely red, globose or depressed-globose, usually ribbed. Seeds brown, minute, areolate.

1.6 Taxonamy of various *Saurauia* Genus in Asia

1.6.1. *Saurauia griffithii* [26]

Trees ca. 8 m tall. Branchlets stout, densely brown tomen-tose, hairs intermixed with subulate scales. Petiole 4.5–5 cm, stout, scaly, tomentose; leaf blade broadly or narrowly elliptic-oblong, 20–40 × 8–17 cm, leathery, abaxially flocculently brown or ferruginous tomentose, adaxially glabrous, midvein and lateral veins with subulate scales buried in tomentum, lat-eral veins 37–40 pairs, base obtuse to rounded, symmetrical to oblique, margin remotely setose-serrate, apex abruptly acumi-nate. Inflorescences 15–33 cm, axillary, densely tomentose, sparsely scaly; pedicels 1–1.7 cm; bracteoles 2, above middle, narrowly elliptic, ca. 8 × 3 mm, abaxially tomentose. Sepals broadly elliptic, 4–5 mm wide, abaxially sparsely to densely tomentose. Petals unknown. Stamens 52–54. Ovary subglobose, glabrous; styles 5, connate below middle. Fruit unknown. **Flowers:** and **Fruits:** unknown. Broad-leaved forests; 600–1300 m.

1.6.2. *Saurauia miniata* [21]

Small trees or shrubs, 2–8 m tall. Branchlets densely fer-ruginous tomentose, hairs intermixed with unguiculate scales, scales occasionally truncate-tipped or 2-fid. Petiole 2–2.5 cm, densely ferruginous tomentose and sparsely scaly; leaf blade oblong-elliptic, 19–24 × 6–14 cm, leathery, abaxially thickly tomentose, hairs intermixed with subulate scales on midvein and lateral veins, adaxially glabrous, with subulate scales on midvein, lateral veins 23–30 pairs, base obtuse to subrounded, margin mucronate-serrate, apex acute or shortly acuminate.

Inflorescences 2.5–7 cm, axillary, 3- or 4-fascicled on old branches, ferruginous tomentose and sparsely scaly; peduncles 5–10 mm, with 4 or 5 bracts at apex; bracts broadly elliptic to ovate-triangular, 2–6 mm, ciliate; pedicels ca. 1.2 cm, ferrugi-nous tomentose and sparsely scaly, with 2 bracteoles at base; bracteoles ca. 1 mm. Flowers pink, small, ca. 8 mm in diam. Sepals elliptic to broadly elliptic, 3–4 mm. Petals oblong, ca. 5 mm. Stamens 45–75. Ovary subglobose, glabrous; styles 5,connate below middle. Fruit green to white, depressed-globose, 3–5 mm in diam. **Flowers:** May–Jun, **Fruits:** Oct. *2n* = 78.Mountain forests, thickets on riverbanks, valleys; 500–1500 m.

1.6.3. *Saurauia napaulensis* [27]

Trees 4–20 m tall. Branchlets brown pubescent to glabres-cent, with setose hairs intermixed with subulate scales. Petiole 2.5–5 cm, pubescent to glabrescent, with minute hairs inter-mixed with subulate and unguiculate scales; leaf blade narrowly elliptic to oblong-obovate, 13–36 × 7–15 cm, thinly leathery, abaxially sparsely ferruginous tomentose, indumentum cadu-cous or not, sparsely scaly on midvein and lateral veins, adax-ially glabrescent, sometimes with scales at base of midvein, lateral veins 28–40(–46) pairs, base obtuse to subrounded to cu-neate, margin finely serrate, apex acuminate to acute. Inflores-cences 12–33 cm, axillary, sparsely scaly and puberulent; pe-duncleca. 1/2 as long as inflorescence, with 1 or 2 bracts at base of each branch; pedicels 1.7–2.5 cm, with 2 nearly oppo-site bracteoles below middle; bracteoles caducous, lanceolate, 2–4 mm. Flowers pink to purplish, 0.8–1.5 cm in diam. Sepals unequal, outer 3 smaller, elliptic to broadly elliptic; inner 2 larger, broadly elliptic to orbicular, 5–7 mm. Petals oblong, ca. 8 mm, connate at base, recurved at apex. Stamens 50–90. Ovary globose to depressed-globose; styles 4 or 5, connate below middle. Fruit green to yellowish, globose to depressed-globose, ribbed or slightly ribbed. **Flowers:** Jun–Dec, **Fruits:** Jun–Dec. Sparse mountain forests, thickets, valleys; 400–3200 m.

1.6.4. Saurauia erythrocarpa[21]

Trees or shrubs, 1–6 m tall. Branchlets with subulate or unguiculate scales. Petiole 1.5–4.5 cm, scaly; leaf blade elliptic-obovate to elliptic-oblong, 15–25 × 5–10 cm, abaxially brown tomentose, scaly on midvein and lateral veins, even on retic-ulate veins, adaxially glabrous, sparsely scaly, lateral veins 22– 28 pairs, base obtuse to subrounded, margin biserrate, apex shortly acuminate. Inflorescences 2.5–5 cm, 1–3-fascicled in axils of leaves on current year or older branches, scaly, brown puberulent to glabrous; bracts 1 or 2 at base of each branch, broadly to narrowly ovate, 5–9 mm; pedicels 1.2–1.7 cm; bracteoles 2 below middle or at

base of pedicel, broadly ovate, 2–3 mm. Flowers pink, 1–1.3 cm in diam. Sepals broadly ellip-tic, outer 3 small, 7–8 mm, inner 2 large, 7–10 mm. Petals broadly elliptic to suborbicular, 7–10 mm. Stamens 70–80.Ovary subglobose, glabrous; styles 4 or 5, connate below mid-dle.Fruit pink, depressed-globose to subglobose, 7–8 mm in diam. **Flowers:** Sep–Oct, **Fruits:** Oct–Nov.

1.6.5. *Saurauia rubricalyx* [21]

Shrubs ca. 3 m tall. Branchlets with fine subulate scales.Petiole 1.3–3.5 cm, scaly; leaf blade ovate, 9–23 × 5–10 cm,leathery, abaxially glandular-puberulent, sparsely scaly on mid-vein and lateral veins, adaxially glabrous, sparsely scaly on midvein, lateral veins ca. 26 pairs, reticulate veins conspicuous,base broadly cuneate, margin biserrate, apex shortly acuminate.Inflorescences 3.5–4.5 cm, 1–3-fascicled on old branches, 2- or3-flowered; bracts 2 at base of branches, elliptic, 2–3 mm;pedicels ca. 1.5 cm, with 2 bracteoles at middle; bracteoles ca. 2mm. Flowers small, 5–8 mm in diam. Sepals red, elliptic, un-equal.Petals and stamens unknown. Ovary subglobose; styles 5, connate below middle. Fruit unknown. **Flowers:** and **Fruits:** unknown. Margins of broad-leaved forests, mountain slopes; 1600–2000m.

1.6.6. *Saurauia thyrsiflora* [21]

Small trees or shrubs, 2–4 m tall. Branchlets with scurfy tomentum, intermixed with subulate scales. Petiole 1.5–4 cm, brown puberulent, with subulate scales; leaf blade oblong-ellip-tic,14–26 × 5.5–11 cm, membranous, both surfaces sparsely brown tomentose when young, puberulent on midvein and lat-eral veins abaxially when mature, sparsely setose only on mid-vein and lateral veins abaxially, sparsely setose on midvein and lateral veins and occasionally in between veins adaxially, lateral veins 12–15 pairs, base obtuse to rounded, margin finely ser-rate, apex shortly acuminate to acute. Inflorescences axillary,8–12 cm, brown puberulent and subulate-scaly, ca. 13-flow-ered; bracts more than 2 at base of each branch, elliptic, 2–5 mm, sometimes leaflike, to 1.6 cm; pedicels 1–1.7 cm, with 2 bracteoles at base. Flowers pink, 0.8–1 cm in diam. Sepals white to greenish white, outer 3 broadly elliptic, inner 2 nar-rowly elliptic, ca. 5 mm, abaxially brown tomentose, margin occasionally ciliate. Petals oblong, connate at base. Stamens 48–65. Ovary subglobose; styles 3 or 4(or 5), connate below middle.Fruit green, subglobose, 0.8–1.2 cm in diam., slightly 5-ribbed. **Flowers:** May–Jul, **Fruits:** Aug–Dec.

1.6.7. *Saurauia punduana* [28]

Trees ca. 6 m tall. Branchlets pubescent to glabrescent with minute scurfy hairs intermixed with unguicular scales.Petiole 3–5 cm, with similar pubescence as branchlets; leaf blade narrowly elliptic to narrowly obovate, ca. 33 × 11 cm, thinly leathery, abaxially densely brown scurfy-puberulent, with sparse scales on midvein and lateral veins, adaxially glabrous but scaly on midvein, lateral veins 25–34 pairs, base cuneate, margin finely biserrate, apex shortly acuminate. Inflorescences ca. 5 cm, axillary, 1–3-fascicled, 2- or 3-flowered, glabrous, scaly; pedicels slender; bracts broadly elliptic, ca. 4 mm. Flowers pinkish white, large, 1.8–2 cm in diam. Sepals: outer 2 broadly elliptic, inner 3 narrowly elliptic to orbicular, enlarged when in fruit. Petals oblong, ca. 1.2 × 1.1 cm. Stamens ca. 90.Ovary subglobose, ribbed; styles 5, connate below middle.

1.6.8. *Saurauia tristyla* [24]

Small trees or shrubs, 3–6(–12) m tall. Branchlets tomen-tose to glabrescent, with unguiculate hairs or subulate scales.Petiole tomentose or not, with unguiculate hairs or subulate scales; leaf blade obovate to broadly elliptic-obovate, 10–28 × 4–11 cm, papery, both surfaces sparsely pubescent with ap-pressed setose to unguiculate hairs on midvein and lateral veins, with sparse and appressed setose hairs especially on veins adax-ially, lateral veins 8–20 pairs, base cuneate to broadly so, margin setose-serrate, apex shortly acuminate to caudate. Inflores-cences 1–4-fascicled, axillary, 1–5 cm, hairy and scaly, 1–3-flowered; bracts 2 or 3 at base of branches, ovate; bracteoles 2, nearly opposite at base of pedicel, 1–5 mm. Flowers pink to white, 0.7–1.6 cm in diam. Sepals broadly ovate to elliptic, 3–4mm. Petals ovate, ca. 8 mm, recurved at apex. Stamens 25–34.Ovary ovoid to globose; styles 3 or 4(or 5), connate below middle. Fruit green to white to pale yellow, globose, 6–10 mm in diam. **Flowers:** Mar–Jul, **Fruits:** Aug–Dec. Broad-leaved forests, sparse mountain forests, thickets, valleys;100–1700 m.

1.6.9. *Saurauia polyneura* [21]

Trees 3–6 m tall. Branchlets unguiculate-scaly. Petiole1.5–3 cm, sparsely subulate-scaly; leaf blade obovate-lanceo-late to elliptic, 12–32 × 5–10 cm, membranous to leathery, both surfaces glabrous, except with scalelike hairs on midvein and lateral veins abaxially, lateral veins 22–40 pairs or more, base cuneate to obtuse, margin finely serrate, apex acute to rounded. Inflorescences solitary, axillary, 7–33 cm, with sparsely dis-tributed scales; pedicels 0.8–1.5 cm, with 2 bracteoles below middle; bracteoles narrowly ovate, ca. 2 mm. Flowers

pink to white, 6–10 mm in diam. Sepals elliptic to broadly elliptic, 4–5 mm, abaxially glabrous to very sparsely tomentose. Petals ob-long, 5–6 mm, connate at base. Stamens 50–64. Ovary subglo-bose, 6–8 mm in diam. **Flowers:** Jul–Sep, **Fruits:** Sep–Nov. Forests, valleys; 1200–3200 m.

1.6.10. *Saurauia cerea* [29]

Trees 5–15 m tall. Branchlets stout with dense unguiculate hairs or subulate scales, not tomentose. Petiole 1.1–3.5 cm, stout, with subulate scales; leaf blade obovate, 17–36 × 12–20 cm, leathery, abaxially yellowish tomentose when young, gla-brous when old, unguiculate-scaly on midvein and lateral veins on both surfaces, lateral veins 23–29 pairs, base cuneate, rarely obtuse, margin densely setose-serrate, apex acute. Inflores-cences 1-flowered, fascicled on old branches; pedicels to 1.5 cm, somewhat thick, with yellowish indumentum and scales,with 2 bracts below middle; bracts ovate, 5–7 mm, abaxially hairy and scaly. Flowers large, 3.5–4 cm in diam. or larger. Sepals ca. 1 cm, outer 3 elliptic, inner 2 orbicular, abaxially yel-lowish tomentose and scaly, adaxially yellowish white puberu-lent at base. Petals white to pink, purple at base, oblong, ca. 1.9 × 1.2 cm. Stamens 120–130. Ovary subglobose, yellowish brown tomentose; styles 4 or 5, distinct. Fruit greenish white, depressed-globose, ca. 8 mm in diam., 5-ribbed, yellowish brown tomentose. **Flowers:** Jul–Sep, **Fruits:** Oct–Nov. Moist mountain forests, valleys; 400–2200 m.

1.6.11. *Saurauia yunnanensis* [21]

Small trees or shrubs, 4–5 m tall. Branchlets with sparse fine unguiculate scales, with tomentose indumentum or not. Petiole 1.5–2.5 cm, sparsely scaly; leaf blade narrowly obovate-lanceolate, 6–22 × 1.2–5.2 cm, thinly leathery, midvein and lateral veins with sparse unguiculate scales on both surfaces, lateral veins 12–14 pairs, base obtuse, margin serrate, apex shortly acuminate. Inflorescences 2.5–3.3 cm, few flowered, sparsely scaly, with 2 bracts at base of branches; bracts lanceo-late, 1.5–3 mm; pedicels ca. 1.2 cm, with 2 bracteoles proxi-mally;bracteoles ovate-triangular, 1–2 mm. Flowers pink, ca. 8mm in diam. Sepals: outer 2 elliptic, ca. 3.5 mm, inner 3 broadly elliptic to suborbicular, 3.5–5 mm. Petals unknown. Stamens ca. 45. Ovary depressed-globose; styles 4 or 5, connate below middle. Fruit white when mature, ca. 5 mm in diam. **Flower:**Apr–Jul, **Fruits:** Aug–Nov.. Shaded moist places in forests, thickets, valleys, by marshes;400–1700 m.

1.6.12. *Saurauia macrotricha* [29,30]

Small trees or shrubs, 1–5 m tall. Branchlets stout, dark reddish ferruginous pubescent, hairs setose, long. Petiole 2– 3.8 cm, densely ferruginous pubescent with long setose hairs;leaf blade narrowly lanceolate, 20–28 × 3.7–6.8 cm, papery,both surfaces setose, lateral veins 17 pairs, base obtuse, margin setose-serrate, apex acuminate. Inflorescences ca. 2.5 cm, axillary, 2- or 3-fascicled, 1–3-flowered, on young branchlets; peduncles ca. 8 mm, with 2 bracts at base of branches; bracts nearly opposite, lanceolate, 2–3 mm; pedicels 8–11 mm,densely setose with long, ferruginous hairs. Flowers pink, ca.8 mm in diam. Sepals elliptic to suborbicular, ca. 5 mm, con-nate at base. Petals suborbicular, connate at base. Stamens ca. 39. Ovary depressed-globose, glabrous; styles 5, connate be-low middle. Fruit unknown. **Flowers:** and **Fruits:** unknown. Mountain forests, valleys; 900–1400 m.

1.6.13. *Saurauia sinohirsuta*

Replaced synonym: *Saurauia hirsute* [21] not *Saurauia hirsuta* [21]

Trees small, 3–5 m tall. Young branches hirsute, old branches glabrescent to glabrous. Leaf blade obovate, 15–25 × 9–15 cm, softly leathery, abaxially densely appressed-hirsute,hairs on midvein and lateral veins thicker, adaxially sparsely setulose, hairs on midvein and lateral veins longer, lateral veins ca. 18 pairs, base obtuse, margin densely setose-serrate, apex shortly acute. Flowers solitary, axillary on young branchlets; pedicels 1–1.3 cm, hirsute; bracts ca. 4 mm. Sepals sanguine-ous, ovate to oblong, 8–10 mm, densely hirsute. Petals pink, spatulate-oblong, 1.1–1.2 cm. Stamens 50–90, 3–4 mm. Ovary subglobose, hirsute; styles 4. Fruit unknown. **Flowers:** and **Fruits:** un-known. Semi-evergreen forests on mountains; 800–1700 m.

1.7 The plant *Saurauia roxburghii* [25, 31]

Tree 5–8 m tall. Branchlets scattered strigose, younger parts scurfy pubescent. Petioles 2.5–4.5 cm long, scattered strigose. Lamina membranaceous to chartaceous, obovate to broadly elliptic, 10–26 by 5–10.5 cm, apex acuminate, acumen 0.5–1.5 cm long, margins finely serrulate to serrate, base cuneate to rounded-cuneate, adaxially glabrous, abaxially scattered strigose along major veins, lateral veins 9–17 pairs. Inflorescences 1.5–8 cm long, normally densely crowded, 3–12-flowered; peduncles 0.6–5.5 cm long; bracts ovate to linear, 1–2 mm long; pedicels 0.4–2.75 cm long; peduncles, pedicels and bracts scurfy pubescent. Flowers

pink; sepals 5, ovate to rounded ovate to obovate, 3.5–5.5 by 2.5–4.5 mm, puberulent at base outside, glabrous on the upper part; petals 5, pink, obovate, 5–7 by 2.5–3.8 mm; stamens 20–45, filaments 1–2 mm long, anthers 0.5–0.85 mm long, ovary ovoid, (3- or) 4- (or 5-)loculed; styles (3 or) 4 (or 5), 1.5–3.2 mm long, connate at base half to three-fourth their length.Its fruit is glabrous and edible[34].

Flowering: April to June; **Fruiting:** August to October [31, 35].

Distribution: Throughout Vietnam. Also in Nepal, India, Myanmar, Thailand, Cambodia, Laos, China (including Taiwan), Japan, Malaysia and the species less frequently occurs in the greater districts of Sylhet, Chittagong and Chittagong Hill Tracts in Bangladesh[31, 32, 33].

Habitat & Ecology: Secondary & primary evergreen tropical rain forest.Altitude 150-1700m.

Medicinal Uses: The stems and leaves of the plants are extensively used as herbal medicines against a large number of severe diseases[31, 36] like asthma, bronchitis, hepatitis B, ulcers, fever, gout, eczema, Piles and central nerves system (CNS) depression.

1.7.1 Botanical features of *Saurauia roxburghii*

Scientific name	:	*Saurauia roxburghii*
Family	:	*Actinidiaceae.*
Synonyms	:	*Saurauja roxburghii, Ternstroemia serrata*
Common names	**:**	Singkrang, Sing khau (Bengali name: Pannikomari, Pahari Kadam or Bhola Kadam)

❖ Classification

- **Domain:** *Eukaryota*
  - **Kingdom:** *Plantae*
    - **Phylum:** *Magnoliophyta*
      - **Subphylum:** *Euphyllophytina*
        - **Class:** *Magnoliopsida*
          - **Subclass:** *Actinididae*
            - **Superorder:** *Actinidianae*
              - **Order:** *Actinidiales*
                - **Family:** *Actinidiaceae*
                  - **Genus:** *Saurauia*
                    - **Specific epithet:** *roxburghii* - Wall.
                      - **Botanical name:** *Saurauia roxburghii* Wall.

1.7.2 Chemistry of *Saurauia roxburghii*

It has been reported that the plant extracts contain steroids/triterpenes at moderate and low levels amounts of alkaloids and saponins [37]. The plant extracts also showed anti-tumor and antifungal activities [38, 39]. Compounds isolated from ***Saurauia roxburghii*** are mainly terpenes and steroid. From the n-Hexane extracts of the leaves of ***Saurauia roxburghii*** are Stigmasterol, β-sitosterol and 3β-hydroxy-urs-12-en-28-oic acid and chloroform extracts are 3β-hydroxy-olea-12-en (β-amyrin), 3β-hydroxy-olea-12-en-28-oic acid and 2α, 3β-dihydroxy-olea-12-en-28-oic acid respectivelly has been first isolated.

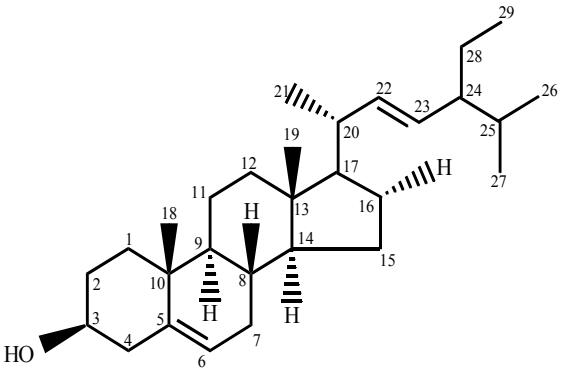


Fig 1.18: Stigmasterol

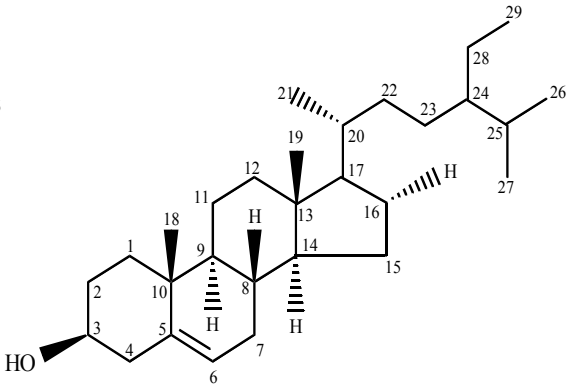


Fig 1.19: β-sitosterol

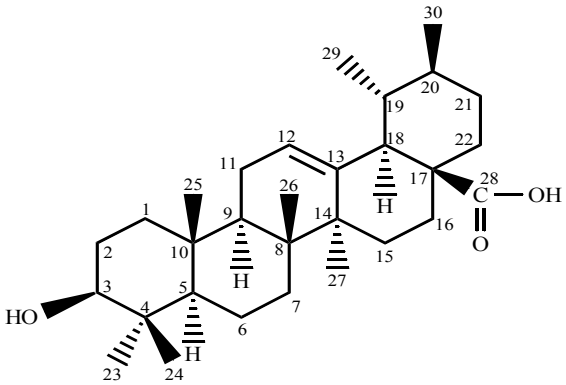


Fig 1.20: 3β-hydroxy-urs-12-en-28-oic acid

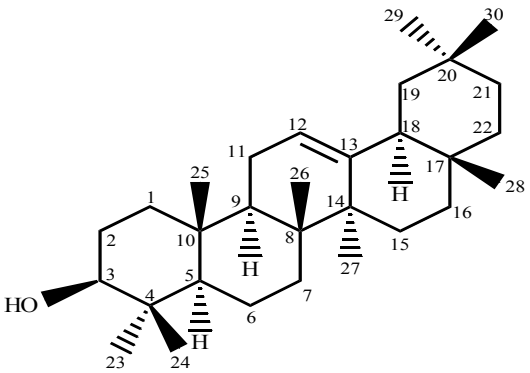


Fig 1.21: 3β-hydroxy-olea-12-en (β-amyrin)

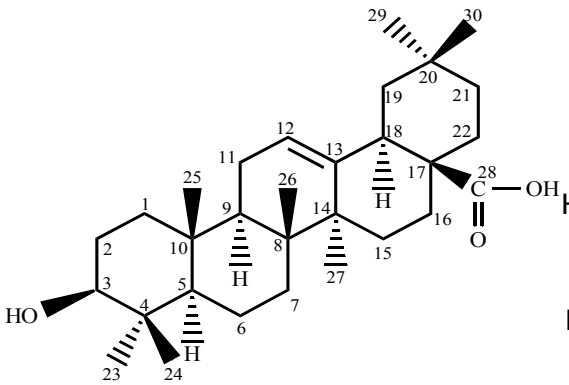


Fig 1.22: 3β-hydroxy-olea-12-en-28-oic acid

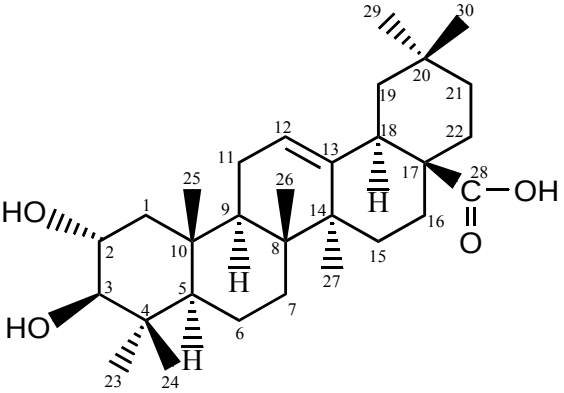
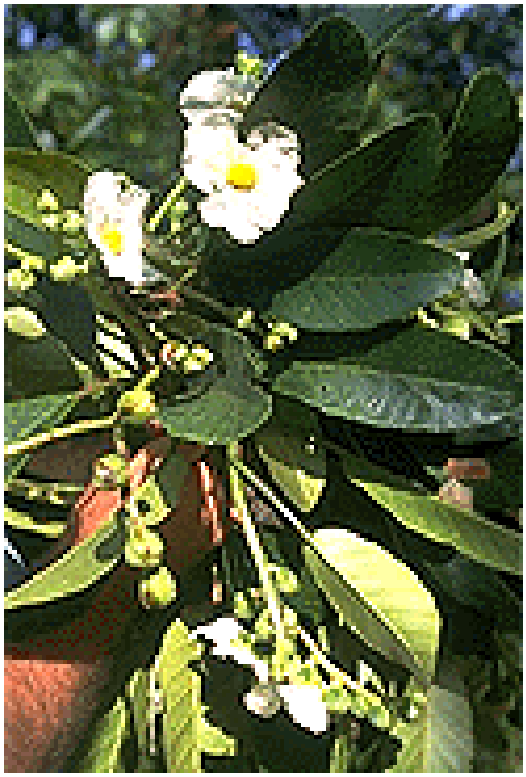


Fig 1.23: 2α, 3β-dihydroxyolea-12-en-28-oic acid

(a)

(b)



(c)

(d)

Figure 1.24: Whole plant (a), leaves (b), Flower (c) and Fruits (d) of ***Saurauia roxburghii.***

1.8 Aim of the work

Bangladesh is a good repository of medicinal plants belonging to various families, including *Actinidiaceae*. The *Actinidiaceae* plants contain wide range of chemical and unique pharmacologically active compounds, including antiseptic, astringent, febrifuge, stomachic, anti-inflammatory, anti-rheumatic, anti-diarrhea and anti-emetic activities.

Saurauia is a genus of *Actinidiaceae* family.It comprises about three genera (*Actinidia*, *Clematoclethra* and *Saurauia*) and and 250 species distributed in the tropics and subtropics of Asia, and South and Central America.The *Saurauia roxburghii* Willd. (Bengali name: Pannikomari or Bhola Kadam) is a very important medicinal plant of Bangladesh & through out the world as well.

Although uses of some of these species are based on old and new experiences and clinical data, many of them have no foundation what so ever. Therefore an attempt has been taken to study systematically the chemical constituents and biological activities of *Saurauia roxburghii*, a member of the *Actinidiaceae* family, growing in Bangladesh and evaluate its pharmacological profiles.

These investigations may provide some interesting compounds, which may be pharmacologically active. If significant results are obtained these can be used for the treatment of some diseases. Since this plant is available in Bangladesh and a lot of herbal centers and herbal industries are using such related herbal plants for treatment, so if the biological activity of this plant can be studied thoroughly, this may be a cost- effective treatment. So, the objective is to explore the possibility of developing new drug candidate from this plant for the treatment of various diseases.

1.9 Present Study Protocol

Our present study was designed to isolate pure compounds as well as to observe biological activities of the isolated pure compounds with crude extract and their different fractions. The study protocol consisted of the following steps:

- The leaves of the plant was collected from the Chittagong University campus.
- The whole plants was dried at room temperature and ground into powder.
- The dried powder was extracted by ethanol.
- The ethanol extract was dissolved in water and partitioned with organic solvents e.g. n-Hexane, chloroform and ethylacetate.
- Fractionation of the different extracts was done by column chromatography (CC).
- Isolation and purification of the pure compounds from different column chromatographic (CC) fractions were carried out.
- Determination of the structure of the isolated compounds with the help of different spectroscopic methods like UV, FT-IR, ^{1}H & ^{13}C NMR.
- Brine shrimp lethality bioassay and determination of LC_{50} for crude extracts and column chromatographic (CC) fractions.
- Observed the *in vitro* antimicrobial activity of crude extracts, column chromatographic (CC) fractions and pure compounds.

CHAPTER-2

EXPERIMENTALS

2.1 General methods

The chemical investigation of a plant can be divided roughly into the following major steps:

a) Collection and proper identification of the plant materials

b) Preparation of plant sample

c) Extraction

d) Fractionation and isolation of compounds

e) Structural characterization of purified compounds

The last step will be discussed in Chapter-3 (Result and discussion).However, other steps will be presented here initially as general procedure and then in connection with concerned plants.

2.1.1 Collection and proper identification of the plant sample

At first with the help of a comprehensive literature review a plant was selected for investigation and then the whole plant/plant part(s) was collected from an authentic source and was identified by a taxonomist. A voucher specimen that contains the identification characteristics of the plant was submitted to the herbarium for future reference.

2.1.2 Preparation of plant sample

The leaves of the plant were collected in fresh condition. It was sun-dried and then, dried in an oven at reduced temperature (not more than 40^0C) to make it suitable for grinding purpose. The coarse powder was then stored in air-tight container with marking for identification and kept in cool, dark and dry place for future use.

2.1.3 Extraction procedures

2.1.3.1 Solvents and Chemicals

Analytical or laboratory grade solvents and chemicals had used in the experiments. All solvents and reagents used in the experiments were produced from E. Merk (Germany), BDH (England). The commercial grade solvents (ethyl acetate, chloroform, methanol, ethanol, n-hexane, DMSO, dichloromethane and 1-butanol) were distilled before use.

2.1.3.2 Distillation of the solvents

The analytical grade solvents (ethyl acetate, chloroform, methanol, ethanol, n-hexane, DMSO, acetone and dichloromethane) were distilled. Distilled solvents were used throughout the investigation.

2.1.3.3 Evaporation

All evaporations were carried out under reduced pressure using a rotary evaporator at a bath temperature not more than 40^0C. The residual solvent in the extract and compounds were removed under high vacuum.

2.1.3.4 Initial extraction

Extraction can be done in two ways such as

a) Cold extraction
b) Hot extraction

a) Cold extraction : In cold extraction the powdered plant materials is submerged in a suitable solvent or solvent systems in an air-tight flat bottomed container for several days, with occasional shaking and stirring. The major portion of the extractable compounds of the plant material will be dissolved in the solvent during this time and hence extracted as solution.

b) Hot extraction: In hot extraction the powdered plant material is successively extracted to exhaustion in a Soxhlet at elevated temperature with several solvents of increasing polarity. The individual extractives are then filtered through several means, e.g., cotton, cloth, filter paper etc. All the extractives are concentrated with a rotary evaporator at low temperature (40^0C - 50 ^{0}C) and reduced pressure. The concentrated extract thus obtained is termed as crude extract.

2.1.3.5 Solvent-solvent partitioning of crude extract

The crude extract is diluted with sufficient amount of aqueous alcohol (90%) and then gently shaken in a separating funnel with almost equal volume of a suitable organic solvent (such as petroleum ether) which is immiscible with aqueous alcohol. The mixture is kept undisturbed for several minutes for separation of the organic layer from the aqueous phase. The materials

of the crude extract will be partitioned between the two phases depending on their affinity for the respective solvents. The organic layer is separated and this process is carried out thrice for maximum extraction of the samples. After separating of the organic phase, the aqueous phase thus obtained is successively extracted with other organic solvents, usually of the increasing polarity (such as carbon-tetrachloride, dichloromethane, chloroform, ethyl-acetate, methanol etc). Finally, all the fractions (organic phases as well as the aqueous phase) are collected separately and evaporated to dryness. These fractions are used for isolation of compounds.

2.1.4 Fractionation and isolation of compounds

Pure compounds are isolated from the crude and fractionated extracts using different chromatographic and other techniques. A brief and general description of these is given below.

2.1.4.1 Chromatographic techniques

Chromatographic techniques are the most useful in the isolation and purification of compounds from plant extracts. The advent of relatively new chromatographic media e.g. Sephadex and Polyamide, have improved the range of separations that can be performed.

2.1.4.1.1 Column Chromatography

Column Chromatography is the most common separation technique based on the principle of distribution (partition/adsorption) of compounds between a stationary and mobile phase. A normal Chromatographic column is packed with silica gel (Kiesel gel 60, mesh 70-230). A slurry of silica gel in a suitable solvent is added into a glass column of appropriate height and diameter. When the desired height of adsorbent bed is obtained, a few hundred milliliters of solvent is run through the column for proper packing of the column. After packing, the sample to be separated is applied as a concentrated solution in a suitable solvent or the sample is adsorbed onto silica gel (Kiesel gel 60, mesh 70-230), allowed to dry and subsequently applied on top of the adsorbent layer. Then the column is developed with suitable solvent mixtures of increasing polarity. The elutes are collected either in test tubes or in beakers.

2.1.4.1.2 Vacuum Liquid Chromatography (VLC)

Vacuum Liquid Chromatography is a relatively recent separation technique which involves short column chromatography under reduced pressure, the column being packed with fine TLC grade silica (Kiesel gel 60H, mesh 70-230). Details of the method have been published by Pelletier [40] and by Coll and Bowden[41]. This technique is used for the initial rapid fractionation of crude extracts.

The column is packed with silica gel (Kiesel gel 60H, mesh 70-230) under vacuum. The size of the column and the height of the adsorbent layer are dependent upon the amount of extract to be analyzed. The column is initially washed with a non-polar solvent (petroleum ether) to facilitate compact packing. The sample to be separated was adsorbed onto silica gel (Kiesel gel 60, mesh 70-230), allowed to dry and subsequently applied on top of the adsorbent layer. The column is then eluted with a number of organic solvents of increasing polarity and the fractions are collected.

2.1.4.1.3 Thin layer chromatography (TLC)

Ascending one-dimensional thin layer chromatographic technique is used for the initial screening of the extracts and column fractions and checking the purity of isolated compounds. For the latter purpose commercially available precoated silica gel (Kiesel gel 60 PF_{254}) plates are usually used. For initial screening, TLC plates are made on glass plates with silica gel (Kiesel gel 60 PF_{254}).

A number of glass plates measuring 20cm ×5cm are thoroughly washed and dried in an oven. The dried plates are then swabbed with acetone-soaked cotton in order to remove any fatty residue. To make the slurry required amount of silica gel 60 PF_{254} and appropriate volume of distilled water (2ml/gm of silica gel) are mixed in a conical flask and the flask is gently shaken. The slurry is then evenly distributed over the plates using TLC spreader. After air drying the coated plates are subjected to activation by heating in an oven at 110 ^{0}C for 70 minutes [42]. Table 2.1 shows the amount of silica gel required for preparing plates of varying thicknesses.

Table 2.1: Amount of silica gel required preparing TLC plates of various thicknesses

Size (cm × cm)	Thickness (mm)	Amount of silica gel/plate (gm)
20 × 5	0.3	0.9
	0.4	1.2
	0.5	1.5

Cylindrical glass chamber (TLC tank) with air-tight lid is used for the development of chromatoplates. The selected solvent system is poured in sufficient quantity into the tank. A smooth sheet of filter paper is introduced into the tank and allowed to soak in the solvent. The tank is then made airtight and kept for few minutes to saturate the internal atmosphere with the solvent vapor. A small amount of dried extract is dissolved in a suitable solvent to get a solution (approximately 1%)[43,44]. A small spot of the solution is applied on the activated silica plate with a capillary tube just 1 cm above the lower edge of the plate. The spot is dried with a hot air blower and a straight line is drawn 2cm below the upper edge of the activated plate which marks the upper limit of the solvent flow.

The spotted plate is then placed in the tank in such a way as to keep the applied spot above the surface of the solvent system and the cap/lid is placed again. The plate is left for development. When the solvent front reaches up to the given mark, the plate is taken out and air-dried. The properly developed plates are viewed under UV light of various wavelengths as well as treated with suitable reagents to detect the compounds.

Preparative thin layer chromatographic technique is routinely used in separating and for final purification of the compounds. The principle of preparative TLC is same as that of TLC. Here larger plates (20cm × 20cm) are used. Table 2.2 shows the amount of silica gel required for preparing plates of varying thicknesses.

Table 2.2: Amount of silica gel required preparing PTLC plates of various thicknesses

Size (cm × cm)	Thickness (mm)	Amount of silica gel/plate (gm)
20 × 20	0.3	3.6
	0.4	4.8
	0.5	6.0

The sample to be analyzed is dissolved in a suitable solvent and applied as a narrow uniform band rather than spot. The plates are then developed in an appropriate solvent system previously determined by TLC. In some cases multiple development technique was adopted for improved separation. After development, the plates are allowed to dry and the bands of compounds are visualized under UV light (254 nm and 365 nm) or with appropriate spray reagents on both edges of the plates. The required bands are scraped from the plates and the compounds are eluted from the silica gel by treating with suitable solvent or solvent mixtures.

2.1.4.2 Solvent treatment

Solvent treatment is a process by which a compound consisting of the major portion of a mixture of compounds can be purified utilizing selective solvent washing. Initially, a solvent or a solvent mixture in which the desired compound is practically insoluble and other components are soluble is chosen. The undesired components are separated with repeated washing with this solvent or solvent mixture. If required other solvent or solvent mixture can be used until a pure compound is obtained.

2.1.5 Visualization/ detection of compounds

Detection of compounds in TLC plates is a very important topic in analyzing extractives to isolate pure compounds. The following techniques are used for detecting the compounds in TLC/PTLC plates.

2.1.5.1 Visual detection

The developed chromatogram is viewed visually to detect the presence of coloured compounds.

2.1.5.2 UV light

The developed and dried plates are observed under UV light of both long and short wavelength (254 nm and 365 nm) to detect the spot/band of any compound. Some of the compounds appear as fluorescent spots while the others as dark spots under UV light.

2.1.5.3 Iodine chamber

The developed chromatogram is placed in a closed chamber containing crystals of iodine and kept for few minutes. The compounds that appeared as brown spots are marked. Unsaturated compounds absorb iodine. Bound iodine is removed from the plate by air blowing.

2.1.5.4 Spray reagents

Different types of spray reagents are used depending upon the nature of compounds expected to be present in the fractions or the crude extracts.

a) Vanillin/H_2SO_4[42]:

1% vanillin in concentrated sulfuric acid is used as a general spray reagent followed by heating the plates to 100 ^{0}C for 10 minutes.

b) Modified Dragendorff's reagent[44]:

Modified Dragendorff's reagent was used to detect alkaloids. Some coumarins also give a positive test with modified Dragendorff's reagent. The reagent is prepared by mixing equal parts (v/v) of 1.7% bismuth subnitrate dissolved in 20% acetic acid in water and a 40% aqueous solution of potassium iodide.

c) Ferric chloride/EtOH[45]:

Some of the phenolic compounds were detected by spraying the plates with ferric chloride (5% ferric chloride in absolute ethanol) reagent.

d) Perchloric acid reagent[44]:

2% aqueous perchloric acid produces brown spots with steroids after heating at 150 ^{0}C for 10 minutes.

e) Potassium permanganate reagent[45]:

Only the oxidizable compounds were detected by this reagent. After spraying with the reagent the compound appeared as yellow or pale yellow spot on the colored (color of permanganate) plate.

2.1.6 Determination of R_f (retardation factor) value

Retardation factor (R_f) is the ratio of the distance the compound travels and the distance the solvent front moves.

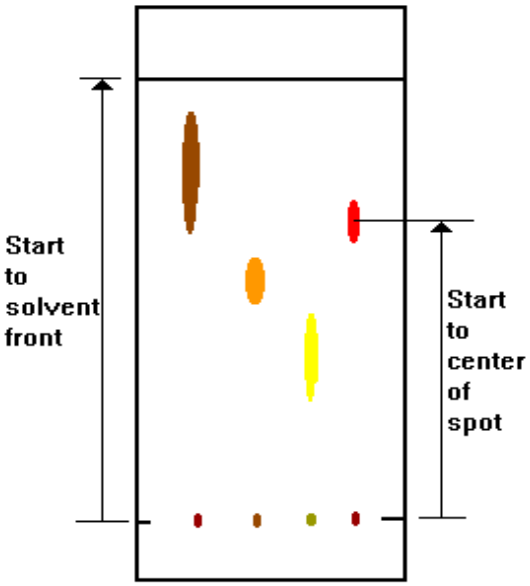


Fig 2.1: Calculation of R_f value

Usually, the R_f value is constant for any given compound and it corresponds to a physical property of that compound.R_f value is characteristic of a compound in a specific solvent system. It helps in the identification of compounds. R_f value of a compound can be calculated by the following formula:

$$\mathbf{R_f}\text{ value} = \frac{\text{Distance traveled by the compound}}{\text{Distance traveled by the solvent system}}$$

2.1.7 Stationary Phases of Column Chromatography

For normal phase column chromatography, silica gel of particle size 230-400 mesh (Merck) was used and separation was performed by gravitational flow with solvents of increasing polarity. The sample was applied into the column either as a solution or in a powdered form or by adsorbing samples by the silica gel. The eluted samples were collected in several test tubes and were monitored by TLC to make different fractions on the basis of R_f values.

For preparation of Sephadex LH-20 column, the required amount of Sephadex LH-20 gel (25-100μm, Pharmacia, Sweden) was suspended in chloroform or DCM or methanol and the column was packed with this suspended gel.

2.1.8 Procedure for Micro Scale Column Chromatography

In micro scale chromatography, the column does not need either a pinch clamp or a stopcock at the bottom of the column to control the flow, nor does it need air-pressure connection at the top of the column, the solvent flows very slowly through the column by gravity until we apply air pressure at the top of the column with an ordinary Pasteur Pipette Bulb.

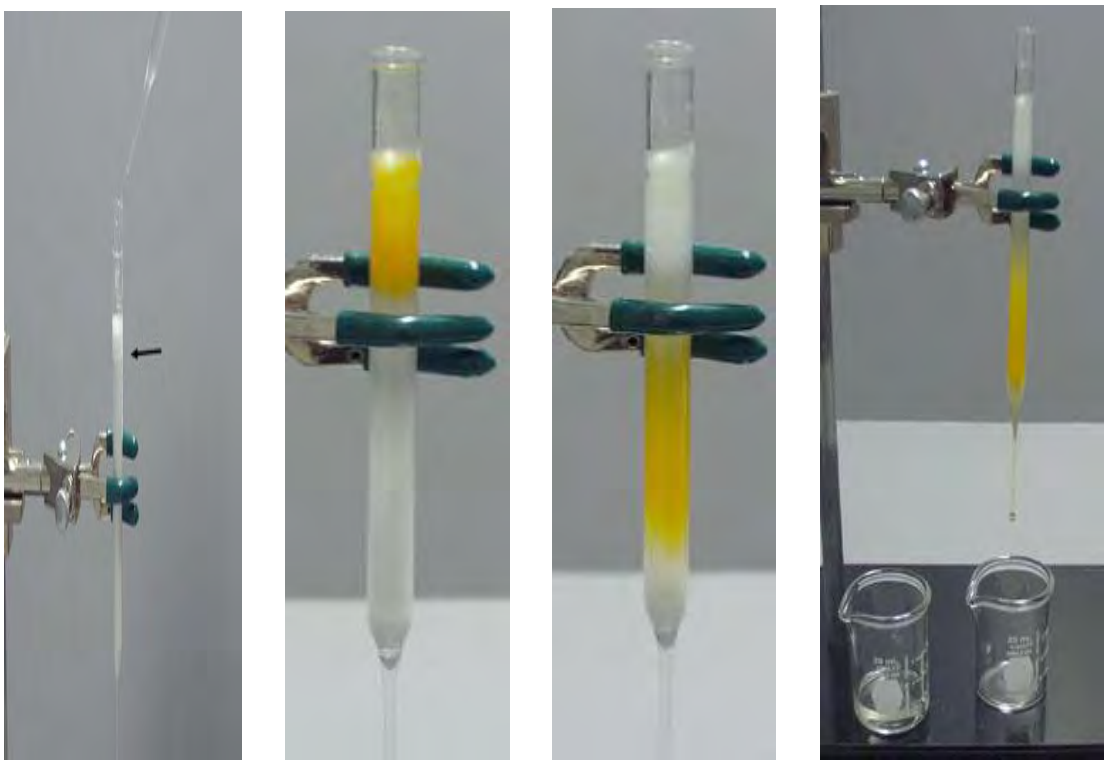


Fig 2.2: Various stages in micro scale column.

2.1.8.1 Preparation of column (For micro scale operation)

A Pasteur pipette was plugged with a small amount of cotton to prevent the adsorbent from leaking. The Pasteur Pipette was filled with the slurry of column grade silica gel with a stream of solvent using a dropper. It was ensured that the "Sub Column" is free from air bubbles by recycling the solvents several times. The samples were applied at the top of the column. Elution was started with petroleum ether or n-Hexane followed by increasing polarity.

2.1.9 Re-crystallization

Re-crystallization was employed as a final purification process. A solution of the compound in a minimum volume of the solvent in which it is soluble was prepared in hot condition. It was then left for crystallization. Sometimes, a mixture of solvents was used.

2.2 Spectroscopic Techniques

2.2.1 Ultra-Violet Spectroscopy

UV absorbance for obtaining the λ-max of the isolated compounds was taken by Shimadzu UV-1601 spectrophotometer.

2.2.2 Infra-Red Spectroscopy (IR)

A Shimadzu IR Affinity-1(FT-IR) spectrophotometer was used for recording infrared spectrum. Major bands (ν_{max}) were recorded in wave number (cm^{-1}) as KBr pellets.

2.2.3 Nuclear Magnetic Resonance (NMR) Spectroscopy

^{1}H and ^{13}C NMR spectra were recorded on a BRUKER NMR DBX-400 MHz and Varian Mercury-VX500 Fourier spectrometer. Instrument with chemical shift data reported in ppm relative to the solvent used. The spectra were taken by using $CDCl_3$ & CD_3OD with tetra methyl silane (TMS) as standard reference.

2.3 Determination of melting point

Melting point (m. p.) was determined by using an electro- thermal melting point apparatus (Mel-temp, OGAWA, SEIKICO and Japan).

2.4 Investigation of *Saurauia roxburghii*

2.4.1 Collection of the plant

The leaves of plant of *Saurauia roxburghii* was collected from Chittagong University Campus, Chittagong in the month of January, 2011.

2.4.2 Identification of species

The taxonomy of the plant was confirmed consulting with the National Herbarium's Botanist. It was identified by **Sardar Nasir Uddin, Scientific officer, Bangladesh National Herbarium, Dhaka**. A voucher specimen has been deposited in the Bangladesh National Herbarium, Dhaka (DACB Accession no. 32,567), for the collection. The leaves with the barks of this plant were separated and dried under in open air. Finally the dried leaves were ground into a coarse powder (~200 mesh) using a grinding machine. This powder (1.25Kg) was used throughout this investigation.

2.4.3 Test of steroids

Salkowski reaction[43]: A few crystals or extract was dissolved in chloroform and a few drops of concentrated sulfuric acid were added to the solution. A reddish color was seen in the upper chloroform layer. When a reddish color is developed, indicating the presence of steroidal compound.

Liebermann-burchard reaction[43]: A few crystals or extract was dissolved in chloroform and a few drops of concentrated sulfuric acid were added to it followed by addition of 2-3 drops of aceticanhydride. Solution turned violet blue and finally green. When a greenish color is developed, indicating the presence of steroidal compound.

2.4.4 Test of alkaloid

4gm of cerric ammonium nitrate was dissolved in 10ml of 2N HNO_3, on mild heating. A few crystals of isolated compound were dissolved in 0.5ml of dioxane. The solution was added to 0.5ml of cerric ammoinium nitrate reagent and diluted to 1ml with dioxane and shaken well. The developed yellow to red color indicates the presence of an alcoholic hydroxyl group.

Extracted 2gm of powder drug by warming for two minutes with 20ml 1% H_2SO_4 in a 50ml conical flask on a water bath, with intermittent shaking, centrifuge; pipette off supernatant into a small conical flask. Make an initial test for alkaloids by adding to 0.1ml in a semi-micro tube, one drop of Meyer's reagent. It gives a cream precipitate with alkaloids.

2.4.4.1 Preparation of Meyer's reagent:

It is prepared by dissolving 1.36 gm of mercuric chloride in 60ml distilled water (A) & 5gm of potassium iodide in 10ml of distilled water (B). A & B are mixed together and the volume is adjusted to 100ml with water.

