Chapter – 3

PHYTOCHEMICAL STUDIES ON THE LEAVES OF *BISCHOFIA JAVANICA* AND *FRAXINUS FLORIBUNDA*

3.1 Introduction:

Phytochemical studies are vital in medicinal plant research to explore the specific phytoconstituents responsible for specific pharmacological actions. During isolation of phytoconstituents the researcher should follow the systematic procedures and precautionary measures, so that the phytoconstituents are not degraded in any of the steps and are extracted in maximum amount.

The precautionary measures to be taken are as follows:

- Collection of plant material has to be performed in the appropriate season, time and method
- Drying of the plant materials has to be performed for thermolabile constituents with specific care
- Method of extraction and solvent selection for extraction has got important role to obtain the phytoconstituents in maximum quantity.
- Selection of solvent ratio for mobile phase in column chromatography has to be performed systematically from non-polar to polar.

Schematic representation of the method of phytochemical studies is shown in **Figure 3.1.**

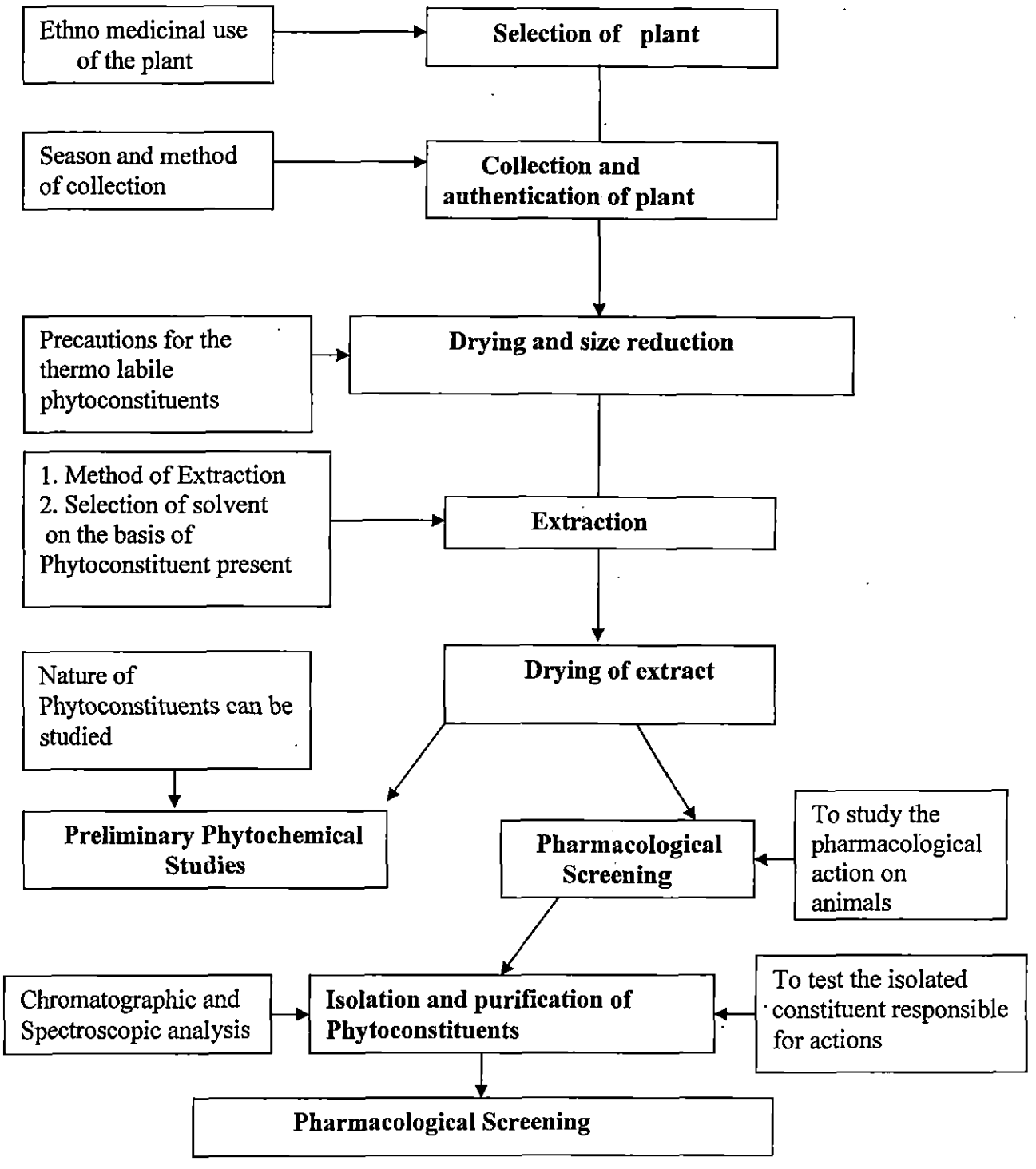


Figure 3.1 Schematic representation of phytochemical studies

3.1.1 Extraction of Plant Material:

The Plant material:

Plants are selected on the basis of ethno-medicinal importance and then collected and authenticated by a Botanist. Plant materials may require drying before extraction, drying operation is carried out under controlled conditions to avoid chemical changes of the constituents. [1] It should be dried as quickly as possible, without using high temperature, preferably in a good air draft. Once thoroughly dried, plants can be stored for a long period of time. The dried plant material is reduced to coarse powder before extraction to improve the efficiency of extraction procedure.