2.4.5 Test of terpenoids

A few mg of sample was dissolved in a mixture of $CHCl_3$-CH_3OH, and then few drops of conc. H_2SO_4 were added to it followed by 4-6 drops of Ac_2O. A red-violet color is developed, indicating the presence of terpenoid type compounds.

2.4.6 Extraction of the Plant Material

About 1.0 Kg of the powdered material was taken in a clean, round bottomed flask (5 liters) and soaked in 3 liter of methanol. The container with its content was sealed by foil and kept for a period of 7 days accompanying occasional shaking and stirring. The whole mixture was then filtered through filter paper and the filtrate. The filtrate was then evaporated under reduced pressure at $(40\text{-}50)^0$C using a Buchii Rotary Evaporator and gummy concentrate of the crude extract was obtained.

2.4.7 Solvent-solvent partition of crude extract

Solvent -solvent partitioning of the crude concentrated methanolic extract was done using the protocol designed by Kupchan and modified by Van Wagenen[46].The extract was dissolved in 90% methanol. It was extracted with n-Hexane ($60\text{-}80^0$C), then with chloroform ($CHCl_3$), and finally with ethylacetate (EA) .The whole partition process is schematically shown in **Figure 2.3**. All of the fractions were concentrated with a Buchii rotary evaporator a low temperature ($40^0\text{-}50^0$ C) and reduced pressure. These were collected for further analysis.

2.4.7.1 Partitioning with n-Hexane

The mother solution was taken in a separating funnel and 100 ml of the n-Hexane was added to it and the funnel was shaken and then kept undisturbed. The organic portion was collected. The process was repeated thrice; n-Hexane fractions were collected together (2.5 gm) and evaporated. The aqueous fraction was preserved for the next step.

2.4.7.2 Partitioning with chloroform

The mother solution left after washing n-Hexane was mixed with 12.5 ml of distilled water. The mother solution was then taken in a separating funnel and extracted with $CHCl_3$ (100ml×3). The $CHCl_3$ fractions were collected together (2.0gm) and evaporated. The aqueous fraction was preserved for the next step.

2.4.7.3 Partitioning with ethyl-acetate

The mother solution that left after washing n-Hexane and $CHCl_3$ was mixed with 16 ml of distilled water uniformly. The mother solution was then taken in a separating funnel and extracted with ethylacetate (EA) (100 ml×3). The ethylacetate (EA) soluble fractions were collected together (1.0gm) and evaporated. The aqueous ethanolic fraction was preserved as aqueous fraction.

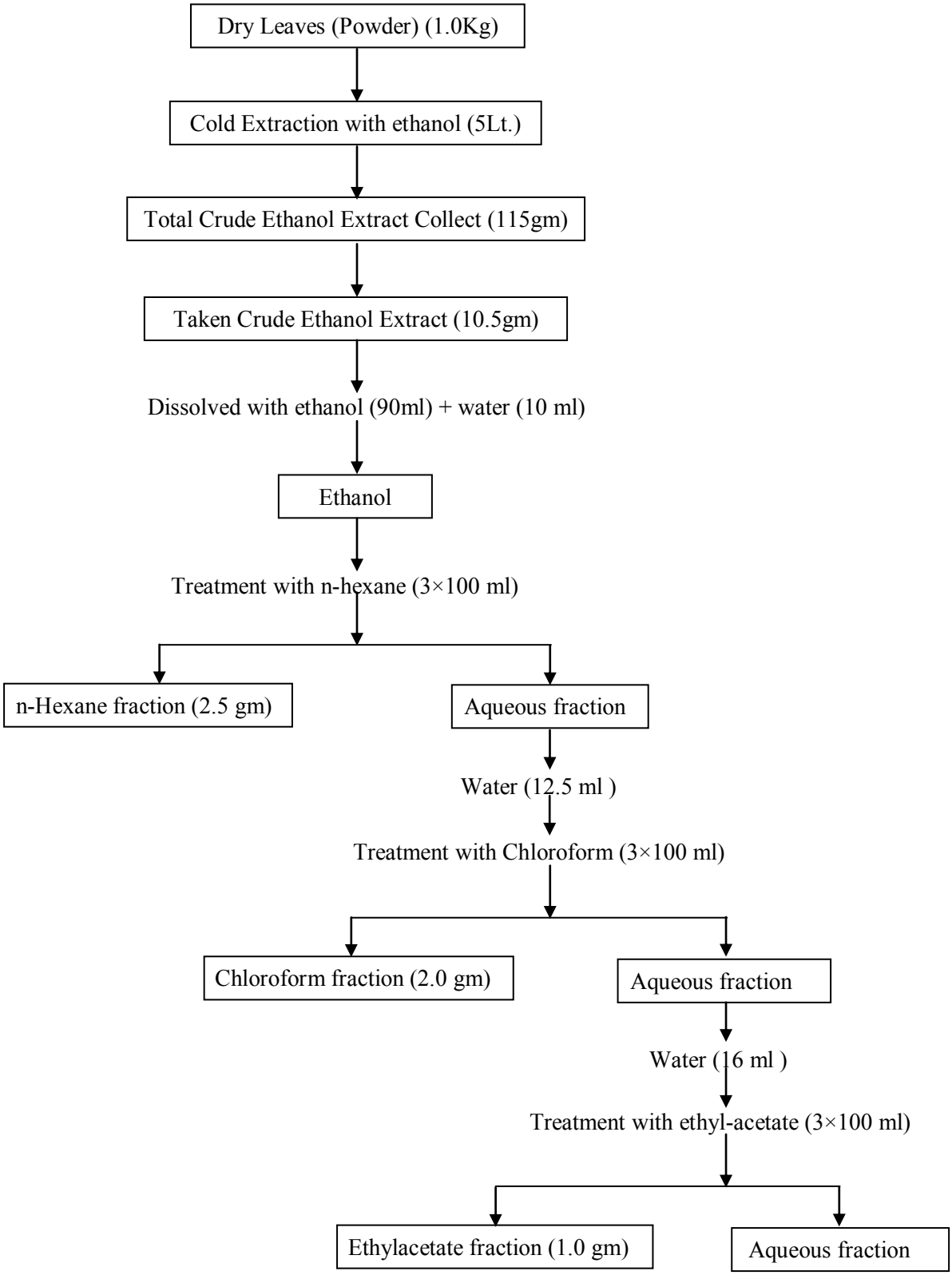


Figure 2.3: Schematic representation of modified Kupchan partitioning of the crude ethanol extract of ***Saurauia roxburghii.***

Thus four types of crude extracts were found:

a) n-Hexane extract (2.5 gm)

b) Chloroform extract (2.0 gm)

c) Ethyl-acetate extract (1.0 gm)

d) Aqueous fraction (5.0)

2.4.8 Investigation of the n-Hexane soluble extract

The n-Hexane extract was subjected to TLC screening to see the type of compounds present in the extract. A portion of the n-Hexane extract (2.5 gm) was subjected to Column Chromatography (CC) for rapid fractionation. The CC fractions were screened by TLC to find out interesting fractions.

2.4.8.1 Column Chromatography (CC) of n-Hexane extract

The column was packed with fine TLC grade silica gel (Kiesel gel 60H, mesh 70-230) was used as the packing material. A column having 40 cm length and 3 cm in diameter was packed with the silica gel (70 gm) up to a height of 23 cm under reduced pressure. The column was washed with n-Hexane to facilitate compact packing. The sample was prepared by adsorbing 3.5 gm of n-Hexane soluble extract onto silica gel (Kiesel gel 60H, mesh 70-230), allowed to dry and subsequently applied on top of the adsorbent layer. The column was then eluted with n-Hexane followed by mixtures of n-Hexane and dichloromethane and then dichloromethane and methanol. The polarity was gradually increased by adding increasing proportions of dichloromethane and methanol. Solvent systems used as mobile phases in the column chromatography analysis of n-Hexane soluble extractive are listed in **Table: 2.4.** A total of 30 fractions were collected each in 100 ml beakers.

Table 2.4: Different solvent systems used for Column Chromatography (CC) analysis of n-Hexane extract

Fraction no.	Solvent systems	Volume Collected (ml)
H_1	n-Hexane (100%)	100
H_2	n-Hexane + dichloromethane (97.5 : 2.5)	50
H_3	n-Hexane +dichloromethane (95 : 5)	100
H_4	n-Hexane +dichloromethane (92.5 : 7.5)	100
H_5	n-Hexane +dichloromethane (90 : 10)	50
H_6	n-Hexane +dichloromethane (87.5 : 12.5)	100
H_7	n-Hexane + dichloromethane (85 : 15)	50
H_8	n-Hexane + dichloromethane (82.5 : 17.5)	50
H_9	n-Hexane + dichloromethane (80 : 20)	100
H_{10}	n-Hexane + dichloromethane (77.5 : 22.5)	50
H_{11}	n-Hexane + dichloromethane (75 : 25)	100
H_{12}	n-Hexane + dichloromethane (70 : 30)	100
H_{13}	n-Hexane + dichloromethane (65 : 35)	100
H_{14}	n-Hexane + dichloromethane (60 : 40)	100
H_{15}	n-Hexane + dichloromethane (55 : 45)	100
H_{16}	n-Hexane + dichloromethane (50 : 50)	100
H_{17}	n-Hexane + dichloromethane (40 : 60)	100
H_{18}	n-Hexane + dichloromethane (30 : 20)	100
H_{19}	n-Hexane + dichloromethane (20 : 80)	100
H_{20}	n-Hexane + dichloromethane (10 : 90)	100
$H_{21(a+b)}$	Dichloromethane (100%)	200
H_{22}	Methanol +dichloromethane (1 : 99)	100
H_{23}	Methanol +dichloromethane (5 : 95)	100
H_{24}	Methanol +dichloromethane (10 : 90)	100
H_{25}	Methanol +dichloromethane (25 : 75)	100
H_{26}	Methanol +dichloromethane (50 : 50)	100
H_{27}	Methanol +dichloromethane (75 : 25)	100
$H_{28(a+b)}$	Methanol (100%)	200

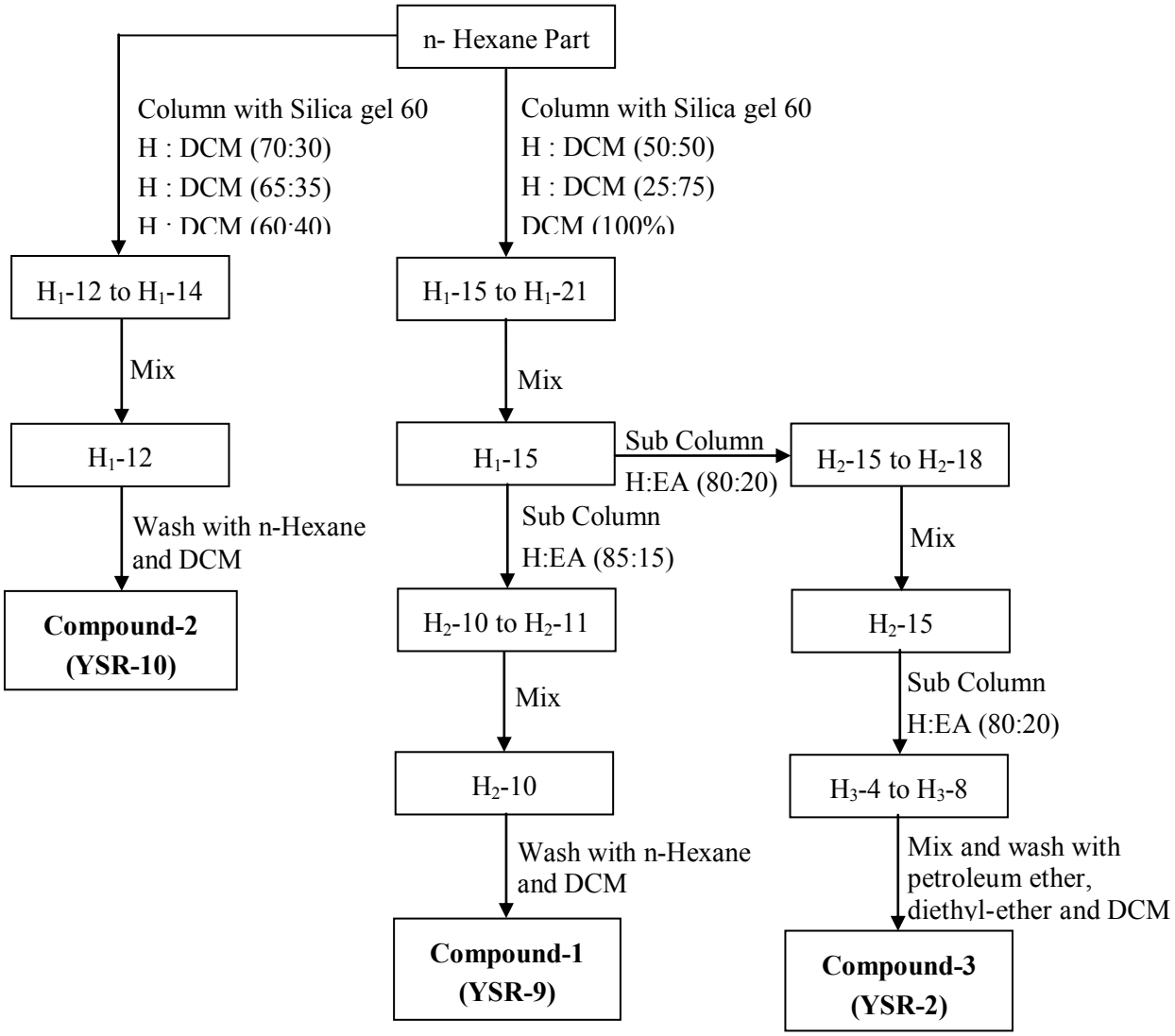


Figure 2.4: Scheme for the isolation of compounds from n-Hexane part.

2.4.8.2 Analysis of the fractions by Column Chromatography of n-Heexane

Fraction H_1-1 to H_1-11 & H_1-22 to H_1-28 shows no prominent spot on TLC analysis. So, they were discarded.

The fractions H_1-15 to H_1-21 shows similar spots in TLC analysis (R_f = 0.82; n-Hexane: ethyl acetate =1:1); they were got mixed and named as H_1-15. As they contain chlorophyll and fatty materials, so they were done sub-column using silica gel (Kiesel gel 60H, mesh 70-230) as stationary phase and n-Hexane: EA ≡ 85:15 as a mobile phase. Among the fractions, the TLC analyses of H_2-10 to H_2-11 were similar. So they got mixed and named H_2-10. After solvent evaporation of fraction H_2-10 white needle shaped crystal was formed which was further purified by washing with different solvents. As a result mother solution was

obtained leaving back the white needle shaped crystals. This needle shape crystal was transferred to a vial and was designated as **Compound-1 (YSR-9)**, which was soluble in dichloromethane, chloroform and ethyl acetate.

Fractions H_1-12 to H_1-14 were mixed together for their similar R_f value (R_f = 0.81; n-Hexane: ethyl acetate =1:1) and named H_1-12. Then this fraction was washed with n-Hexane to remove rest of fatty materials and dust thus the white crystalline compound was obtained and designated as **Compound-2(YSR-10)**, which was soluble in dichloromethane, chloroform and ethyl acetate.

On the other hand, the second fraction H_2-15 to H_2-18 shows similar spots in TLC analysis (R_f = 0.76; n-Hexane: ethyl acetate =1:1). with a little bit contamination. So, it was done a sub-column using n-Hexane: EA ≡ 80:20. But still containing some tailing in the TLC analysis, it was washed with petroleum ether, diethyl-ether carefully. As a result mother solution was obtained leaving back the white amorphous powder. This amorphous powder was transferred to a vial and was designated as **Compound-3 (YSR-2)**, which was soluble in dichloromethane or chloroform with few drops of methanol.

2.4.9 Investigation of the chloroform soluble extract

The chloroform extract was subjected to TLC screening to see the type of compounds present in the extract. A portion of the chloroform extract (2.0 gm) was subjected to Column Chromatography (CC) for rapid fractionation. The CC fractions were screened by TLC to find out interesting fractions.

2.4.9.1 Column Chromatography (CC) of chloroform extract

A column having 40 cm length and 3 cm in diameter was packed with the silica gel (60 gm) up to a height of 20 cm under reduced pressure. The sample was prepared by adsorbing 2.0 gm of chloroform soluble extract onto silica gel (Kiesel gel 60H, mesh 70-230), allowed to dry and subsequently applied on top of the adsorbent layer. The column was then eluted with n-Hexane followed by mixtures of n-Hexane and dichloromethane and then dichloromethane and methanol. The polarity was gradually increased by adding increasing proportions of dichloromethane and methanol. Solvent systems used as mobile phases in the column chromatography analysis of n-Hexane soluble extractive are listed in **Table: 2.5.** A total of 29 fractions were collected each in 100 ml beakers.

Table 2.5: Different solvent systems used for Column Chromatography (CC) analysis of chloroform extract

Fraction no.	Solvent systems	Volume Collected (ml)
C_1	n-Hexane (100%)	100
C_2	n-Hexane + dichloromethane (99.5 : 0.5)	50
C_3	n-Hexane +dichloromethane (99 : 1)	100
C_4	n-Hexane + dichloromethane (97.5 : 2.5)	100
C_5	n-Hexane +dichloromethane (95 : 5)	50
C_6	n-Hexane +dichloromethane (90 : 10)	100
C_7	n-Hexane + dichloromethane (85 : 15)	50
C_8	n-Hexane + dichloromethane (80 : 20)	50
C_9	n-Hexane + dichloromethane (75 : 25)	100
C_{10}	n-Hexane + dichloromethane (70 : 30)	50
C_{11}	n-Hexane + dichloromethane (55 : 45)	100
C_{12}	n-Hexane + dichloromethane (50 : 50)	100
C_{13}	n-Hexane + dichloromethane (40 : 60)	100
C_{14}	n-Hexane + dichloromethane (30 : 20)	100
C_{15}	n-Hexane + dichloromethane (20 : 80)	100
C_{16}	n-Hexane + dichloromethane (10 : 90)	100
$C_{17(a+b)}$	Dichloromethane (100%)	200
C_{18}	Methanol +dichloromethane (0.5 : 99.5)	100
C_{19}	Methanol +dichloromethane (1 : 99)	100
C_{20}	Methanol +dichloromethane (2 : 98)	100
C_{21}	Methanol +dichloromethane (5 : 95)	100
C_{22}	Methanol +dichloromethane (10 : 90)	100
C_{23}	Methanol +dichloromethane (15 : 85)	100
C_{24}	Methanol +dichloromethane (25 : 75)	100
C_{25}	Methanol +dichloromethane (30 : 70)	100
C_{26}	Methanol +dichloromethane (50 : 50)	100
$C_{27(a+b)}$	Methanol (100%)	200

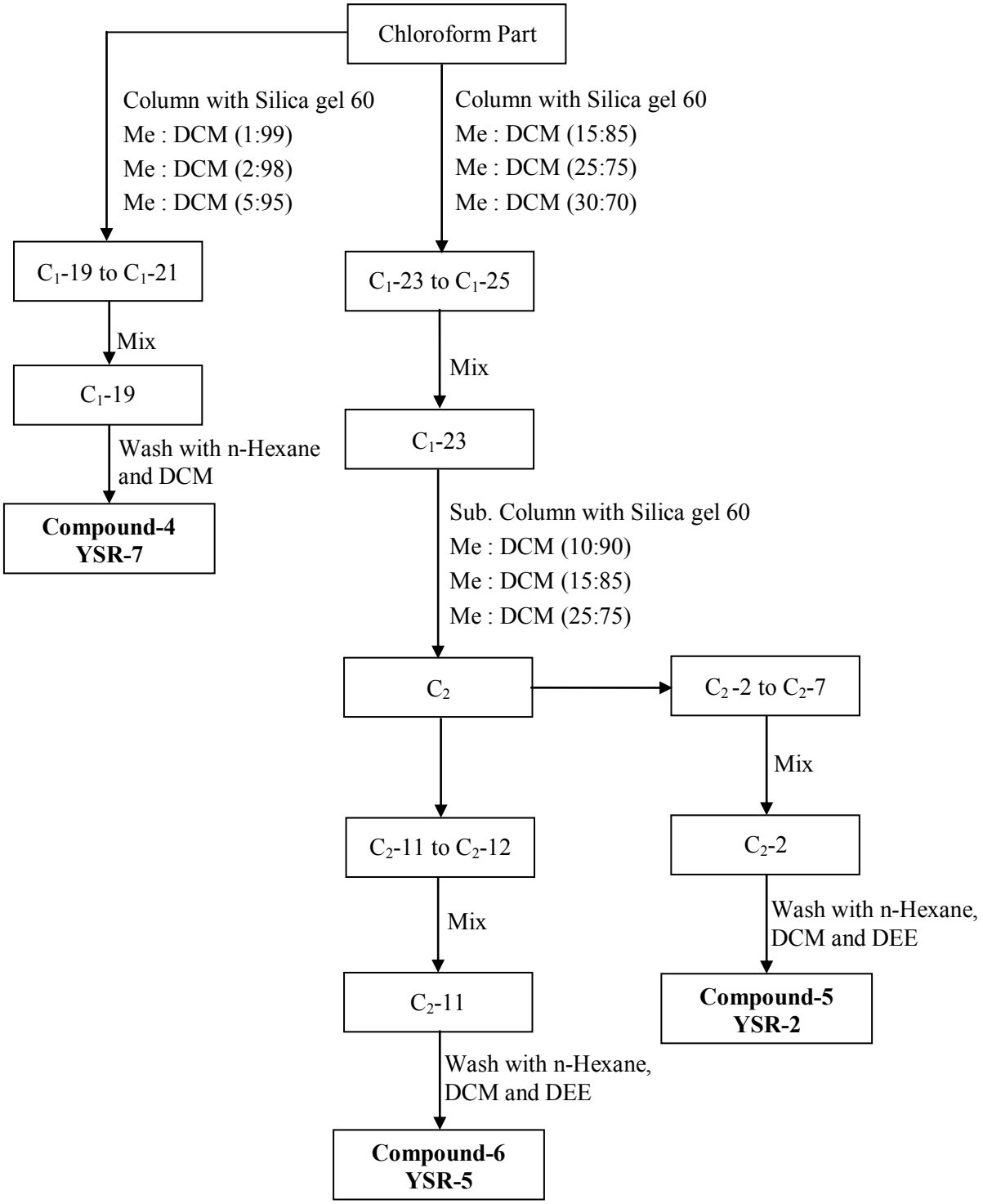


Figure 2.5: Scheme for the isolation of compounds from chloroform part.

2.4.9.2 Analysis of the fractions by Column Chromatography of chloroform extract

Fraction C_1-1 to C_1-18 shows no prominent spot on TLC analysis. So, they were discarded.

Fractions C_1-19 to C_1-21 were mixed together for their similar R_f value (R_f = 0.54; Toluene: ethyl acetate=9:1) and named C_1-18. Then this fraction was washed with n-Hexane to remove rest of fatty materials and dust. As a result mother solution was obtained leaving back the colorless sharp crystals. This sharp crystal was transferred to a vial and was designated as **Compound-4 (YSR-7)** was obtained, which was soluble in dichloromethane, chloroform and ethyl acetate.

The rest fractions C_1-23 to C_1-25 shows similar spots in TLC analysis with a little bit contamination; they were got mixed and named C_1-23. As they contain chlorophyll and fatty materials, so they were done sub-column using silica gel (Kiesel gel 60H, mesh 70-230) as stationary phase and chloroform: methanol of different percentage as a mobile phase. Among the fractions, the TLC analyses of C_2-2 to C_2-7 were similar R_f value (R_f = 0.73; chloroform: methanol =9:1). So they got mixed and named C_2-2. After solvent evaporation of fraction C_2-2 white amorphous powder was formed which was further purified by washing with different solvents. As a result mother solution was obtained leaving back the white amorphous powder which was transferred to a vial and designated as **Compound-5 (YSR-3).**

On the other hand, the fractions C_2-11 and C_2-12 shows similar spots in TLC analysis with the same R_f value. (R_f = 0.87; n-Hexane: ethyl acetate =2:3). It was washed with n-Hexane, chloroform, diethyl-ether carefully and finally which is soluble in chloroform with methanol. As a result mother solution was obtained leaving back the white amorphous powder. This amorphous powder was transferred to a vial and was designated as **Compound-6 (YSR-5)**.

2.5 Characterization of isolated compounds from *Saurauia roxburghii*

2.5.1 Properties of compound-1 (YSR-9)

2.5.1.1 Physical properties

The compound-1 (YSR-9) was obtained as a white needle shaped crystal. The R_f value of the compound was 0.86 in n-hexane: EA = 50:50. It was soluble in chloroform, dichloromethane, ethyl acetate, methanol & ethanol. It was tested by Salkawoski and Liebermann-burchard method which developed a reddish color and greenish color respectively indicating that the compound may be steroid. The melting point of this compound was found to be 138-140^{0}C.

2.5.1.2 Characterization of compound-1 (YSR-9) by spectroscopic method

2.5.1.2.1 Ultraviolet (UV) spectroscopy of compound-1 (YSR-9)

UV spectrum of the compound had absorption at λ_{max} 219, 280, 415, 545 and 640 nm in methanol.

2.5.1.2.2 Infrared (IR) spectroscopy of compound-1 (YSR-9)

IR (KBr) $\upsilon_{max}cm^{-1}$: 3446.79, 2935.66, 2866.22, 1653.0, 1458.18, 1375.25, 1055.06 and 883.4.

2.5.1.2.3 ^{1}H-NMR spectroscopy of compound-1 (YSR-9)

^{1}H-NMR (400 MHz,$CDCl_3$) δ_H (ppm): 5.34 (1H, d, 5.2 Hz, H-6), 5.16 (1H, dd, *J*=15.0, 8.4 Hz, H-22), 5.03 (1H, dd, *J*=15.0, 8.4 Hz, H-23), 3.52 (1H, dd, 9.6, 4.8 Hz, H-3), 1.00, 0.67 (3H, s, Me-19 and Me-18), 0.92 (3H, d,6.0 Hz, Me-21), 0.85 (3H, d, 8.0 Hz, Me-29), 0.81 (3H, d, 7.2 Hz, Me-26) and 0.79 (3H, d, 7.2 Hz, Me-27).

2.5.1.2.4 ^{13}C-NMR spectroscopy of compound-1 (YSR-9)

^{13}C-NMR (400 MHz, $CDCl_3$) δ_C (ppm): 37.29 (CH_2,C-1), 28.28 (CH_2,C-2), 71.85 (CH,C-3), 42.36 (CH_2,C-4), 140.81 (Cq,C-5), 121.74 (CH,C-6), 31.72 (CH_2,C-7), 34.01 (CH,C-8), 50.2 (CH,C-9), 36.54 (Cq,C-10), 26.17 (CH_2,C-11), 39.83 (CH_2,C-12), 42.37 (Cq,C-13), 56.82 (CH,C-14), 24.33 (CH_2,C-15), 29.23 (CH_2,C-16), 56.12 (CH,C-17), 12.02 (CH_3,C-18), 19.41 (CH_3,C-19), 40.50 (CH_2,C-20), 21.13 (CH_3, C-21), 138.33 (CH,C-22), 129.0 (CH,C-23), 51.28 (CH,C-24), 45.91 (CH,C-25), 19.42 (CH_3, C-26), 19.84 (CH_3,C-27), 24.34 (CH_2,C-28), 12.26 (CH_3,C-29).

2.5.2 Properties of compound-2 (YSR-10)

2.5.2.1 Physical properties

The compound-2 (YSR-10) was obtained as white powdered crystal. The R_f value of the compound was 0.33 in toluene: ethyl acetate = 95:5. It was soluble in chloroform, dichloro-methane, ethyl acetate, methanol and ethanol. It was tested by Salkawoski and Liebermann-burchard method which developed a reddish color and greenish color respectively indicating that the compound may be steroid. The melting point of this compound was found to be 130^0-135^0C.

2.5.2.2 Characterization of compound-2 (YSR-10) by spectroscopic method

2.5.2.2.1 Ultraviolet (UV) spectroscopy of compound-2 (YSR-10)

The UV spectrum of the compound-2 had absorption at λ_{max} 503, 545, 615 and 670 nm in methanol.

2.5.2.2.2 Infrared (IR) spectroscopy of compound-2 (YSR-10)

IR (KBr) υ_{max} cm^{-1}: 3421.72, 2935.66, 2866.22, 1653.00, 1458.18, 1375.25, 1062.78, 883.40 and 800.46.

2.5.2.2.3 ^{1}H-NMR spectroscopy of compound-2 (YSR-10)

^{1}H-NMR (400 MHz, $CDCl_3$) δ_H (ppm): 5.34 (1H, d, *J*=5.2 Hz, H-6), 3.51 (1H, m, H -3), 1.00, 0.67 (3H, each, s, Me-19 and Me-18), 0.92 (3H, d, 6.0 Hz, Me-21), 0.85 (3H, d, 8.0 Hz, Me-29), 0.83 (3H, d, 7.2 Hz, Me-26) and 0.79 (3H, d, 7.2 Hz, Me-27).

2.5.2.2.4 ^{13}C-NMR spectroscopy of compound-2 (YSR-10)

^{13}C-NMR (400 MHz, $CDCl_3$) δ_C (ppm): 37·29 (CH_2,C-1), 31.95 (CH_2,C-2), 71·84 (CH,C-3), 42·36 (CH_2,C-4), 140·80 (Cq,C-5), 121·73 (CH,C-6), 31·71 (CH_2,C-7), 31·95 (CH,C-8), 50·19 (CH,C-9), 36·18 (Cq,C-10), 21·12 (CH_2,C-11), 39·82 (CH_2,C-12), 42·36 (Cq,C-13), 56·81 (CH,C-14), 24·33 (CH_2,C-15), 28.26 (CH_2,C-16), 56·11 (CH,C-17), 11·88 (CH_3,C-18), 19·41 (CH_3,C-19), 36.54 (CH,C-20), 19·07 (CH_3,C-21), 34·00 (CH_2,C-22), 26·16 (CH_2,C-23), 45·89 (CH,C-24), 29.23 (CH,C-25), 19·83 (CH_3,C-26), 18.81 (CH_3,C-27), 23.12 (CH_2,C-28), 12·01 (CH_3,C-29).

2.5.3 Properties of compound-3 (YSR-2)

2.5.3.1 Physical properties

The compound-3 (YSR-2) was obtained as white amorphous powder compound. The R_f value of the compound was 0.73 in chloroform: methanol: 90:10. It was soluble in chloroform with 2-3 drops of methanol. It was tested for terpenoid & a red-violet color confirms that the compound-3 (YSR-2) was terpenoid type compound. The melting point was found to be 271-273^{0}C.

2.5.3.2 Characterization of compound-3 (YSR-2) by spectroscopic method

2.5.3.2.1 Ultraviolet (UV) spectroscopy of compound-3 (YSR-2)

The UV spectrum of the compound-3 (YSR-2) had absorption at λ_{max} 202 nm in methanol.

2.5.3.2.2 Infrared (IR) spectroscopy of compound-3 (YSR-2)

IR (KBr) υ_{max} cm^{-1}: 3446.79, 3101.54, 2926.01, 2854.65, 1734.01, 1653.00, 1458.18, 1375.25, 1163.08, 1035.77 and 815.89.

2.5.3.2.3 ^{1}H-NMR spectroscopy of compound-3 (YSR-2)

^{1}H-NMR (500 MHz, $CDCl_3$ + CD_3OD) δ_H (ppm) : 5.18 (1H, brs, H-12), 3.13 (1H, dd,10.5, 5.5 Hz H-3), 2.14(1H, d, 11.5 Hz, H-18), 0.88 (3 H, d, 5.5 Hz, H-29), 0.81 (3 H, d, 6.5 Hz, H-30), 1.05, 1.04, 0.92, 0.90 and 0.77 (3H, each, s, Me).

2.5.3.2.4 ^{13}C-NMR spectroscopy of compound-3 (YSR-2)

^{13}C-NMR (125 MHz, $CDCl_3$ + CD_3OD) δ_C (ppm) : 39.3 (CH_2,C-1), 27·3 (CH_2,C-2), 79.3 (CH,C-3), 39.7 (Cq,C-4), 55.9 (CH,C-5), 18.9(CH_2, C-6), 33.7 (CH_2,C-7), 40.1 (Cq,C-8), 49.5 (CH,C-9), 37·5 (Cq,C-10), 23·8 (CH_2,C-11), 126.1 (CH,C-12), 138.8 (Cq,C-13), 42.6 (Cq,C-14), 28.6 (CH_2,C-15), 24.8 (CH_2,C-16), 49.7 (Cq,C-17), 53.5 (CH,C-18), 39.5 (CH_2,C-19), 39.3 (Cq,C-20), 31.2 (CH_2,C-21), 37.4 (CH_2,C-22),28. 5(CH_3, C-23), 16.1 (CH_3,C-24), 15.9 (CH_3,C-25), 17.4 (CH_3,C-26), 24.0 (CH_3,C-27), 181.3 (Cq,C-28), 17.5 (CH_3,C-29), 21.6 (CH_3,C-30)

2.5.4 Properties of compound-4 (YSR-7)

2.5.4.1 Physical properties

The compound-4 (YSR-7) was obtained as white crystalline powder. It appeared as dark quenching spot on the TLC plate under UV light at 254 nm and also exhibited blue fluorescence at 365 nm (R_f = 0.54; Toluene/10% ethyl acetate). It was soluble in DCM, chloroform and ethyl acetate. It was appeared as a purple spot on the TLC plate when sprayed with vanillin-sulfuric acid spray reagent, followed by heating at 110^0C, for 5 minutes, which gave positive Lieberman-Burchard test for triterpenes. The melting point was found to be 197-198^{0}C.

2.5.4.2 Characterization of compound-4 (YSR-7) by spectroscopic method

2.5.4.2.1 Ultraviolet (UV) spectroscopy of compound-4 (YSR-7)

The UV spectrum of the compound-4 (YSR-7) had absorption at λ_{max} 265 nm in methanol.

2.5.4.2.2 Infrared (IR) spectroscopy of compound-4 (YSR-7)

IR (KBr) υ_{max} cm^{-1}: 3446.79, 2920.23, 2848.86, 1653.00, 1458.18, 1375.25, 1174.65, 1035.77 and 821.68.

2.5.4.2.3 ^{1}H-NMR spectroscopy of compound-4 (YSR-7)

^{1}H-NMR (400 MHz, $CDCl_3$) δ_H (ppm) : 5.12 (1H, t, *J*=3.2 Hz, H-12), 3.21 (1H, dd, 10.0, 5.0 Hz, H -3), 1.07, 1.00, 0.99, 0.95, 0.91, 0.86, 0.79 and 0.79 (3H, each, s, Me).

2.5.4.2.4 ^{13}C-NMR spectroscopy of compound-4 (YSR-7)

^{13}C-NMR (125 MHz, CD_3OD) δ_C (ppm) : 39.3 (CH_2, C-1), 21.5 (CH_2, C-2),79.3 (CH, C-3), 39.7 (Cq, C-4), 55.9 (CH, C-5), 18.9 (CH_2, C-6), 33.7 (CH_2, C-7), 40.1 (Cq, C-8), 49.5 (CH, C-9), 37·5 (Cq, C-10), 23·8 (CH_2, C-11), 124.1 (CH, C-12), 143.5 (Cq, C-13), 42.6 (Cq, C-14), 28.6 (CH_2, C-15), 24.8 (CH_2, C-16), 48.2 (Cq, C-17), 41.9 (CH, C-18), 48.4 (CH_2, C-19), 31.3 (Cq, C-20), 39.2 (CH_2, C-21), 37.4 (CH_2, C-22), 28.5 (CH_3, C-23), 16.1 (CH_3, C-24), 15.9 (CH_3, C-25), 17.4 (CH_3, C-26), 26.5 (CH_3, C-27), 28.5 (CH_3, C-28), 33.1 (CH_3, C-29), 24.0 (CH_3, C-30).

2.5.5 Properties of compound-5 (YSR-3)

2.5.5.1 Physical properties

The compound-5 (YSR-3) was obtained as a white amorphous powder compound. The R_f value of the compound was 0.76 in n-hexane: ethylacetate = 50:50. It was soluble in chloroform, dichloromethane, ethyl acetate & ethanol. It was tested for terpenoid & a red-violet color confirms that the compound-5 (YSR-3) was terpenoid type compound. The melting point was found to be 303-305^{0}C.

2.5.5.2 Characterization of compound-5 (YSR-3) by spectroscopic method

2.5.5.2.1 Ultraviolet (UV) spectroscopy of compound-5 (YSR-3)

The UV spectrum of the compound YSR-3 had absorption at λ_{max} 215 and 322 nm in methanol.

2.5.5.2.2 Infrared (IR) spectroscopy of compound-5 (YSR-3)

IR (KBr) υ_{max} cm^{-1}: 3298.28, 3122.75, 2920.23, 2852.72, 1716.65, 1653.00, 1458.18, 1375.25 1188.15, 1035.77 and 827.46.

2.5.5.2.3 ^{1}H-NMR spectroscopy of compound-5 (YSR-3)

^{1}H-NMR (500 MHz, $CDCl_3$) δ_H (ppm): 5.06 (1H, brs, H-12), 3.52 (1H, m, H -3), 3.14 (1H, dd, 8.0, 8.0 Hz, H-18), 1.01, 0.95, 0.92, 0.89, 0.85, 0.81 and 0.73 (3H, each, s, Me).

2.5.5.2.4 ^{13}C-NMR spectroscopy of compound-5 (YSR-3)

^{13}C-NMR (125 MHz, $CDCl_3$ + CD_3OD) δ_C (ppm): 39.3 (CH_2,C-1), 21.5 (CH_2,C-2), 79.3 (CH,C-3), 39.7 (Cq,C-4), 55.9 (CH,C-5), 18.9 (CH_2, C-6), 33.7 (CH_2,C-7), 40.1 (Cq,C-8), 49.5 (CH,C-9), 37·5 (Cq,C-10), 23·8 (CH_2,C-11), 124.1 (CH,C-12), 143.5 (Cq,C-13), 42.6 (Cq,C-14), 28.6 (CH_2,C-15), 24.8 (CH_2,C-16), 48.2(Cq, C-17), 41.9 (CH,C-18), 48.4 (CH_2,C-19), 31.3 (Cq,C-20), 39.2 (CH_2,C-21), 37.4 (CH_2,C-22), 28.5 (CH_3,C-23), 16.1 (CH_3,C-24), 15.9 (CH_3,C-25), 17.4 (CH_3,C-26), 26.5 (CH_3,C-27), 181.9 (Cq,C-28), 33.1 (CH_3,C-29), 24.0 (CH_3,C-30) .

2.5.6 Properties of compound-6 (YSR-5)

2.5.6.1 Physical properties

The compound-6 (YSR-5) was obtained as a white amorphous powder compound. The R_f value of the compound was 0.66 in n-hexane: ethyl-acetate = 50:50. It was soluble in chloroform, dichloromethane, ethyl acetate & ethanol. It was tested for terpenoid and a red-violet color confirms that the compound-6 (YSR-5) was terpenoid type compound. The melting point was found to be 295-298^{0}C.

2.5.6.2 Characterization of compound-6 (YSR-5) by spectroscopic method

2.5.6.2.1 Ultraviolet (UV) spectroscopy of compound-6 (YSR-5)

The UV spectrum of the compound-6 (YSR-5) had absorption at λ_{max} 255, 295, 355 and 422 nm in methanol.

2.5.6.2.2 Infrared (IR) spectroscopy of compound-6 (YSR-5)

IR (KBr) υ_{max} cm^{-1}: 3446.79, 2924.09, 2852.72, 1701.22, 1653.00, 1458.18, 1375.25, 1166.93, 1055.06 and 813.96.

2.5.6.2.3 ^{1}H-NMR spectroscopy of compound-6 (YSR-5)

^{1}H-NMR (500 MHz, CD_3OD) δ_H (ppm): 5.23 (1H, like t, H-12), 3.60 (1H, m, H -2), 2.89 (1H, d, 10.0 Hz, H-3), 2.82 (1H, dd, 10.0, 2.0 Hz, H-18), 1.14, 0.99, 0.98, 0.92, 0.89, 0.82 and 0.78 (3H, each, s, Me).

2.5.6.2.4 ^{13}C-NMR spectroscopy of compound-6 (YSR-5)

^{13}C-NMR (125 MHz, CD_3OD) δ_C (ppm): 40.06 (CH_2, C-1), 67.18 (CH, C-2), 78.29 (CH, C-3), 37.49 (Cq, C-4), 48.39 (CH, C-5), 17.48 (CH_2, C-6), 31.24 (CH_2, C-7),39.27 (Cq, C-8), 48.18 (CH, C-9), 37.42 (Cq, C-10), 21.55 (CH_2, C-11), 122.14 (CH, C-12), 144.01(Cq, C-13), 42.64 (Cq, C-14), 27.26 (CH_2, C-15), 24.01(CH_2, C-16), 48.64 (Cq, C-17), 39.69 (CH, C-18), 42.64 (CH_2, C-19), 28.59(Cq, C-20), 33.65 (CH_2, C-21), 31.24 (CH_2, C-22), 28.50 (CH_3, C-23), 23.84 (CH_3, C-24), 16.11 (CH_3, C-25),17.38 (CH_3, C-26), 26.1 (CH_3, C-27), 182.17(Cq, C-28), 33.1 (CH_3, C-29), 24.78 (CH_3, C-30).

CHAPTER-3

RESULTS AND DISCUSSION

3.1 Preliminary investigation of the plant material

3.1.1 Plant material

A species of the *Actinidiaceae* family, *Saurauia roxburghii* has been investigated in this work. The leaves of the plant was used for investigation.

3.1.2 Extraction of the plant material

The air-dried and powdered plant material (1.0 kg) was suspended in 3 litres of ethanol for seven days for the purpose of cold extraction. The extract was filtered through fresh cotton bed and finally with Whatman No.1 filters paper. The volume of the filtrate was concentrated with a rotary evaporator at low temperature (40^0-50^0C) and reduced pressure.

3.1.3 Isolation and characterization of compounds

From the various crude extracts, pure compounds were isolated applying various chromatographic techniques. The isolated pure compounds were then characterized using various spectroscopic techniques.

3.2 Characterization of isolated compounds from *Saurauia roxburghii*

3.2.1 Characterization of compound-1 (YSR-9) as Stigmasterol

3.2.1.1 Physical properties

The compound-1(YSR-9) was obtained as a white needle shaped crystal. The R_f value of the compound was 0.86 in n-hexane: EA = 50:50. It was soluble in chloroform, dichloromethane, ethyl acetate, methanol & ethanol. It was tested by Salkawoski and Liebermann-burchard method which developed a reddish color and greenish color respectively indicating that the compound may be steroid. The melting point of this compound was found to be 138-140^{0}C[48].

3.2.1.2 Characterization of compound-1 (YSR-9) by spectroscopic method

The structure of the compound-1(YSR-9) has been established by UV, FT-IR, ^{1}H-NMR and ^{13}C-NMR spectral evidences.

3.2.1.2.1 Ultraviolet (UV) spectroscopy of compound-1 (YSR-9)

UV spectrum of the compound had absorption at λ_{max} 219, 280, 415, 545 and 640 nm in methanol.

3.2.1.2.2 Infrared (IR) spectroscopy of compound-1 (YSR-9)

The IR spectrum (in KBr) of compound-1(YSR-9) exhibit characteristic absorption at 3446.79cm^{-1} that was characteristic of O-H stretching. Absorption at 2935.55 cm^{-1} and 2855.22 cm^{-1} is due to aliphatic C-H stretching and bending vibrations of methyl group. Other absorption frequencies include 1653.00 cm^{-1} as a result C=C stretching however this band was weak, at 1447.18 cm^{-1} was a bending frequency for cyclic (CH_2)n and 1375.25 cm^{-1} for C-H bending. The absorption frequency at 1055.06 cm^{-1} due to C-C vibration signified cycloalkane. The out of plane C-H vibrations of unsaturated part was observed at 883.40 cm^{-1}.These absorption frequencies resemble the absorption frequencies observed for Stigmasterol[47].

3.2.1.2.3 ^{1}H-NMR spectroscopy of compound-1 (YSR-9)

The ^{1}H NMR spectrums showed two one proton multiplets at δ 3.53and δ 5.36 typical for H-3 and H-6 of a steroidal nucleus. The olefinic protons H-22 and H-23 appeared as characteristic downfield signals at δ 5.16 (1H, dd, *J*=15.0, 6.5 Hz) and 5.03 (1H, dd, *J*=15.0, 9.0 Hz)

respectively. Each of the signals were observed as double doublet, which indicated coupling with the neighbouring olefinic and methine protons. The ^{1}H NMR spectrums displayed two three-proton singlets at δ 1.00 and δ 0.67 assignable for Me-19 and Me-18 respectively. In addition, two doublets at δ 0.82 (3H, d, 7.2 Hz) and 0.80 (3H, d, 7.2 Hz) could be recognized to the two methyl groups at Me-26 and Me-27 and another three-proton doublet at δ 0.91 (3H, d, 6.8 Hz) for Me-21. On the other hand, one three-proton triplet at δ 0.85(3H,t, 7.2 Hz) could be assigned to the primary methyl group attached for Me-29[48-51].

3.2.1.2.4 ^{13}C-NMR spectroscopy of compound-1 (YSR-9)

The ^{13}C NMR spectrum showed 29 carbons including an oxymethine carbon signal at δ 71·85 was due to C-3β hydroxyl group and two signals at δ 140·81 and δ 121·74 ppm, which were assigned C5 and C6 double bonds respectively as in Δ^5 spirostene[49]. The signal at 19.41 and 12.02 ppm corresponds to angular carbon atom (C-19 and C-18 respectively). The value for C18 is lower due to γ-gauche interaction that increases the screening of the C-18 hence lower chemical shift. However, the loss of H in C-6 results in decrease in screening of the C19 leading to increase in C19 chemical shift to higher frequency. This is also tenable as in chemical shift of 19.41 and 12.02 ppm (for C19 and C18 respectively). Spectra showed twenty nine carbon signal including six methyls, nine methylenes, eleven methines and three quaternary carbons which were showed in **Table-3.1** The alkene carbons appeared at δ140·81, 138.33, 129.0 and 121·74 ppm. The physical and spectral data of this compound was in complete agreement to the reported data in literature [47-55], the compound-1 (YSR-9) was identified as Stigmasterol. This compound-1 (YSR-9) was first time isolated so far from the species *Saurauia roxburghii.*

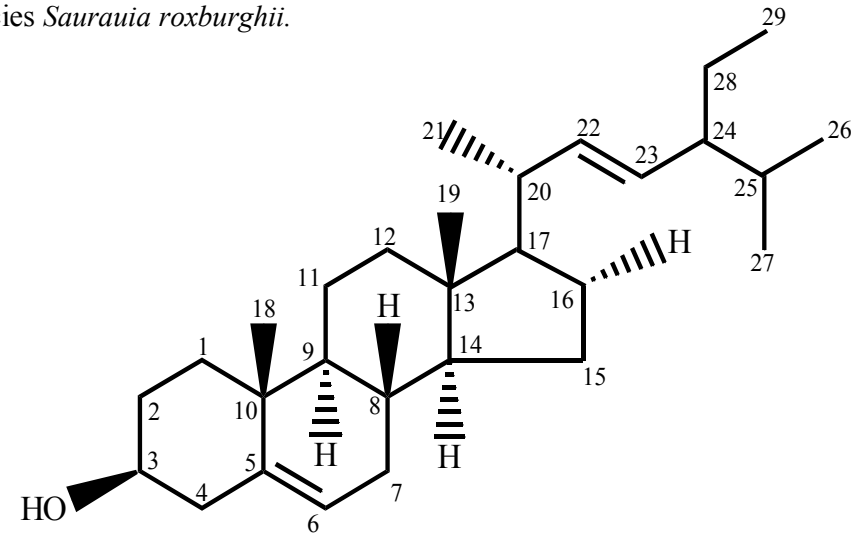


Figure 3.1: Stigmasterol

Table-3.1: ^{1}H-NMR & ^{13}C-NMR Spectral data of compound-1 (YSR-9)

C/H	^{1}H (δppm) Obtained	^{1}H (δppm) Literature[48]	^{13}C (δppm) Obtained	^{13}C (δppm) Literature[48]
1			37·29 (CH_2)	37.2
2			28·28 (CH_2)	28.2
3	3·52(1H,dd,*J*=9.6,4.8Hz)	3.53 (1H, m)	71·85 (CH)	71.82
4			42·36 (CH_2)	42.8
5			140·81 (Cq)	140.77
6	5·34 (1H, d, *J*=5·2 Hz)	5.35 (1H, m)	121·74 (CH)	121.73
7	1.99 (2H, m)	2.00 (2H, m)	31·72 (CH_2)	31.9
8			34.01 (CH)	33.96
9			50·20 (CH)	50.14
10			36·54 (Cq)	36.5
11			26·17 (CH_2)	26.08
12			39·83 (CH_2)	39.7
13			42·37 (Cq)	42.2
14			56·82 (CH)	56.88
15			24·33 (CH_2)	24.31
16	1.84 (2H, m)	1.86 (2H, m)	29.23 (CH_2)	29.16
17			56·12 (CH)	56.06
18	0·67 (3H, s, Me)	0.68 (3H, s, Me)	12.02 (CH_3)	12.05
19	1.00 (3H, s, Me)	1.02 (3H, s, Me)	19·41 (CH_3)	19.0
20	2.28 (1H, m)	2.30 (1H, m)	40·50 (CH)	40.5
21	0·92 (3H,d,J=6.0Hz,Me)	0.93(3H,d,J=8.0Hz,Me)	21.13 (CH_3)	21.22
22	5.16(1H,dd,*J*=15.0,8.4Hz)	5.18 (1H, m)	138.33 (CH_2)	138.33
23	5.03(1H,dd,*J*=15.0,8.4Hz)	5.00(1H, m)	129.0 (CH_2)	129.28
24			51.28 (CH)	51.25
25			45·91 (CH)	45.84
26	0·81 (3H,d,J=7.2Hz,Me)	0.82(3H,d,J=6.7Hz,Me)	19·42 (CH_3)	19.4
27	0·79 (3H,d,J=7·2Hz,Me)	0.79(3H,d,J=6.7Hz,Me)	19.84 (CH_3)	20.21
28			24·34 (CH_2)	24.4
29	0·85 (3H,d,J=8.0Hz,Me)	0.85 (3H,t,J=7.4Hz,Me)	12·26 (CH_3)	12.26

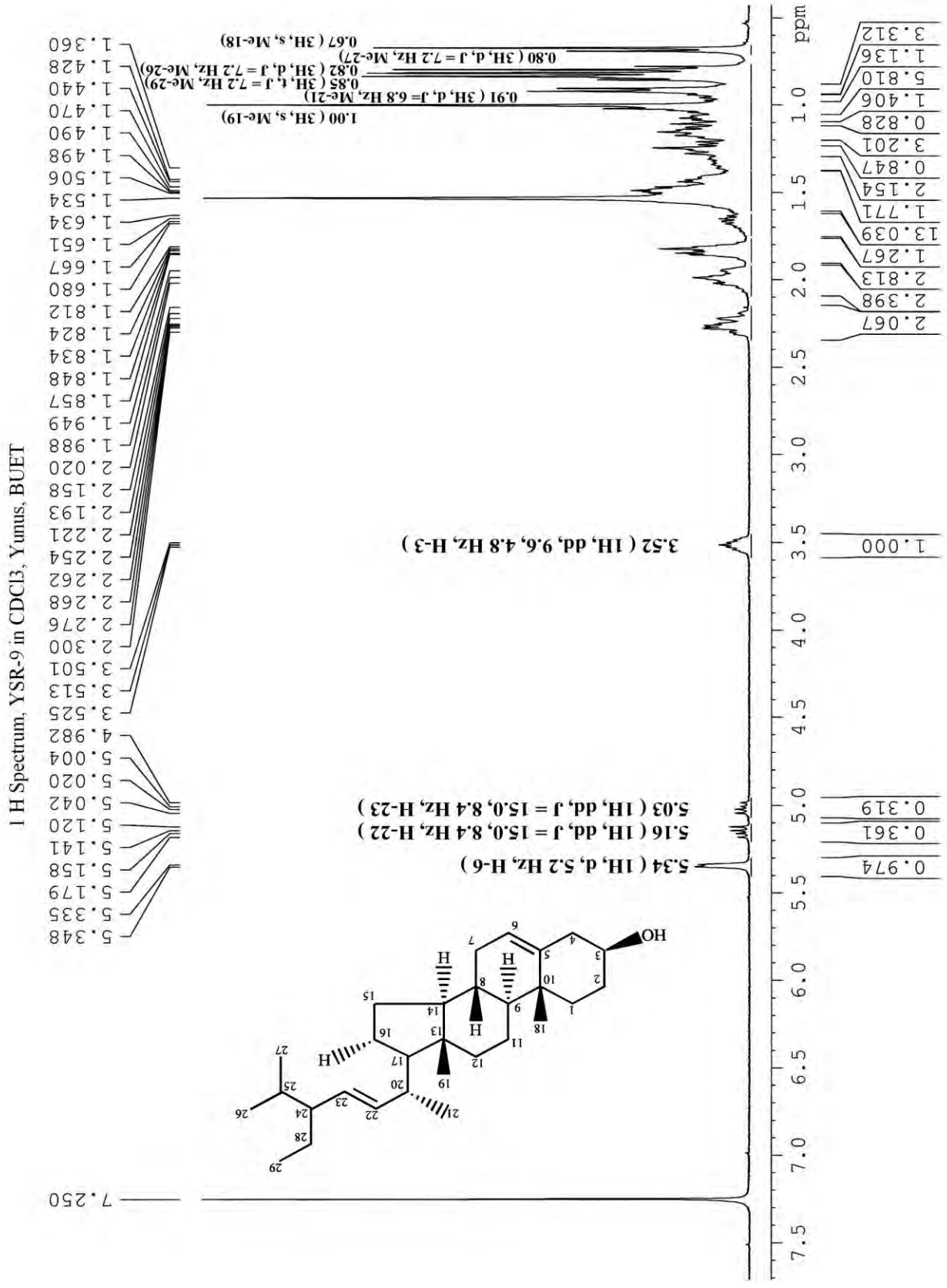


Figure 3.2: ^{1}H NMR spectrum of compound-1 (YSR-9) in $CDCl_3$.

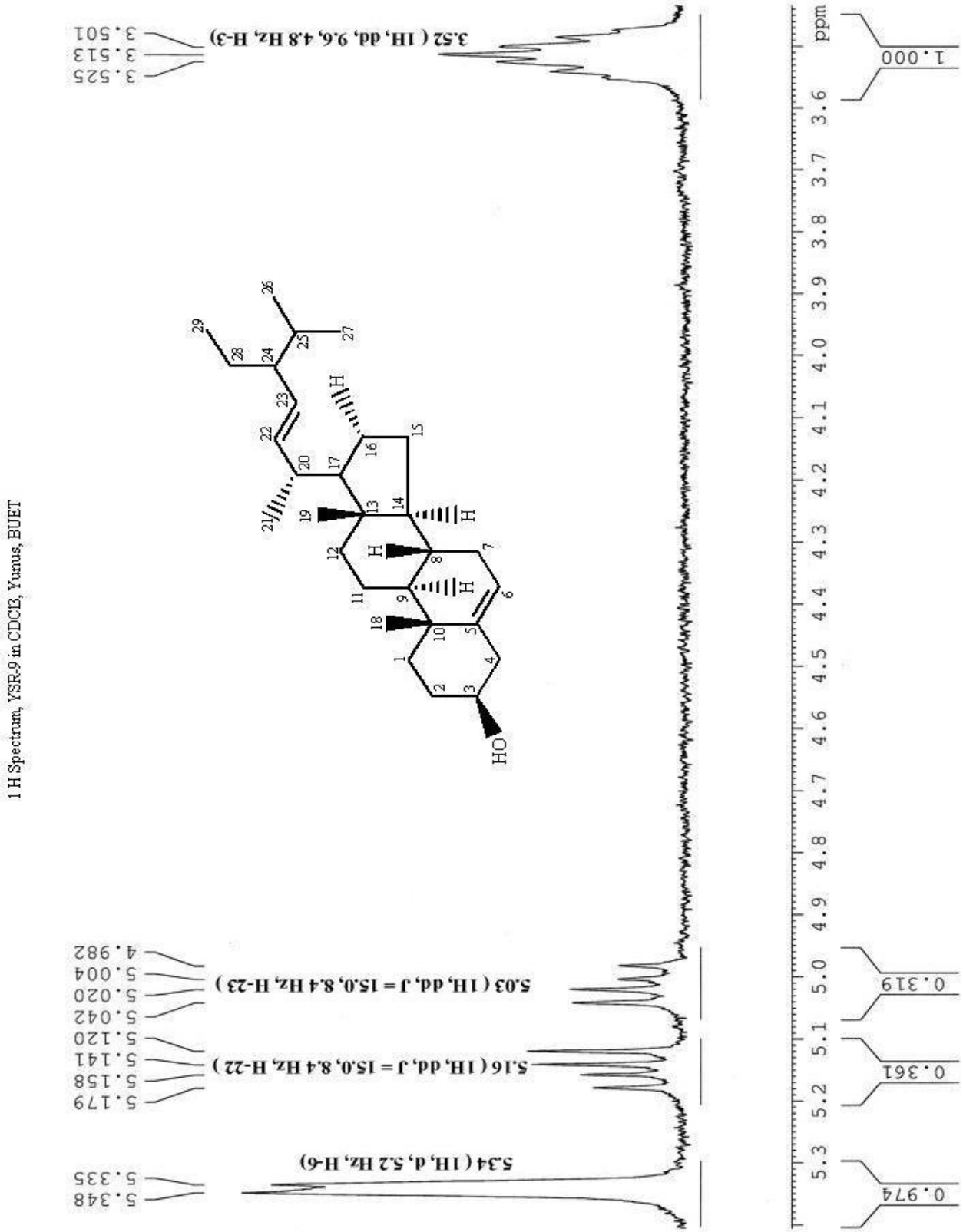


Figure 3.3: Partially expanded ^{1}H NMR spectrum of compound-1 (YSR-9) in $CDCl_3$.

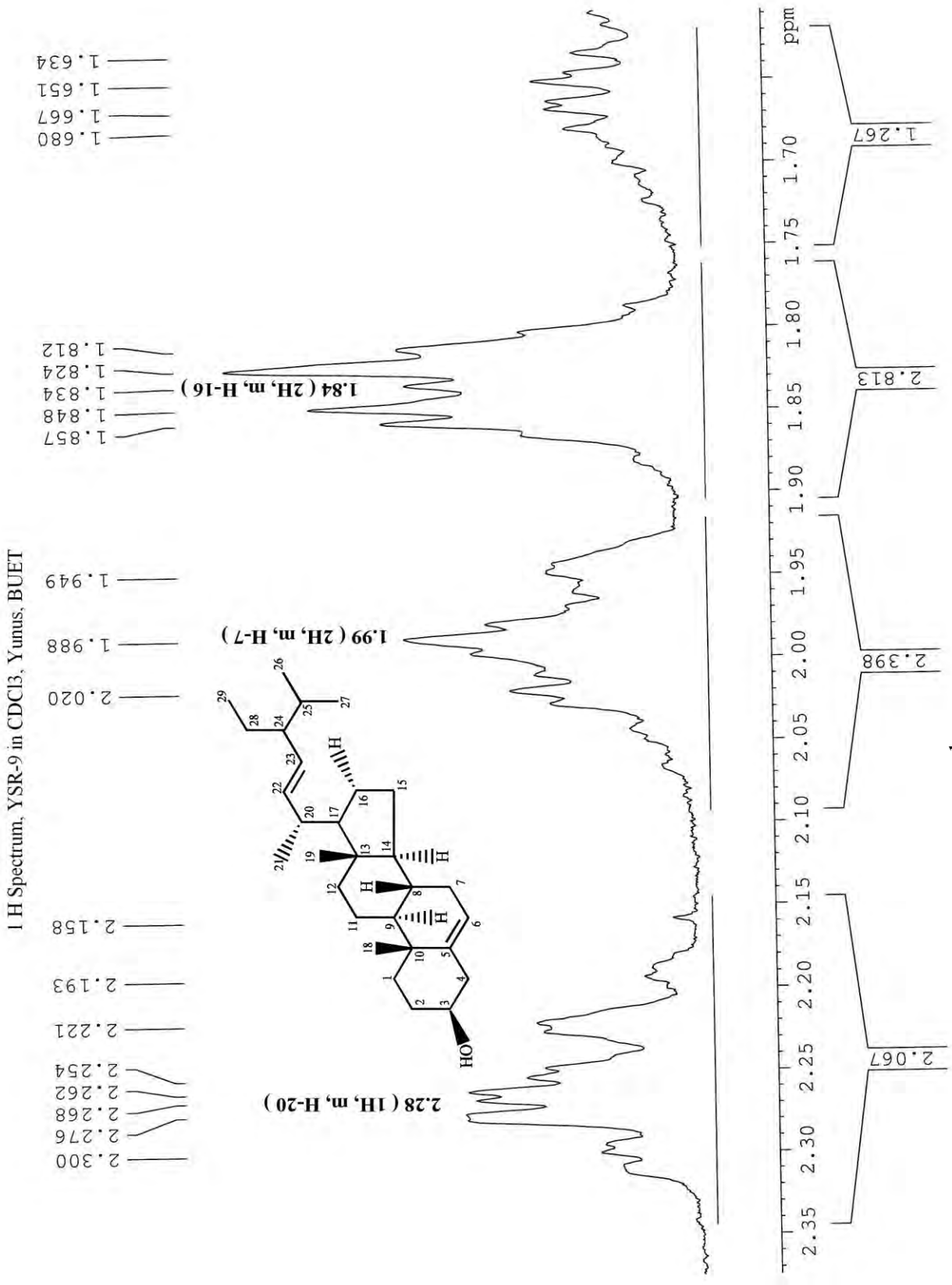


Figure 3.4: Partially expanded ^{1}H NMR spectrum of compound-1 (YSR-9) in $CDCl_3$.

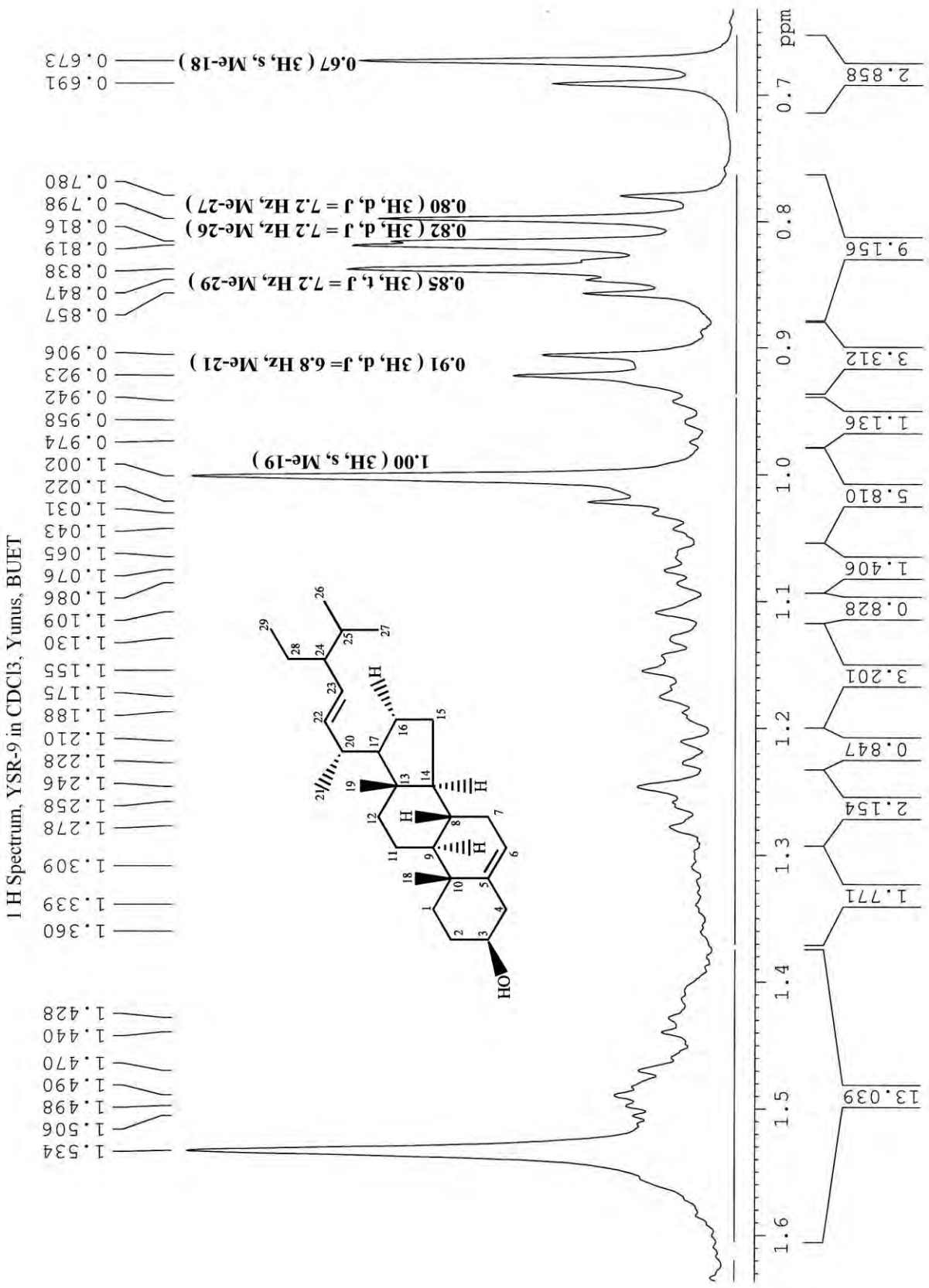


Figure 3.5: Partially expanded ^{1}H NMR spectrum of compound-1 (YSR-9) in $CDCl_3$.

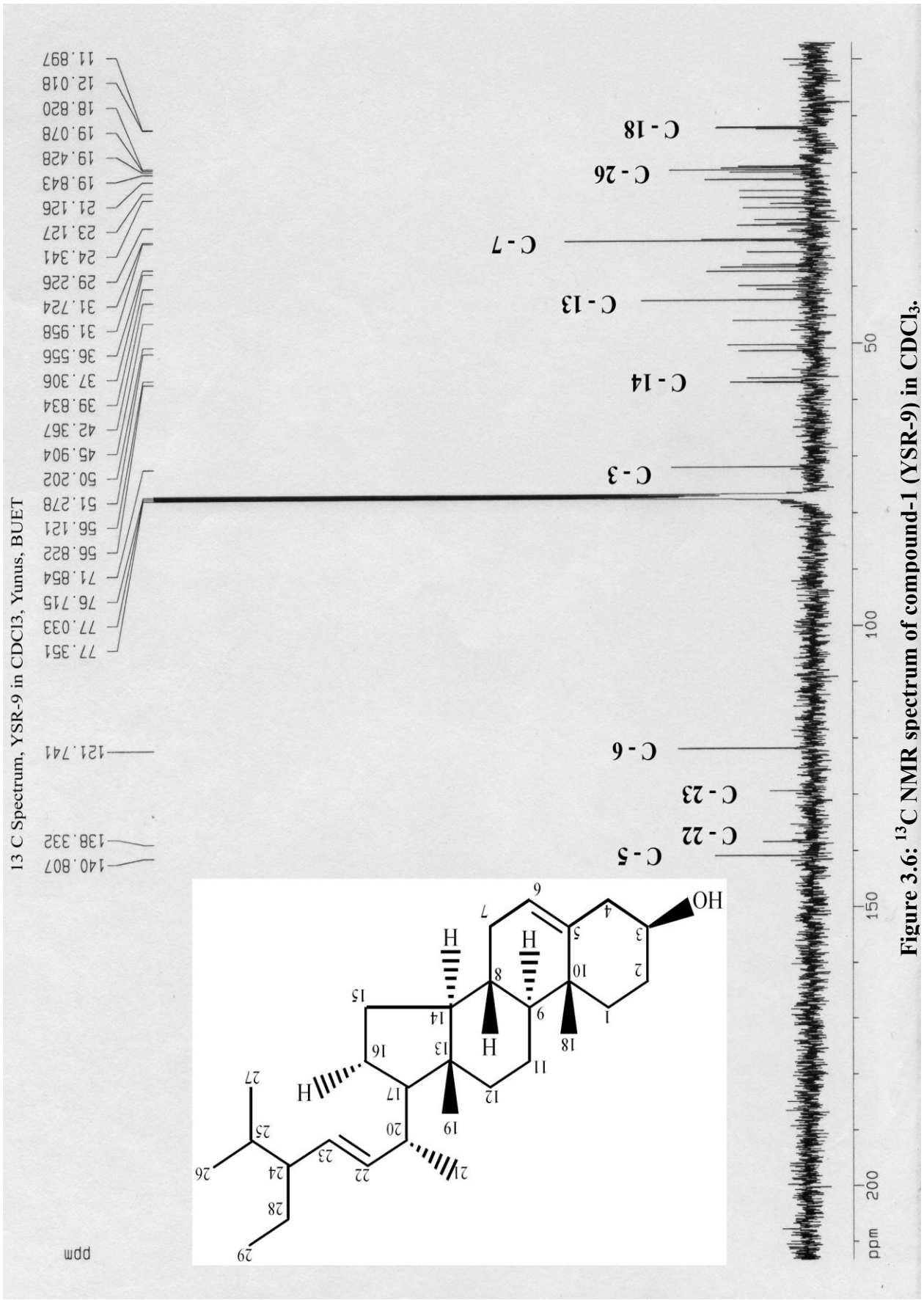


Figure 3.6: ^{13}C NMR spectrum of compound-1 (YSR-9) in $CDCl_3$.

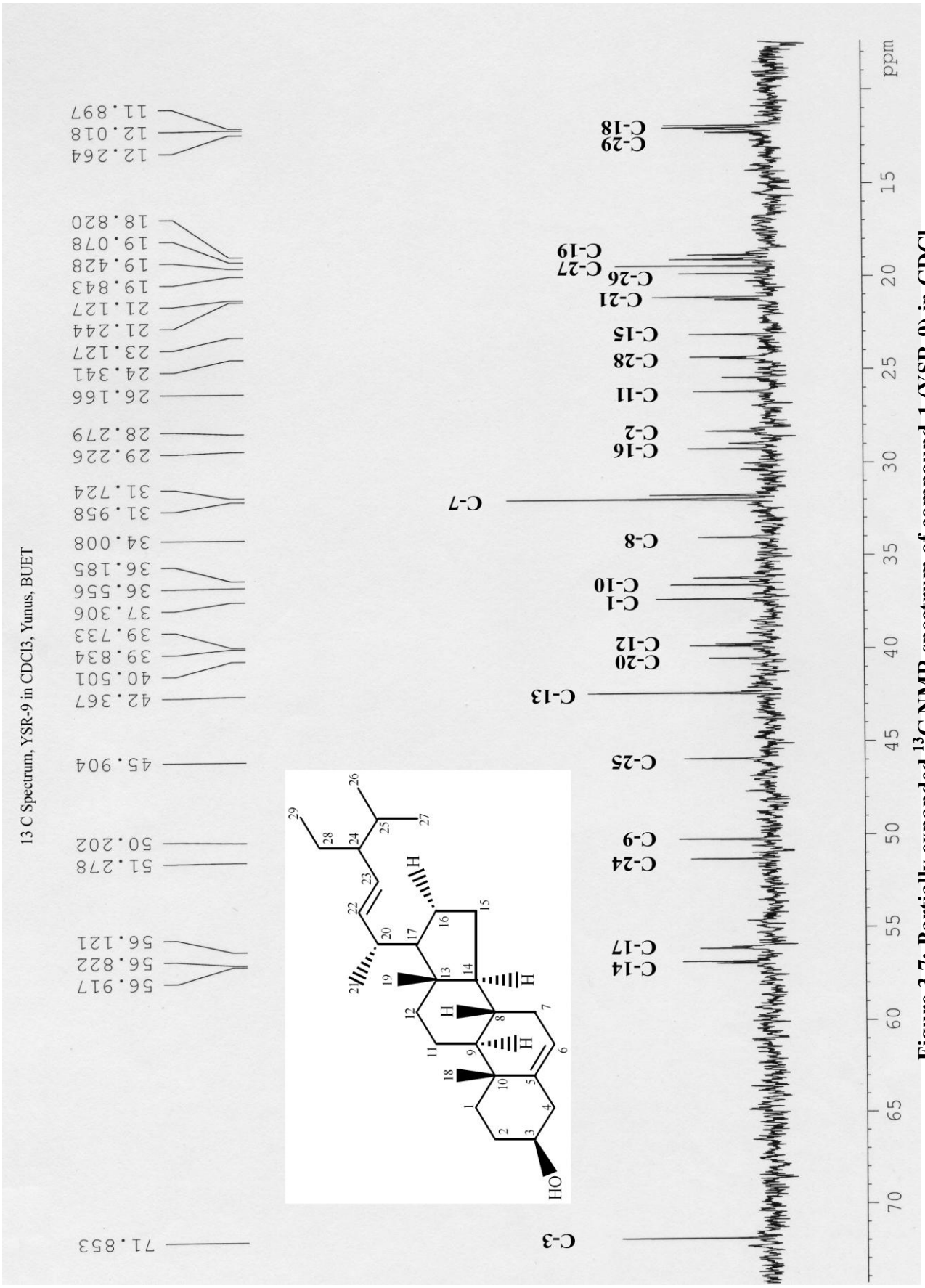


Figure 3.7: Partially expanded ^{13}C NMR spectrum of compound-1 (YSR-9) in $CDCl_3$.

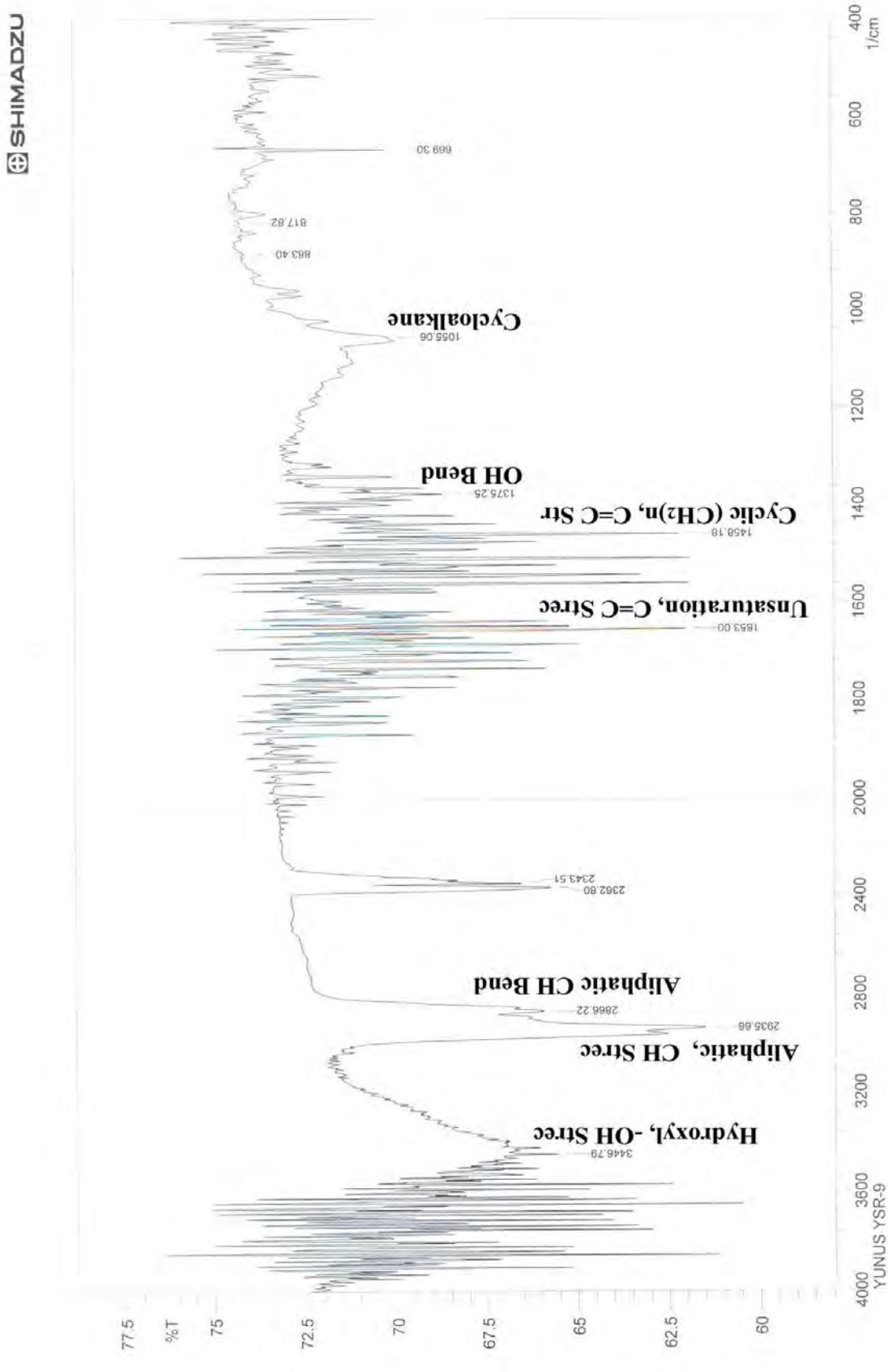


Figure 3.8: IR spectrum of compound-1 (YSR-9) in KBr.

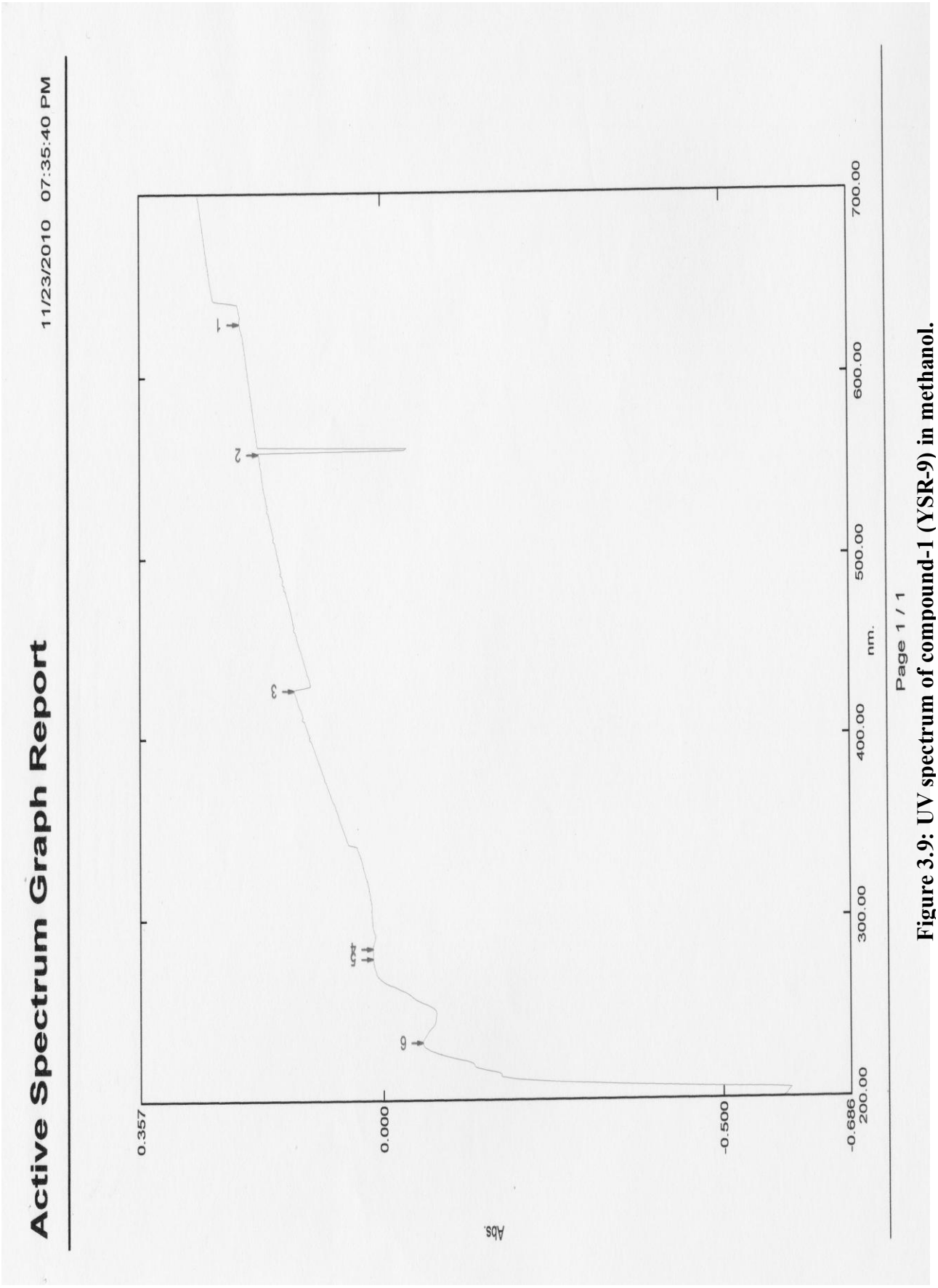


Figure 3.9: UV spectrum of compound-1 (YSR-9) in methanol.

3.2.2 Characterization of compound-2 (YSR-10) as *β*-sitosterol.

3.2.2.1 Physical properties

The compound-2 (YSR-10) was obtained as white powdered crystal. The R_f value of the compound was 0.33 in toluene: ethyl acetate = 95:5. It was soluble in chloroform, dichloro-methane, ethyl acetate, methanol and ethanol. It was tested by Salkawoski and Liebermann-burchard method which developed a reddish color and greenish color respectively indicating that the compound may be steroid. The melting point of this compound was found to be 130^0-135^0C[52].

3.2.2.2 Characterization of compound-2 (YSR-10) by spectroscopic method

The structure of the compound-2(YSR-10) has been established by UV, FT-IR, ^{1}H-NMR, ^{13}C-NMR, DEPT-90 and DEPT-135 spectral evidences.

3.2.2.2.1 Ultraviolet (UV) spectroscopy of compound-2 (YSR-10)

The UV spectrum showed absorption band at λmax 503, 515, 545 and 670 nm in methanol.

3.2.2.2.2 Infrared (IR) spectroscopy of compound-2 (YSR-10)

The IR spectrum (in KBr) of compound-2 (YSR-10) exhibit characteristic absorption band at 3421.72 cm^{-1} that was characteristic of O-H stretching. Absorption at 2935.55 cm^{-1} and 2855.22 cm^{-1} is due to aliphatic C-H stretching and bending vibrations of methyl group. Other absorption frequencies include 1653.00 cm^{-1} as a result C=C stretching however this band was weak at 1447.18 cm^{-1} was a bending frequency for cyclic $(CH_2)n$ and 1375.25 cm^{-1} for C-H bending. The absorption frequency at 1051.78 cm^{-1} due to due to C-C vibration signified cycloalkane. These absorption frequencies resemble the absorption frequencies observed for *β*-sitosterol[52].

3.2.2.2.3 ^{1}H-NMR spectroscopy of compound-2 (YSR-10)

The ^{1}H NMR spectrum of the compound-2(YSR-10) showed two one-proton multiplet at δ 3.51 ppm and δ 5.34 ppm typical for H-3 and H-6 of a steroidal nucleus. The spectrum further revealed two singlets at δ 0.67 and δ 1.00 each integrating for three protons assignable to two tertiary methyl groups at Me-18 and Me-19 respectively. The spectrum also showed two doublets at δ 0.79 ppm (*J* =7.2 Hz) and δ 0.83 ppm (*J* =7.2 Hz) which could be attributed

to two methyl groups at Me-27 and Me-26 respectively. On the other hand, two doublet at δ 0.92 ppm (J = 6.0 Hz) and δ 0.85 (J = 8.0 Hz) could be assigned to the primary methyl groups attached at Me-21 and Me-29 respectively[52-55].

3.2.2.2.4 ^{13}C-NMR spectroscopy of compound-2 (YSR-10)

The ^{13}C NMR spectrum showed 29 carbons including an oxymethine carbon signal at δ 71.84 and two olefinic carbons at δ 140.80 and δ 121.73.The double bonded unsaturation at δ 140·80 and δ 121·73 which were assigned C5 and C6 double bonds respectively as in Δ^5 spirostene[49]. If we compare DEPT 135 & DEPT 90 experiments for compound-2(YSR-10) then we confirmed that this compound was having six methyl (CH_3) groups, eleven methylene (CH_2) groups, nine methine (CH) groups and three quaternary carbons (C) groups which are shown in **Table-3.2**. The physical and spectral data of the compound was in complete agreement to the reported data in literature[47,50,52-55], the compound-2 (YSR-10) was identified as β-sitosterol. The compound-2 (YSR-10) was first time isolated so far from the species *Saurauia roxburghii.*

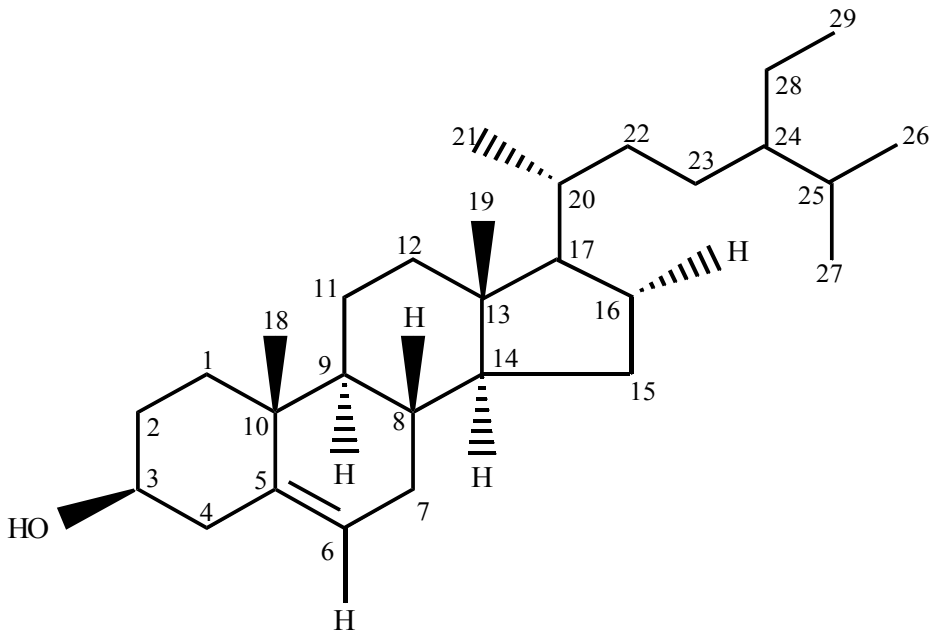


Figure 3.10: β-sitosterol.

Table-3.2: ^{1}H-NMR & ^{13}C-NMR Spectral data of compound-2 (YSR-10)

C/H	^{1}H (δppm) Obtained	^{1}H (δppm) Literature[52]	^{13}C (δppm) Obtained	^{13}C (δppm) Literature[52]
1			37·29 (CH_2)	37.28
2			31.95 (CH_2)	31.93
3	3·51 (1H,m)	3.52 (1H, m)	71·84 (CH)	71.82
4			42·36 (CH_2)	42.33
5			140·80 (Cq)	140.70
6	5·34 (1H,d,*J*=5·2Hz)	5.36 (1H, m)	121·73 (CH)	121.72
7			31·71 (CH_2)	31.69
8			31·95 (CH)	31.93
9			50·19 (CH)	50.17
10			36·18 (Cq)	36.12
11			21·12 (CH_2)	21.10
12			39·82 (CH_2)	39.80
13			42·36 (Cq)	42.33
14			56·81 (CH)	56.79
15			24·33 (CH_2)	24.37
16			28.26 (CH_2)	28.25
17			56·11 (CH)	56.09
18	0·67 (3H, s, Me)	0.68 (3H, s, Me)	11·88 (CH_3)	11.86
19	1.00 (3H, s, Me)	1.02 (3H, s, Me)	19·41 (CH_3)	19.40
20			36.54 (CH)	36.52
21	0·92 (3H,d,J=6.0Hz,Me)	0.94 (3H,d,J=6.5Hz,Me)	19·07 (CH_3)	18.79
22			34·00 (CH_2)	33.98
23			26·16 (CH_2)	26.14
24			45·89 (CH)	45.88
25			29.23 (CH)	28.91
26	0·83 (3H,d,J=7.2Hz,Me)	0.85 (3H,d,J=6.7Hz,Me)	19·83 (CH_3)	19.80
27	0·79 (3H,d,J=7·2Hz,Me)	0.83 (3H,d,J=6.7Hz,Me)	18.81 (CH_3)	18.79
28			23.12 (CH_2)	23.10
29	0·85 (3H,d,J=8.0Hz,Me)	0.88 (3H,t,J=7.4Hz,Me)	12·01 (CH_3)	11.99

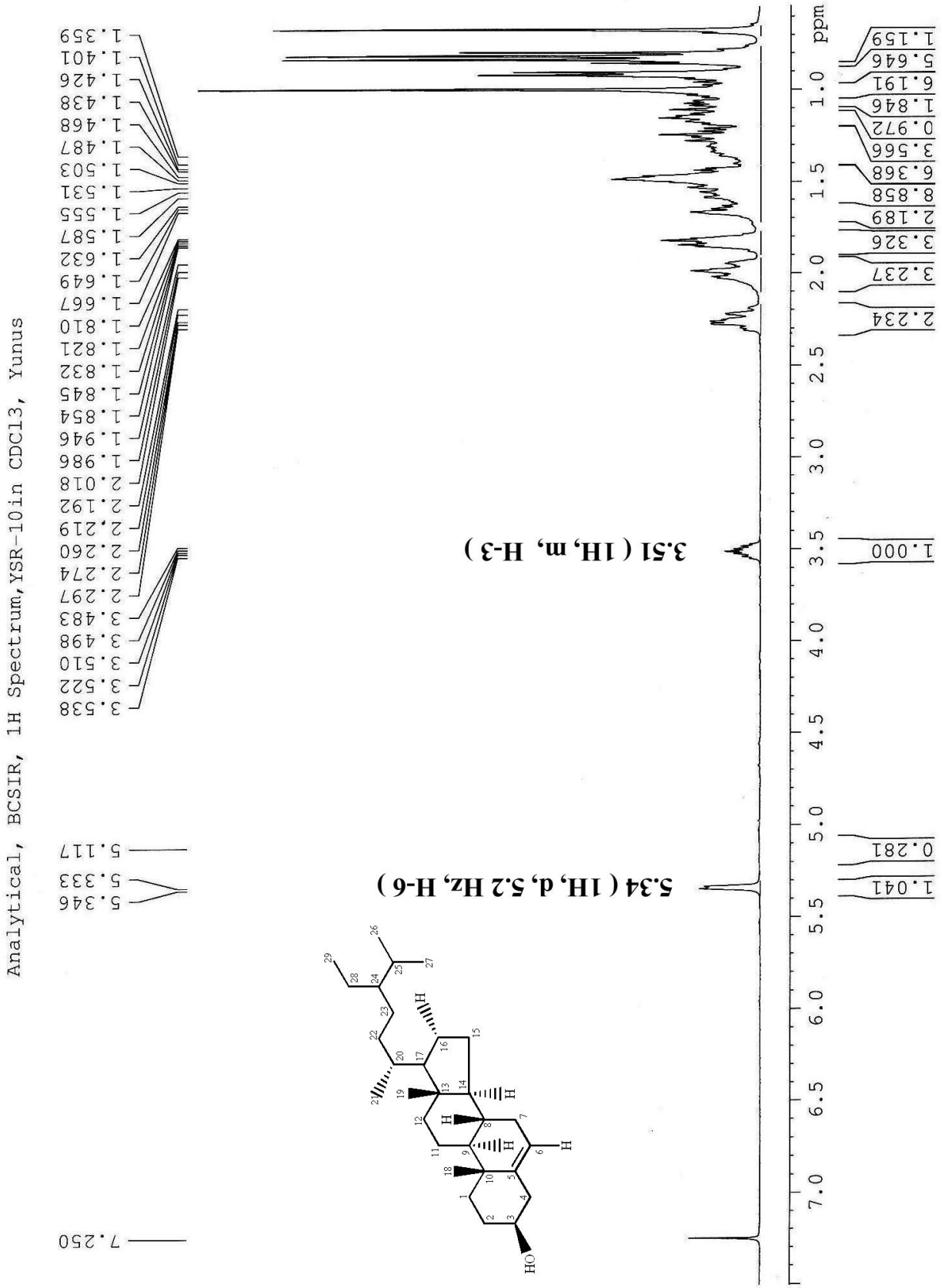


Figure 3.11: ^{1}H NMR spectrum of compound-2 (YSR-10) in $CDCl_3$.

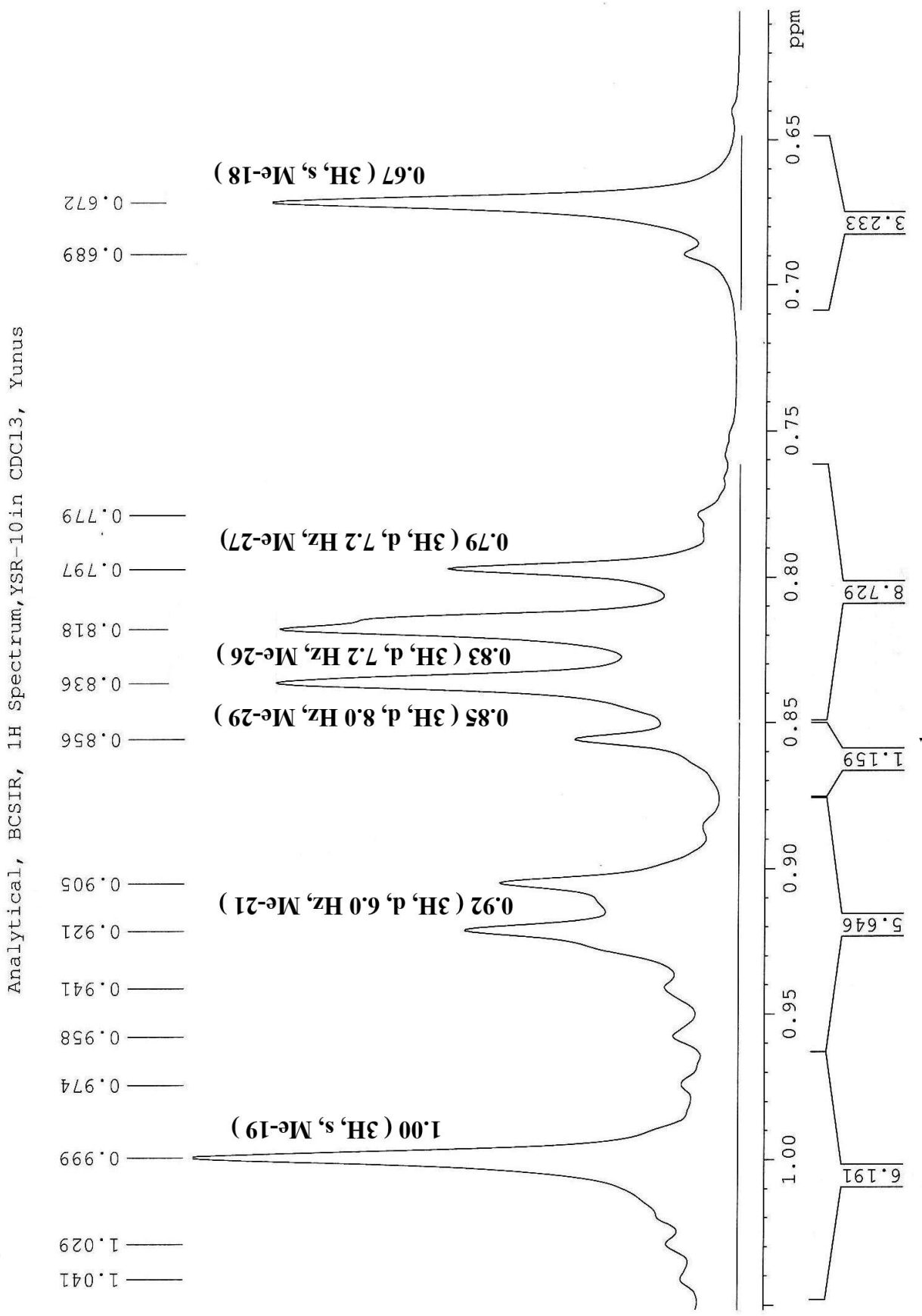


Figure 3.12: Partially expanded ^{1}H NMR spectrum of compound-2 (YSR-10) in $CDCl_3$.