Extraction:

The precise method of extraction normally depends on the texture and water content of the plant material being extracted and on the type of substance that is to be isolated. Alcohol in any case is a good all purpose solvent for preliminary extraction. Subsequently the material can be macerated in a blender and filtered but this is only necessary if exhaustive extraction is being attempted.

The common procedure for obtaining organic constituents from the dried plant tissue (heartwood, dried seeds, root, leaf) is to continuously extract the powdered material in Soxhlet apparatus with series of solvents, starting with petroleum ether, and chloroform, (to separate lipids and terpenoids) and then using alcohol and ethyl acetate (for more polar compounds).

The extract obtained is clarified by filtration through celite then concentrated in vacuo. This can be carried out in a rotary evaporator, which concentrates the bulky solutions, without bumping, at temperatures between 30-40°C.When investigating the complete phytochemical profile of a given plant species, fractionation of a crude extract is desirable in order to separate the main class of constituent from other, prior to chromatographic analysis. [2]

3.1.2 Isolation of Phytoconstituents:

Separation and isolation of phytoconstituents is done by the chromatographic techniques. Adsorption chromatography is based on the principle that the compounds, which have more affinity towards stationary phase, are separated (eluted) later and the compounds, which have least affinity towards stationary phase, are eluted initially.

Column Chromatography:

The equipment required in column chromatography is very simple and inexpensive. A chromatographic column need only glass tube. The dimensions are not critical, with diameters ranging from a few mm to few cms and length from a few cm to meter. The greater efficiency of elution is obtained with a long narrow column than that of short and wide one. The stationary phase is prepared as a slurry in mobile phase and packed in the column supported at the bottom by a perforated disk or by a plug of a glass wool. The stationary phase is chosen on the basis of adsorption property of the constituents. In general the adsorbents used are Fuller's earth, Activated charcoal, Activated alumina, Activated silica, Magnesium oxide, Calcium carbonate, Potassium carbonate, Talc, Starch etc.

The mobile phase usually starts gradually from non polar to polar by combination of them depending on the secondary metabolites. Eluents used are in the order of increasing eluent power eg petroleum ether, carbon tetrachloride, benzene, ether, chloroform, acetone, ethyl acetate, ethanol, methanol and water

The sample is applied to the column on the top of the stationary phase. The mobile phase solvent is added to the column and the lower opening is opened. The eluent percolates through the packing and the column effluent are collected in small fractions for analysis.

Thin Layer Chromatography (TLC):

Thin layer chromatography is a form of adsorption chromatography in which the adsorbent is spreaded in a thin layer on a glass plate. TLC is a simple technique widely used for the separation of phytoconstituents and also TLC using different ratios of mobile phases can easily identify the impurities of the extract. The development in TLC is carried out by the ascending technique. After development is complete, the plates are allowed to air dry before examination of migrated constituents. The constituents are detected by an adequate physical technique or chemical reaction. Retention factor R_f is the distance moved by a compound in chromatography relative to the movement of the solvent front. It is obtained by measuring the distance from the origin to the centre of the spot produced by the compound, and this is divided by the distance traveled by the solvent from the origin to the final (i.e. the distance traveled by the solvent).This always appears as a fraction and lies between 0.01 and 0.99. R_f value differs with different compounds e.g. for kaempferol it is 0.91, quercetin 0.76, myricetin 0.41 in Butanol: Acetic acid: Water system. [3]

High Performance Liquid Chromatography (HPLC):

In HPLC stationary phase bounded to a porous polymer held in a narrow-bore stainless steel coloumn and the liquid mobile phase is forced through under considerable pressure. The apparatus of HPLC is more expensive. The mobile phase is a miscible solvent mixture, which either remains constant (isocratic separation) or may be changed continuously in proportions including a mixing chamber in to the set-up (gradient elution). The compounds are monitored as they elute off the coloumn by means of a detector usually measuring in the UV range. A computing integrator may be added to handle the data and the whole operation can be controlled through microprocessor.

HPLC is mainly used for those classes of compounds, which are non-volatile, e.g. higher terpenoids, phenolic compounds, alkaloids, lipids and sugars. It works best for compounds which can be detected in the ultraviolet or visible regions of the spectrum. In most model of HPLC separations, prepared, pre packed coloumns are employed and

different types are available from the manufacturers. However, it is possible to carry out most separations using either a silica micro porous particle column (for non-polar compounds) or a reverse phase C_{18} bounded phase coloumn (for polar compounds) [4]

HPLC is the latest chromatographic technique to be added to the phytochemists. Apart from the expense of the apparatus and the solvents, it is most important and versatile method of quantitative analysis.

3.1.3 Characterization of isolated compounds:

It can be performed with the help of UV-Visible, IR, NMR, Mass spectroscopic techniques.