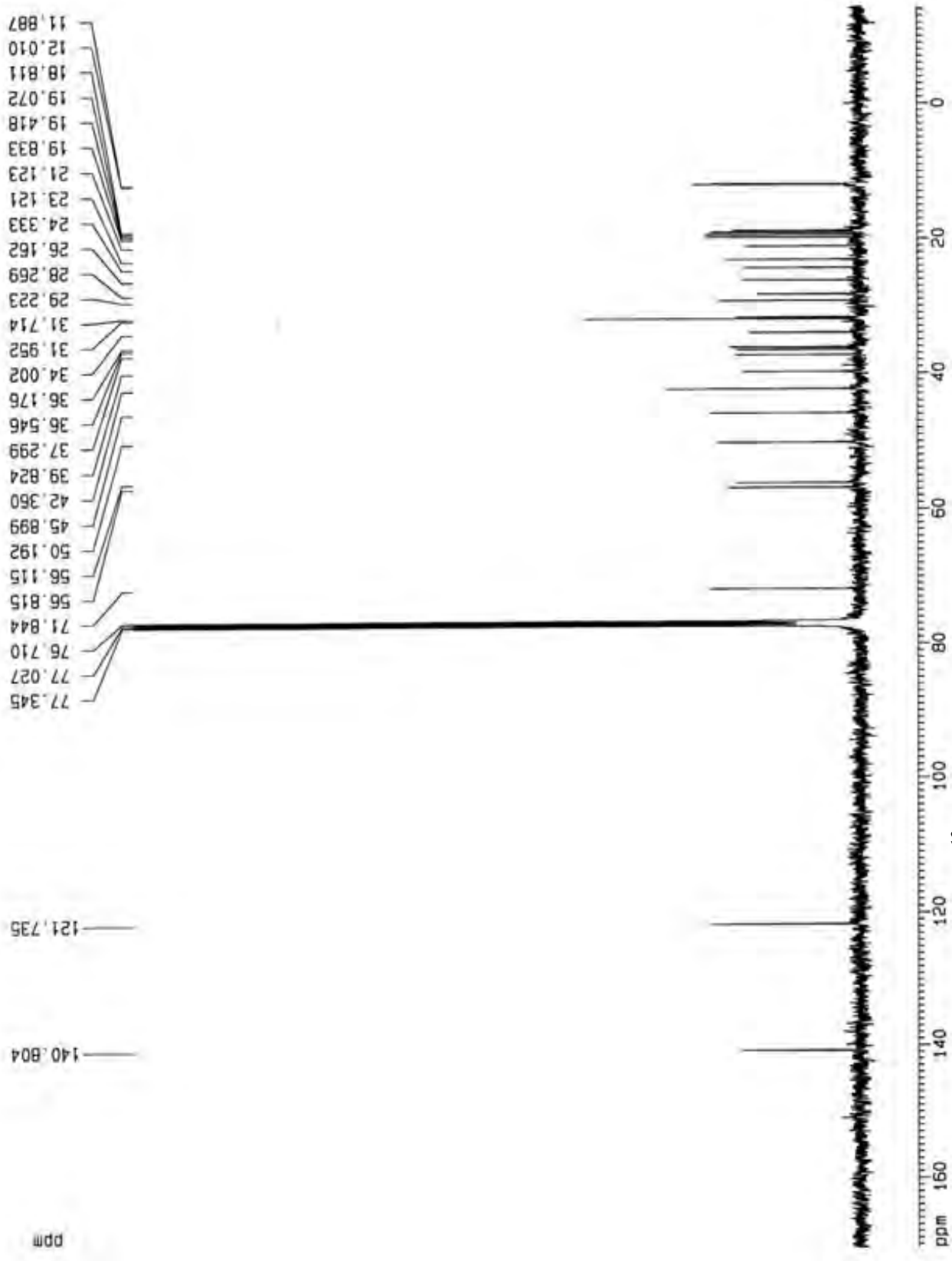


Figure 3.13: ^{13}C NMR spectrum of compound-2 (YSR-10) in $CDCl_3$.

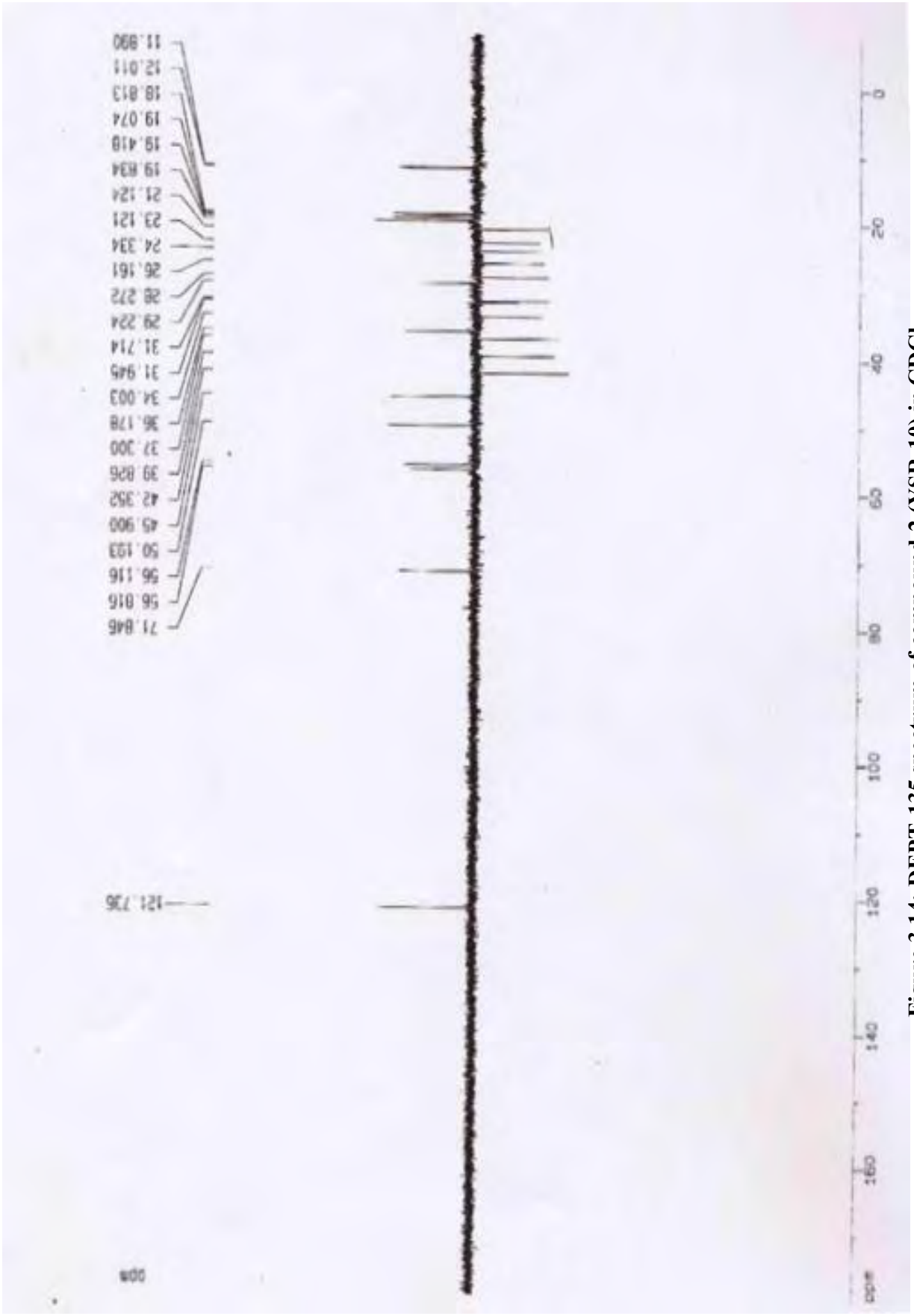


Figure 3.14: DEPT-135 spectrum of compound-2 (YSR-10) in $CDCl_3$.

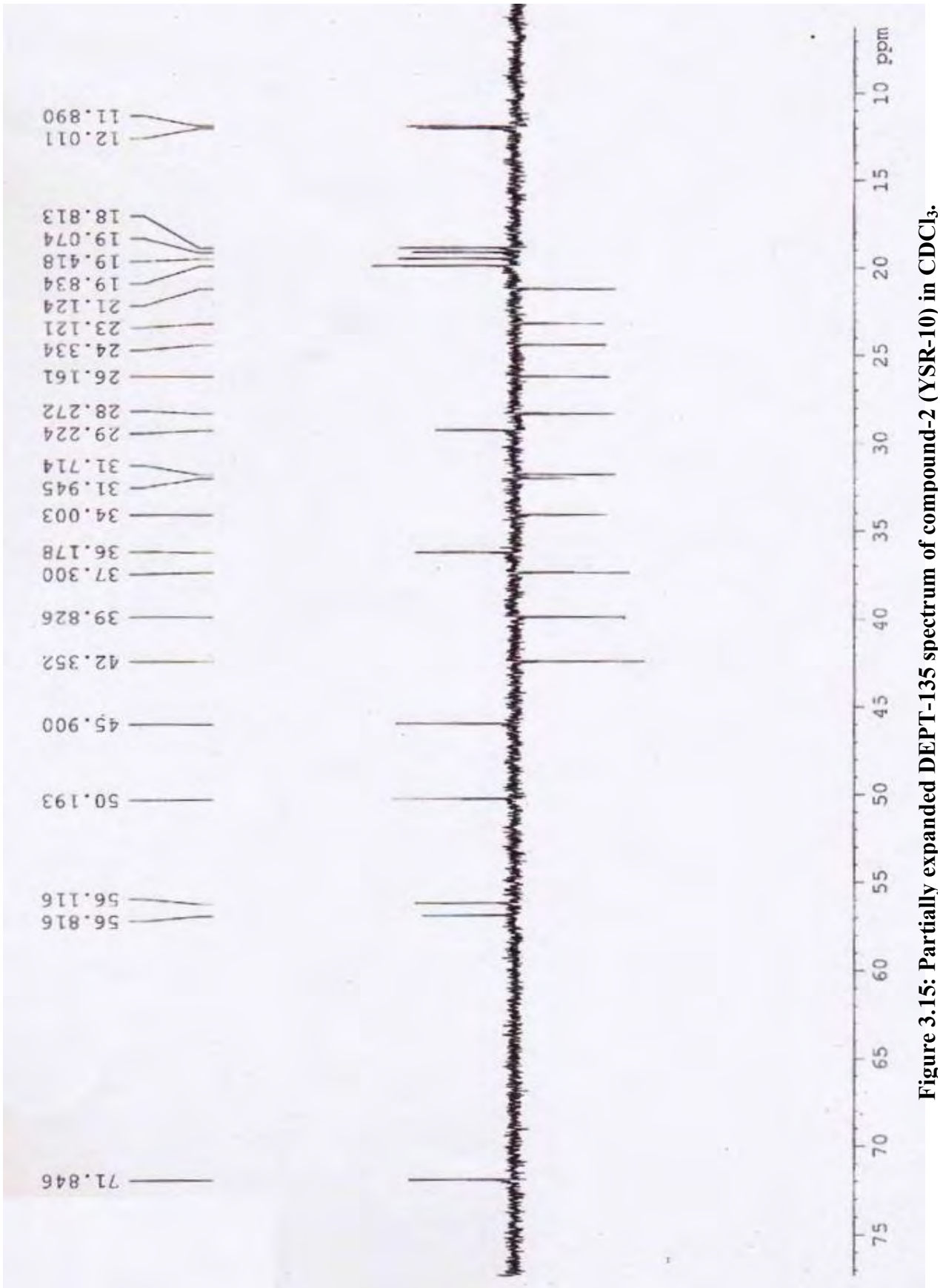


Figure 3.15: Partially expanded DEPT-135 spectrum of compound-2 (YSR-10) in $CDCl_3$.

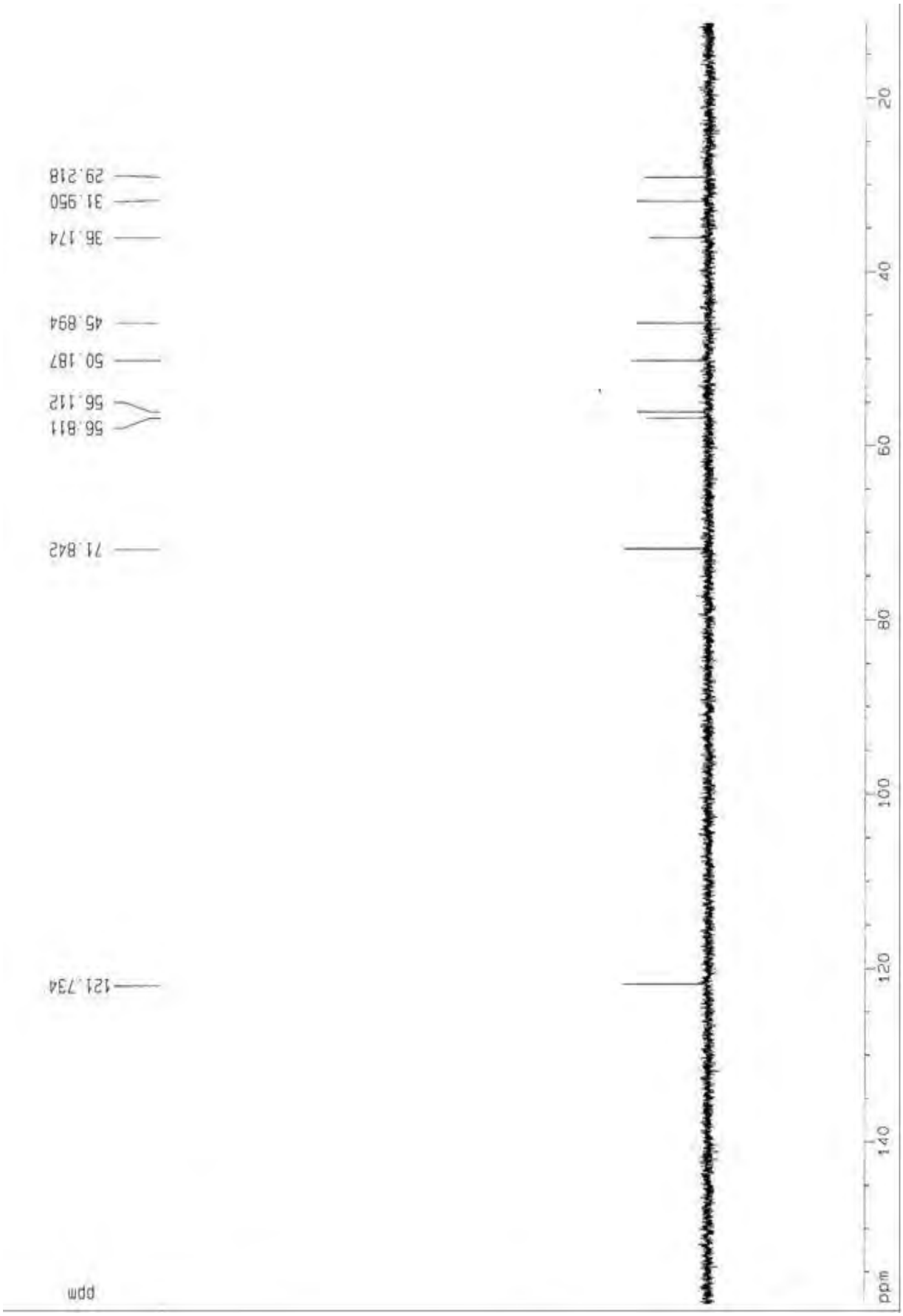


Figure 3.16: DEPT-90 spectrum of compound-2 (YSR-10) in $CDCl_3$.

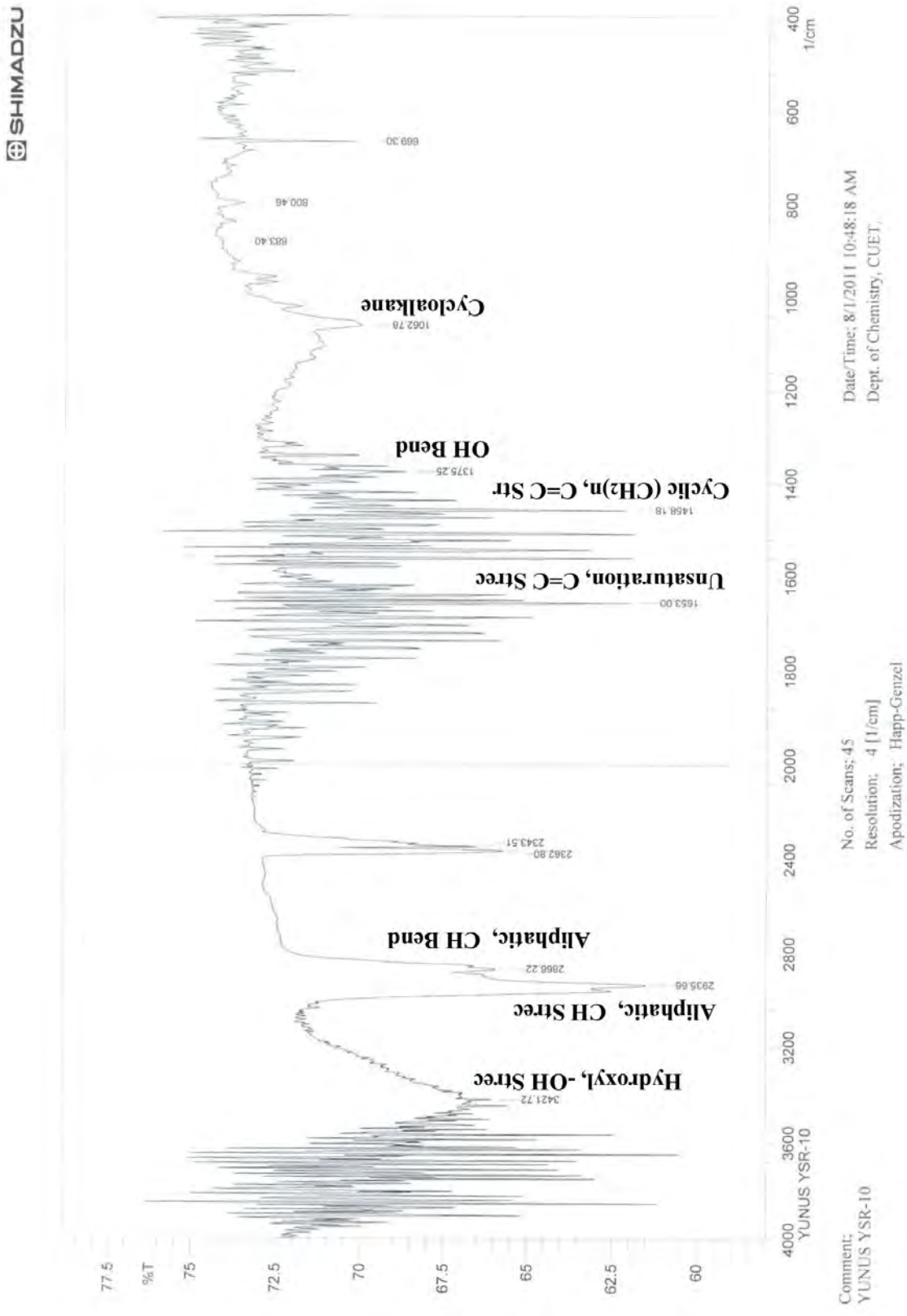


Figure 3.17: IR spectrum of compound-2 (YSR-10) in KBr.

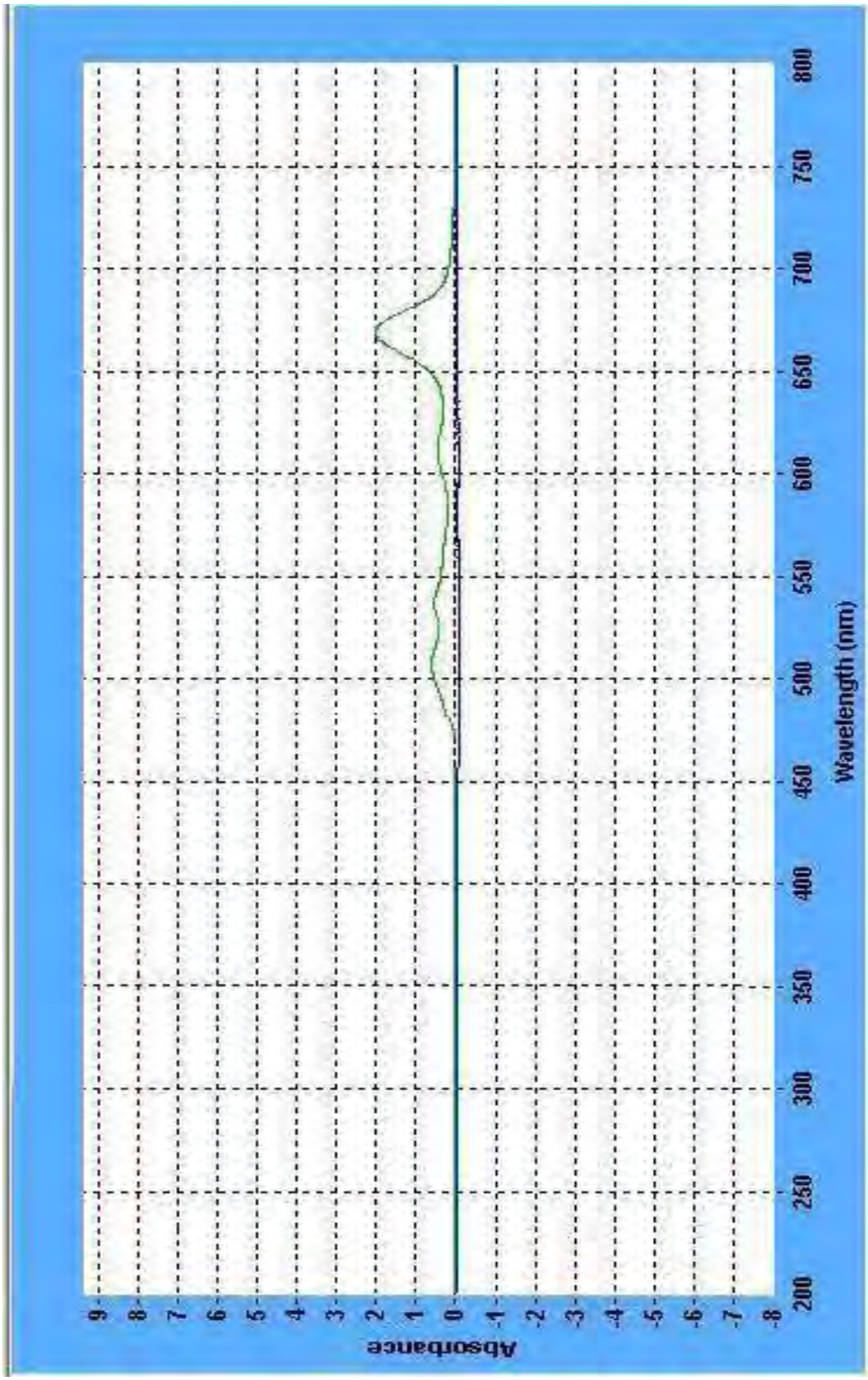


Figure 3.18: UV spectrum of compound-2 (YSR-10) in methanol.

3.2.3 Characterization of compound-3(YSR-2) as 3*β*-hydroxy-urs-12-en-28-oic acid

3.2.3.1 Physical properties

The compound-3 (YSR-2) was obtained as white amorphous powder compound. The R_f value of the compound was 0.73 in chloroform: methanol: 90:10. It was soluble in chloroform or dichloromethane with 2-3 drops of methanol. It was tested for terpenoid and a red-violet color confirms that the compound-3 was terpenoid type compound. The melting point was found to be 271-273^{0}C[56].

3.2.3.2 Characterization of compound-3 (YSR-2) by spectroscopic method

The structure of the compound-3(YSR-2) has been established by FT-IR, ^{1}H-NMR, ^{13}C-NMR and DEPT-135 spectral evidences.

3.2.3.2.1 Ultraviolet (UV) spectroscopy of compound-3 (YSR-2)

No characteristics absorption was observed in the UV spectrum in methanol.

3.2.3.2.2 Infrared (IR) spectroscopy of compound-3 (YSR-2)

The IR spectrum (in KBr) of compound-3 (YSR-2) exhibit characteristic absorption band for hydroxyl (-OH) group at 3446.79 cm^{-1}, OH of carboxylic function at 3101.54 cm^{-1}, Absorption at 2926.01 cm^{-1} and 2854.65 cm^{-1} was due to aliphatic C-H stretching and bending vibrations of methyl group, carbonyl (C=O) of the carboxyl group at 1734.01 cm^{-1}, Other absorption frequencies include 1653.00 cm^{-1} as a result C=C stretching however this band was weak at 1458.18 cm^{-1} was a bending frequency for cyclic (CH_2)n and 1375.25 cm^{-1} for C-H bending, carboxy (C-O stretching) function at 1163.08 cm^{-1}, hydroxy (O-H bending) function at 1035.77 cm^{-1}and presence of trisubstituted double bond at 815.89 cm^{-1}[56].

3.2.3.2.3 ^{1}H-NMR spectroscopy of compound-3 (YSR-2)

The ^{1}H NMR spectrum of the compound-2 (YSR-2) showed five methyls singlets at 1.05, 1.04, 0.92, 0.90 and 0.77. In this spectrum all signals are shifted 0.31 ppm due to of two solvent effects CD_3OD and $CDCl_3$ when we compare with standard literature value then we obserbed. The tertiary natures of these methyls were evident from their sharp singlets in the ^{1}H NMR. The ^{1}H NMR spectrum also showed the presence of a proton germinal to the hydroxyl group was observed at δ 3.13 (1H, dd, 10.5, 5.5 Hz) corresponding to H-3 and δ 2.14 (1H, d, 11.5 Hz) corresponding to H-18 and a characteristic olefinic proton of C12-C13

double bonded pentacyclic triterpenoid at δ 5.18. In the ^{1}H NMR spectrum of this compound, the signal of H-18 permitted the distinction between the oleane and ursane skeletons. The H-18 signal appears at δ 2.1-2.6 ppm in the ursane skeleton and at δ 3.1-3.3 ppm in the oleane skeleton[57]. The proton signals of H-29 and H-30 in the ursane skeletons appears as doublet, 0.88 (3 H, d, 5.5 Hz, H-29) and 0.81 (3 H, d, 6.5 Hz, H-30) but in oleane as a singlet[58].

3.2.3.2.4 ^{13}C-NMR spectroscopy of compound-3 (YSR-2)

The ^{13}C NMR assignments of various carbon atoms were substantiated by DEPT experiments, which revealed the presence of seven methyls, nine methylenes, seven methines and seven quaternary carbon atoms which showed in **Table-3.3**. It revealed the presence of signals due to an oxygenated carbon signal at δ 79.3 (C-3), one tri-substituted double bond at δ 126.12(C-12) and 138.80 (C-13) and one carboxyl group at 181.27 (C-28). In general, for the polyols C-l2 is deshielded (~2 p.p.m.) and C-l3 shielded (~5 p.p.m.) in urs-12-enes compared to the corres-ponding olean-12-enes. The chemical shift of C-l3 appears to be about 138-140 p.p.m. in the urs-12-enes and 144-145 p.p.m. in the olean-12-enes[59].The chemical shifts of 126.12 (C-12) and 138.80 (C-13) and H-12 (δ 5.20-5.4 ppm) suggests that this compound was Δ12-unsaturated triterpenoid. The spectra clearly exhibited the difference in the chemical shifts of C-12, C-13, C-17, C-18, C-19, C-20, C-22, C-27, C-29 and C-30 between the ursane group and the oleane group. Moreover ^{13}C-NMR signals due to C-18 to C-22 suggested that YSR-2 was an urs-12-en derivative. The physical and spectral data of the compound was in complete agreement to the reported data in literature[56-60]. The compound-3(YSR-2) was identified as 3*β*-hydroxy-urs-12-en-28-oic acid. This was the first time isolated compound so far on this plant *Saurauia roxburghii.*

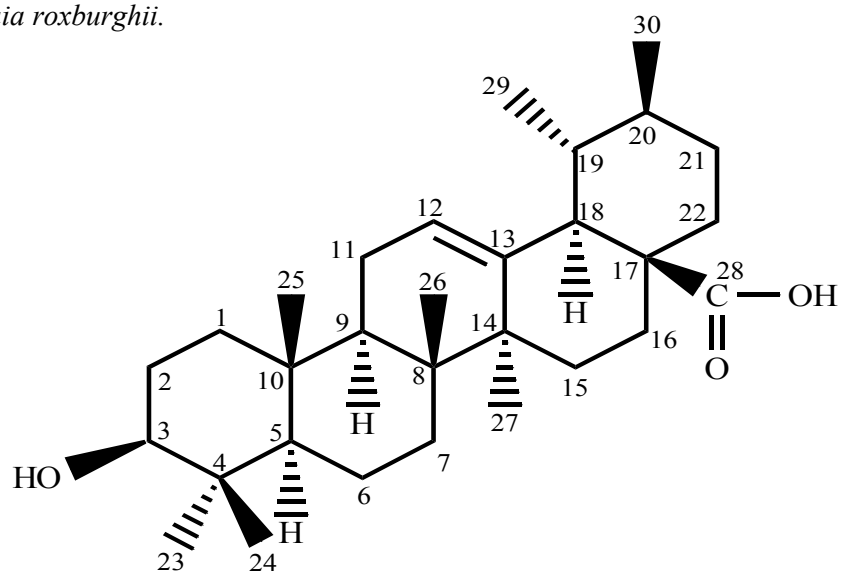


Figure-3.19: 3*β*-hydroxy-urs-12-en-28-oic acid.

Table-3.3: ^{1}H-NMR & ^{13}C-NMR Spectral data of compound-3 (YSR-2)

C/H	^{1}H (δ ppm) Obtained	^{1}H (δ ppm) Literature[60]	^{13}C(δ ppm) Obtained	^{13}C(δppm) Literature[58]
1			39.3 (CH_2)	39.2
2			27·3 (CH_2)	28.2
3	3·13(1H,dd,*J*=10.5, 5.5Hz)	3.07(1H,dd, *J*= 11.0,5.0 Hz)	79.3 (CH)	78.2
4			39.7 (Cq)	39.6
5			55.9 (CH)	55.9
6			18.9 (CH_2)	18.8
7			33.7 (CH_2)	33.7
8			40.1 (Cq)	40.1
9			49.5 (CH)	48.1
10			37·5 (Cq)	37.5
11			23·8 (CH_2)	23.7
12	5.18 (1H, brs)	5.11 (1H, t, *J*= 3.3 Hz)	126.1 (CH)	125.7
13			138.8 (Cq)	139.3
14			42.6 (Cq)	42.6
15			28.6 (CH_2)	28.8
16			24.8 (CH_2)	25.0
17			49.7 (Cq)	48.1
18	2.14 (1H, dd, 11.5 Hz)	2.08 (1H, d, *J*= 11.4 Hz)	53.5 (CH)	53.6
19			39.5 (CH_2)	39.5
20			39.3 (Cq)	39.4
21			31.2 (CH_2)	31.1
22			37.4 (CH_2)	37.4
23	1.04 (3H, s, Me)	0.97 (3H, s, Me)	28.5 (CH_3)	28.8
24	0·90 (3H, s, Me)	0.78 (3H, s, Me)	16.1 (CH_3)	16.5
25	0·77 (3H, s, Me)	0.65 (3H, s, Me)	15.9 (CH_3)	15.7
26	0·92 (3H, s, Me)	0.84 (3H, s, Me)	17.4 (CH_3)	17.5
27	1.05 (3H, s, Me)	1.02 (3H, s, Me)	24.0 (CH_3)	24.0
28			181.3 (Cq)	179.7
29	0·88 (3H, d, 5.5 Hz, Me)	0.88 (3H, d, 5.4 Hz, Me)	17.5 (CH_3)	17.5
30	0·81 (3H, d, 6.5 Hz, Me)	0·78 (3H, d, 6.2 Hz, Me)	21.6 (CH_3)	21.4

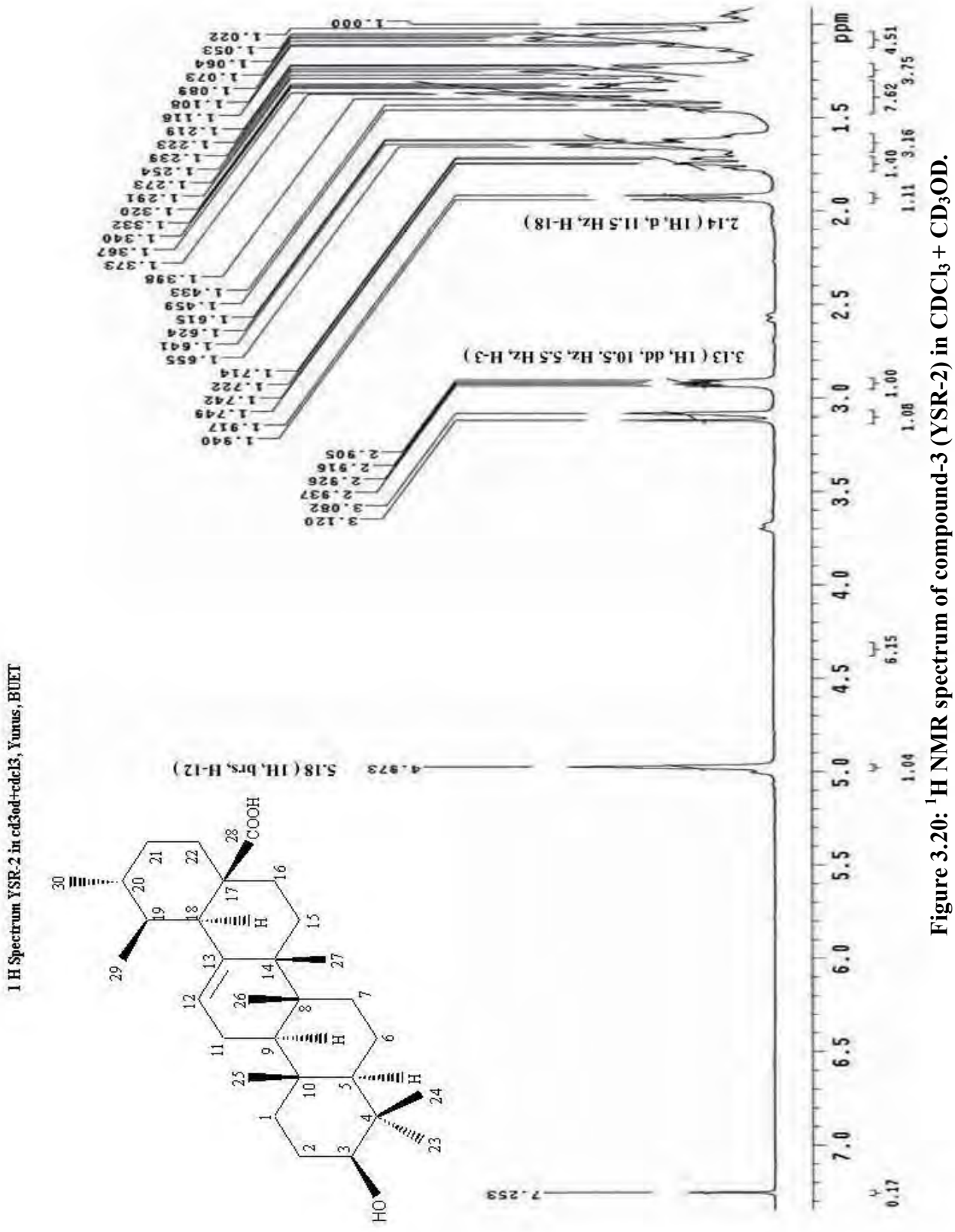


Figure 3.20: ^{1}H NMR spectrum of compound-3 (YSR-2) in $CDCl_3$ + CD_3OD.

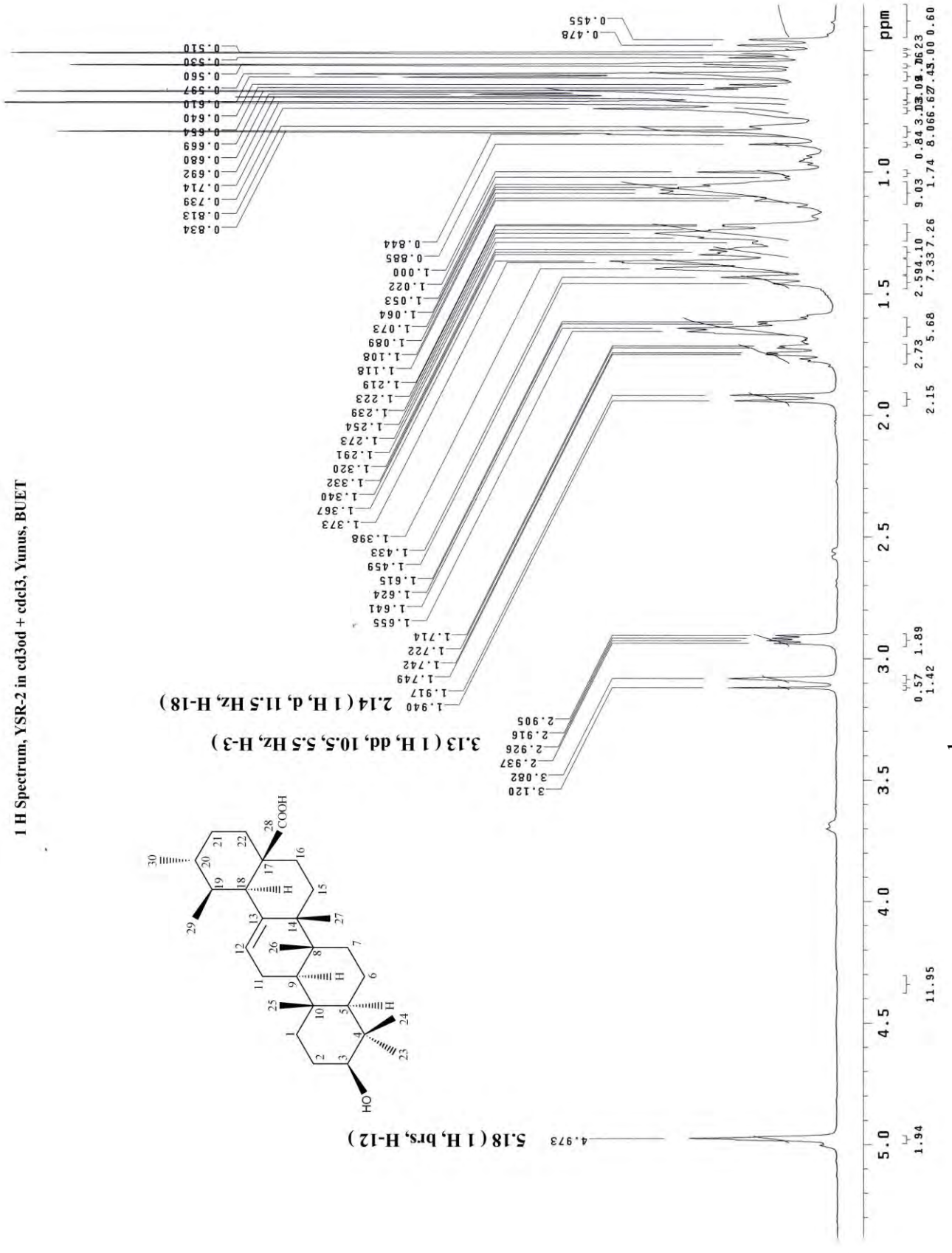


Figure 3.21: Partially expanded ^{1}H NMR spectrum of compound-3 (YSR-2) in $CDCl_3 + CD_3OD$.

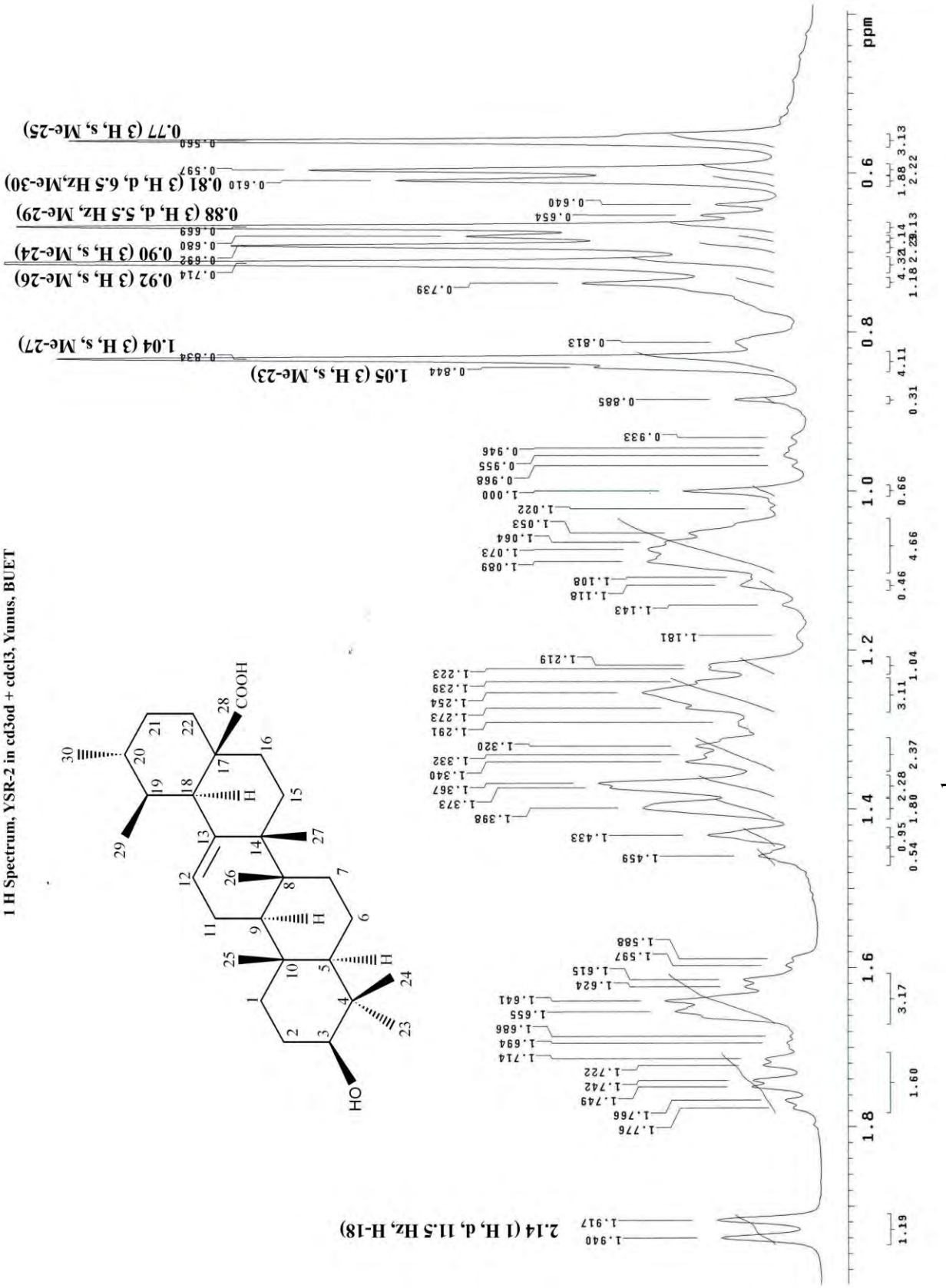


Figure 3.22: Partially expanded ^{1}H NMR spectrum of compound-3 (YSR-2) in $CDCl_3$ + CD_3OD.

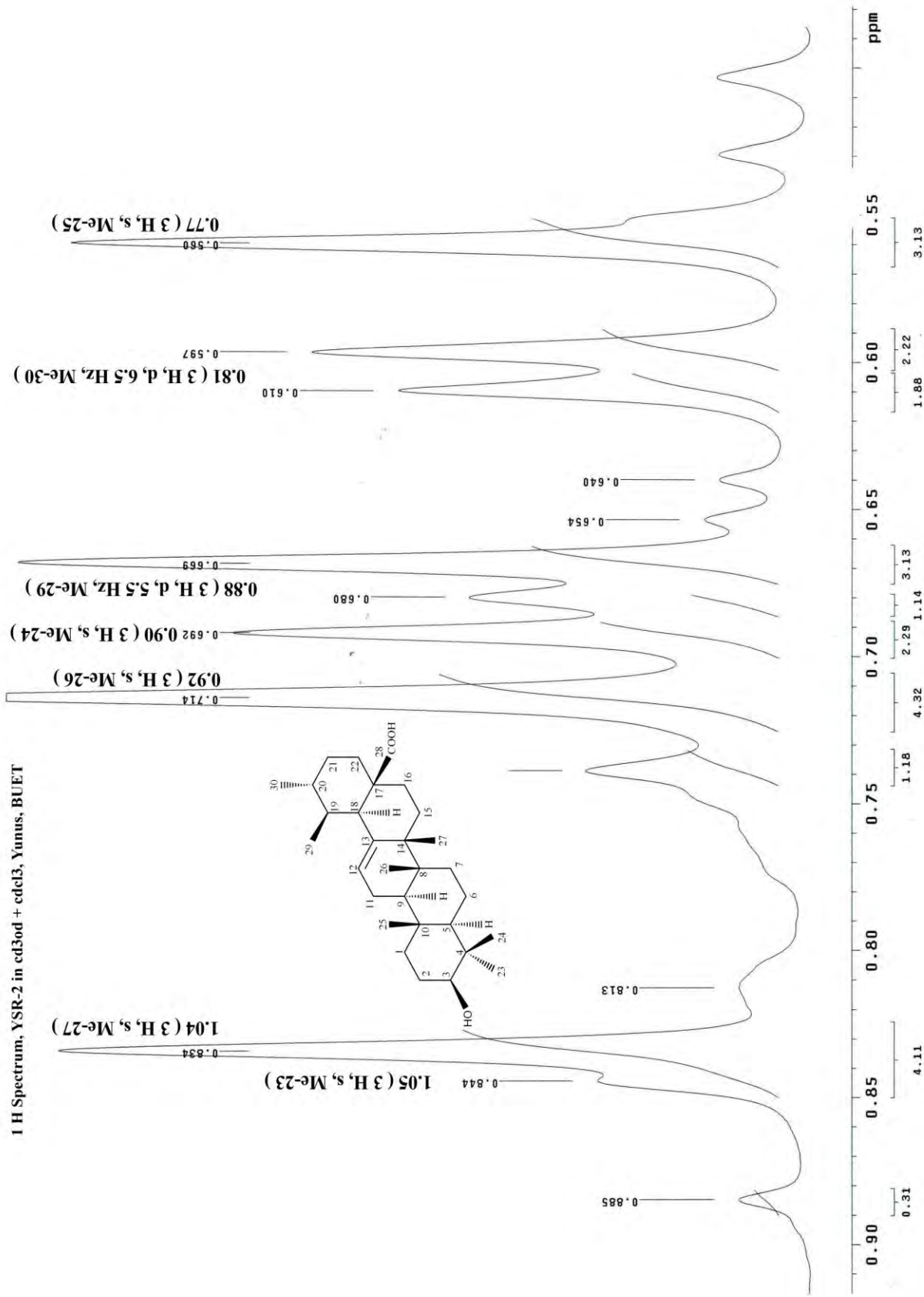


Figure 3.23: Partially expanded ^{1}H NMR spectrum of compound-3 (YSR-2) in $CDCl_3$ + CD_3OD.

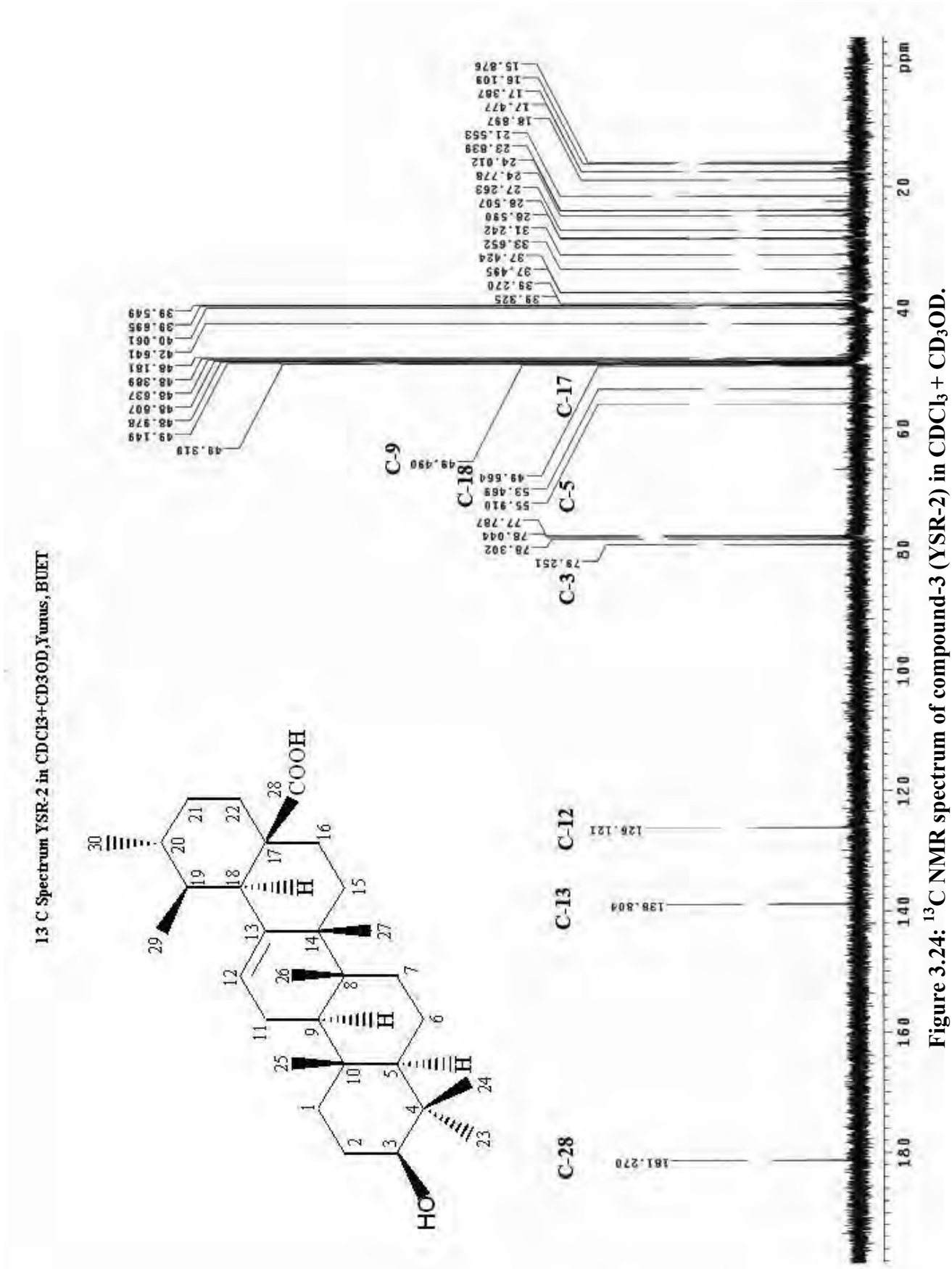


Figure 3.24: ^{13}C NMR spectrum of compound-3 (YSR-2) in $CDCl_3$ + CD_3OD.

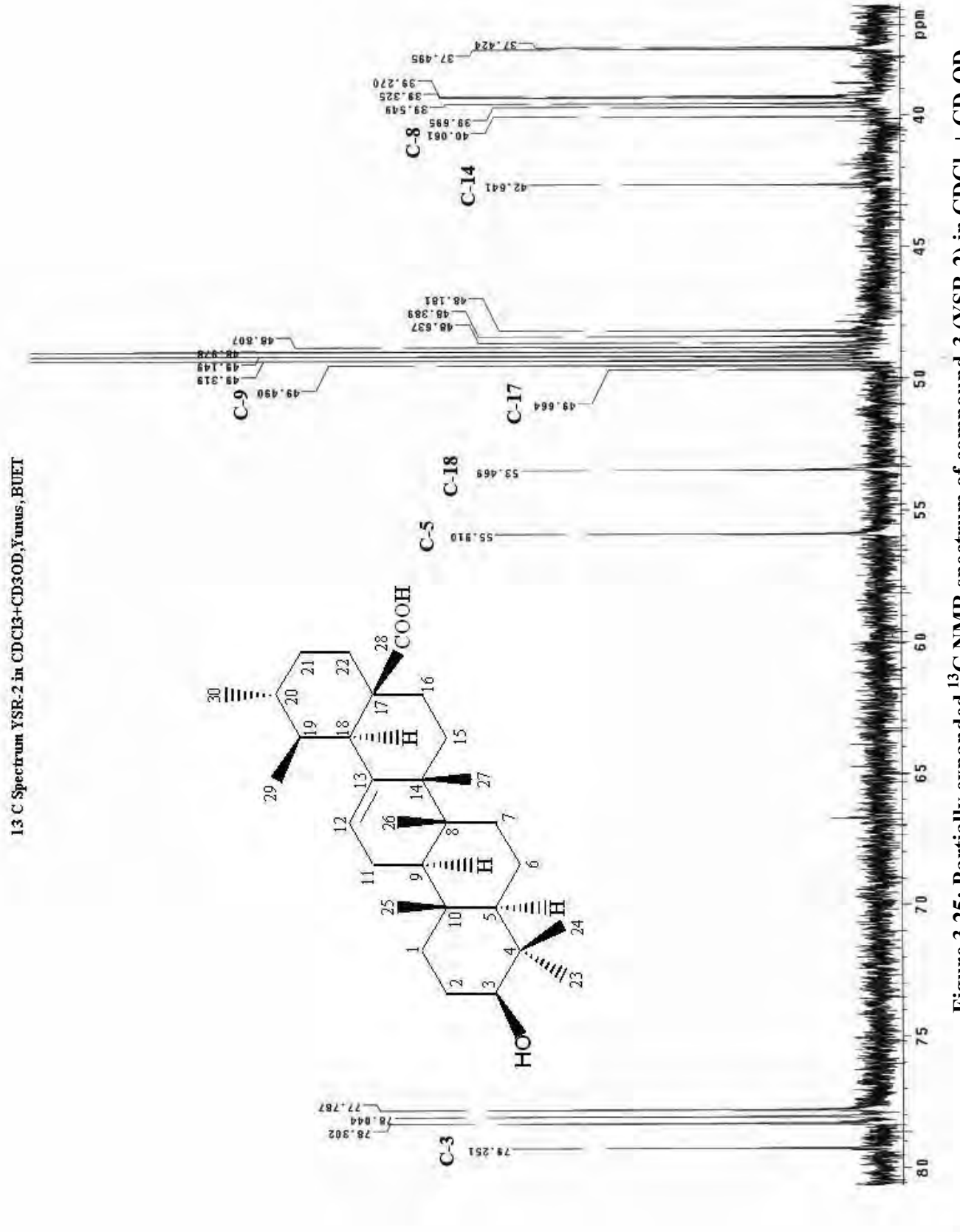


Figure 3.25: Partially expanded ^{13}C NMR spectrum of compound-3 (YSR-2) in $CDCl_3 + CD_3OD$.

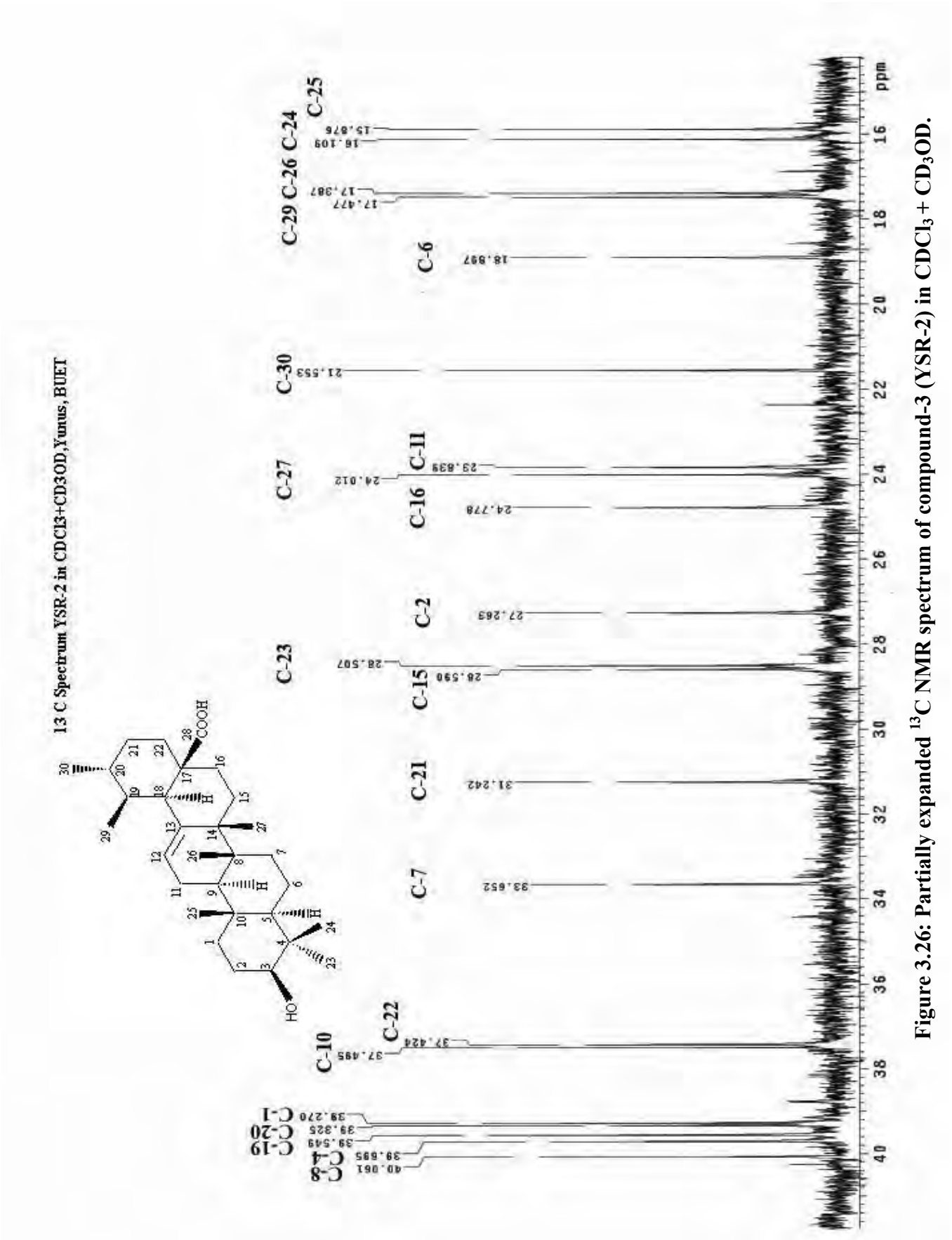


Figure 3.26: Partially expanded ^{13}C NMR spectrum of compound-3 (YSR-2) in $CDCl_3$ + CD_3OD.

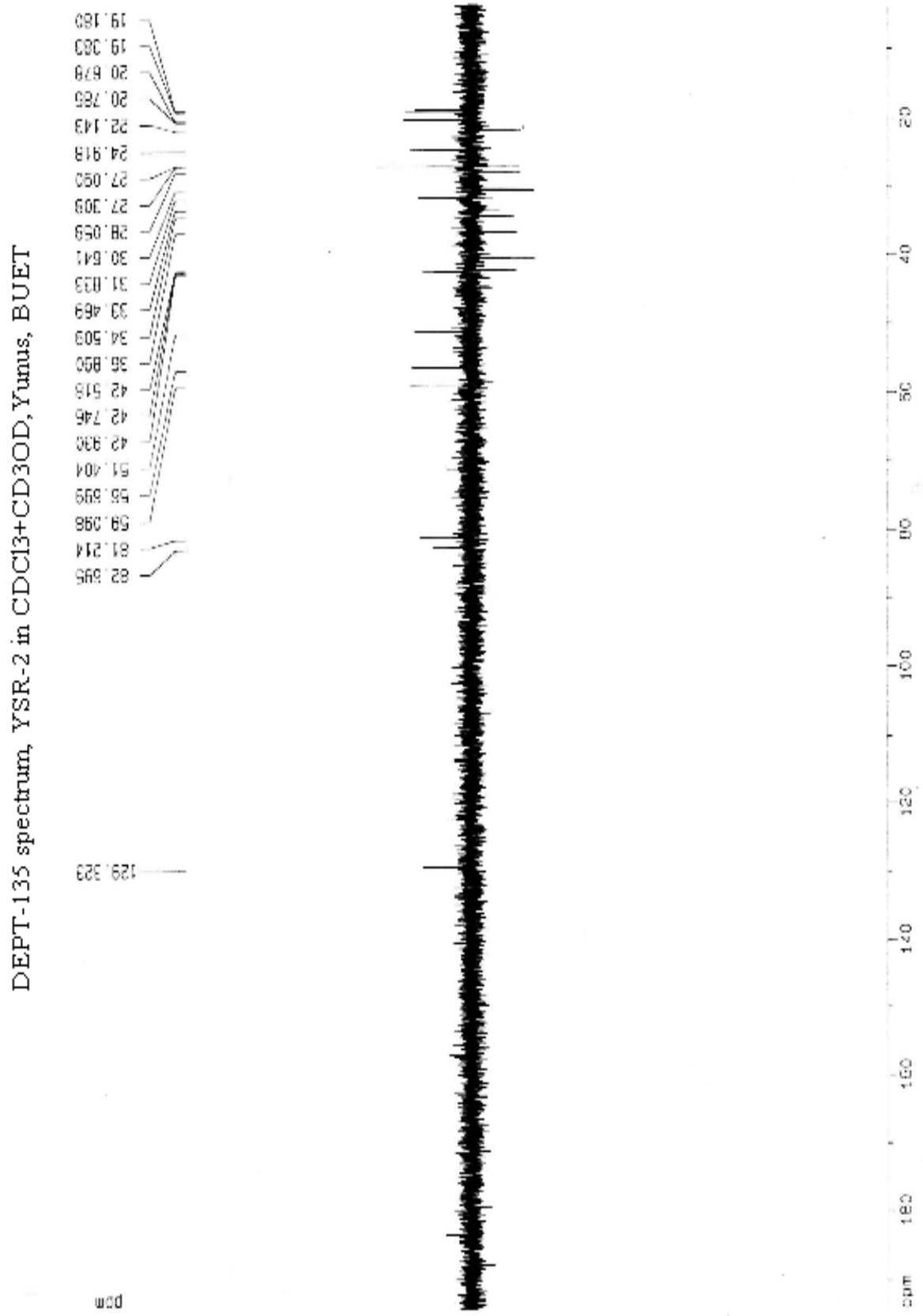


Figure 3.27: DEPT-135 spectrum of compound-3 (YSR-2) in $CDCl_3$ + CD_3OD.

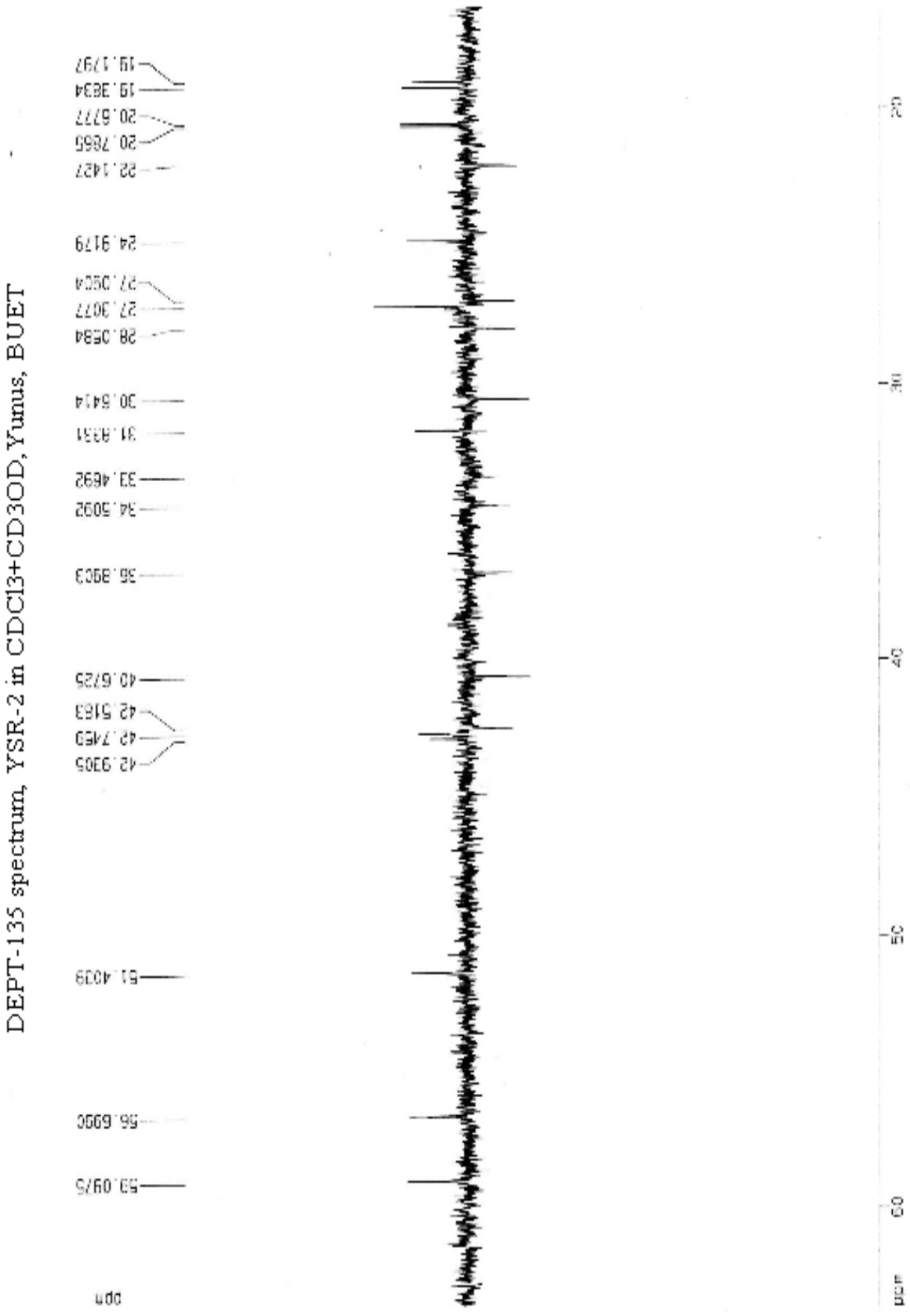


Figure 3.28: Partially expanded DEPT-135 spectrum of compound-3 (YSR-2) in $CDCl_3$ + CD_3OD.

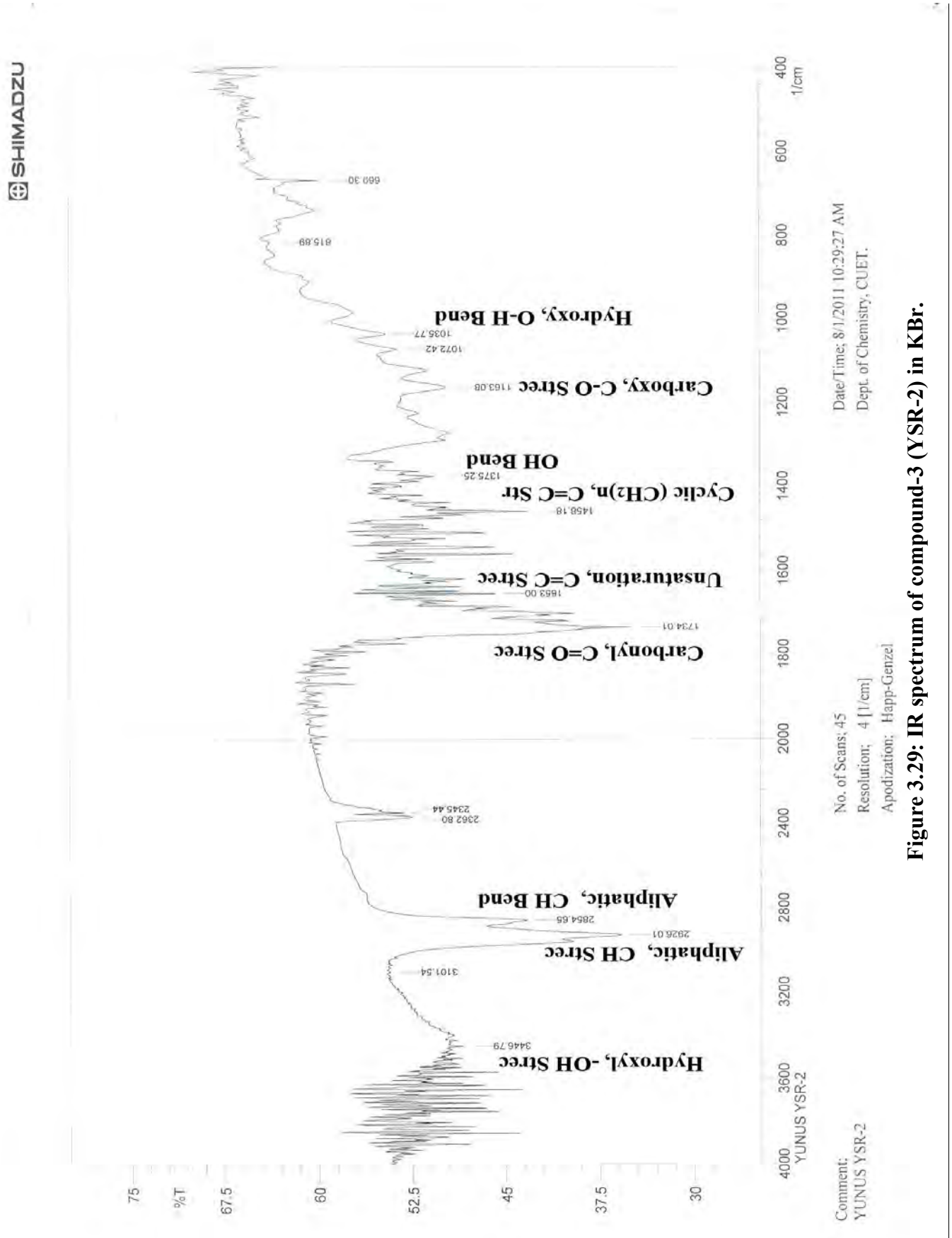


Figure 3.29: IR spectrum of compound-3 (YSR-2) in KBr.

3.2.4 Characterization of compound-4(YSR-7) as 3*β*-hydroxy-olea-12-en (*β*-amyrin)

3.2.4.1 Physical properties

The compound-4 (YSR-7) was obtained as white crystalline powder. It appeared as dark quenching spot on the TLC plate under UV light at 254 nm and also exhibited blue fluorescence at 365 nm (R_f = 0.54; toluene/10% ethyl acetate). It was soluble in DCM, chloroform and ethyl acetate. It was appeared as a purple spot on the TLC plate when sprayed with vanillin-sulfuric acid spray reagent, followed by heating at 110^0C, for 5 minutes, which gave positive Lieberman-Burchard test for triterpenes. The melting point was found to be 197-198^{0}C[57].

3.2.4.2 Characterization of compound-4 (YSR-7) by spectroscopic method

The structure of the compound-4 (YSR-7) has been established by UV, FT-IR, ^{1}H-NMR and ^{13}C-NMR spectral evidences.

3.2.4.2.1 Ultraviolet (UV) spectroscopy of compound-4 (YSR-7)

The UV spectrum showed absorption band at λmax 265 nm in methanol

3.2.4.2.2 Infrared (IR) spectroscopy of compound-4 (YSR-7)

The IR spectrum (in KBr) of compound-4(YSR-7) exhibit characteristic absorption band for hydroxyl (-OH) group at 3446.79 cm^{-1}, Absorption at 2920.23 and 2848.86cm^{-1} was due to aliphatic C-H stretching and bending vibrations of methyl group, Other frequencies include 1653.00 cm^{-1} as a result C=C stretching however this band was weak at1447.18 cm^{-1} was a bending frequency for cyclic (CH_2)n and 1375.25 cm^{-1} for C-H bending, hydroxyl (O-H bending) function at1035.77cm^{-1} and presence of tri-substituted double bond at 821.68 cm^{-1} [57].

3.2.3.2.3 ^{1}H-NMR spectroscopy of compound-4 (YSR-7)

The ^{1}H NMR spectrum of the compound-4 showed eight methyl singlet at 1.07, 1.00, 0.99, 0.95, 0.91, 0.86, 0.79 and 0.79.The tertiary nature of these methyls was evident from their sharp singlets in the ^{1}H NMR. The proton signals of H-29 and H-30 in the ursane skeletons appears as a doublet, but in oleane as a singlet[58]. The carbonyl proton resonated at δ 3.21 in α and axial configuration as confirmed by double doublet (*J*ax.ax = 10.0 Hz, *J*ax.eq 5.0 Hz H-3) and a characteristic olefinic proton of C12-C13 pentacyclic triterpenoid at δ 5.12 [66].

3.2.4.2.4 ^{13}C-NMR spectroscopy of compound-4 (YSR-7)

The ^{13}C-NMR spectrum revealed the presence of signals due to an oxygenated carbon signal at δ 79.3 (C-3), one tri-substituted double bond at δ 124.11 (C-12) and 143.47 (C-13) in the ring-C. In general, for the polyols, C-l2 is deshielded (~2 p.p.m.) and C-l3 shielded (~5 p.p.m.) in urs-12-enes compared to the corresponding olean-12-enes. The chemical shift of C-l3 appears to be about 138-140 p.p.m. in the urs-12-enes and 144-145 p.p.m. in the olean-12-enes[59]. The chemical shifts of 124.11 (C-12) and 143.47 (C-13) and H-12 (δ 5.20-5.4 ppm) suggests that this compound was Δ12-unsaturated triterpenoid. The ^{13}C NMR spectra clearly exhibited the difference in the chemical shifts of C-12, C-13, C-17, C-18, C-19, C-20, C-22, C-27, C-29 and C-30 between the ursane group and the oleane group which showed in **Table-3.4**. Moreover ^{13}C-NMR signals due to C-18 to C-22 and C-28 to C-30 suggested that YSR-7 was an olean-12-en derivative. The physical and spectral data of the compound was in complete agreement to the reported data in literature[57, 66], the compound-4 (YSR-7) was identified as 3*β*-hydroxy-olea-12-en (*β*-amyrin). The compound-4 (YSR-7) was reported here for the first time so far from the species *Saurauia roxburghii.*

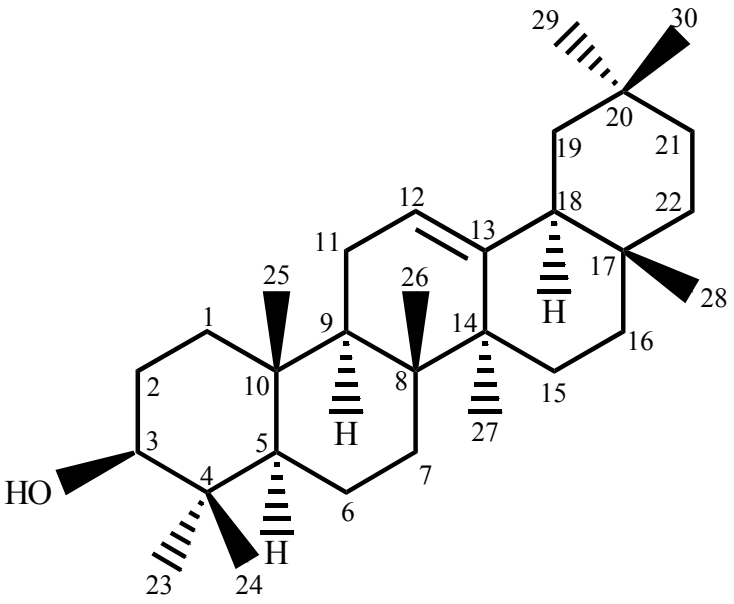


Figure-3.30: 3*β*-hydroxy-olea-12-en (*β*-amyrin)

Table-3.4: ^{1}H-NMR & ^{13}C-NMR Spectral data of compound-4 (YSR-7)

C/H	^{1}H (δ ppm) Obtained	^{1}H (δ ppm) Literature[57]	^{13}C(δ ppm) Obtained	^{13}C(δ ppm) Literature[57]
1			39.3 (CH_2)	39.0
2			21.5 (CH_2)	27.4
3	3·21(1H, dd, J=10.0,5.0Hz)	3.19(1H, dd, J =10.0,4.5Hz)	79.3 (CH)	78.8
4			39.7 (Cq)	39.1
5			55.9 (CH)	54.4
6			18.9 (CH_2)	18.5
7			33.7 (CH_2)	33.2
8			40.1 (Cq)	39.2
9			49.5 (CH)	48.7
10			37·5 (Cq)	37.10
11			23·8 (CH_2)	23.6
12	5.12 (1H, t, *J*=3,2Hz)	5.11 (1H, m)	124.1 (CH)	124.4
13			143.5 (Cq)	144.3
14			42.6 (Cq)	42.3
15			28.6 (CH_2)	27.9
16			24.8 (CH_2)	24.5
17			48.2 (Cq)	47.6
18			41.9 (CH)	41.3
19			48.4 (CH_2)	46.9
20			31.3 (Cq)	31.7
21			39.2 (CH_2)	38.9
22			37.4 (CH_2)	36.6
23	0·91 (3H, s, Me)	0·93 (3H, s, Me)	28.5 (CH_3)	28.2
24	0·86 (3H, s, Me)	0·88 (3H, s, Me)	16.1 (CH_3)	15.5
25	1.07 (3H, s, Me)	1.08 (3H, s, Me)	15.9 (CH_3)	15.6
26	1.00 (3H, s, Me)	1.02 (3H, s, Me)	17.4 (CH_3)	16.9
27	0.99 (3H, s, Me)	1.01 (3H, s, Me)	26.5 (CH_3)	26.0
28	0.95 (3H, s, Me)	0.96 (3H, s, Me)	28.5 (CH_3)	28.0
29	0·83 (3H, s, Me)	0·85 (3H, s, Me)	33.1 (CH_3)	32.7
30	0·79 (3H, s, Me)	0·80 (3H, s, Me)	24.0 (CH_3)	23.3

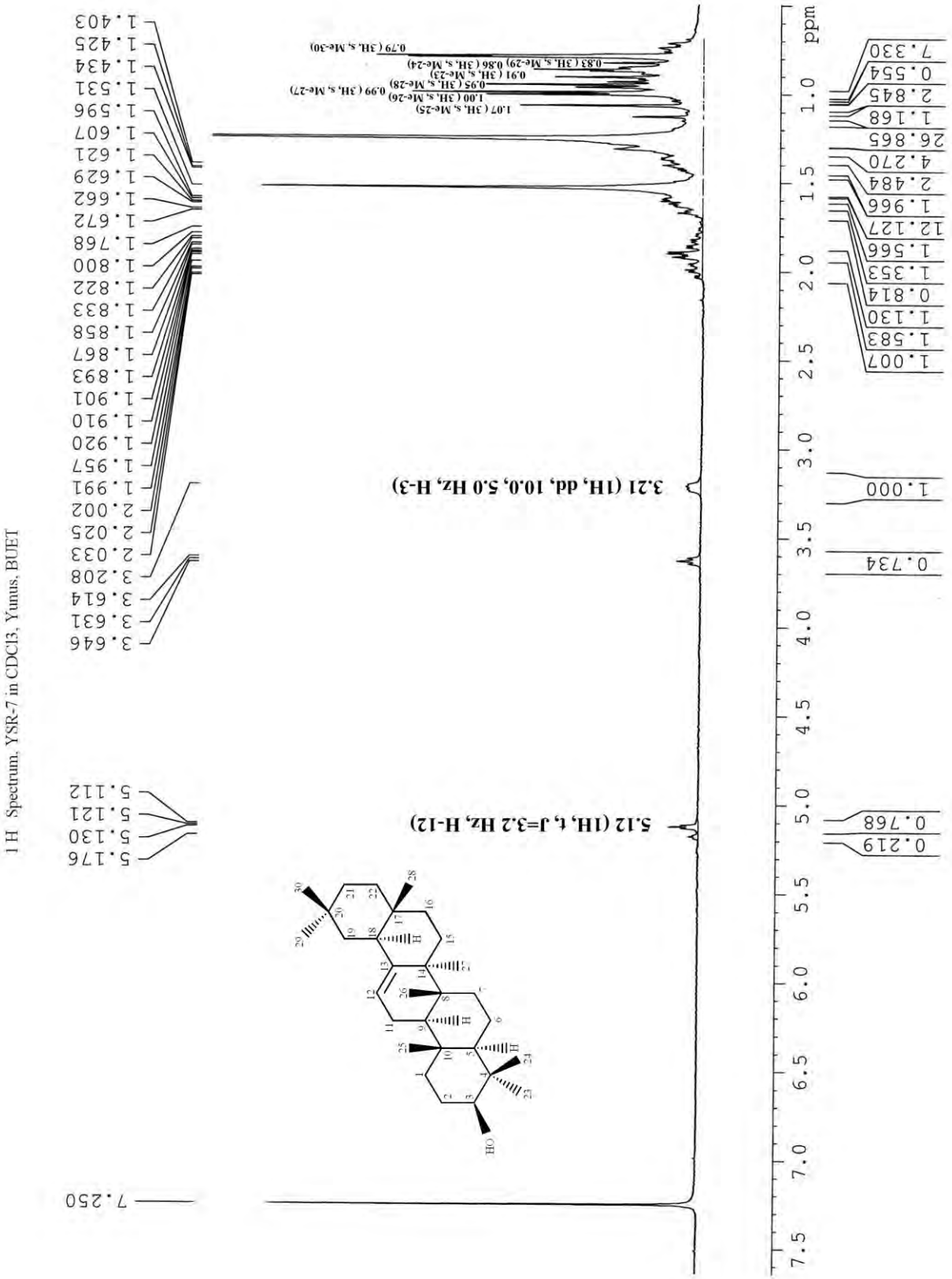


Figure 3.31: ^{1}H NMR spectrum of compound-4 (YSR-7) in $CDCl_3$.

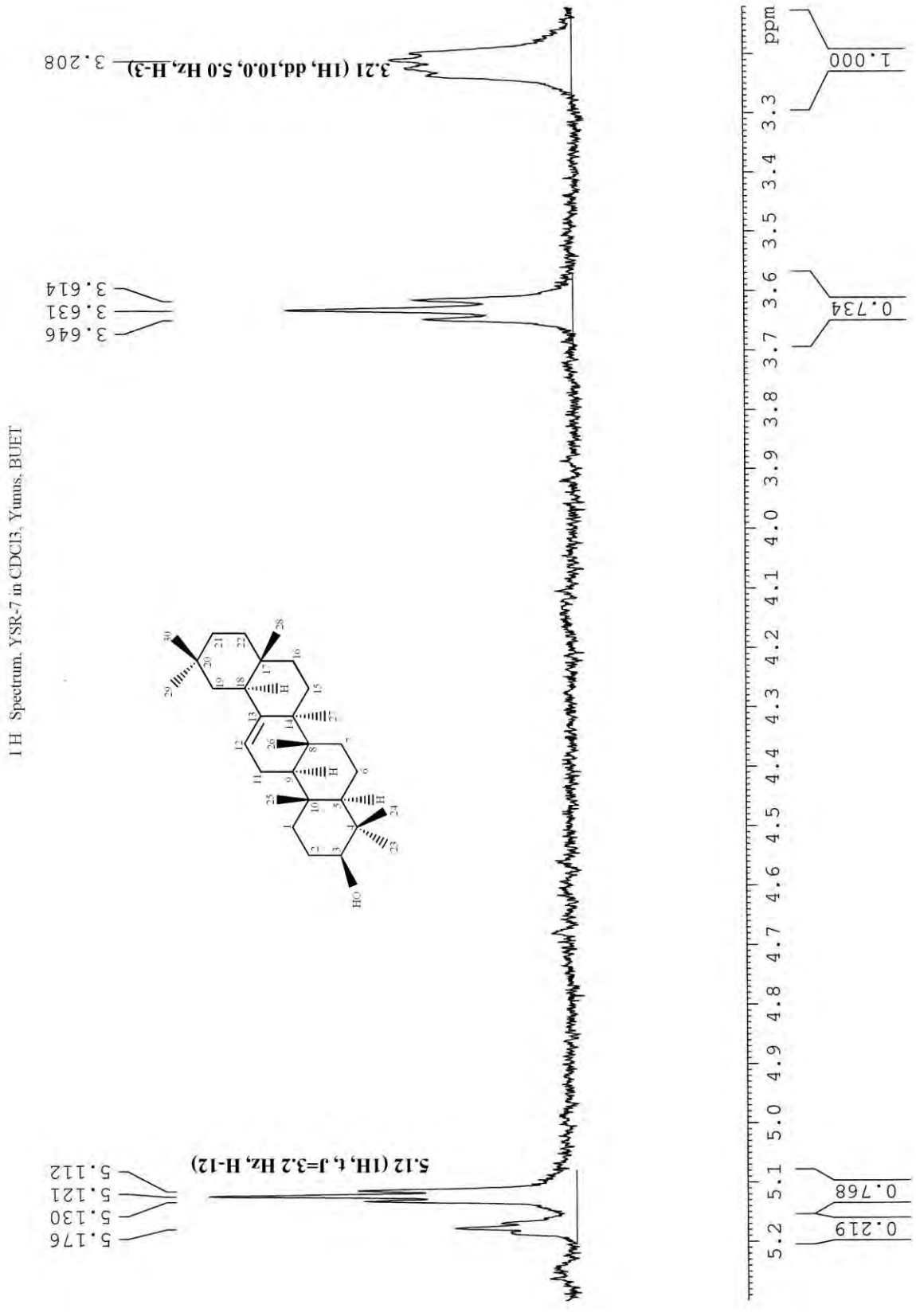


Figure 3.32: Partially expanded ^{1}H NMR spectrum of compound-4 (YSR-7) in $CDCl_3$.

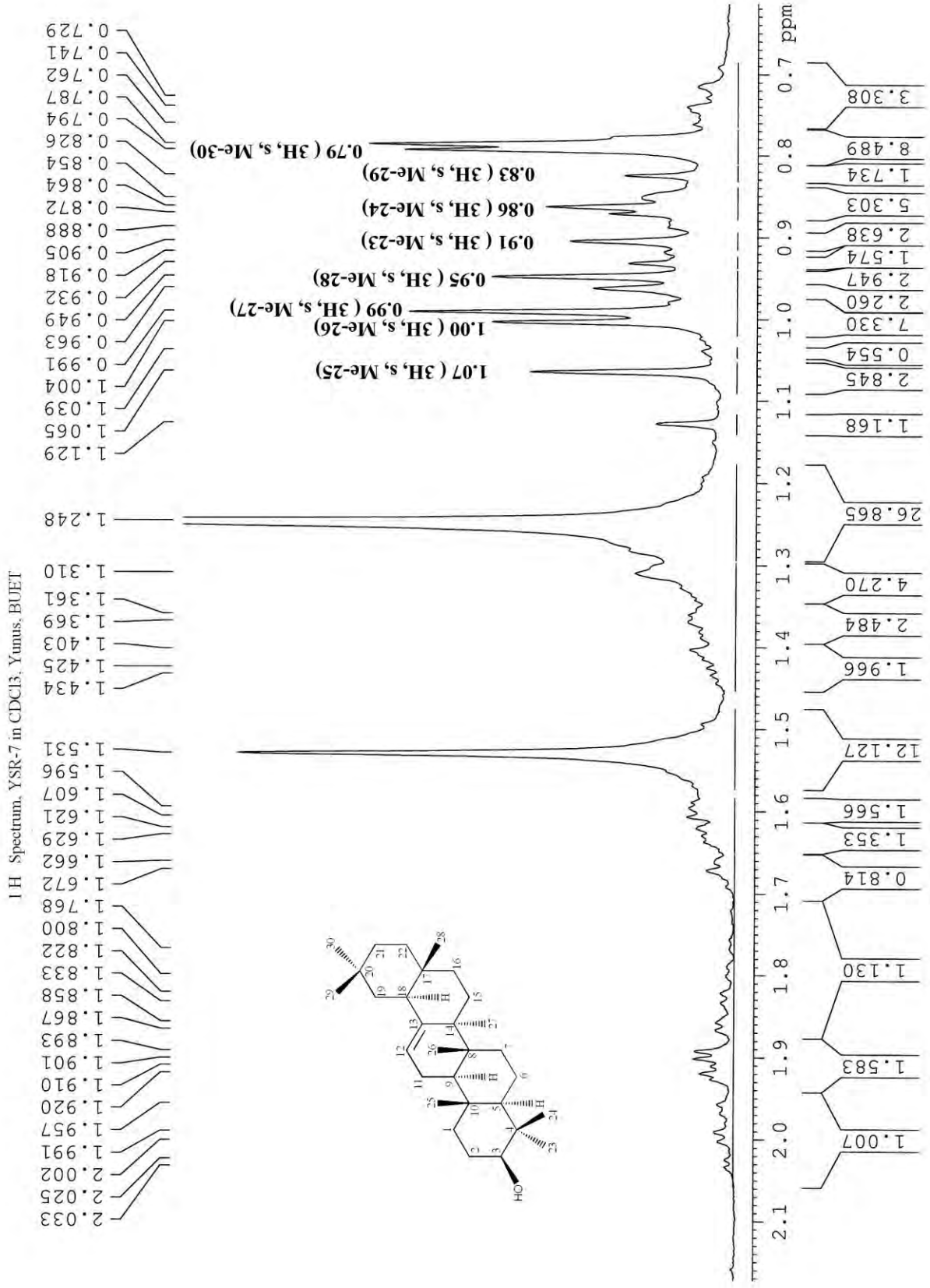


Figure 3.33: Partially expanded ^{1}H NMR spectrum of compound-4 (YSR-7) in $CDCl_3$.

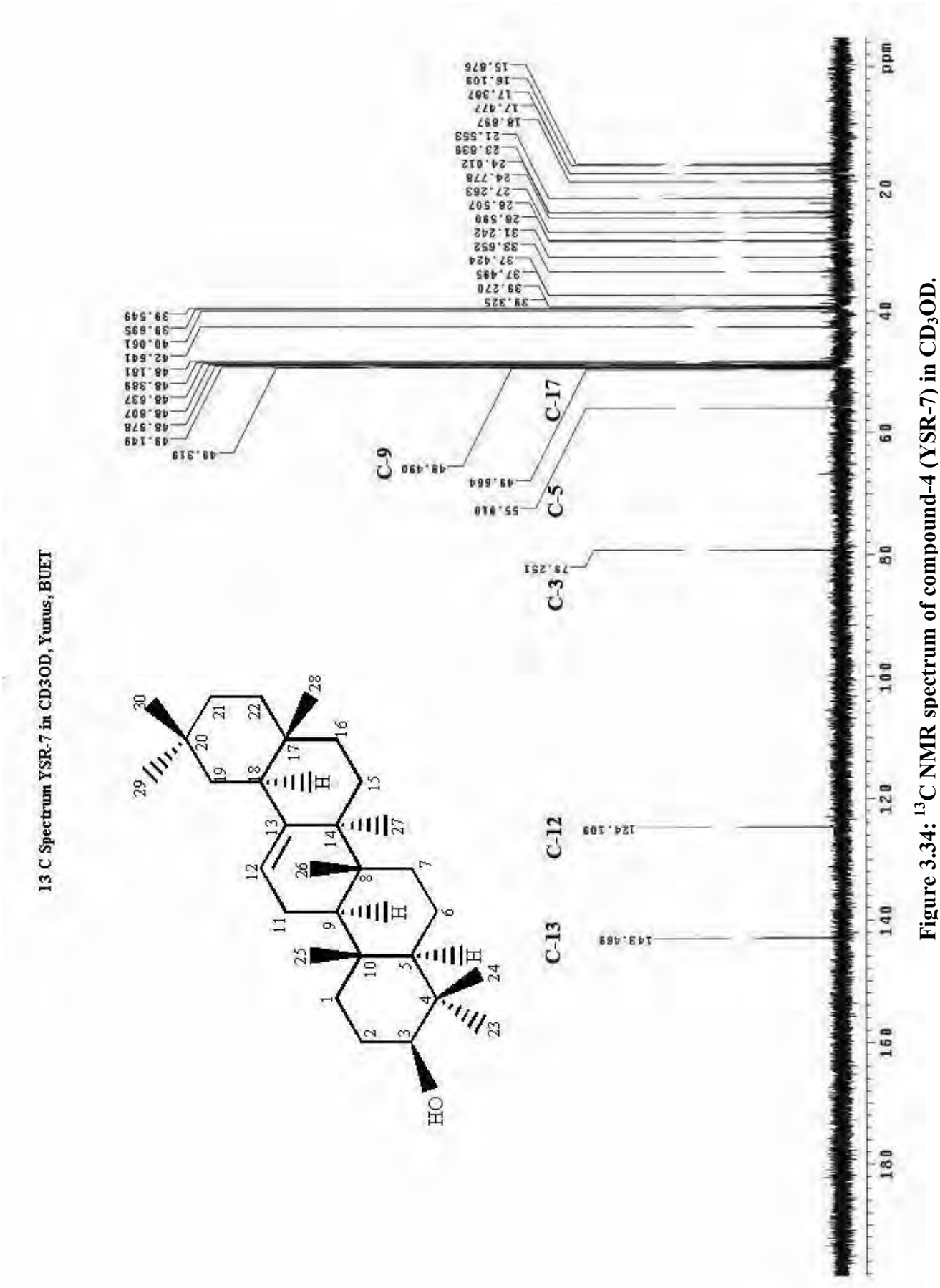


Figure 3.34: ^{13}C NMR spectrum of compound-4 (YSR-7) in CD_3OD.

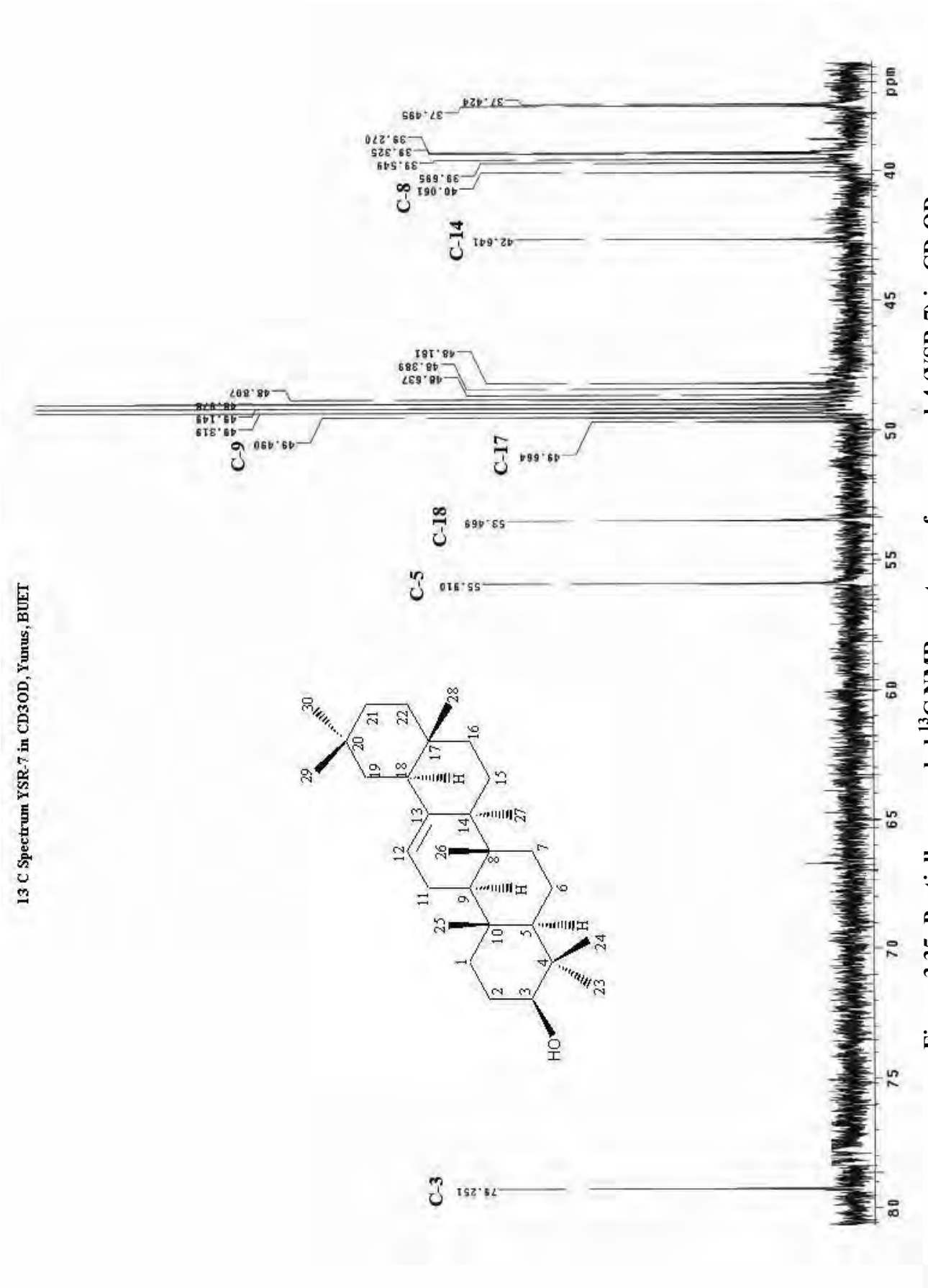


Figure 3.35: Partially expanded ^{13}C NMR spectrum of compound-4 (YSR-7) in CD_3OD.