UV-Visible Spectrophotometery:

Molecular absorption in the ultraviolet and visible region of the spectrum is dependent on the electronic structure of the molecule. Absorption of energy is quantized, resulting in the elevation of electrons from orbitals in the ground state to higher energy orbitals in an excited state. The principal characteristics of an absorption band are its position and intensity. The position of absorption corresponds to the wavelength of radiation whose energy is equal to that required for an electronic transition. Absorption intensity is that derived from the Beer-Lambert's law, which establishes a relationship between the absorbance, the sample thickness and the concentration of the absorbing species. UV spectrophotometer measurement is useful in the study of impurities in the sample and can be compared with that of standard raw material; also it is useful in the structure elucidation of a organic molecules. The presence of hetero atoms like S, O, N or halogens can be determined. Structure analysis of organic compounds like effect of conjugation, effect of geometric isomerism and effect of alkyl substitution can be studied with the help of λ_{max} of the compounds [5]. Spectral properties of plant pigments were given below in the **Table 3.1.**

Table 3.1 Spectral range of some plant constituents

Pigment class	Visible spectral range (nm)	Ultraviolet range (nm)
Chlorophyll (green)	640-660 and 430-470	Intense short UV absorption due to protein attachment
Phycobilins (red and blue)	615-650 and 540-570	Intense short UV absorption due to protein attachment
Cytochromes (yellow)	545-605 and 415-440	Intense short UV absorption due to protein attachment
Anthraquinones (yellow)	420-460	3-4 intense peaks between 220 and 290
Carotenoids (yellow to orange)	400-500	-----
Flavanols (yellow)	365-390	250-270

Infra Red (IR) Spectroscopy:

Infrared spectroscopy is concerned with the study of absorption of infrared radiation, which results in vibrational transitions. IR spectra are mainly used in structure elucidation to determine the functional groups. Infrared radiation in the range from about 10,000-100cm^{-1} is absorbed and converted by an organic molecule into energy of molecular vibration. This absorption is also quantized, but vibrational spectra appear as bands rather than as lines because a single vibrational energy change is accompanied by a number of rotational energy changes. The frequency or wavelength of absorption depends on the relative masses of the atoms, the force constants of the bonds and the geometry of the atoms.

In any molecule it is known that bonds connect atoms or groups of atoms. These bonds are analogous to springs and not rigid in nature. Because of the continuous motion of the molecule, they maintain some vibrations with some frequency,

characteristic to every portion of the molecule. This is called the natural frequency of vibration. When energy in the form of infrared radiation is applied and when applied infrared frequency equals to natural frequency of vibration, absorption of IR radiation takes place and a peak is observed. Every bond or portion of a molecule or functional group requires different frequency for absorption. Hence characteristic peak is observed for every functional group or part of the molecule. An IR spectrum is useful in identification of functional group and structure elucidation. eg two trace components of tobacco smoke are identified as the bases harmane and norharmane using the KBr disc procedure in IR spectroscopy. [6]

Mass Spectroscopy (MS):

Mass or molecular weight of a compound can be determined in several ways. One such technique is using Mass spectrometer. Not only for determination of mass, the technique can be used for structure elucidation. Using Mass spectrum of the compounds can do quantitative analysis and even advanced studies. It is also called as positive ion spectra or line spectra. Thus, Mass spectroscopy is useful in structure elucidation, detection of impurities, quantitative analysis and drug metabolism studies.[7] New technical developments continue to emerge in mass spectroscopy and modern spectrometers may be provided with a Fast Atom Bombardment (FAB) source and are then capable of analyzing fragile or in volatile organic compounds including salts and higher molecular weight materials.

Nuclear Magnetic Resonance (NMR) Spectroscopy:

NMR spectroscopy is the study of spin changes at the nuclear level when radio-frequency energy is absorbed in the presence of magnetic field. Nuclei with odd mass number only give NMR spectra eg.^{1}H, ^{13}C, ^{19}F, ^{35}Cl etc, because they have asymmetrical charge distribution. Any proton or nucleus with odd mass number spins on its own axis. By the application of an external magnetic field, the nucleus spins on its own axis a magnetic moment is created, resulting in a precessional orbit with a frequency called as precessional frequency. This state is called as ground state or parallel orientation. In this

state the magnetic field caused by the spin of nuclei is aligned with the externally applied magnetic field. When energy in the form of radio-frequency is applied and when, applied frequency equals to precessional frequency, absorption of energy occurs and a NMR signal is recorded. Because of the absorption of energy, the nucleus moves from ground state to excited state, which results in spin reversal or anti-parallel orientation in which the magnetic field caused by the spin of nucleus opposes the externally applied magnetic field. When the application of radio-frequency energy is stopped, the nucleus returns to ground state or parallel orientation. It should be noted that increasing of the strength field does not cause transition from ground state to excited state, but it merely increases the precessional frequency. Without application of magnetic field, there are no two spin states and there is only one average spin, hence radio-frequency radiation cannot be absorbed. Therefore, application of magnetic field and radio frequency is necessary to cause NMR spectra to be useful for structure elucidation of organic compounds, investigation of dynamic properties like conformational isomerism, hydrogen bonding, molecular asymmetry, keto-enol tautomerism, study of molecular interactions like micelle formation and drug macromolecule or drug receptor interactions. [8]

3.2 Instruments and Chemicals:

The ^{1}H (600 MHz) and ^{13}C (600 MHz) NMR data were obtained using $CDCl_3$ as the reference solvent, employing BRUKER DRX-600 NMR-spectrometers. ES-MS was recorded on a Micromass Quattro II instrument. IR spectra were recorded on a Perkin-Elmer, FTIR 1750 spectrophotometer. Column and Thin layer chromatography were performed on silica gel (60–120 mesh) and silica gel-G (Merck) respectively. Other chemicals and solvents used in the phytochemical study were procured from the local firm and were of analytical grade.