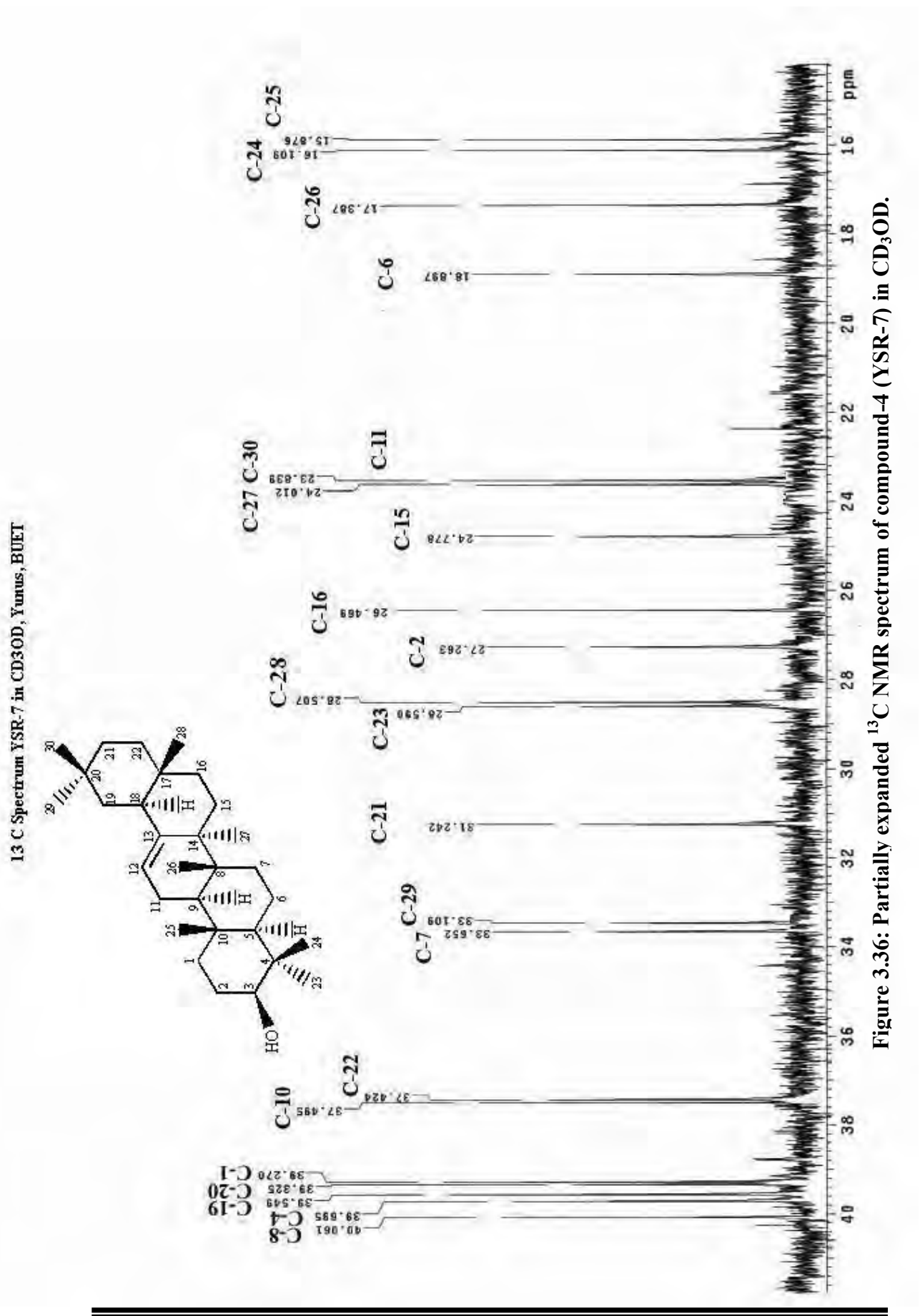


Figure 3.36: Partially expanded ^{13}C NMR spectrum of compound-4 (YSR-7) in CD_3OD.

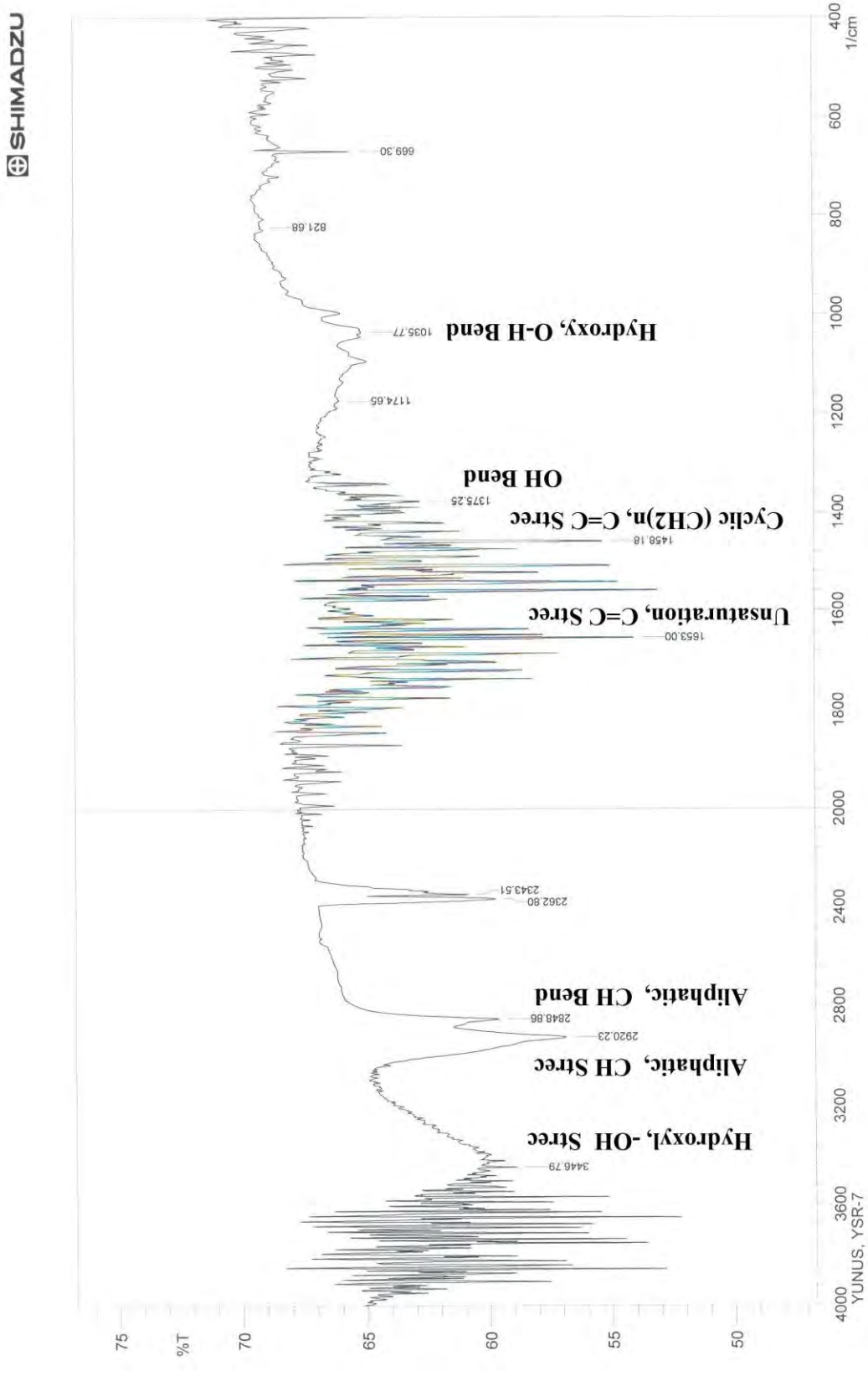


Figure 3.37: IR spectrum of compound-4 (YSR-7) in KBr.

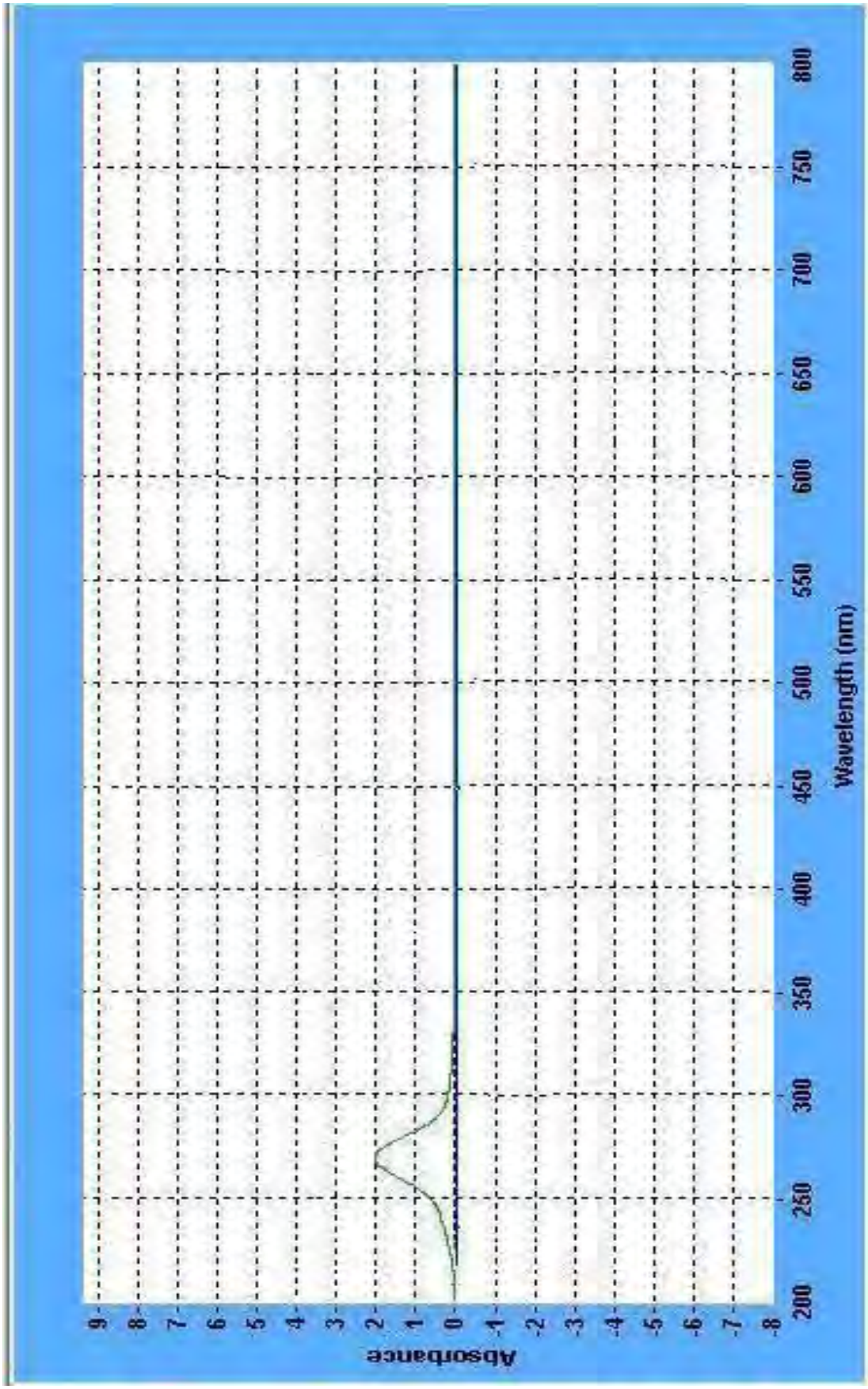


Figure 3.38: UV spectrum of compound-4 (YSR-7) in methanol.

3.2.5 Characterization of compound-5(YSR-3) as 3*β*-hydroxy-olea-12-en-28-oic acid

3.2.5.1 Physical properties

The compound-5 (YSR-3) was obtained as a white amorphous powder compound. The R_f value of the compound was 0.76 in n-hexane: ethylacetate = 50:50. It was soluble in chloroform, dichloromethane, ethyl acetate and methanol. It was tested for terpenoid and a red-violet color confirms that the compound-5(YSR-3) was terpenoid type compound. The melting point was found to be 303-305^{0}C[61].

3.2.5.2 Characterization of compound-5 (YSR-5) by spectroscopic method

The structure of the compound-5 (YSR-3) has been established by UV, FT-IR, ^{1}H-NMR, ^{13}C-NMR and DEPT-135 spectral evidences.

3.2.5.2.1 Ultraviolet (UV) spectroscopy of compound-5 (YSR-3)

The UV spectrum showed absorption band at λmax 215 and 322 nm in methanol

3.2.5.2.2 Infrared (IR) spectroscopy of compound-5 (YSR-3)

The IR spectrum (in KBr) of compound-5 (YSR-3) exhibit characteristic absorption band for hydroxyl (-OH) group at 3298.28 cm^{-1}, OH of carboxylic function at 3122.75 cm^{-1}, Absorption at 2920.23 and 2852.72 cm^{-1} was due to aliphatic C-H stretching and bending vibrations of methyl group, carbonyl (C=O) of the carboxyl group at 1716.65 cm^{-1}, Other absorption frequencies include 1653.00 cm^{-1} as a result C=C stretching however this band was weak at 1447.18 cm^{-1} was a bending frequency for cyclic (CH_2)n and 1375.25 cm^{-1} for C-H bending, carboxy (C-O stretching) function at 1188.15 cm^{-1}, hydroxy (O-H bending) function at 1035.77 cm^{-1} and presence of trisubstituted double bond at 827.46 cm[61].

3.2.5.2.3 ^{1}H-NMR spectroscopy of compound-5 (YSR-3)

The ^{1}H NMR spectrum of the compound showed for seven methyl singlets at 1.01, 0.95, 0.92, 0.89, 0.85, 0.81 and 0.73. The tertiary nature of these methyls was evident from their sharp singlets in the ^{1}H NMR. The ^{1}H NMR spectrum showed a downfield signal for oxygenated methine proton at δ3.52 (1H, m), which was assigned for H-3 proton and a characteristic olefinic proton of C12-C13 double bonded pentacyclic triterpenoid at δ 5.06. The spectrum of this compound, the signal at δ 3.14 (1H, dd, 8.0, 8.0Hz) for H-18 permitted the distinction

between the oleane and ursane skeletons. The H-18 signal appears at δ 2.1-2.6 ppm in the ursane skeleton and at δ 2.8-3.3 ppm in the oleane skeleton[57].The proton signals of H-29 and H-30 in the ursane skeletons appears as a doublet, but in oleane as a singlet[58, 60].

3.2.5.2.4 ^{13}C-NMR spectroscopy of compound-5 (YSR-3)

The ^{13}C NMR assignments of various carbon atoms were substantiated by DEPT experiments, which revealed the presence of seven methyls, ten methylenes, five methines and seven quaternary carbon atoms which showed in **Table-3.5**. The ^{13}C-NMR spectrum also revealed the presence of signals due to an oxygenated carbon signal at δ 79.3 (C-3), one tri-substituted double bond at δ 124.11 (C-12) and 143.47 (C-13) in the ring-C and the most downfield signal due to the carboxyl carbon displayed its resonance at 181.31 (C-28). In general, for the polyols C-l2 is deshielded (~2 p.p.m.) and C-l3 shielded (~5 p.p.m.) in urs-12-enes compared to the corresponding olean-12-enes. The chemical shift of C-l3 appears to be about 138-140 p.p.m. in the urs-12-enes and 144-145 p.p.m. in the olean-12-enes[59].The chemical shifts of 124.11 (C-12) and 143.47 (C-13) and H-12 (δ 5.20-5.4 ppm) suggests that this compound was Δ12-unsaturated triterpenoid[57]. The ^{13}C NMR spectra clearly exhibited the difference in the chemical shifts of C-12, C-13, C-17, C-18, C-19, C-20, C-22, C-27, C-29 and C-30 between the ursane group and the oleane group. Moreover ^{13}C-NMR signals due to C-18 to C-22 suggested that compound-5 (YSR-3) was an olean-12-en derivative. The physical and spectral data of this compound was in complete agreement to the reported data in literature[57, 58, 61]. The compound-5 (YSR-3) was identified as 3*β*-hydroxy-olea-12-en-28-oic acid. This compound was the first isolated so far on the plant *Saurauia roxburghii.*

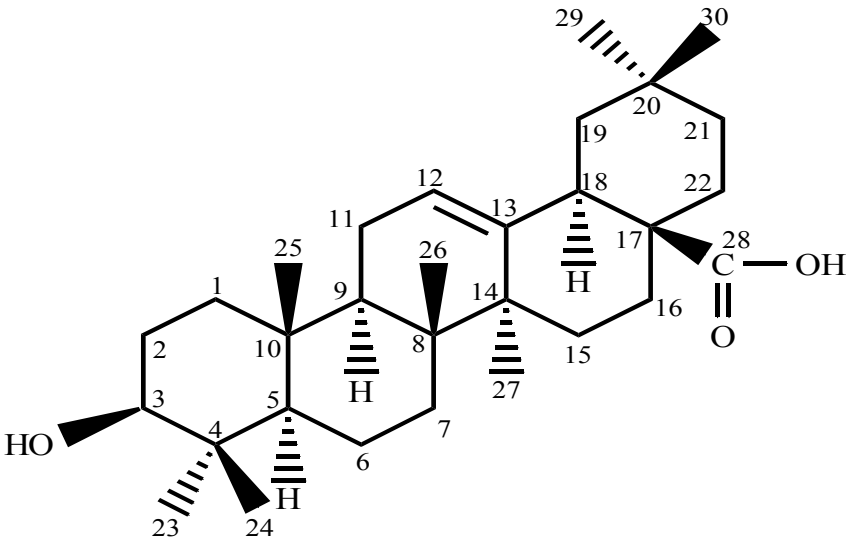


Fig 3.39: 3*β*-hydroxy-olea-12-en-28-oic acid.

Table-3.5 : ^{1}H-NMR & ^{13}C-NMR Spectral data of compound-5 (YSR-3)

C/H	^{1}H (δ ppm) Obtained	^{1}H (δ ppm) Literature[57]	^{13}C(δ ppm) Obtained	^{13}C(δ ppm) Literature[58]
1			39.3 (CH_2)	39.0
2			21.5 (CH_2)	21.1
3	3·52 (1H, m)	3.60 (1H, dd, *J* = 9.9,4.1Hz)	79.3 (CH)	78.2
4			39.7 (Cq)	39.4
5			55.9 (CH)	55.9
6			18.9 (CH_2)	18.8
7			33.7 (CH_2)	33.4
8			40.1 (Cq)	39.8
9			49.5 (CH)	48.2
10			37·5 (Cq)	37.4
11			23·8 (CH_2)	23.8
12	5.06 (1H, brs)	5.24 (1H, t, *J* = 3.45 Hz)	124.1 (CH)	122.6
13			143.5 (Cq)	144.8
14			42.6 (Cq)	42.2
15			28.6 (CH_2)	28.4
16			24.8 (CH_2)	23.8
17			48.2 (Cq)	46.7
18	3.14(1H, dd, *J*=8.0, 8.0 Hz)	3.22(1H, dd, *J*=10.0, 6.0 Hz)	41.9 (CH)	42.1
19			48.4 (CH_2)	47.9
20			31.3 (Cq)	31.0
21			39.2 (CH_2)	39.3
22			37.4 (CH_2)	33.2
23	0·95 (3H, s, Me)	1.03(3H, s, Me)	28.5 (CH_3)	28.8
24	0·89 (3H, s, Me)	0·98 (3H, s, Me)	16.1 (CH_3)	16.5
25	0·73 (3H, s, Me)	0·90 (3H, s, Me)	15.9 (CH_3)	15.6
26	0·92 (3H, s, Me)	0·98 (3H, s, Me)	17.4 (CH_3)	17.5
27	1.01 (3H, s, Me)	1.12 (3H, s, Me)	26.5 (CH_3)	26.2
28			181.9 (Cq)	180.0
29	0·81 (3H, s, Me)	0·91 (3H, s, Me)	33.1 (CH_3)	33.4
30	0·85 (3H, s, Me)	0·97 (3H, s, Me)	24.0 (CH_3)	23.8

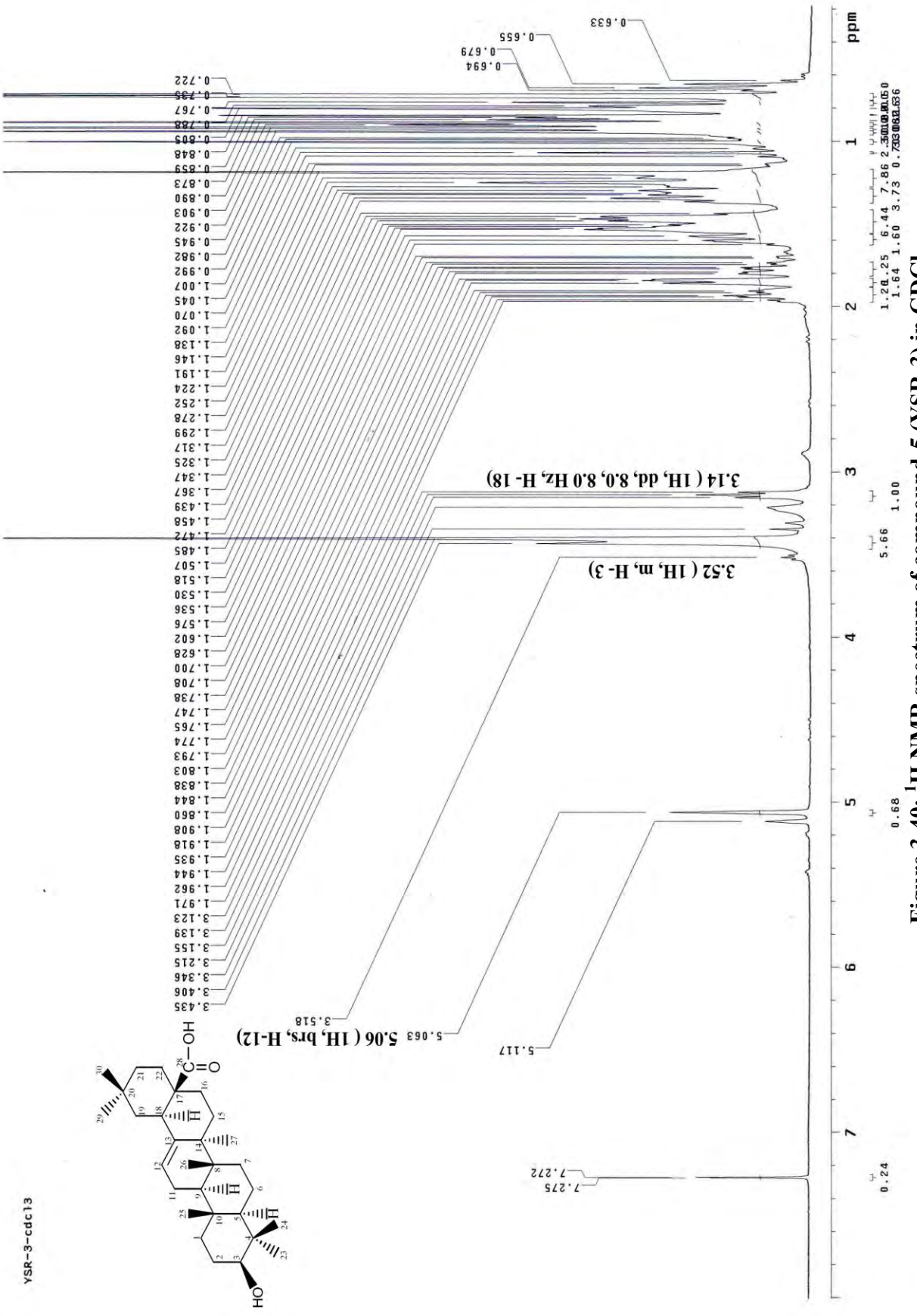


Figure 3.40: ^{1}H NMR spectrum of compound-5 (YSR-3) in $CDCl_3$.

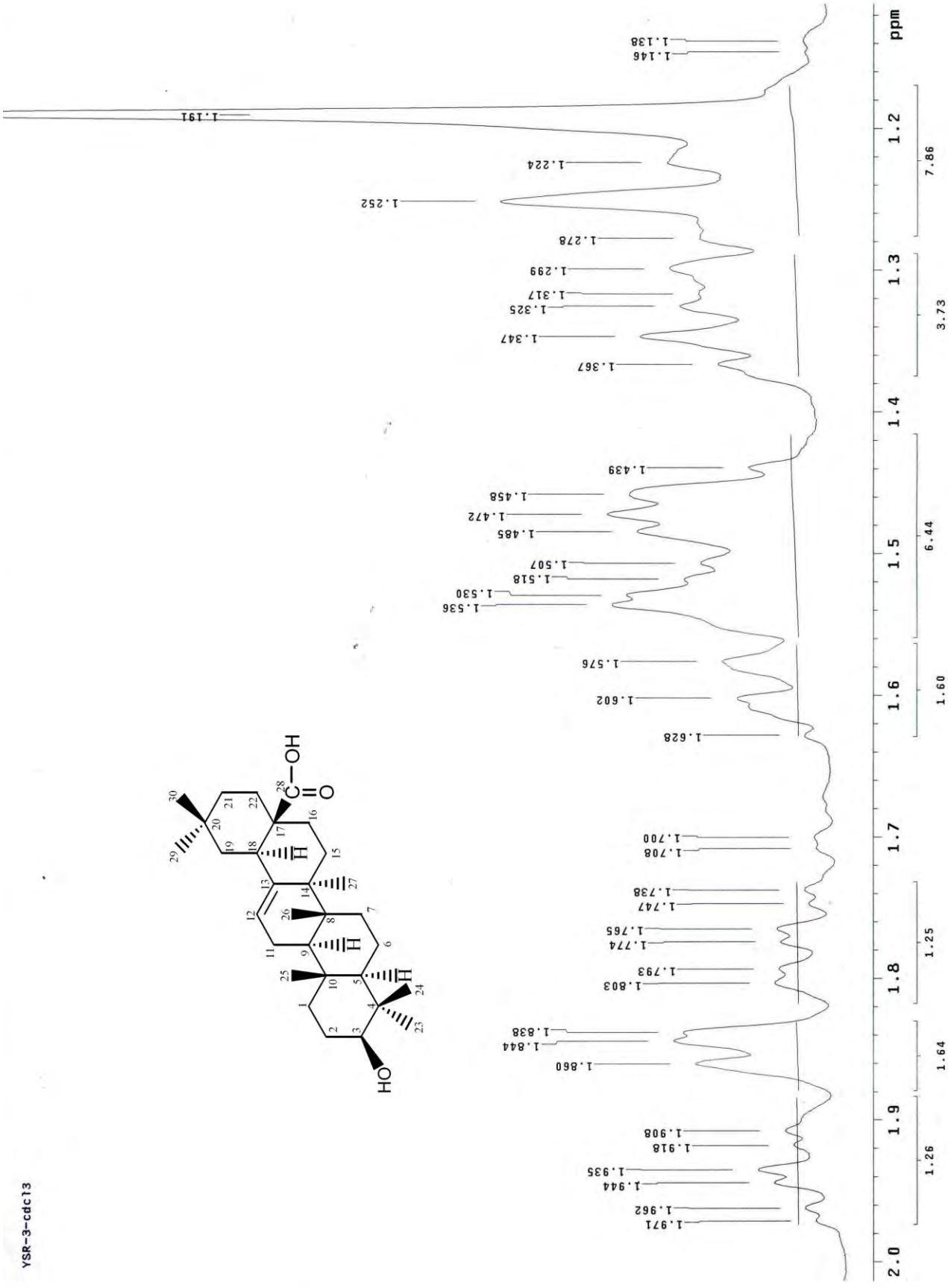


Figure 3. 41: Partially expanded ^{1}H NMR spectrum of compound-5 (YSR-3) in $CDCl_3$.

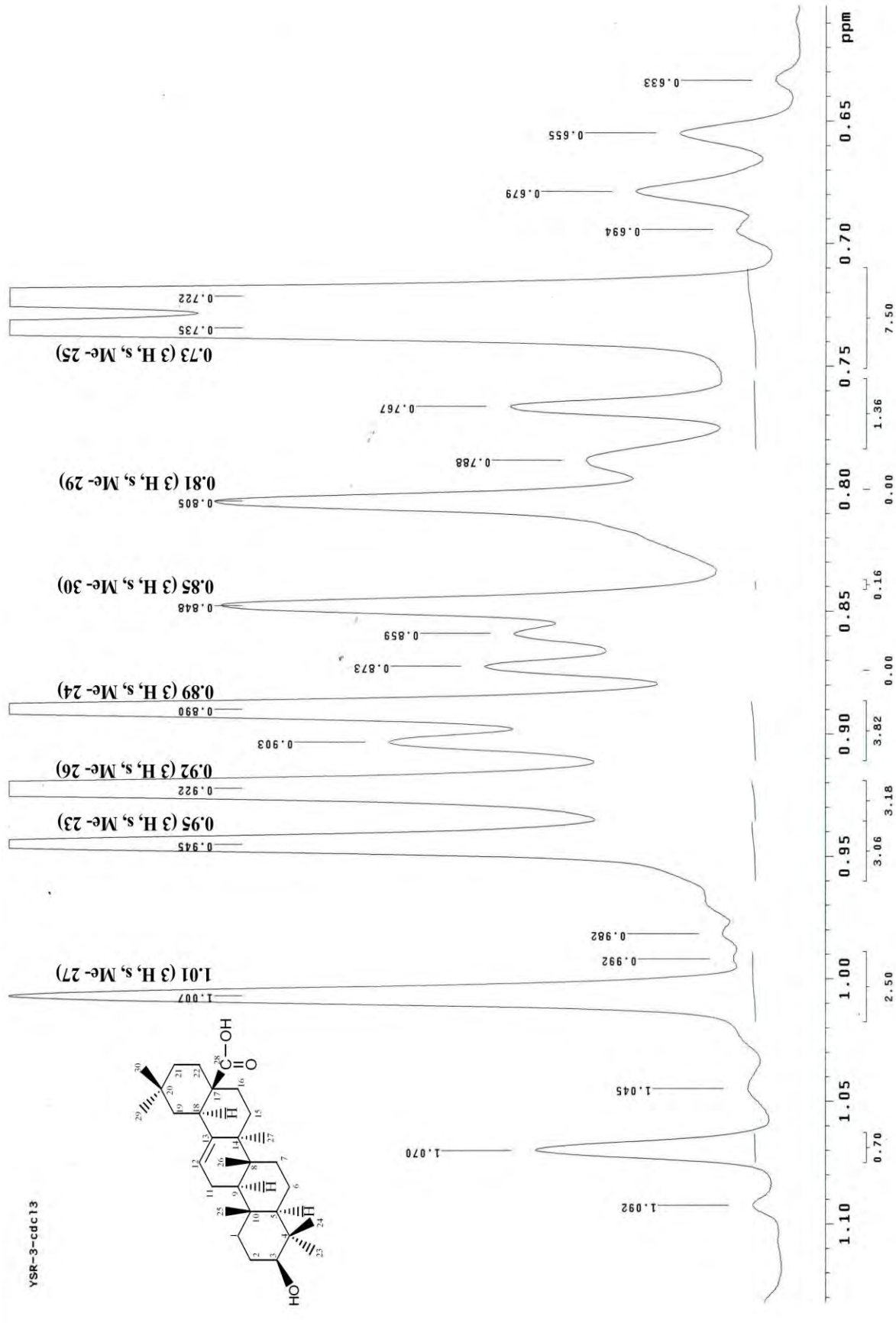


Figure 3.42: Partially expanded ^{1}H NMR spectrum of compound-5 (YSR-3) in $CDCl_3$.

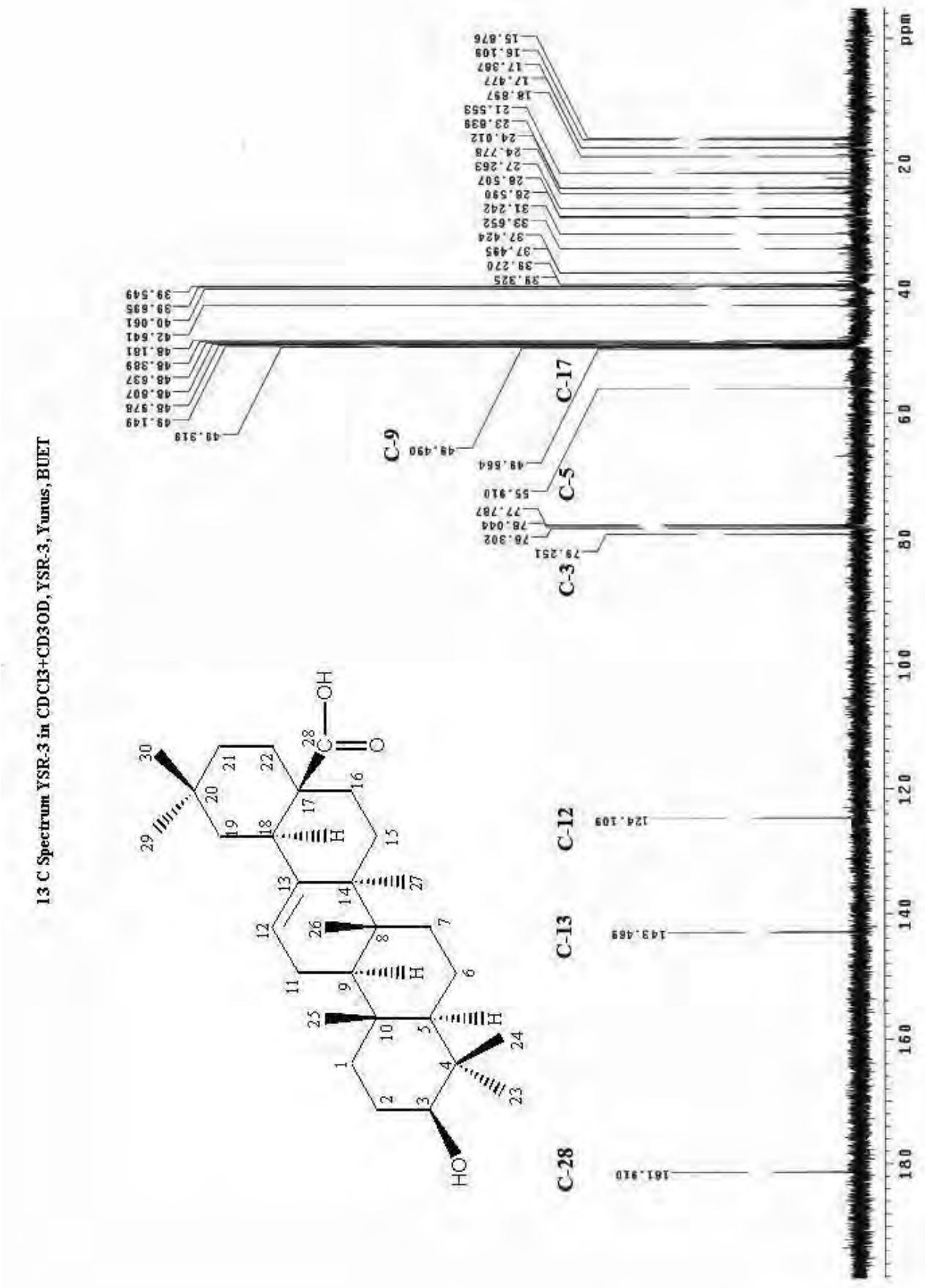


Figure 3.43: ^{13}C NMR spectrum of compound-5 (YSR-3) in $CDCl_3$+CD_3OD.

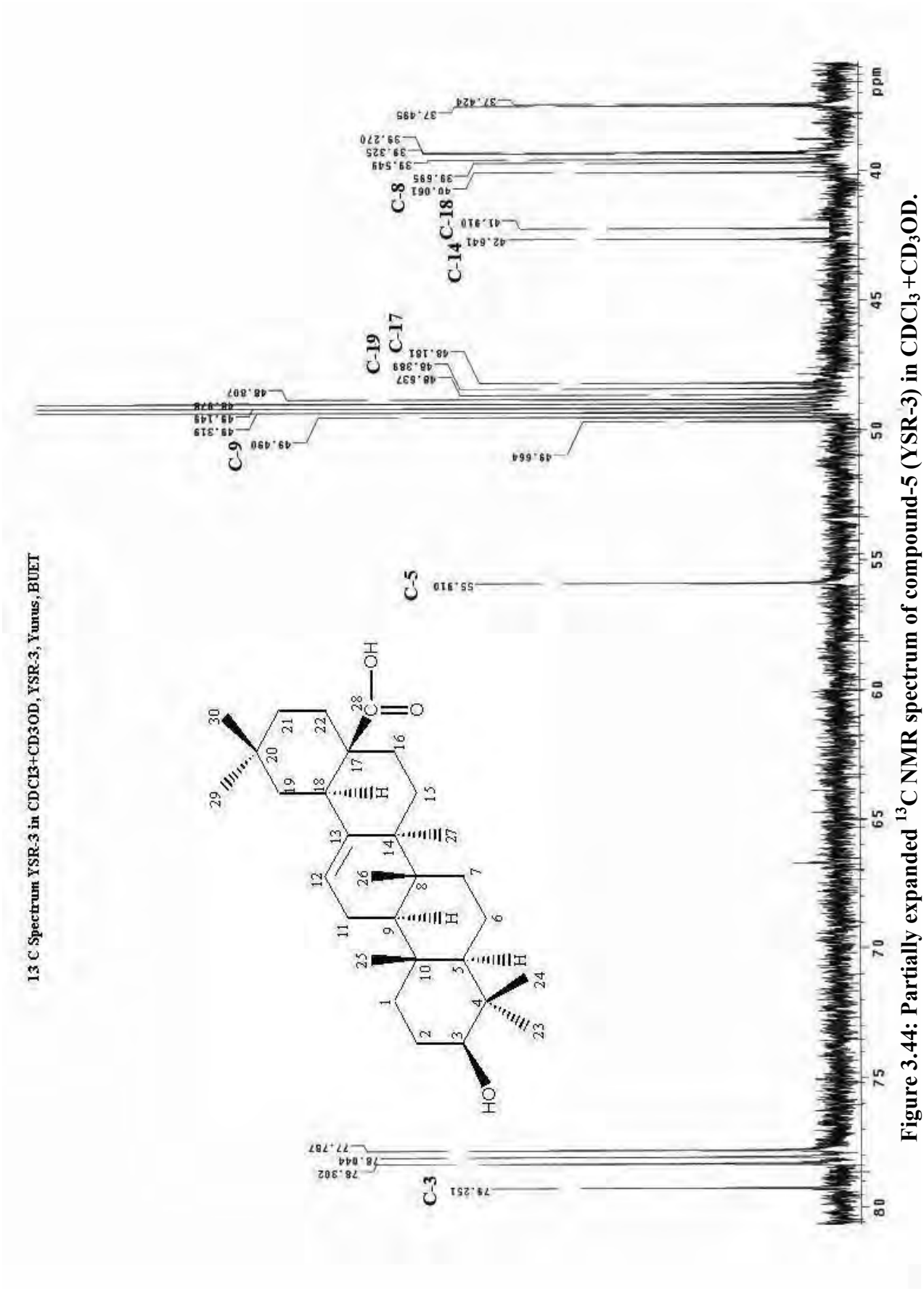


Figure 3.44: Partially expanded ^{13}C NMR spectrum of compound-5 (YSR-3) in $CDCl_3$+CD_3OD.

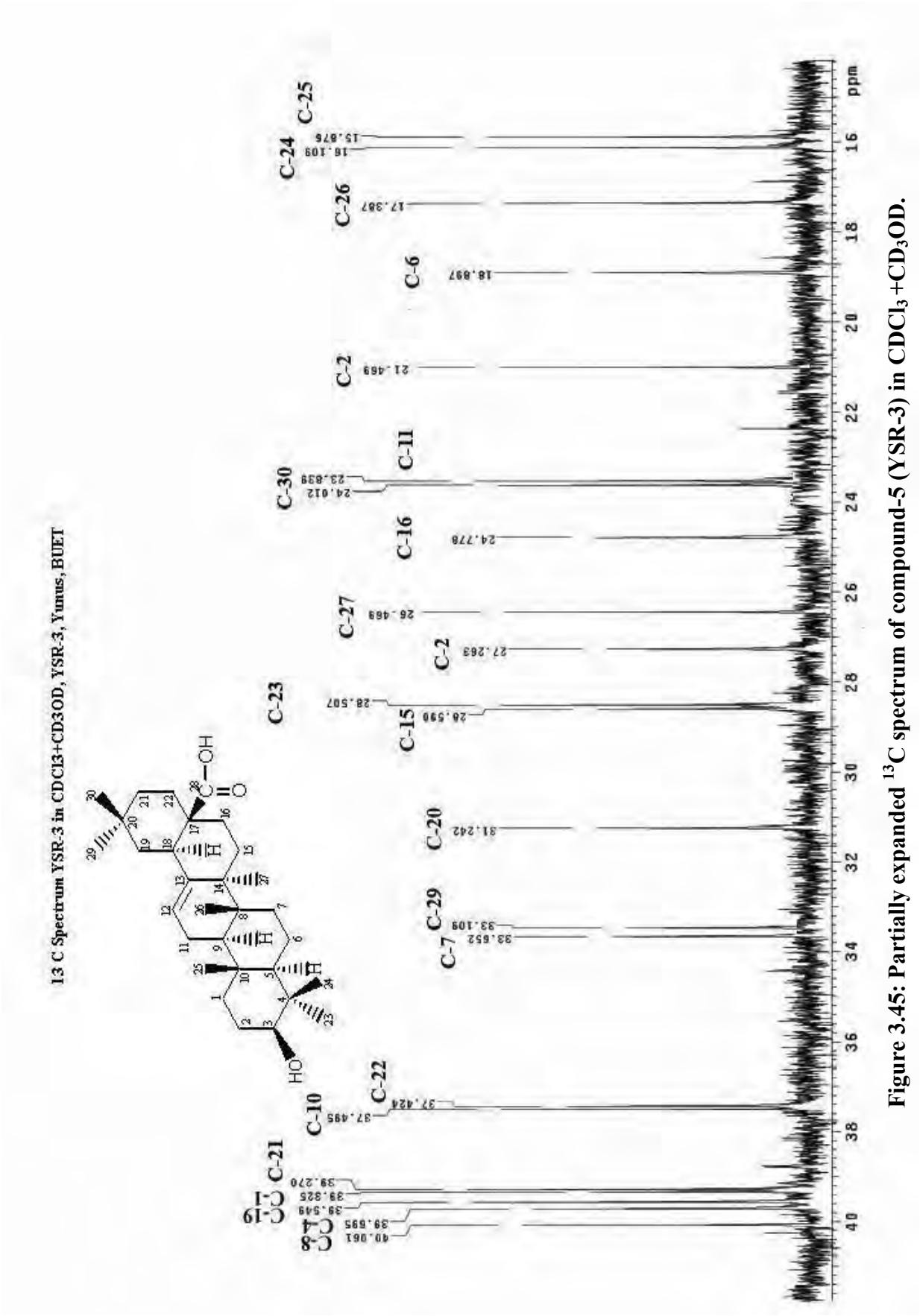


Figure 3.45: Partially expanded ^{13}C spectrum of compound-5 (YSR-3) in $CDCl_3$+CD_3OD.

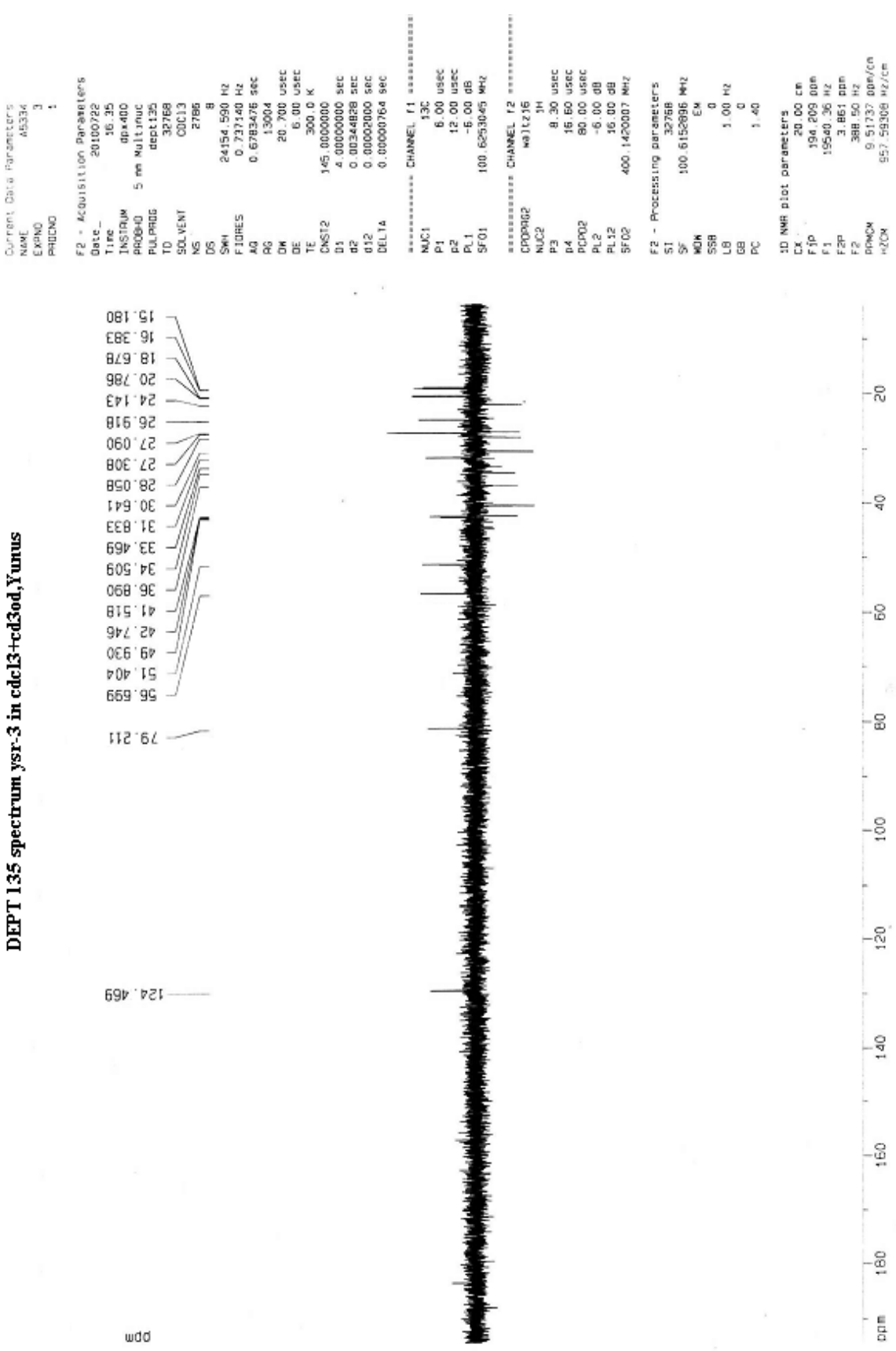


Figure 3.46: DEPT-135 spectrum of compound-5 (YSR-3) in $CDCl_3$ +CD_3OD.