3.3 Extraction and Purification of Phytoconstitutents from *Bischofia javanica*:

Bischofia javanica Blume (Euphorbiaceae), known as kainjal in Nepali a evergreen tree widely distributed over the Southeast Asia, Japan and Australia. In India it is distributed over the Sub-Himalayan region, Orissa and south west coast from Konkan to Nilgiris. [9]

This plant has been utilized significantly for various ailments like topical treatment for ulcer, sores and boils. [10] It possesses antiulcer, antimicrobial, anthelmintic and antidysenteric activities. Tribes of Chhattisgarh and Sikkim use the leaf juice of the plant for the treatment of cancerous Wound. [11] *B. javanica* have been reported to contains the phytoconstituents like tannin, β-amyrins, betulinic acid, chrysoeriol, ellagic acid, fiestin, friedelan-3-alpha-ol, epifriedelinol, friedelin, luteolin and glucoside, quercetin, quercitrin, β-sitosterol, stigmosterol, ursolic acid, triacontane, fatty acids including linoleic acid, stearic acid, iscchofianin, corigalin, furosin, geranin, punicalagin [12],[13]

3.3.1 Extraction of Phytoconstituents from *Bischofia javanica*:

3.3.1.1 Collection of Plant Material:

The leaves of *Bischofia javanica* were collected from Melli region of Sikkim (India) in the month of June. The plant material was identified and authenticated at Botanical Survey of India (BSI), Sikkim branch. A herbarium was also kept in the parent institute for future reference (LS/BJ/03/RPS). The collected leaves of *B.javanica* was shade dried for 15 days and size reduced using laboratory grinder in to coarse powder. It was stored in a well closed container free from environmental changes till usage.

3.3.1.2 Extraction Procedure:

The powdered leaves (5 Kg) were extracted with methanol in soxhlet apparatus at 50-60°C. After exhaustive extraction the extract was concentrated by distilling the solvent for further use. The concentrated extract was kept in the desiccator. Yields of the prepared extract were 5.0 %w/w (250g) of the dried leaf powder. The extract was used for the study of preliminary chemical analysis.

The methanol extract was concentrated, suspended in distilled water and partitioned with petroleum ether, chloroform. [14-15] The chloroform soluble fraction was subjected to thin layer chromatographic analysis. The aqueous and petroleum ether fraction did not show any positive pharmacological activities under perview of this investigation and was discarded. Flow chart of the extraction procedure is shown in **Figure 3.2.**