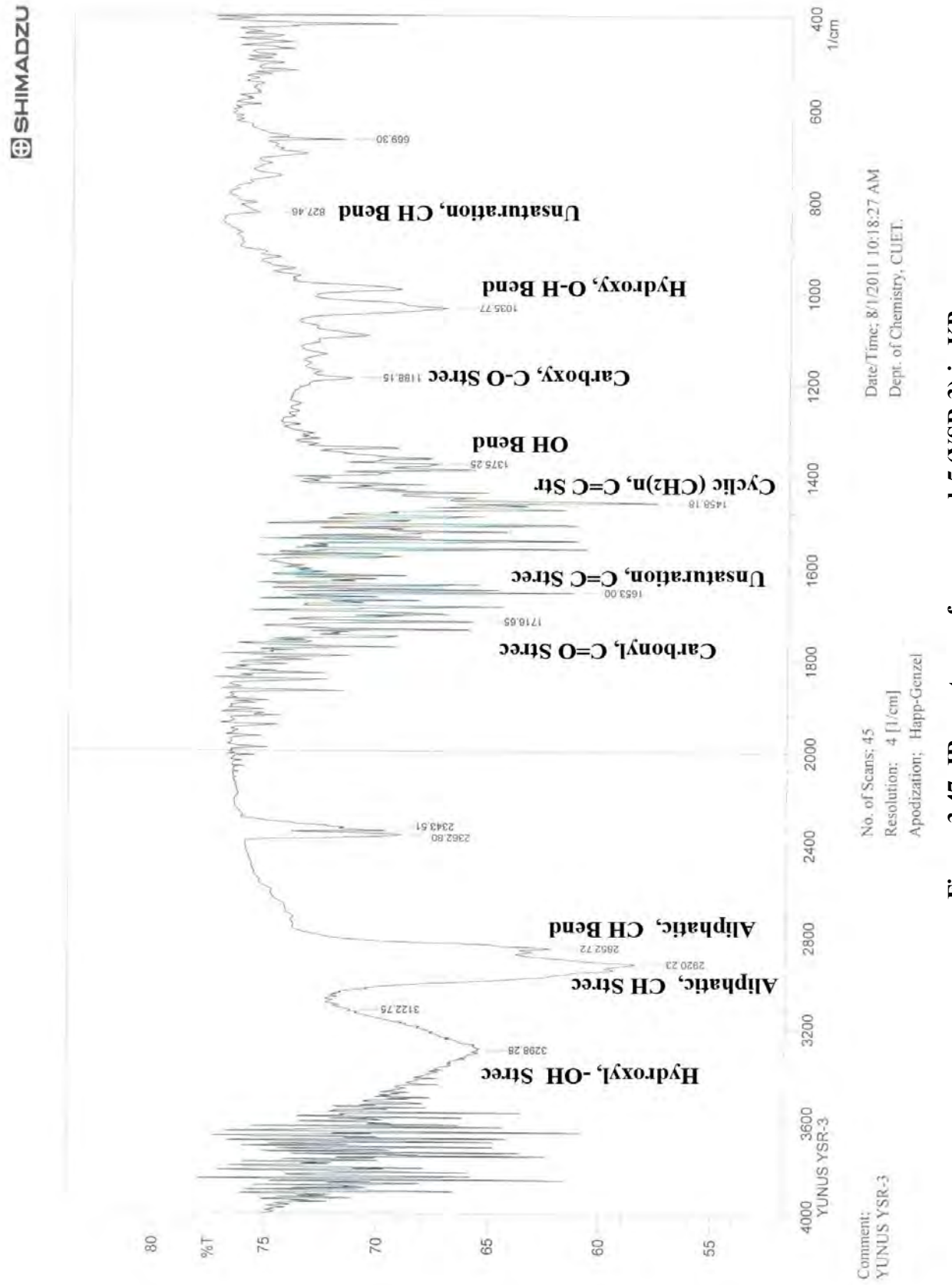


Figure 3.47: IR spectrum of compound-5 (YSR-3) in KBr.

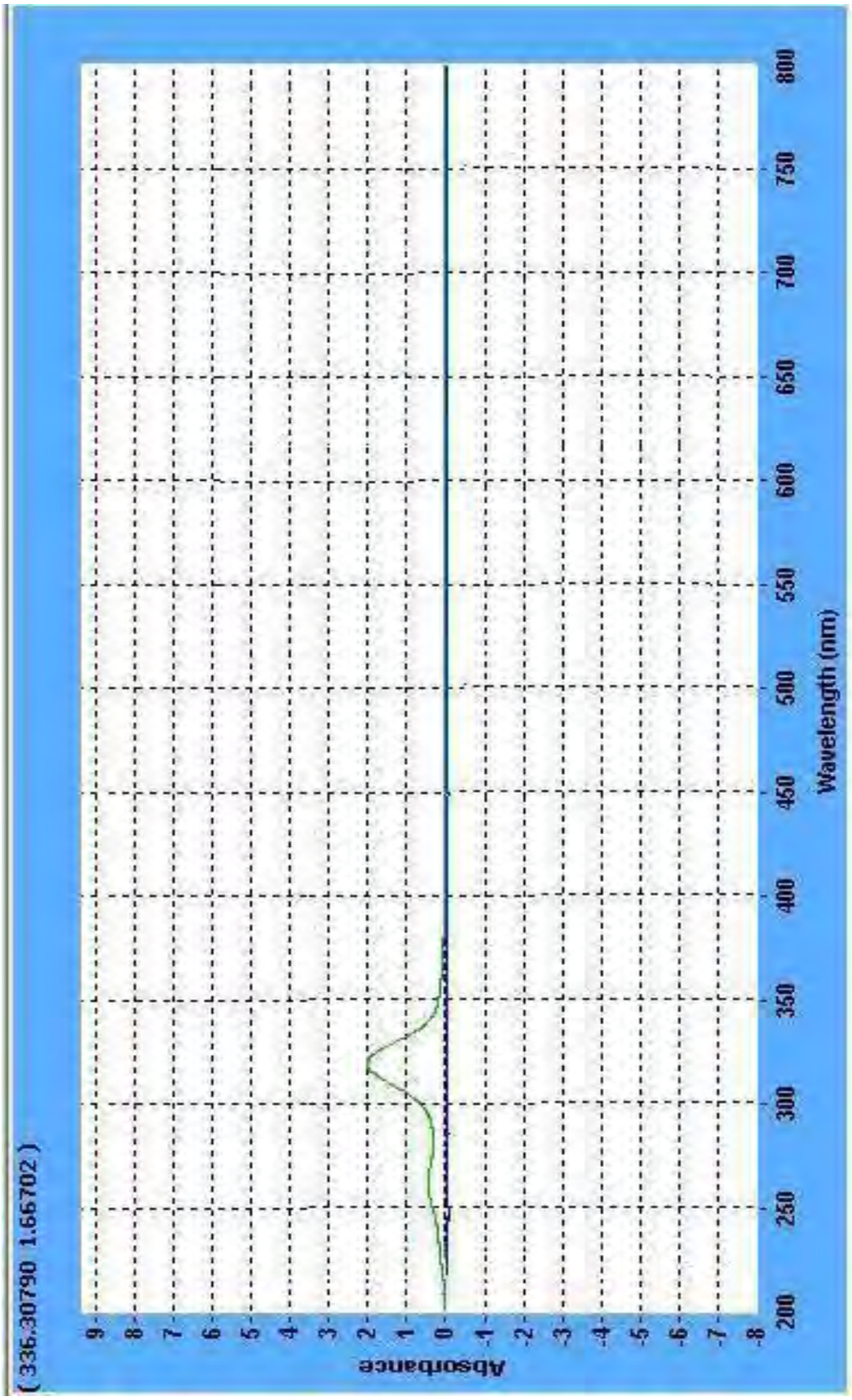


Figure 3.48: UV spectrum of compound-5 (YSR-3) in methanol.

3.2.6 Characterization of compound-6 (YSR-5) as 2*α*,3*β*-dihydroxy-olea-12-en-28-oic acid.

3.2.6.1 Physical properties

The compound-6 (YSR-5) was obtained as a white amorphous powder compound. The R_f value of the compound was 0.55 in n-hexane: ethyl-acetate = 50:50. It was soluble in chloroform, dichloromethane, ethyl acetate & ethanol. It was tested for terpenoid and a red-violet color confirms that the compound-6 (YSR-5) was terpenoid type compound. The melting point was found to be 295-298^{0}C[63].

3.2.6.2 Characterization of compound-6 (YSR-5) by spectroscopic method

The structure of the compound-6 (YSR-5) has been established by UV, FT-IR, ^{1}H-NMR and ^{13}C-NMR spectral evidences.

3.2.6.2.1 Ultraviolet (UV) spectroscopy of compound-6 (YSR-5)

The UV spectrum showed absorption band at λmax 255, 295, 355 and 422 nm in methanol.

3.2.6.2.2 Infrared (IR) spectroscopy of compound-6 (YSR-5)

The IR spectrum (in KBr) of compound-6 (YSR-5) exhibit characteristic absorption band for hydroxyl (-OH) group at 3446.79 cm^{-1}, Absorption at 2924.09 and 2852.72 cm^{-1} was due to aliphatic C-H stretching and bending vibrations of methyl group, carbonyl (C=O) of the carboxyl group at 1701.22 cm^{-1}, Other absorption frequencies include 1653.00 cm^{-1} as a result C=C stretching however this band was weak at 1447.18 cm^{-1} was a bending frequency for cyclic (CH_2)n and 1375.25 cm^{-1} for C-H bending, carboxy (C-O stretching) function at 1155.93 cm^{-1}, hydroxy (O-H bending) function at 1055.06 cm^{-1} and presence of trisubstituted double bond at 813.96 cm^{-1}[62].

3.2.6.2.3 ^{1}H-NMR spectroscopy of compound-6 (YSR-5)

The ^{1}H NMR spectrum of the compound showed seven methyls singlets at 1.14, 0.99, 0.98, 0.92, 0.89, 0.82 and 0.78. The tertiary nature of these methyls was evident from their sharp singlets in the ^{1}H NMR. The ^{1}H NMR spectrum also showed the presence of a proton vicinal to the hydroxyl group was observed at δ 3.60 (1H, m) corresponding to H-2 and δ 2.89 (1H, d, 10.0 Hz) and 2.82 (1H, dd, 10.0, 2.0 Hz) corresponding to H-3 and H-18 respectively and a

characteristic olefinic proton of C12-C13 double bonded pentacyclic triterpenoid at δ 5.23. The proton signals of H-29 and H-30 in the ursane skeletons appears as a doublet, but in oleane as a singlet[58]. From the above spectral data of compound-6 (YSR-5) suggested that it was an olean-12-en derivative.

3.2.6.2.4 ^{13}C-NMR spectroscopy of compound-6 (YSR-5)

The ^{13}C-NMR spectrum revealed the presence of signals due to an oxygenated carbon signal at δ 78.29 (C-3), one tri-substituted double bond at δ 122.14 (C-12) and 144.01 (C-13) in the ring-C and the most downfield signal due to the carboxyl carbon displayed its resonance at 182.17 (C-28). In general, for the polyols C-l2 is deshielded (~2 p.p.m.) and C-l3 shielded (~5 p.p.m.) in urs-12-enes compared to the corresponding olean-12-enes. The chemical shift of C-l3 appears to be about 140 p.p.m. in the urs-12-enes and 144-145 p.p.m. in the olean-12-enes.[59]. The chemical shifts of 122.14 (C-12) and 144.01 (C-13) and H-12 (δ 5.20-5.4 ppm) suggests that this compound was Δ12-unsaturated triterpenoid. The ^{13}C NMR spectra clearly exhibited the difference in the chemical shifts of C-12, C-13, C-17, C-18, C-19, C-20, C-22, C-27, C-29 and C-30 between the ursane group and the oleane group which showed in **Table-3.6**. Moreover ^{13}C-NMR signals due to C-18 to C-22 suggested that compound-6 (YSR-5) was an olean-12-en derivative. The coupling constant of H-3 (*J* =2.3-2.5 Hz) suggested that two OH groups at C-2 and 3 were at the *cis* position. However, the coupling constant of H-3 (J=9.4-10.0 Hz) suggested that the two OH groups at C-2 and C-3 were in *trans* position. The physical and spectral data of the compound was in complete agreement to the reported data in literature[63-65].The compound-6 (YSR-5) was identified as 2*α*, 3*β*-dihydroxy-olea-12-en-28-oic acid. This was the first isolated compound so far on this plant *Saurauia roxburghii.*

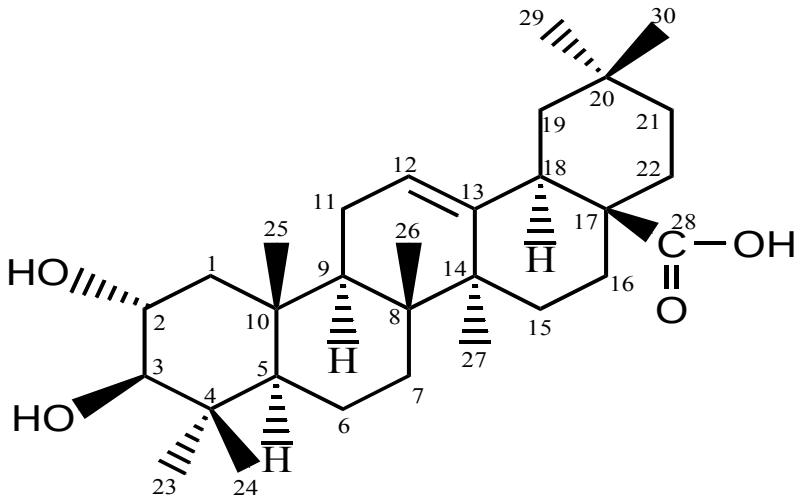


Fig 3.49: 2*α*,3*β*-dihydroxy-olea-12-en-28-oic acid.

Table-3.6: ^{1}H-NMR & ^{13}C-NMR Spectral data of compound-6 (YSR-5)

C/H	^{1}H (δ ppm) Obtained	^{1}H (δ ppm) Literature[63]	^{13}C(δ ppm) Obtained	^{13}C(δ ppm) Literature[64]
1			40.06 (CH_2)	41.7
2	3.60 (1H, m)	3.45 (1H, m)	67.18 (CH)	68.5
3	2.89 (1H, d, 10.0 Hz)	2.75 (1H, d, J=10.0Hz)	78.29 (CH)	78.9
4			37.49 (Cq)	38.3
5			48.39 (CH)	49.1
6			17.48 (CH_2)	18.0
7			31.24 (CH_2)	32.4
8			39.27 (Cq)	39.5
9			48.18 (CH)	49.3
10			37.42 (Cq)	38.2
11			21.55 (CH_2)	22.9
12	5.23 (1H, br t)	5.05 (1H, t)	122.14 (CH)	122.5
13			144.01(Cq)	143.7
14			42.64 (Cq)	43.1
15			27.26 (CH_2)	27.6
16			24.01(CH_2)	24.4
17			48.64 (Cq)	49.5
18	2.83(1H, dd,10.0, 2.0 Hz)	2.60 (1H, dd, 10.0, 2.5 Hz)	39.69 (CH)	41.0
19			42.64 (CH_2)	43.9
20			28.48(Cq)	30.7
21			33.65 (CH_2)	36.7
22			31.24 (CH_2)	32.8
23	0·99 (3H, s, Me)	0.97 (3H, s, Me)	28.50 (CH_3)	28.9
24	0·82 (3H, s, Me)	0·80 (3H, s, Me)	23.84 (CH_3)	24.5
25	0·98 (3H, s, Me)	0·96 (3H, s, Me)	16.11 (CH_3)	16.5
26	0·78 (3H, s, Me)	0·70 (3H, s, Me)	17.38 (CH_3)	17.9
27	1.14 (3H, s, Me)	1.11 (3H, s, Me)	26.1 (CH_3)	26.1
28			182.17(Cq)	186.9
29	0·89 (3H, s, Me)	0·87 (3H, s, Me)	33.1 (CH_3)	33.1
30	0·92 (3H, s, Me)	0·90 (3H, s, Me)	24.78 (CH_3)	25.6

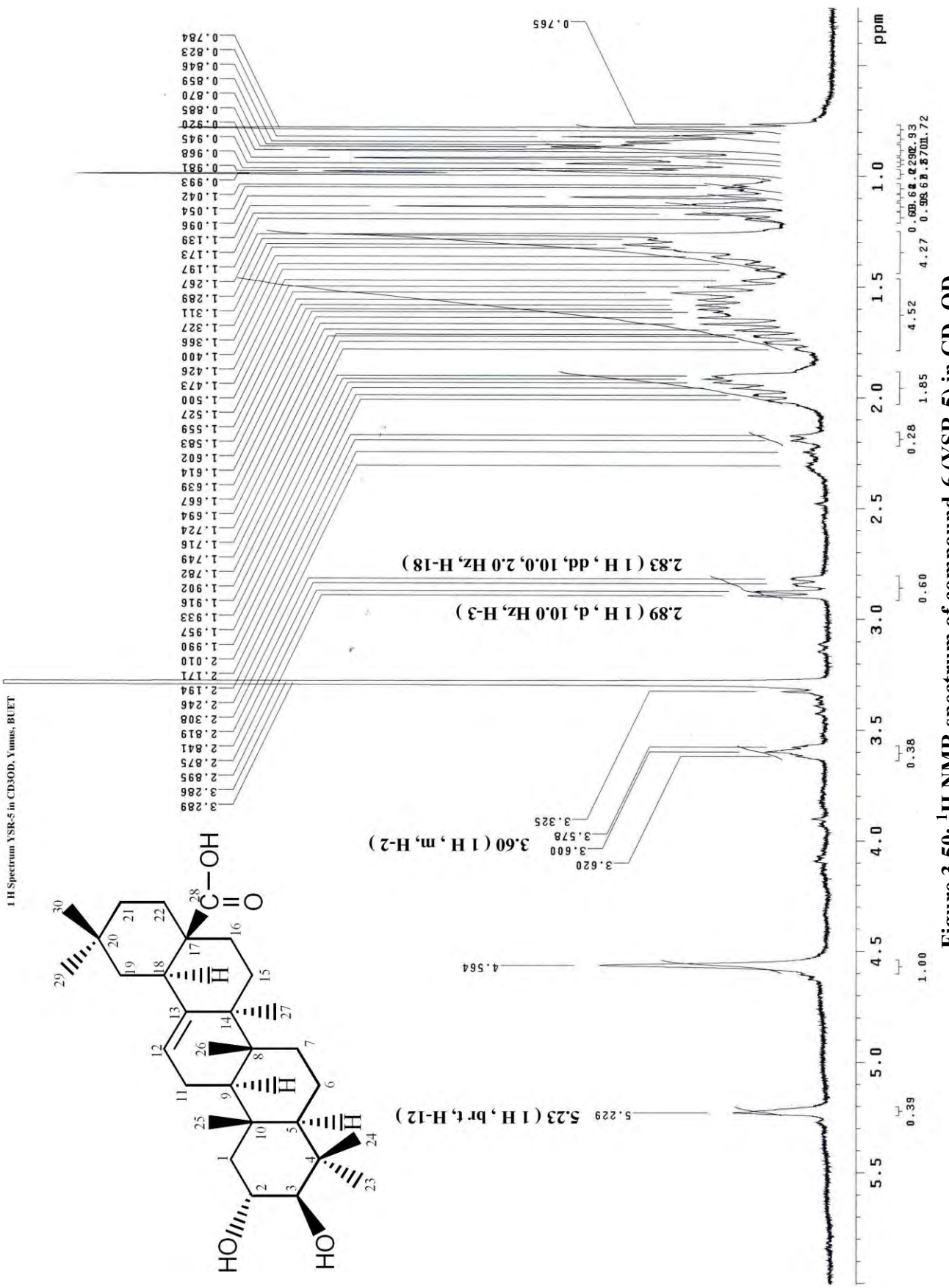


Figure 3.50: ^{1}H NMR spectrum of compound-6 (YSR-5) in CD_3 OD.

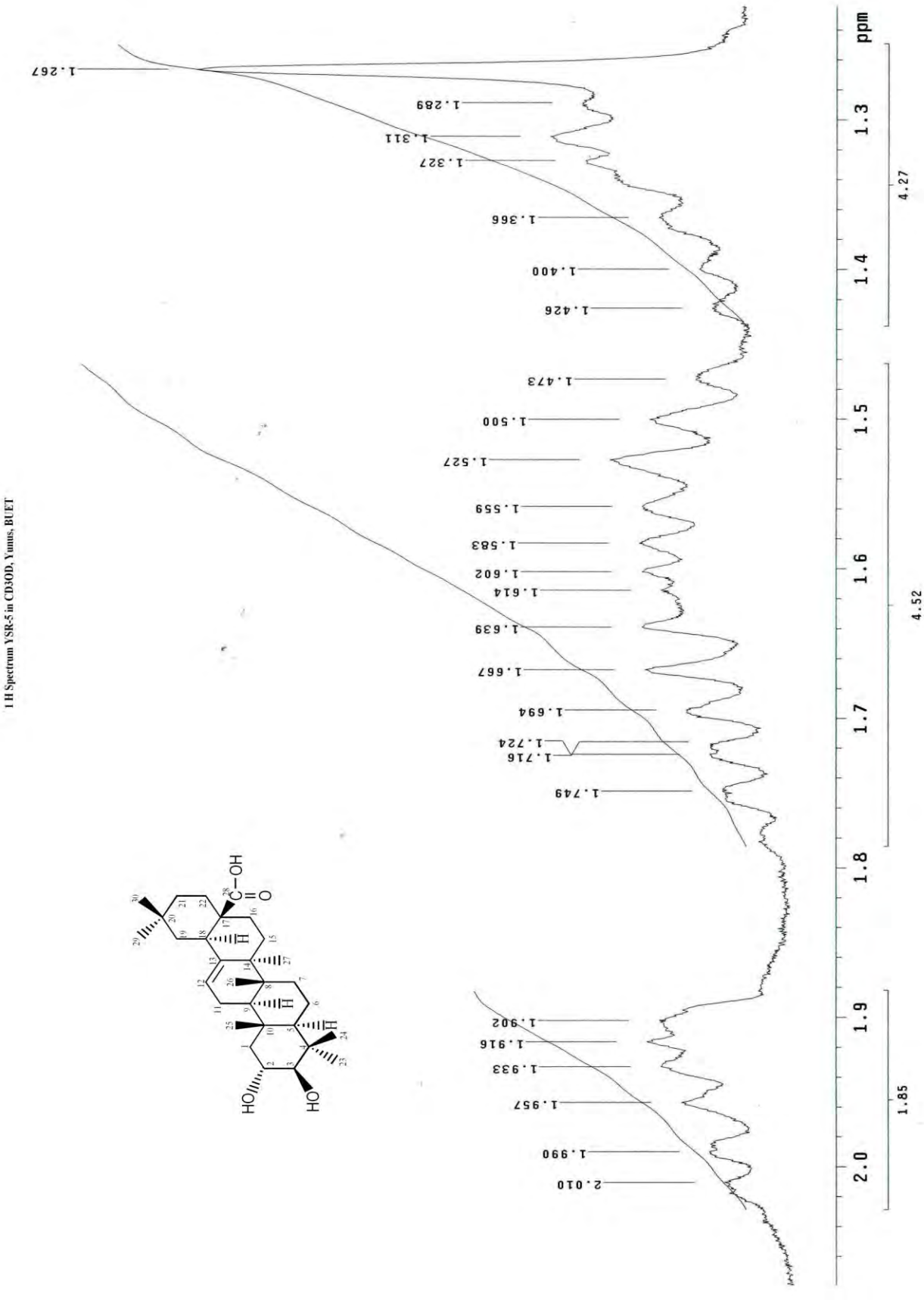


Figure 3.51: Partially expanded ^{1}H NMR spectrum of compound-6 (YSR-5) in CD_3OD.

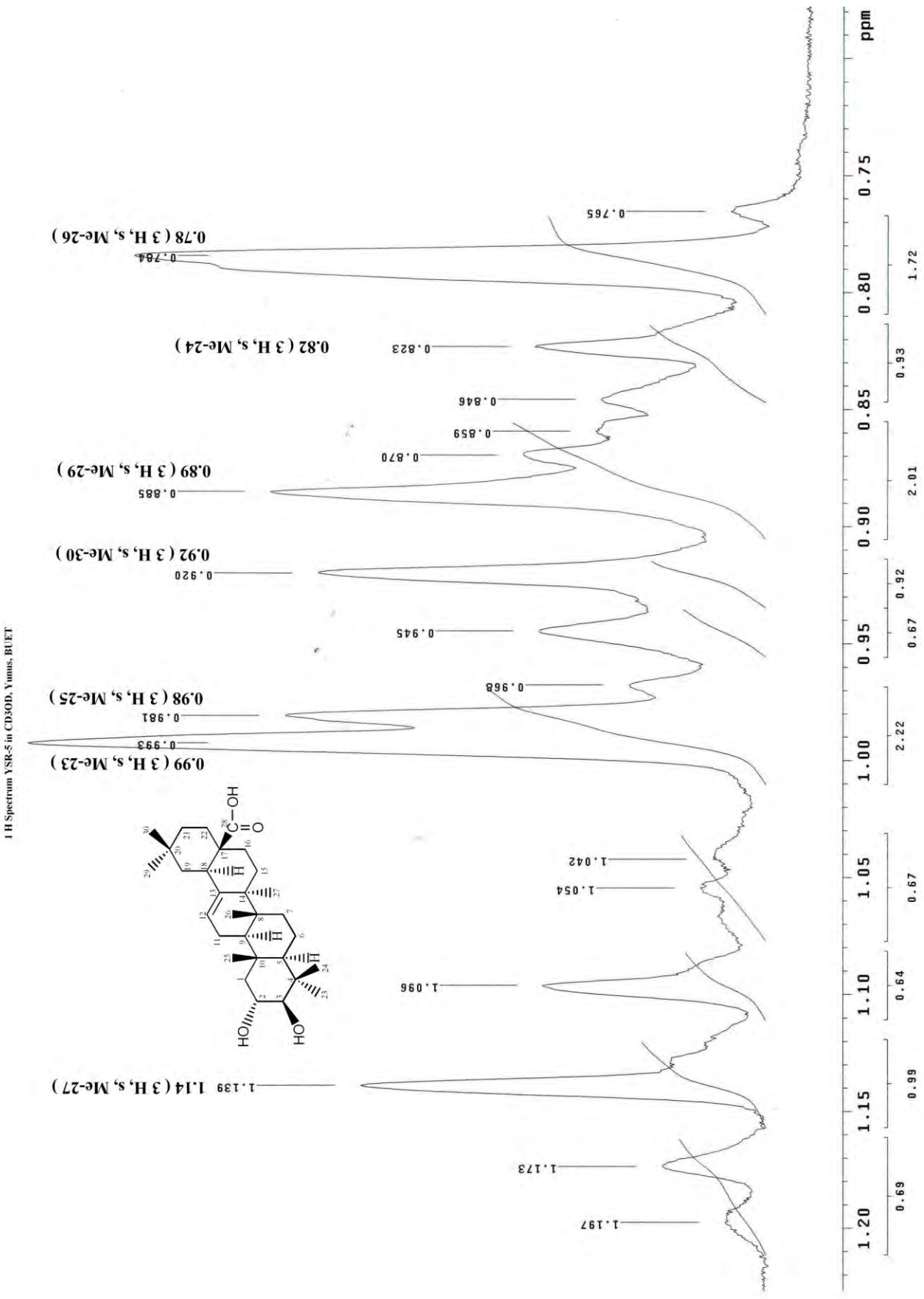


Figure 3.52: Partially expanded ^{1}H NMR spectrum of compound-6 (YSR-5) in CD_3OD.

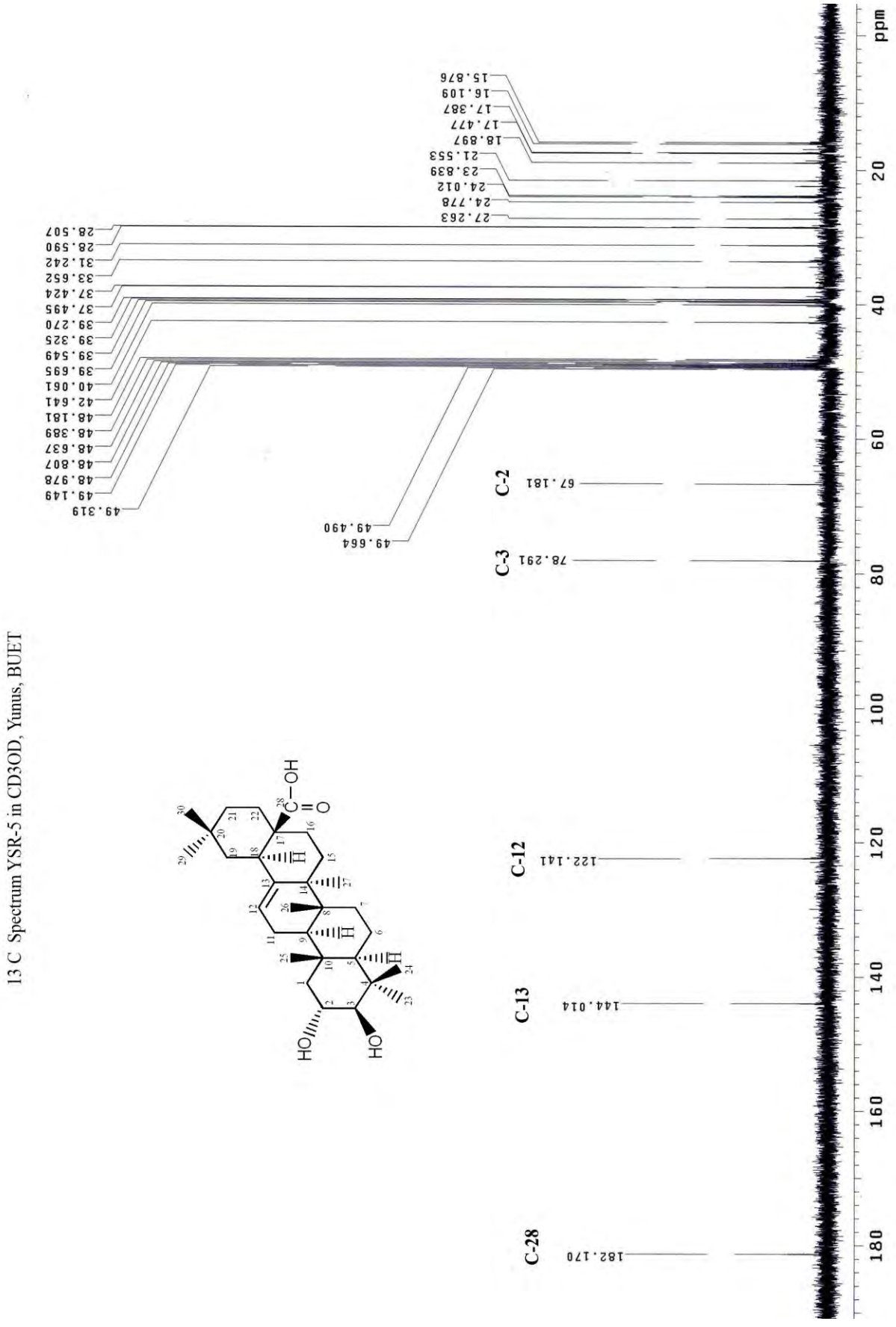


Figure 3.53: ^{13}C NMR spectrum of compound-6 (YSR-5) in CD_3OD.

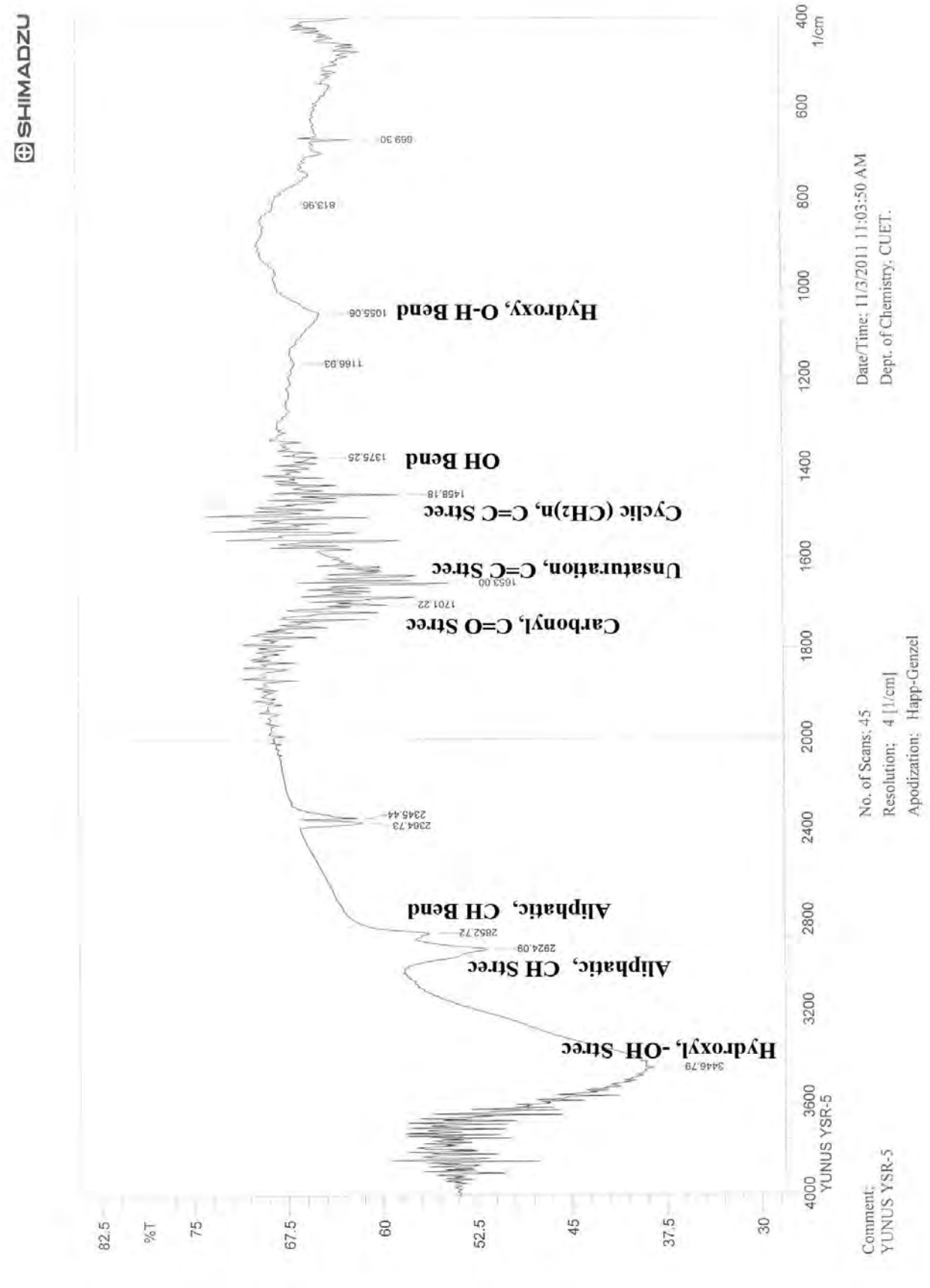


Figure 3.54: IR spectrum of compound-6 (YSR-5) in KBr.

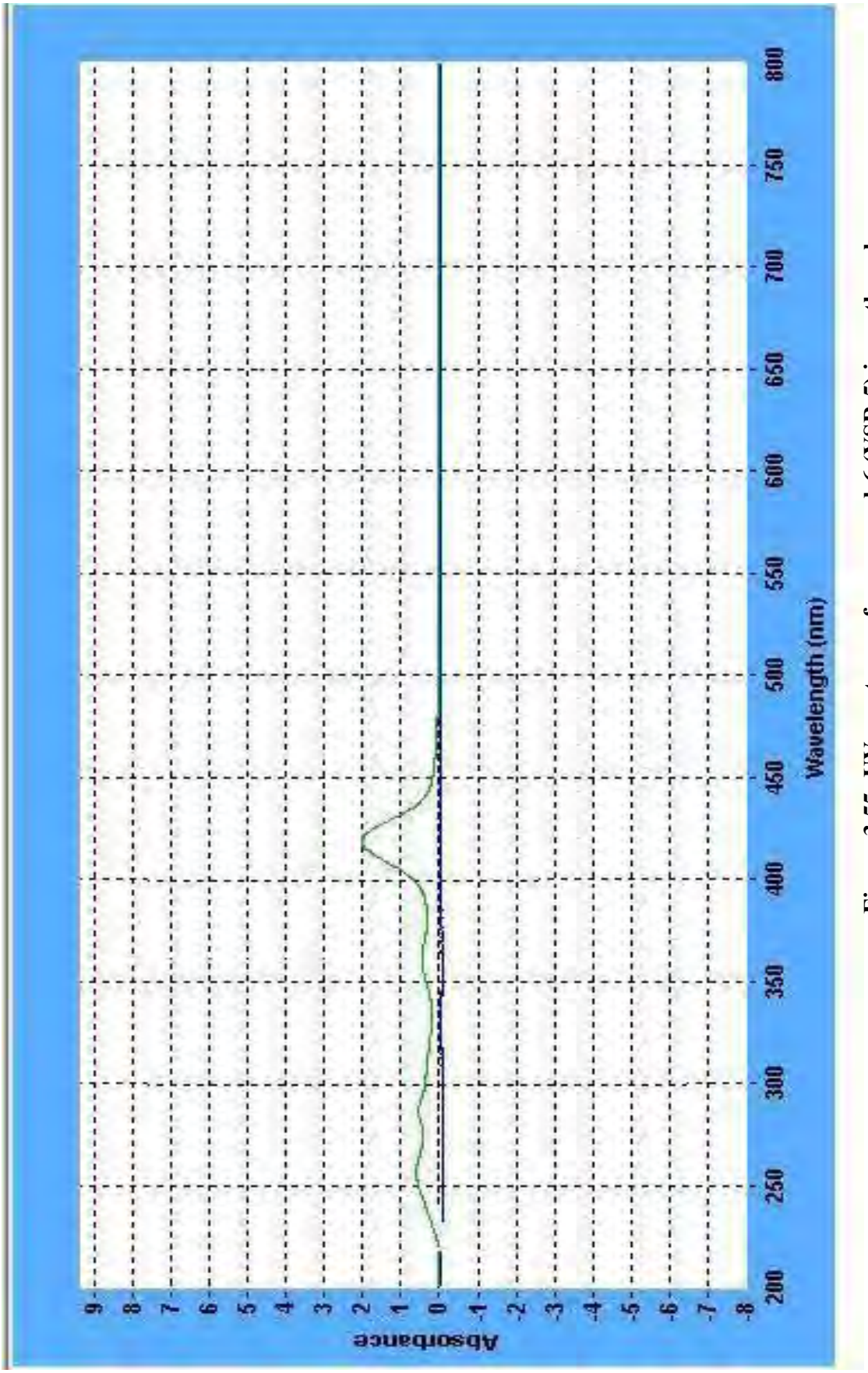


Figure 3.55: UV spectrum of compound-6 (YSR-5) in methanol.

CHAPTER-1

BRINE SHRIMP LETHALITY BIOASSAY

4.1 Introductions

Bioactive compounds are always toxic to living body at some higher doses and it justifies the statement that 'Pharmacology is simply toxicology at higher doses and toxicology is simply pharmacology at lower doses. Brine shrimp lethality bioassay[75,76] is a rapid and comprehend-sive bioassay for the bioactive compound of the natural and synthetic origin. By this method, natural product extarcts, fractions as well as the pure compounds can be tested for their bioactivity. In this method, *in vivo* lethality in a simple zoological organism (Brine shrimp nauplii) is used as a favorable monitor for screening and fractionation in the discovery of new bioactive natural products.

This bioassay indicates cytotoxicity as well as a wide range of pharmacological activities such as antimicrobial, antiviral, pesticidal & anti-tumor etc. of the compounds[75, 77].

Brine shrimp lethality bioassay technique stands superior to other cytotoxicity testing procedures because it is rapid in process, inexpensive and requires no special equipment or aseptic technique. It utilizes a large number of organisms for statistical validation and a relatively small amount of sample. Furthermore, unlike other methods, it does not require animal serum.

4.2 Materials

a. *Artemia salina* leach (brine shrimp eggs)
b. Sea salt (NaCl)
c. Small tank with perforated dividing dam to hatch the shrimp
d. Lamp to attract shrimps
e. Pipettes
f. Micropipette
g. Glass vials
h. Magnifying glass
i. Test samples of experimental plants.

Test samples of *Saurauia roxburghii*

1. Three crude extracts (**crude ethanol, n-Hexane** and **chloroform**).
2. Five column fractions (**H-13, C-19, E-13, E-18** and **E-24**) of various extract.

4.3 Principle

Brine shrimp eggs are hatched in simulated sea water to get nauplii. Test samples are prepared by dissolving in DMSO and by the addition of calculated amount of DMSO, desired concentration of the test sample is prepared. The nauplii are counted by visual inspection and are taken in vials containing 5 mL of simulated sea water. Then samples of different concentrations are added to the premarked vials through micropipette. The vials are then left for 24 hours and then the nauplli are counted again to find out the cytotoxicity of the test agents.

4.4 Procedure

4.4.1 Preparation of sea water

38 gm sea salt (pure NaCl) was weighed, dissolved in one liter of distilled water and filtered off to get clear solution.

4.4.2 Hatching of brine shrimp

Artemia salina leach (brine shrimp eggs) collected from pet shops was used as the test organism. Seawater was taken in the small tank and shrimp eggs were added to one side of the tank and then this side was covered. Two days were allowed to hatch the shrimp and to be matured as nauplii. Constant oxygen supply was carried out through the hatching time. The hatched shrimps were attracted to the lamp through the perforated dam and they were taken for experiment.

With the help of a pasteur pipette 20 living shrimps were added to each of the test tubes containing 5 mL of seawater.

4.4.3 Preparation of test solutions with samples of experimental plant.

Earlier of this Chapter (**4.2**) detail of test samples of the experimental plant were given.

Test solutions for crude extract

Measured amount of each sample was dissolved in specific volume of DMSO to obtain the desired concentration of the prepared solution as 2000 µg/30 µL. Then a series of solutions of lower concentrations were prepared from this solution by serial dilution with DMSO. Thus the concentrations of the obtained solutions were 1000 µg/30 µL, 500 µg/30 µL, 250 µg/30 µL, 125 µg/30 µL, 62.5 µg /30 µL, 31.25 µg /30 µL, 14.625µg /30 µL and 7.813µg /30 µL from each of these solutions 30 µL were added to premarked glass vials containing 5 mL of seawater and 20 shrimp nauplii. So, the final concentrations of samples in the vial were 200 µg/mL, 100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL, 3.125 µg/mL and 1.563 µg/mL respectively.

Test solutions for column fractions and pure compounds

Measured amount of each column fractions and pure compounds were dissolved in specific volume of DMSO to obtain the desired concentration of the prepared solution as 1000 µg/30 µL. Then a series of solutions of lower concentrations were prepared from this solution by serial dilution with DMSO. Thus the concentrations of the obtained solutions were 1000 µg/30 µL, 500 µg/30 µL, 250 µg/30 µL, 125 µg/30 µL, 62.5 µg /30 µL, 31.25 µg /30 µL, 14.625µg /30 µL and 7.813µg /30 µL from each of these solutions 30 µL were added to premarked glass vials containing 5 mL of seawater and 20 shrimp nauplii. So, the final concentrations of samples in the vial were 200 µg/mL, 100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL, 3.125 µg/mL and 1.563 µg/mL respectively.

4.4.4 Preparation of control group

Control groups are used in cytotoxicity study to validate the test method and ensure that the results obtained are only due to the activity of the test agent and the effects of the other possible factors are nullified. Usually two types of control groups are used

i) Positive control

ii) Negative control

4.4.4.1 Preparation of positive control group

Positive control in a cytotoxicty study is a widely accepted cytotoxic agent and the result of the test agent is compared with the result obtained for the positive control. In the present study vincristine sulphate is used as the positive control. Measured amount of the vincristine

sulphate is dissolved in DMSO to get an initial concentration of 20 µg/mL from which serial dilutions are made using DMSO to get 10 µg/mL, 5µg/mL, 2.5µg/mL, 1.25 µg/mL, 0.625 µg/mL, 0.3125 µg/mL and 0.15625 µg/mL respectively. Then the positive control solutions are added to the premarked vials containing 20 living brine shrimp nauplii in 5 mL simulated sea water to get the positive control groups.

4.4.4.2 Preparation of negative control group

30 µL of DMSO was added to each of three premarked glass vials containing 5 mL of seawater and 20 shrimp nauplii to use as control groups. If the brine shrimps in these vials show a rapid mortality rate, then the test is considered as invalid as the nauplii died due to some reason other than the cytotoxicity of the compounds.

4.4.5 Counting of brine shrimp nauplii

After 24 hours, the vials were inspected using a magnifying glass and the number of survived nauplii in each vial was counted. From this data, the percent (%) of lethality of the brine shrimp nauplii was calculated for each concentration.

4.5 Results and Discussion of Brine Shrimp Lethality Bioassay

Bioactive compounds are almost always toxic at higher dose. Thus, *in vivo* lethality in a simple zoological organism can be used as a convenient informant for screening and fractionation in the discovery of new bioactive natural products.

In the present bioactivity study all the crude extracts, column fractions and pure compounds showed positive results indicating that the test samples are biologically active. Each of the test samples showed different mortality rates at different concentrations. Plotting of log of concentration versus percent mortality for all test samples showed an approximate linear correlation. From the graphs, the median lethal concentration (LC_{50}, the concentration at which 50% mortality of brine shrimp nauplii occurred) was determined for the samples. The positive control groups showed non linear mortality rates at lower concentrations and linear rates at higher concentrations. There was no mortality in the negative control groups indicating the test as a valid one and the results obtained are only due to the activity of the test agents.

4.6 Results and discussion of the test samples of *Saurauia roxburghii.*

Crude ethanol, column fractions E-13, E-18 and E-24 of the crude extract of ethanol, chloroform, column fractions H-13 of the crude hexane, C-19 of the crude extract of chloroform and column fractions E-13, E-18 and E-24 of the crude extract of ethanol extract were screened for antitumor property using brine shrimp lethality bioassay.

From the bioassay, it was found that the LC_{50} of crude ethanol, n-Hexane, chloroform; column fractions H-13 of the crude hexane, C-19 of the crude extract of chloroform and column fractions E-13, E-18 and E-24 of the crude extract of ethanol were found to be 12.59 µg/mL (**Table: 4.1, Figure: 4.2**), 14.79 µg/mL (**Table: 4.2, Figure: 4.3**), 14.06 µg/mL (**Table: 4.2, Figure: 4.4**), 11.75 µg/mL (**Table: 4.3, Figure: 4.5**), 10.96 µg/mL (**Table: 4.3, Figure: 4.6**), 11.88 µg/mL (**Table: 4.4, Figure: 4.7**), 4.37 µg/mL (**Table: 4.4, Figure: 4.8**) and 6.92 µg/mL (**Table: 4.4, Figure: 4.9**) respectively. It is evident that all the test samples were very lethal to brine shrimp nauplii. However, column fraction E-18 of the crude extract of ethanol was comparatively more active with minimum LC_{50} value and the crude extract of n-hexane was less active with maximum LC_{50} value.

Column fractions E-18 and E-24 of the crude extracts of ethanol showed quite potent activity in brine shrimp lethality bioassay. These positive results suggested that column fractions E-18 and E-24 of the crude extracts of ethanol may be antitumor or pesticidal compounds. However, this cannot be confirmed without further higher and specific tests.

Table 4.1: Effects of crude ethanol extract of *Saurauia roxburghii* and positive control (Vincristine Sulphate) on brine shrimp nauplii.

Crude ethanol extract				Vincristine Sulphate			
Conc (C) (µg/mL)	Log C	% Mortality	LC_{50} (µg/mL)	Conc (C) (µg/mL)	Log C	% Mortality	LC_{50} (µg/mL)
200	2.301	80	12.59	20	1.30	100	0.32
100	2	75		10	1	100	
50	1.699	70		5	0.698	90	
25	1.398	65		2.5	0.397	80	
12.5	1.097	50		1.25	0.096	70	
6.25	0.796	40		0.625	-0.204	60	
3.125	0.495	30		0.3125	-0.488	50	
1.563	0.194	25		0.15625	-0.806	40	

Table 4.2: Effects of crude hexane and chloroform extract of *Saurauia roxburghii* on brine shrimp nauplii.

Conc (C) (µg/mL)	Log C	% Mortality		LC_{50} (µg/mL)	
		Hexane	Chloroform	H-13	C-19
200	2.301	80	80	14.79	14.06
100	2	75	70		
50	1.699	65	65		
25	1.398	55	55		
12.5	1.097	45	50		
6.25	0.796	40	40		
3.125	0.495	35	35		
1.563	0.194	25	25		

Table 4.3 : Effects of column fractions H-13 of the crude hexane and C-19 of the crude extract of chloroform of *Saurauia roxburghii* on brine shrimp nauplii.

Conc (C) (µg/mL)	Log C	% Mortality		LC_{50} (µg/mL)	
		H-13	C-19	H-13	C-19
200	2.301	95	90	11.75	10.96
100	2	85	85		
50	1.699	75	75		
25	1.398	65	60		
12.5	1.097	40	50		
6.25	0.796	40	40		
3.125	0.495	30	30		
1.563	0.194	20	25		

Table 4.4: Effects of column fractions E-13, E-18 and E-24 of the crude extract of ethanol of *Saurauia roxburghii* on brine shrimp nauplii.