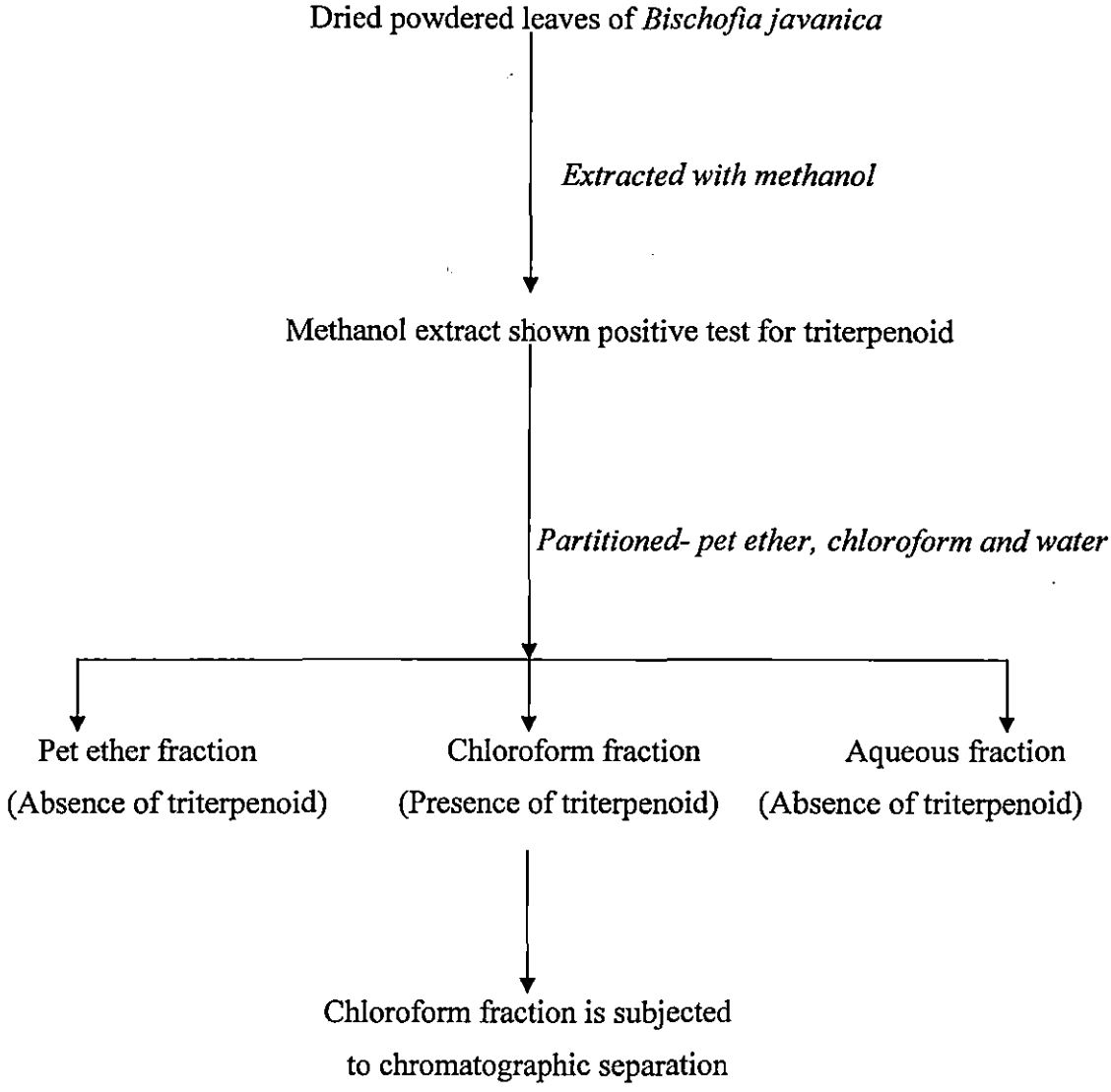


Figure 3.2 Chart showing the method of extraction of *B.javanica*

3.4 Isolation and Purification of Phytoconstituents from the Leaf of *B.javanica*:

3.4.1 Preliminary Phytochemical Test:

The preliminary phytochemical group test of *B.javanica* leaf extract was performed by the standard methods. [16-17]

Test for Flavanoids:

- 5ml of extract solution was hydrolyzed with 10%v/v sulphuric acid and cooled. Then it was extracted with diethyl ether and divided into three portions in three separate test tubes.1ml of dilute ammonia, 1ml of dilute sodium bicarbonate and 1ml of 0.1N sodium hydroxide were added to the first, second and third test tubes respectively. In each test tube, development of yellow colour indicated the presence of flavanoids.
- The extract was dissolved in alcohol. One piece of magnesium followed by concentrated hydrochloric acid was added to the alcoholic solution drop wise and heated. Appearance of magenta color demonstrated the presence of flavanoids.

Test for Steroids and Triterpenoids:

Liebermann-Burchard Test

- 10mg of extract was dissolved in 1ml of choloroform.1ml of acetic anhydride was added following by addition of 2ml of concentrated sulphuric acid. Formation of blue-green colour indicated the presence of steroids and triterpenoids.

Salkowski Test

- 1ml of concentrated sulphuric acid was added to 10 mg of extract dissolved in chloroform. A reddish-blue colour produced in the chloroform layer and green fluorescence in the acid layer indicated the presence of steroids and triterpenoids

Test for Alkaloids:

- To 1ml of solution of the extract in a test tube, 0.2ml of dilute hydrochloric acid and 0.1ml of Mayer's reagent were added. Formation of yellowish-buff precipitate confirmed positive test for alkaloids
- 1ml of dilute hydrochloric acid and 0.1ml of Dragendroff's reagent were added to 2ml solution of the extract in a test tube. Development of orange-brown colour precipitate revealed the presence of alkaloid.
- 2ml of the extract solution was treated with 1 ml of dilute hydrochloric acid and 0.1ml of Hager's reagent. A yellowish precipitate confirmed the presence of alkaloid.

Test for Reducing Sugar:

- To 5ml of the extract solution, 5ml of Fehling's solution was added and boiled for 5 minutes. Formation of brick red coloured precipitate demonstrated the positive test for reducing sugars
- To 5ml of the extract solution, 5ml of Benedict's solution was added in a test tube and boiled for few minutes. Development of brick-red colour precipitate confirmed the presence of reducing sugars

Test for Tannins:

- 5ml of the extract solution was allowed to react with 1ml of 5% ferric chloride solution. Greenish-black coloration indicated the presence of tannins.
- 5ml of the extract solution was treated with 1ml of aqueous potassium dichromate solution. Formation of yellowish-brown precipitate suggested the presence of tannins.
- 5ml of the extract solution was treated with 1ml of aqueous 10% lead acetate solution. Yellow coloured precipitation gave the test for tannins.

Test for Saponins:

- 1ml solution of the extract was diluted with distilled water to 20 ml and shaken in a graduated cylinder for 15 min. Development of stable foam suggested the presence of saponins
- 1ml of the extract solution was treated with 1.0% lead acetate solution. Formation of white precipitate indicated the presence of saponins

Test for Anthraquinones:

- 5ml of the extract solution was hydrolysed with dilute sulphuric acid and then extracted with benzene.1ml of dilute ammonia was added to the extract. No colour change was observed indicating the absence of anthraquinones.

Test for Amino acids and Proteins:

- Small quantity of leaf extract of *B.javanica* was dissolved in a few ml of distilled water and treated with ninhydrin reagent at pH 5.0. The absence of purple coloration suggested the negative test for amino acids.
- Small quantity of leaf extract of *B.javanica* was treated with few drops of 10% sodium hydroxide solution. Few drops of 1% copper sulphate solution was added and mixed. Formation of violet or purple colour demonstrated the presence of proteins.
- Small quantity of leaf extract of *B.javanica* was treated with 1.0 ml of concentrated nitric acid. The sample was boiled and cooled. Few drop of 40% sodium hydroxide was added drop by drop. Appearance of orange coloured solution indicated the presence of proteins.
- A small quantity of leaf extract of *B.javanica* was treated with few drops of Millon's reagent. The samples were boiled and cooled. Few drop of 40% sodium nitrate solution was added to the sample drop by drop. Appearance of red precipitate confirmed the presence of proteins.

3.4.2 Thin Layer Chromatographic Study of the Chloroform Fraction of Methanol Leaf Extract of *B. javanica* :

Thin layer chromatographic study of the chloroform fraction was carried out on silica gel G (Merck) plates with different solvent systems. Glass plates of 15cm × 5 cm were coated with slurry of silica gel prepared by mixing 6 gm of silica gel G with 12 ml of distilled water. The layer was allowed to set for 30 min at room temperature and was then activated at 110°C for 30 min.

The sample of chloroform fraction of methanol extract was spotted on the plates and chromatogram was developed in chromatographic chambers using selected solvent systems at room temperature (28°C) and at an angle of 70°. Since the rate of migration of a compound on a given adsorbent depends upon the solvent used, the solvent system can be arranged in order of elutive power. Mixtures of two or three solvents of different polarities give better separation than the chemically homogeneous solvents. In the present investigation, several solvent systems were studied for effective separation of the components, but the under mentioned solvent systems showed better result. The mobile phase (solvent system) was allowed to run up to a distance of 10 cm from the origin. The plates were removed from the chamber after completion of the run and were allowed to dry in air. The plates were observed under UV light for the appearance of spots before and after spraying with spray reagents.

The chromatograms were run with three solvent systems mentioned below in a chromatography chamber. [18]

3.4.2.1 Solvent Systems Used:

TLC 1. n-hexane: Ethyl acetate (9:1).

TLC 2. n-hexane: Ethyl acetate (8:2)

TLC 3. n-hexane: Chloroform: Ethyl acetate (7:2:1)

3.4.2.2 Spray Reagents Used:

Liebermann Burchard (LB) reagent

3.4.3 Coloumn Chromatography of Leaf Extract of *B. javanica*:

3.4.3.1 Coloumn:

A glass coloumn, 80 cm in length, 3.5 cm diameter. The bottom of the coloumn was plugged with glass wool.

3.4.3.2 Adsorbent:

Silica gel 60-120 (E.Merck).

3.4.3.3 Solvents Used:

n-hexane: Ethyl acetate (with increasing concentration of ethyl acetate).

n-hexane: chloroform: ethylacetate

3.4.3.4 Preparation of Coloumn:

The coloumn was washed with distilled water and finally rinsed with acetone to remove the impurities. It was fixed with a stand and packed with slurry of Silica gel and n-hexane. The solvent n-hexane was adjusted to drip at the rate of 100 drops per minute and a level of 5 cm of the solvent was maintained on the top of the silica gel layer.

3.4.4 Isolation of Compound from Chloroform Fraction of *B.javanica* Leaf

The dried solid chloroform fraction of the leaf extract and silica gel was layered on the top of the coloumn. A thin layer of cotton was placed over it. The solvent was allowed to flow down slowly till the mixture was adsorbed on the top of the coloumn. Gradient elution was carried out using n-hexane and ethyl acetate as solvent by increasing the polarity of n-hexane by adding increments of ethylacetate. [19-20] The fractions obtained from the coloumn chromatography using different ratios of n-hexane: ethyl acetate were numbered as F-1 to F-7 for the ratio 10:0, F-8 to F-17 for the ratio 9:1, F-18 to F-23 for 8:2, F-24 to F-35 for 7:3 and F-36 to F-49 for 6:4. TLC study was performed on the fractions and the fraction F-18 to 22 revealed the elution of the mixture of triterpenoid

amongst all the fractions. So the fractions were combined together and was further purified in coloumn chromatography using n-hexane-chloroform-ethyl acetate (8:1:1, 7:2:1, 6:3:1) as solvent system. The sub fraction (SF-7-10) of F-18 to F-22 in the eluting solvent system of n-hexane-chloroform-ethyl acetate in the ratio of 7:2:1 gave the pure triterpenoid, which was confirmed by TLC studies. The isolated compound LS-1 was further examined by different physico-chemical techniques for its structure elucidation.

3.4.5 Qualitative Study of the Compound Isolated from Chloroform Fraction of *B.javanica* Leaf:

The isolated compound LS-1 was further subjected to chemical tests for confirmation of its chemical nature.

3.4.6 Physical Nature of the Compound Isolated from Chloroform Fraction of *B.javanica* Leaf:

The isolated compound LS-1 was subjected to various physicochemical parameters viz: physical appearance, solubility and melting point.

3.4.7 Thin Layer Chromatographic Study of the Compound Isolated from Chloroform Fraction of *B. javanica* Leaf:

Thin layer chromatographic study of the isolated compound was carried out on silica gel G plates with different solvent systems. The plates were prepared as described in 3.4.2 and used for the study. The chromatograms were run with three solvent systems mentioned below in a chromatography chamber.

3.4.7.1 Solvent Systems Used:

TLC 1. n-hexane: Ethyl acetate (9:1).

TLC 2. n-hexane: Ethyl acetate (8:2)

TLC 3. n-hexane: Chloroform: Ethyl acetate (7:2:1)

3.4.7.2 Spray Reagents Used:

Lieberman-Burchard (LB) reagent

The plates were observed under UV light for the appearance of spots before and after spraying with spray reagents.

3.4.8 Infrared Spectrum of the Compound Isolated from Chloroform Fraction of *B.jacvanica* Leaf:

The infrared (IR) absorption spectra of the isolated compound were taken with Perkin Elmer FTIR-1750 spectrophotometer, in potassium bromide discs. The spectra were recorded in the region of 4000 cm^{-1} to 400 cm^{-1}. The spectra of the isolated compound with absorption bands were recorded and tabulated.