Conc (C) (µg/mL)	Log C	% Mortality			LC_{50} (µg/mL)		
		E-13	E-18	E-24	E-13	E-18	E-24
200	2.301	80	90	90	11.88	4.37	6.92
100	2	65	85	80			
50	1.699	65	80	75			
25	1.398	60	75	65			
12.5	1.097	55	65	60			
6.25	0.796	40	55	50			
3.125	0.495	35	45	40			
1.563	0.194	30	35	30			

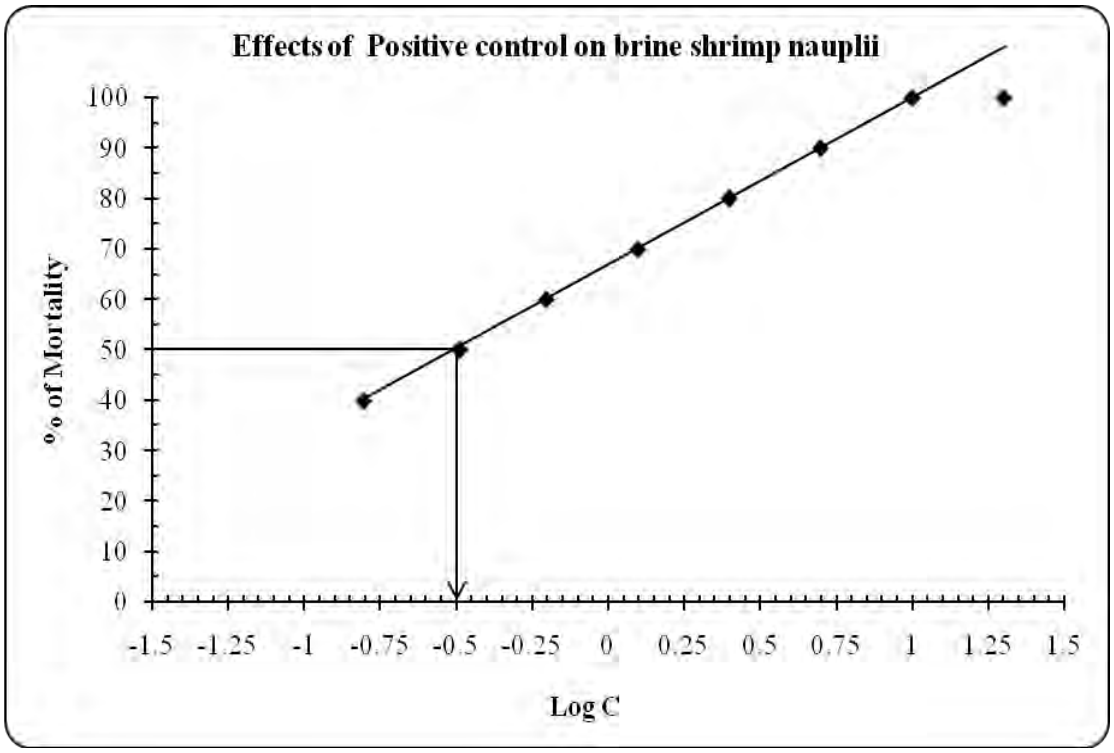


Figure 4.1: Effects of Positive control on brine shrimp nauplii.

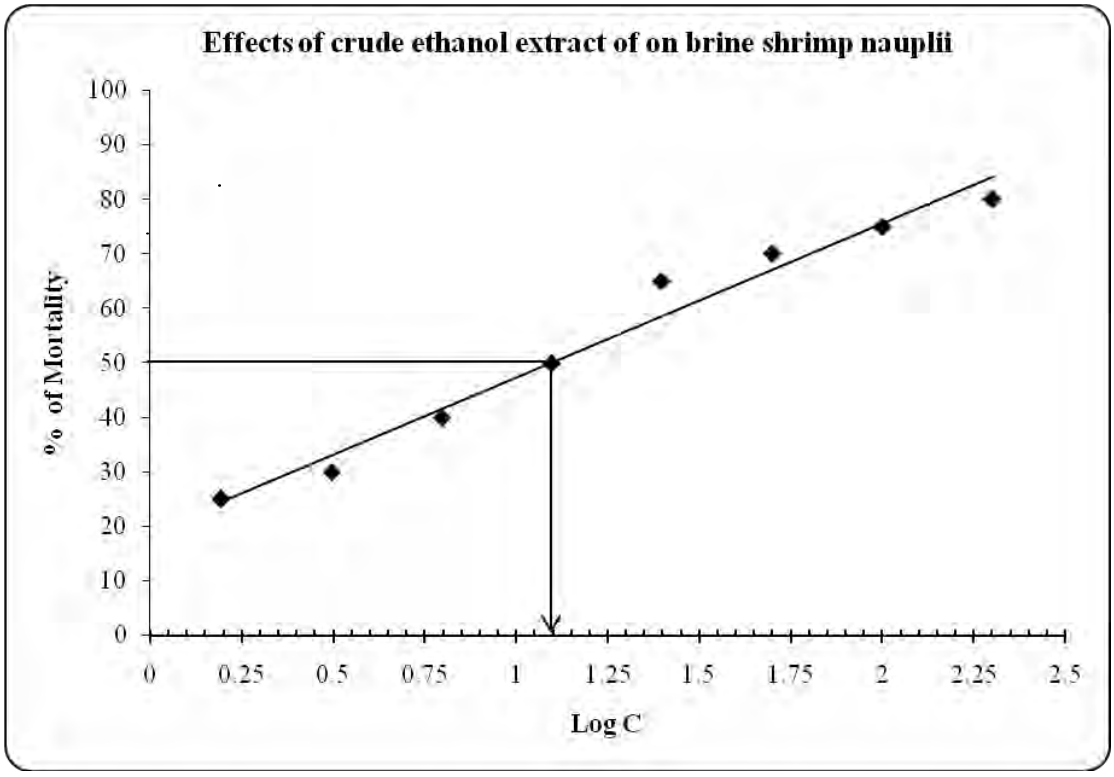


Figure 4.2: Effects of crude ethanol extract on brine shrimp nauplii.

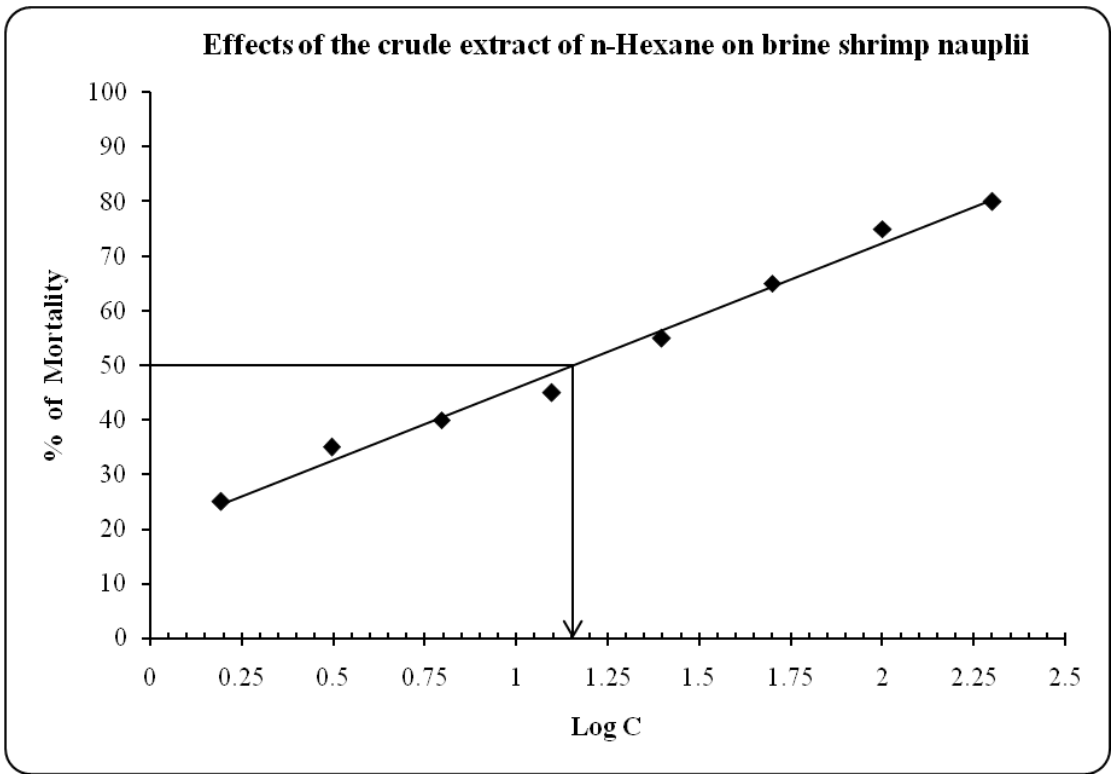


Figure 4.3: Effects of crude extract of n-Hexane on brine shrimp nauplii.

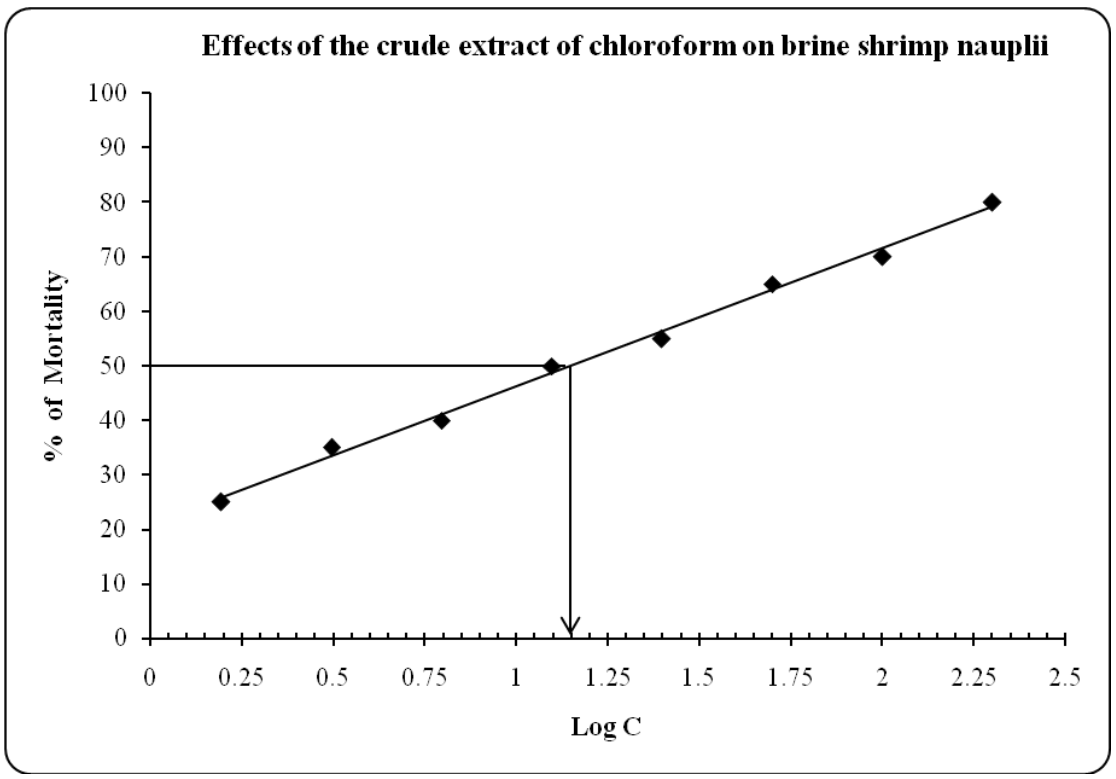


Figure 4.4: Effects of crude extract of chloroform on brine shrimp nauplii.

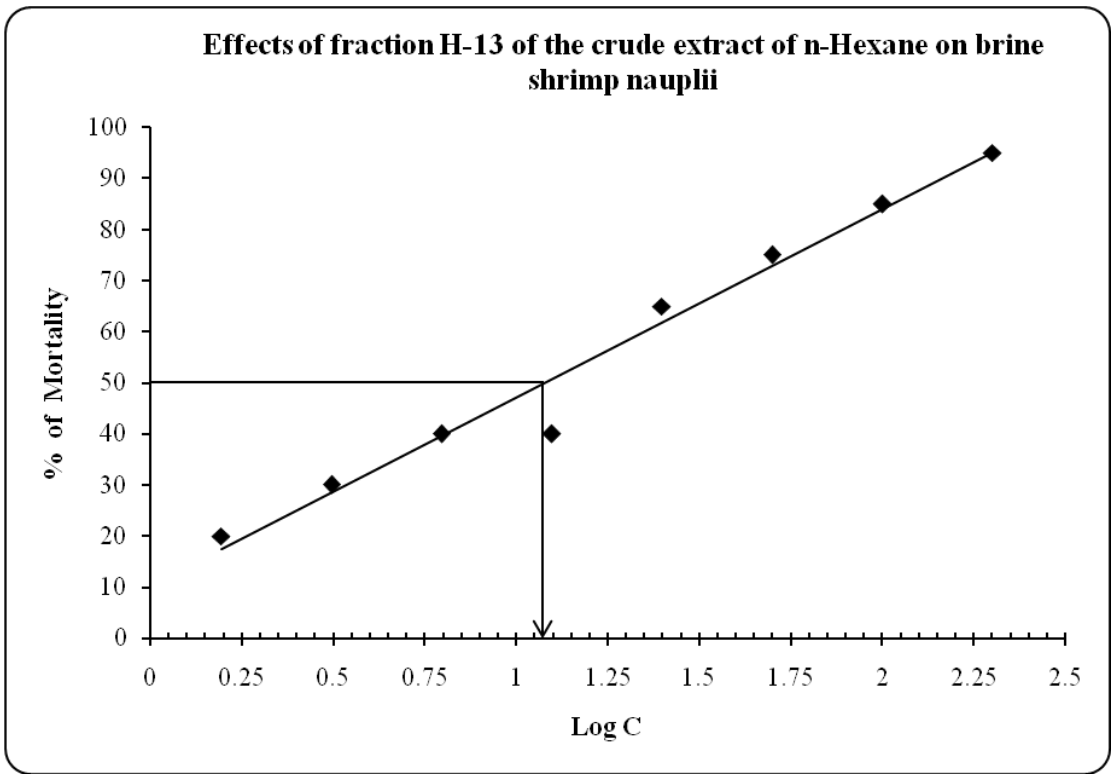


Figure 4.5: Effects of column fraction H-13 of crude hexane on brine shrimp nauplii.

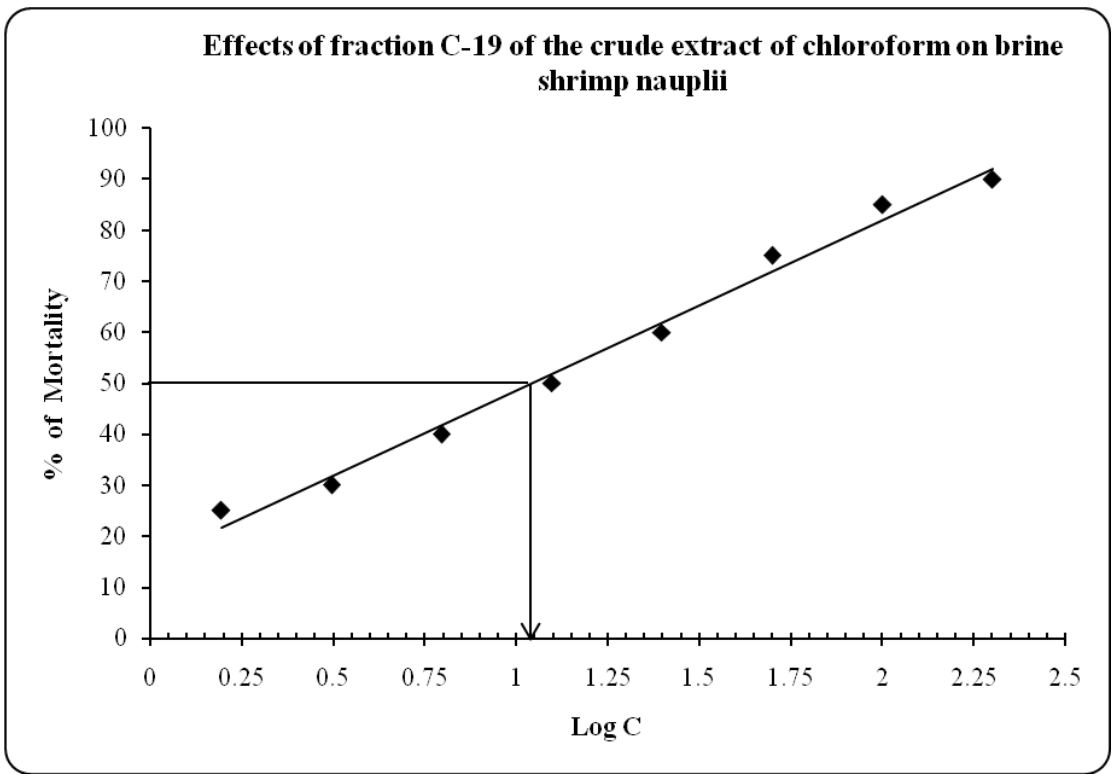


Figure 4.6: Effects of column fraction C-19 of crude chloroform on brine shrimp nauplii.

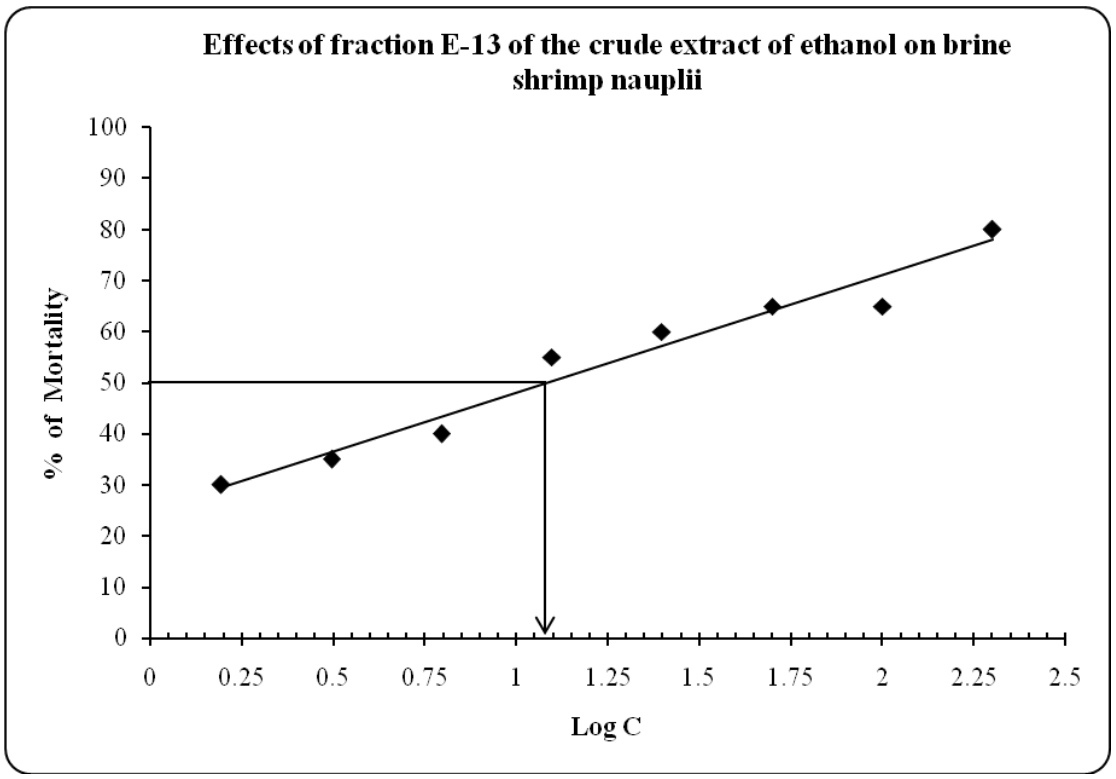


Figure 4.7: Effects of column fraction E-13 of crude ethanol on brine shrimp nauplii.

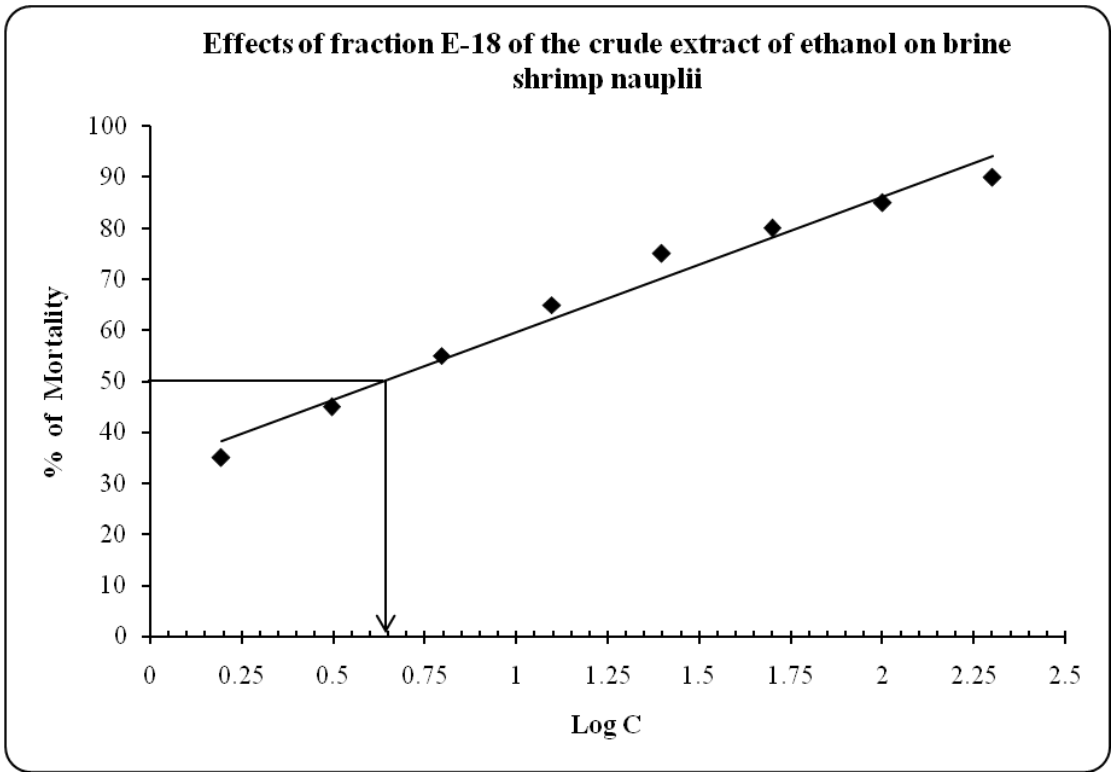


Figure 4.8: Effects of column fraction E-18 of crude ethanol on brine shrimp nauplii.

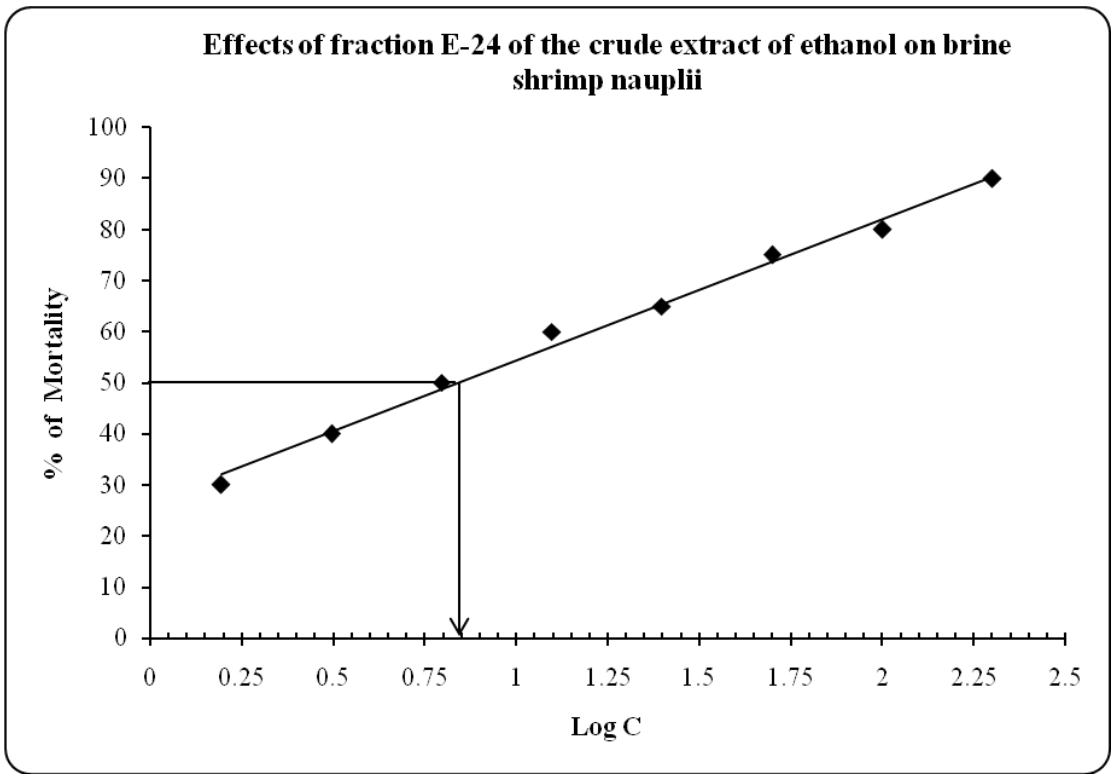


Figure 4.9: Effects of column fraction E-24 of crude ethanol on brine shrimp nauplii.

CHAPTER-2

ANTIMICROBIAL SCREENING

5.1 Introduction

Bacteria and fungi are responsible for many infectious diseases. The increasing clinical implications of drug resistant fungal and bacterial pathogens have lent additional urgency to antimicrobial drug research. The antimicrobial screening which is the first stage of antimicrobial drug research is performed to ascertain the susceptibility of various fungi and bacteria to any agent. This test measures the ability of each test sample to inhibit the *in vitro* fungal and bacterial growth. This ability may be estimated by any of the following three methods.

i) Disc diffusion method

ii) Serial dilution method

iii) Bioautographic method

But there is no standardized method for expressing the results of antimicrobial screening[67]. Some investigators use the diameter of zone of inhibition and/or the minimum weight of extract to inhibit the growth of microorganisms. However, a great number of factors viz., the extraction methods[68, 69], inoculums volume, culture medium composition[70], P^{H}[71] and incubation temperature[72] can influence the results.

Among the above mentioned techniques the disc diffusion[70] is a widely accepted *in vitro* investigation for preliminary screening of test agents which may possess antimicrobial activity. It is essentially a quantitative or qualitative test indicating the sensitivity or resistance of the microorganisms to the test materials. However, no distinction between bacteriostatic and bacteriocidal activity can be made by this method [73].

5.2 Principle of Disc Diffusion Method

Solutions of known concentration (µg/mL) of the test samples are made by dissolving measured amount of the samples in calculated volume of solvents. Dried and sterilized filter paper discs (6 mm diameter) are then impregnated with known amounts of the test substances using micropipette. Discs containing the test material are placed on nutrient agar medium uniformly seeded with the test microorganisms. Standard antibiotic discs and blank discs (impregnated with solvents) are used as positive and negative control. These plates are then kept at low temperature (4^0C) for 24 hours to allow maximum diffusion. During this time dried discs absorb water from the surrounding media and then the test materials are dissolved and diffused out of the sample disc. The diffusion occurs according to the physical law that controls the diffusion of molecules through agar gel[75.] As a result there is a gradual change of test materials concentration in the media surrounding the discs.

The plates are then incubated at 37^0C for 24 hours to allow maximum growth of the organisms. If the test materials have any antimicrobial activity, it will inhibit the growth of the microorganisms and a clear, distinct zone of inhibition will be visualized surrounding the medium. The antimicrobial activity of the test agent is determined by measuring the diameter of zone of inhibition expressed in millimeter.The experiment is carried out more than once and the mean of the readings is required[70].

In the present study the crude extract, some column fractions as well as five purified compounds were tested for antimicrobial activity by disc diffusion method.

5.3 Experimental

5.3.1 Apparatus and Reagents

Filter paper discs	Petridishes	Inoculating loop
Sterile cotton	Sterile forceps	Spirit burner
Micropipette	Screw cap test tubes	Nosemask and Hand gloves
Laminar air flow hood	Autoclave	Incubator
Refrigerator	Nutrient Agar Medium	Ethanol
Chloroform		

5.3.2 Test materials

5.3.2.1 Test materials of *Saurauia roxburghii*

1. Four crude extracts (**crude ethanol, n-Hexane, chloroform** and **ethyl-acetate**).
2. Five column fractions (**H-13, C-15, C-19, E-18** and **E-24**) of various extract.
3. Four pure compounds (Y**SR-2,** Y**SR-3,** Y**SR-7** and Y**SR-9).**

5.3.3 Test Organisms

The bacterial strains used for the experiment were collected as pure cultures from the Institute of Nutrition and Food Science (INFS), University of Dhaka. Both Gram positive and Gram-negative organisms were taken for the test and they are listed in the Table 5.1.

Table 5.1: List of Test Bacteria

Gram-positive Bacteria	Gram-negative Bacteria
Bacillus cereus	*Escherichia coli*
Bacillus megaterium	*Klebsiella sp.*
Bacillus subtilis	*Proteus sp.*
Staphylococcus aureus	*Salmonella typhi*
Bacillus polymyxa	*Shigella sonnei*
Streptococcus pneumoniae	*Pseudomonas Aureus*
Mycobacterium tuberculosis	*Vibrio cholerae*

5.3.4 Culture medium and their composition

The following media is used normally to demonstrate the antimicrobial activity and to make subculture of the test organisms.

a. Nutrient agar medium

Ingredients	**Amounts**
Bacto peptone	0.5 gm
Sodium chloride	0.5 gm
Bacto yeast extract	1.0 gm
Bacto agar	2.0 gm
Distilled water q.s. to	100 mL
P^H	7.2 ± 0.1 at 25 ^{0}C

b. Nutrient broth medium

Ingredients	**Amounts**
Bacto beef extract	0.3 gm
Bacto peptone	0.5 gm
Distilled water q.s.to	100 mL
P^H	7.2 ± 0.1 at 25 ^{0}C

c. Muller – Hunton medium

Ingredients	**Amounts**
Beef infusion	30 gm
Casamino acid	1.75 gm
Starch	0.15 gm
Bacto agar	1.70 gm
Distilled water q.s. to	100 mL
P^H	7.3 ± 0.2 at 25^0 C

***d. Tryptic soya broth medium* (TSB)**

Ingredients	**Amounts**
Bacto tryptone	1.7 gm
Bacto soytone	0.3 gm
Bacto dextrose	0.25 gm
Sodium chloride	0.5 gm
Di potassium hydrogen Phosphate	0.25 gm
Distilled water q.s. to	100 mL
P^H	7.3 ± 0.2 at 25 ^{0}C

Nutrient agar medium (DIFCO) used most frequently for testing the sensitivity of the organisms to the test materials and to prepare fresh cultures.

5.3.4.1 Preparation of medium

To prepare required volume of this medium, calculated amount of each of the constituents was taken in a conical flask and distilled water was added to it to make the required volume. The contents were heated in a water bath to make a clear solution. The P^H (at 25 ^{0}C) was adjusted at 7.2 –7.6 using NaOH or HCl. 10 mL and 5 mL of the medium was then transferred in screw cap test tubes to prepare plates and slants respectively. The test tubes were then capped and sterilized by autoclaving at 15-lbs. pressure/sq. inch at 121 ^{0}C for 20 minutes. The slants were used for making fresh culture of bacteria and fungi that were in turn used for sensitivity study.

Figure 5.1: Autoclave

5.3.5 Sterilization procedures

In order to avoid any type of contamination and cross contamination by the test organisms the antimicrobial screening was done in Laminar Hood and all types of precautions were highly maintained. UV light was switched on one hour before working in the Laminar Hood. Petri-dishes and other glass-wares were sterilized by autoclaving at a temperature of 121 ^{0}C and a pressure of 15-lbs./sq. inch for 20 minutes. Micropipette tips, cotton, forceps, blank discs etc. were also sterilized.

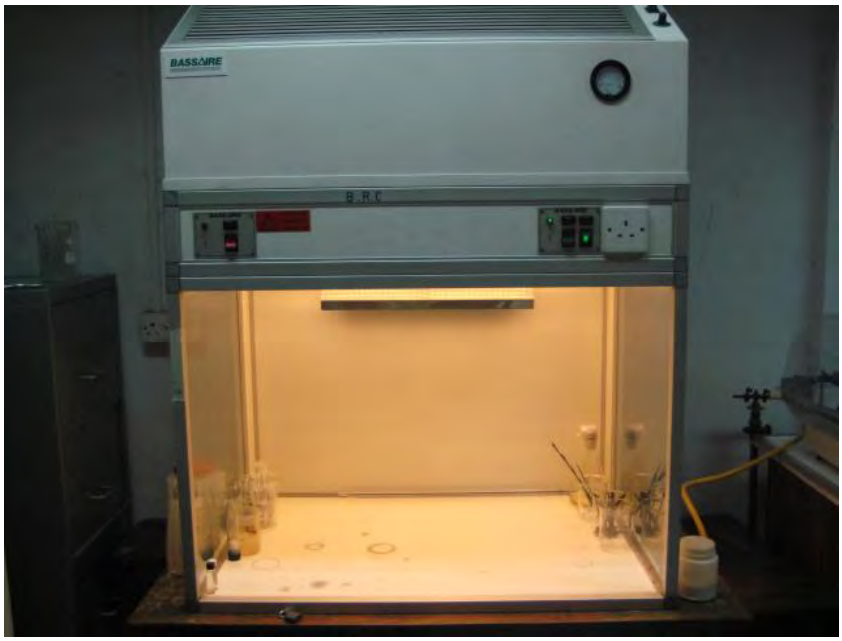


Figure 5.2: Laminar Hood

5.3.6 Preparation of subculture

In an aseptic condition under laminar air cabinet, the test organisms were transferred from the pure cultures to the agar slants with the help of a transfer loop to have fresh pure cultures. The inoculated strains were then incubated for 24 hours at 37 ^{0}C for their optimum growth. These fresh cultures were used for the sensitivity test.

5.3.7 Preparation of the test plates

The test organisms were transferred from the subculture to the test tubes containing about 10 mL of melted and sterilized agar medium with the help of a sterilized transfer loop in an aseptic area. The test tubes were shaken by rotation to get a uniform suspension of the organisms. The bacterial and fungal suspension was immediately transferred to the sterilized petridishes. The petridishes were rotated several times clockwise and anticlockwise to assure homogenous distribution of the test organisms in the media.

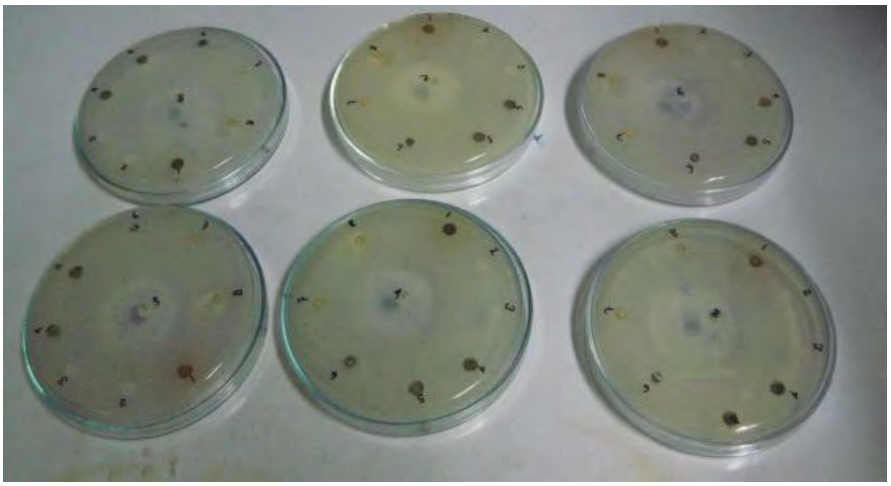


Figure 5.3: Test plate preparation by petridishes

5.3.8 Preparation of discs

Three types of discs were used for antimicrobial screening.

5.3.8.1 Standard discs

These were used as positive control to ensure the activity of standard antibiotic against the test organisms as well as for comparison of the response produced by the known antimicrobial agent with that of the test sample. In this investigation, kanamycin (30μg/disc) disc was used as the reference.

5.3.8.2 Blank discs

These were used as negative controls which ensure that the residual solvent (left over the discs even after air-drying) and the filter paper were not active themselves.

5.3.8.3 Preparation of sample discs with test samples

Measured amount of each test sample was dissolved in specific volume of solvent to obtain the *desired* concentrations in an aseptic condition. Sterilized metrical (BBL, Cocksville, USA) filter paper discs were taken in a blank petridish under the laminar hood. Then discs were soaked with solutions of test samples and dried.

5.3.8.3.1 Preparation of sample discs with test samples of *Saurauia roxburghii.*

Crude ethanol, n-Hexane, chloroform and ethyl-acetate extracts and five column fractions (H-13, C-15, C-19, E-18, and E-24) of various extract and four pure compounds (YSR-2, YSR-3, YSR-7 and YSR-9) were tested for antimicrobial activity against a number of both Gram-positive and Gram-negative bacteria.

a) Test sample for crude extracts

Crude extracts were tested for antimicrobial activity using sample concentration of 500 μg per disc.

b) Test samples for column fractions of the various crude extract

The antimicrobial sensitivity of the column fractions were tested using sample concentration of 400 μg per disc.

c) Test samples for pure compounds

Pure compound was tested for antimicrobial activity using sample concentration of 300 μg per disc.

5.3.9 Preparation and application of the test samples

The test samples were weighed accurately and calculated amounts of the solvents were added accordingly using micropipette to the dried samples to get desired concentrations. The test samples were applied to previously sterilized discs using adjustable micropipette under aseptic conditions.

5.3.9.1 Diffusion and Incubation

The sample discs, the standard antibiotic discs and the control discs were placed gently on the previously marked zones in the agar plates pre-inoculated with test bacteria and fungi. The plates were then kept in a refrigerator at 4 ^{0}C for about 24 hours upside down to allow sufficient diffusion of the materials from the discs to the surrounding agar medium. The plates were then inverted and kept in an incubator at 37 ^{0}C for 24 hours.

Figure 5.4: Incubator before and after inverted petridishes

5.3.10 Determination of antimicrobial activity by the zone of inhibition

The antimicrobial potency of the test agents are measured by their activity to prevent the growth of the microorganisms surrounding the discs which gives clear zone of inhibition. After incubation, the Antimicrobial activities of the test materials were determined by measuring the diameter of the zones of inhibition in millimeter with a transparent scale.

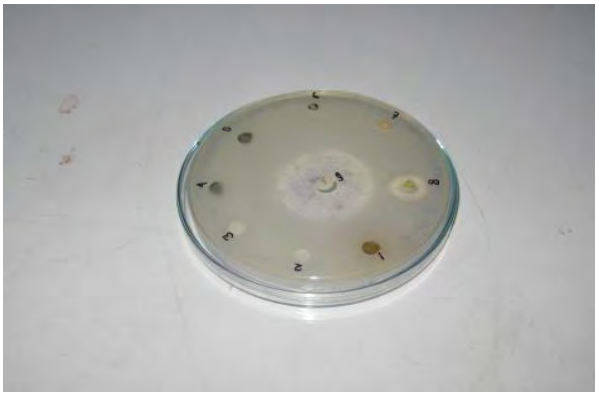


Figure 5.5: Counting of zone of inhibition

5.4 Results and discussion of *in vitro* Antimicrobial screening of *Saurauia roxburghii.*

Crude ethanol, n-Hexane, chloroform and ethyl-acetate extracts and five column fractions (H-13, C-15, C-19, E-18, and E-24) of various extract and four pure compounds (YSR-2, YSR-3, YSR-7 and YSR-9) were tested for antimicrobial activity against a number of both Gram-positive and Gram-negative bacteria. Standard disc of Kanamycin (30 µg/disc) was used for comparison purpose.

The crude ethanol extract exhibited good antimicrobial activity against most of the test organisms **(Table: 5.2)** and n-Hexane, chloroform, ethyl-acetate extracts exhibited moderate antimicrobial activity against the test organisms **(Table: 5.2)**.The five column fractions of exhibited low to mild **(Table: 5.3)** and the four pure compounds exhibited moderate antimicrobial activity against most of the test organisms **(Table: 5.4)**.

The zone of inhibition produced by crude ethanol extract was found to be 8-22 mm and n-Hexane, chloroform, ethyl-acetate extracts were found to be 7-20 at a concentration of 500µg/disc. and Similarly, and five column fractions (H-13, C-15, C-19, E-18, and E-24) of various extract yielded zones of inhibition 7 – 14 mm at a concentration of 400 µg/disc and four pure compounds Compound-1(**YSR-9**), Compound-3(**YSR-2**), Compound-4(**YSR-7**) and Compound-5 (**YSR-3**) yielded zones of inhibition 8 – 20 mm at a concentration of 300 µg/disc

The crude ethanol extract was screened against 14 test bacteria. This extract showed good activity against all the test Gram-positive and Gram-negative bacteria. n-Hexane, chloroform, ethyl-acetate extracts were screened against 14 test bacteria. These extract showed moderate activity against most of the test Gram-positive and Gram-negative bacteria but these extract were found to be resistant to the *Bacillus megaterium, Salmonella typhi, Vibrio cholera* and *Bacillus polymyxa* and *Bacillus megaterium, Streptococcus pneumonia, Vibrio cholera* respectively.

The column fraction H-13 of the crude extract of n-Hexane was screened against 8 test bacteria. This fraction showed poor to mild activity against Gram-positive bacterium *Bacillus cereus,Bacillus subtilis, Staphylococcus aureus, Bacillus polymyxa, Mycobacterium tuberculosis* and Gram-negative bacteria *Escherichia coli, Klebsiella sp., Shigella sonnei.*

The column fractions C-15 and C-19 of the crude extract of chloroform were screened against 8 test bacteria. This fraction showed poor to mild activity against Gram-positive bacterium *Bacillus cereus, Bacillus megaterium, Bacillus subtilis, Staphylococcus aureus*, *Mycobacterium tuberculosis* and Gram-negative bacteria *Escherichia coli, Shigella sonnei, Vibrio cholera.*

The column fractions E-18 and E-24 of the crude extract of ethyl-acetate were screened against 8 test bacteria. This fraction showed poor to mild activity against Gram-positive bacterium *Bacillus cereus, Bacillus subtilis, Staphylococcus aureus*, *Mycobacterium tuberculosis* and Gram-negative bacteria *Escherichia coli, Shigella sonnei.*

Fourteen (14) bacterial strains were taken to study the antimicrobial activity of the pure compounds Compound-3(**YSR-2**), Compound-5 (**YSR-3**), Compound-4(**YSR-7**) and Compound-1(**YSR-9**) at a concentration of 300µg/disc.These compounds showed moderate activity against all the Gram-positive bacteria *Bacillus cereus, Bacillus megaterium, Bacillus subtilis, Staphylococcus aureus, Streptococcus pneumonia, B. polymyxa, Mycobacterium tuberculosis* and Gram-negative bacteria *E.coli, Klebsiella sp., Proteus sp., Salmonella typhi, Shigella sonnei, Pseudomonas Aureus, Vibrio cholera* but Compound-1 (**YSR-9**) resistant to *Streptococcus pneumonia, Mycobacterium tuberculosi;*Compound-3(**YSR-2**) resistant to *Proteus sp., Shigella sonnei;* Compound-4(**YSR-7**) resistant to *Salmonella typhi, Pseudomonas Aureus* and Compound-5(**YSR-3**) resistant to *Salmonella typhi*.

Table-5.2: Antimicrobial activity of crude ethanol, n-Hexane, chloroform and ethyl-acetate extracts of *Saurauia roxburghii.*

Name of the Bacteria	Ethanol	n-Hexane	$CHCl_3$	EA	Kanamycin
	500 (µg/disc)				30 (µg/disc)
Gram-positive bacteria					
Bacillus cereus	16	10	13	8	34
Bacillus megaterium	15	--	13	--	34
Bacillus subtilis	16	12	14	10	32
Staphylococcus aureus	18	11	14	8	35
Bacillus polymyxa	16	10	--	8	33
Streptococcus pneumoniae	16	12	9	--	34
Mycobacterium tuberculosis	22	13	20	9	31
Gram-negative bacteria					
Escherichia coli	18	13	16	12	35
Klebsiella sp.	14	9	12	7	32
Proteus sp.	14	8	12	8	30
Salmonella typhi	15	--	13	10	33
Shigella sonnei	14	7	15	7	34
Pseudomonas Aureus	18	9	16	10	34
Vibrio cholerae	14	--	13	nd	31

"--" Indicates „No activity" and "nd" indicates „Not done".

Table-5.3: Antimicrobial activity of the five column fractions (H-13, C-15, C-19, E-18 and E-24) of various extract of *Saurauia roxburghii.*

Name of the Bacteria	H-13	C-15	C-19	E-18	E-24	Kanamycin
	400 (µg/disc)					30 (µg/disc)
Gram-positive bacteria						
Bacillus cereus	8	9	10	7	12	34
Bacillus megaterium	--	7	9	--	--	34
Bacillus subtilis	9	9	8	8	12	32
Staphylococcus aureus	8	8	10	12	10	35
Bacillus polymyxa	10	--	--	--	--	33
Streptococcus pneumoniae	--	--	--	--	--	34
Mycobacterium tuberculosis	10	10	12	7	12	31
Gram-negative bacteria						
Escherichia coli	12	10	8	7	14	35
Klebsiella sp.	10	--	--	--	--	32
Proteus sp.	--	--	--	--	--	30
Salmonella typhi	--	--	--	--	--	33
Shigella sonnei	10	10	13	7	12	34
Pseudomonas Aureus	--	7	8	14	10	34
Vibrio cholerae	--	9	8	--	--	31

"--" Indicates „No activity" and "nd" indicates „Not done".

Table-5.4: Antimicrobial activity of the four pure compounds (YSR-2, YSR-3, YSR-7 and YSR-9) of *Saurauia roxburghii.*

Name of the Bacteria	YSR-2	YSR-3	YSR-7	YSR-9	Kanamycin
	300 (µg/disc)				30 (µg/disc)
Gram-positive bacteria					
Bacillus cereus	11	13	14	12	34
Bacillus megaterium	8	14	9	8	34
Bacillus subtilis	12	15	16	12	32
Staphylococcus aureus	12	16	14	10	35
Bacillus polymyxa	14	15	12	10	33
Streptococcus pneumoniae	12	16	8	--	34
Mycobacterium tuberculosis	20	20	16	--	31
Gram-negative bacteria					
Escherichia coli	12	12	15	12	35
Klebsiella sp.	7	13	15	11	32
Proteus sp.	--	14	11	12	30
Salmonella typhi	13	nd	--	14	33
Shigella sonnei	--	13	12	10	34
Pseudomonas Aureus	12	16	--	14	34
Vibrio cholerae	8	12	10	10	31

"--" Indicates „No activity" and "nd" indicates „Not done".

CONCLUSION

A detailed phytochemical analysis has been done on the plant of *Saurauia roxburghii*. During this investigation eight compounds were isolated from this plant extract. Among them structural elucidation of the six compounds were performed. The remaining compound which was isolated from the n-hexane part was not elucidated as its ^{1}H-NMR gives evidence to be a fatty material. Compound-1 and 2 were identified as steroids as well as compound-3 to 6 were identified as triterpenes. Compound-1 to 6 were named as Stigmasterol, *β*-sitosterol, 3*β*-hydroxy-urs-12-en-28-oic acid, 3*β*-hydroxy-olea-12-en, 3*β*-hydroxy-olea-12-en-28-oic acid and 2*α*,3*β*-dihydroxy olea-12-en-28-oic acid respectively. All of these compounds were isolated for the first time so far from this species *S. roxburghii*. All the compounds were identified by chemical methods and structure elucidation was done by spectroscopic analysis (UV, FT-IR, ^{1}H & ^{13}C NMR) and comparison of their spectral data with those published in the literature.

The LC_{50} of crude ethanol, n-hexane, chloroform; column fractions H-13 of the crude hexane, C-19 of the crude extract of chloroform and column fractions E-13, E-18 and E-24 of the crude extract of ethanol were found to be 12.59, 14.79, 14.06, 11.75, 10.96,11.88, 4.37 and 6.92 µg/mL respectively. It was evident that all the test samples were very lethal to brine shrimp nauplii. However, column fractions E-18 and E-24 (4.37 and 6.92 µg/mL) of the crude extracts of ethanol might contain bioactive compounds.

The crude ethanol extract exhibited significant antibacterial activity against most of the test organisms and n-hexane, chloroform, ethyl-acetate extracts as well as the compound-1 (**YSR-9**), compound-3 (**YSR-2**), compound-4 (**YSR-7**) and compound-5 (**YSR-3**) showed moderate antibacterial activity against test organisms.

So we can be justified that the plant *Saurauia roxburghii* can be used as a medicinal plant since it contains the bioactive compounds.

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