3.4.9 Nuclear Magnetic Resonance (NMR) Spectra of the Compound Isolated from Chloroform Fraction of *B.javanica* Leaf:

The ^{1}H and ^{13}C NMR spectra of the isolated compound from chloroform fraction of *B.javanica* leaves were undertaken in Brucker DRX 600 Spectrometer in $CDCl_3$ (Duterated chloroform) solution. The spectra are recorded and tabulated.

3.4.10 Mass Spectra of the Compound Isolated from Chloroform Fraction of *B.javanica* Leaf:

Mass spectrum (ES-MS) of the isolated compound from the leaves of *B.javanica* was recorded on a Micromass Quattro II instrument.

3.5 Results:

3.5.1 Phytochemical Study of *B.javanica* Leaf:

The concentrated methanol extract obtained from the shade-dried leaf of *B.javanica* was fractionated successively with petroleum ether, chloroform and water. It was observed that only the Chloroform fraction exhibited significant pharmacological activities under purview of this investigation. So this fraction was subjected to phytochemical analysis.

The preliminary phytochemical group tests indicated the presence of steroids, flavonoids, proteins, tannins, saponins and reducing sugar **(Table 3.2)**. The thin layer chromatographic study of the chloroform fraction of methanol extract of *B.javanica* showed the presence of about five components with selected mobile phases and spraying reagent **(Table 3.3)**. This fraction was subjected to coloumn chromatographic separation on Silica gel coloumn in which forty-nine fractions were collected. The fractions having identical Rf values (F-18-22) from TLC studies were mixed together **(Table 3.4)**. They were again purified with a silica gel (60-120) coloumn, which yielded a pure compound (LS-1) which was again confirmed by TLC studies **(Table 3.5)**. On evaporation of the solvent a white crystalline powder was obtained whose melting point is 292-294°C. The substance is insoluble in water and soluble in organic solvents.

It was further subjected to chemical tests and TLC studies to confirm the chemical nature of the compound LS-1, which are presented in **Table 3.6 & Table 3.7**. The chemical nature of the isolated compound was further characterized from its physical parameters and spectral (IR, Mass, ^{13}C and ^{1}H NMR) data [21-22].

The IR spectrum of the compound is presented in **Figure 3.3**. The IR spectrum shows the presence of absorption bands at 2931, 1738 and 1245 cm^{-1}. Other prominent peaks were 2870, 1461, 1386, 1025 and 526 cm^{-1}. The IR spectrum of the compound LS-1 revealed the presence of carbonyl group (1738 cm^{-1}), C-H stretching and bending were confirmed by absorption at 2870 and 1461 cm^{-1}. The ^{1}H NMR spectrum of compound LS-1 is presented in **(Figure 3.4-3.7)**. The ^{1}H NMR data revealed the eight singlet methyl protons in the compound showing the signals at 1.16, 1.18, 1.13, 1.04, 0.99, 0.82, 0.76 and 0.98 ppm **(Table 3.8)**. The signal at 4.36 ppm revealed the acetyl substitution at C-3 and the singlet at 2.03 ppm confirmed the acetyl group. The methylene link in the skeleton showed the α and β configuration and the signal between 1.24-1.59 ppm.^{13}C NMR data **(Figure 3.8-3.10)** revealed the 32 carbons in the skeleton with eight methyl groups. The acetyl group at C-3 showed the chemical shift at 75.17 and the C=O group

has provided the δ_C at 171 **(Table 3.8)**. Further DEPT (Distortionless Enhancement by Polarization Transfer)-90 and DEPT-135 studies confirmed singlet, doublet, triplet and Quadralet carbons of ^{13}C NMR studies and inferred the pentacyclic triterpenoid skeleton for the test compound LS-1 [23-25]. The Mass spectra (ES-MS) **(Figure 3.11)** showed the m/z (%) 494{M+Na^+} (40), 472{M+1} (20) and confirmed the compound with molecular weight 470.77. From the available literature on Friedelin compounds and the ^1H NMR, and ^{13}C NMR spectral data, [23-26] the structure of the compound LS-1 was found to be **Friedelin-3α-acetate** with molecular formula $C_{32}H_{54}O_2$.**(Figure3.12).**

Table 3.2 Preliminary phytochemical test of methanol extract of *B. javanica* leaf

Phytoconstituents	***B.javanica***	
	Methanol Extract	**Chloroform Fraction**
Alkaloids	+	+
Amino acids	–	–
Proteins	+	–
Reducing sugar	+	–
Steroids and triterpenoids	+	+
Flavanoids	+	+
Tannins	+	–
Saponins	+	+
Anthraquinones	–	–

+ ***ve*** indicates presence and – ***ve*** indicates absence of the phytoconstituents

Table 3.3 Thin layer chromatography study of the chloroform fraction of methanol extract of *B. javanica* leaf.

No of spots	Colour of spots under UV light			Colour of spots under UV light after spraying the spraying reagent			R_f values		
Spots	TLC 1	TLC 2	TLC 3	TLC 1	TLC 2	TLC 3	TLC 1	TLC 2	TLC 3
1	Blue-Green	Blue-Green	Blue-Green	Bright Blue-Green	Bright Blue-Green	Bright Blue-Green	0.43	0.49	0.58
2	Blue-Green	Blue-Green	Blue-Green	Bright Blue-Green	Bright Blue-Green	Bright Blue-Green	0.54	0.57	0.64
3	Green	Green	Green	Green fluorescence	Green fluorescence	Green fluorescence	0.83	0.87	0.86
4	–	Green	Green	–	Green fluorescence	Green fluorescence	–	0.91	0.87
5	–	–	Green	–	–	Green fluorescence	–	–	0.93

Spray reagent: Liebermann Burchard reagent

Solvent system

TLC1 ---- n-hexane: Ethyl acetate (9:1).

TLC2 ---- n-hexane: Ethyl acetate (8:2)

TLC3 ---- n-hexane: Chloroform: Ethyl acetate (7:2:1)

Table 3.4 Coloumn and thin layer chromatographic separation of chloroform fraction of *B. javanica* leaf.

Eluent n-hexane:ethyl acetate	Fraction Number	Colour of TLC spots	Inference
10:0	1-07	NIL	Fatty oil
9:1	08-17	Green	Mixture of Fatty solid with Chlorophyll
8:2	18-22	Blue green (mixture of spots)	Colourless solid (Presence of triterpenoids)
7:3	23-35	Green to Blue green (mixture of spots)	Greenish semisolid
6:4	36-49	Green to Blue green (mixture of spots)	Greenish semisolid

Spray reagent: Liebermann Burchard reagent

Table 3.5 Coloumn and Thin layer chromatographic separation of chloroform Sub-fraction (F 18-22) of *B. javanica* leaf.

Eluent n-hexane: chloroform: ethyl acetate	Fraction Number	Colour of TLC spots	Inference
8:1:1	1-6	Blue -Green (mixture of spots)	Colourless solid (mixture of compounds)
7:2:1	7-10	Blue-Green (single spot)	Colourless crystalline solid (pure compound)
6:3:1	11-13	Blue-Green (mixture of spots)	Colourless solid (mixture of compounds)

Spray reagent: Liebermann Burchard reagent

Table 3.6 Qualitative analysis of the compound LS-1 isolated from chloroform fraction of *B.javanica* leaf.

S. No	Test	Observation	Inference
1	*Liebermann-Burchard test:* 1mg of the isolated compound LS-1 was dissolved in chloroform then 1ml of acetic anhydride was added followed by 0.5ml of concentrated sulphuric acid was added by side of the test tube.	Formation of blue-green colour	Presence of triterpenoid.
2	*Salkowskis Test:* 0.5ml of concentrated sulphuric acid was added to 1mg of the isolated compound LS-1 and dissolved in chloroform.	A reddish-blue colour produced in the chloroform layer and green fluorescence in the acid layer.	Presence of triterpenoid.

Table 3.7 Thin layer chromatography of the compound LS-1 isolated from chloroform fraction of *B.javanica* leaf.

Solvent system	R_f values	Colouration	
		UV_{366nm}	UV_{366nm}+LB Reagent
TLC-1	0.49	Blue-Green	Bright Blue-Green
TLC 2	0.53	Blue-Green	Bright Blue-Green
TLC 3	0.61	Blue-Green	Bright Blue-Green

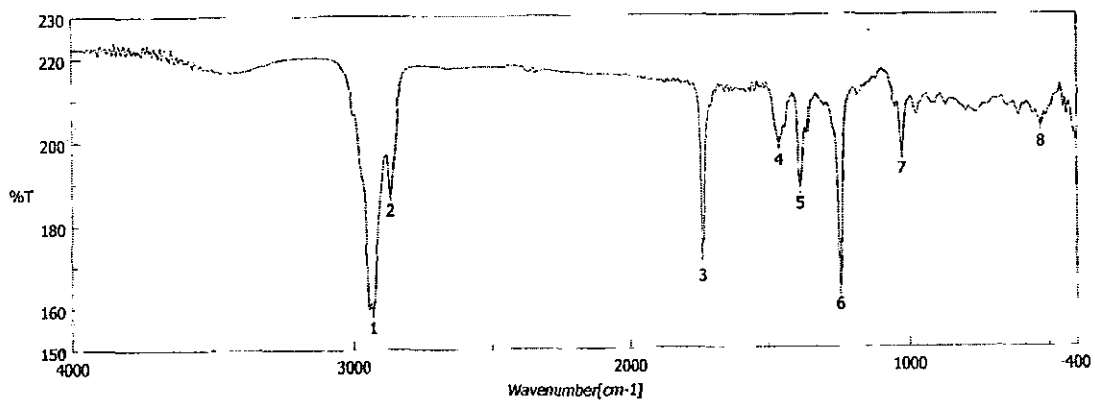
LB Reagent - Liebermann Burchard Reagent

Solvent system

TLC1----- n-hexane: Ethyl acetate (9:1).

TLC2----- n-hexane: Ethyl acetate (8:2)

TLC3----- n-hexane: Chloroform: Ethyl acetate (7:2:1)



Accumulation	34	Resolution	4 cm-1
Zero Filling	ON	Apodization	Cosine
Gain	2	Scanning Speed	2 mm/sec
Date/Time	10/23/06 3:46PM		
Update	10/23/06 3:48PM		
Operator	SATYA		
File Name	Memory#7		
Sample Name	LS-1		
Comment	KBr		

1: 2931.27, 159.124	2: 2869.56, 187.501	3: 1737.55, 171.12	4: 1460.81, 189.136
5: 1385.8, 153.704	6: 1244.83, 163.987	7: 1024.98, 197.022	8: 529.471, 203.307

LS1

Figure 3.3 IR spectrum of compound LS-1 isolated from the leaves of *Bischofia javanica*

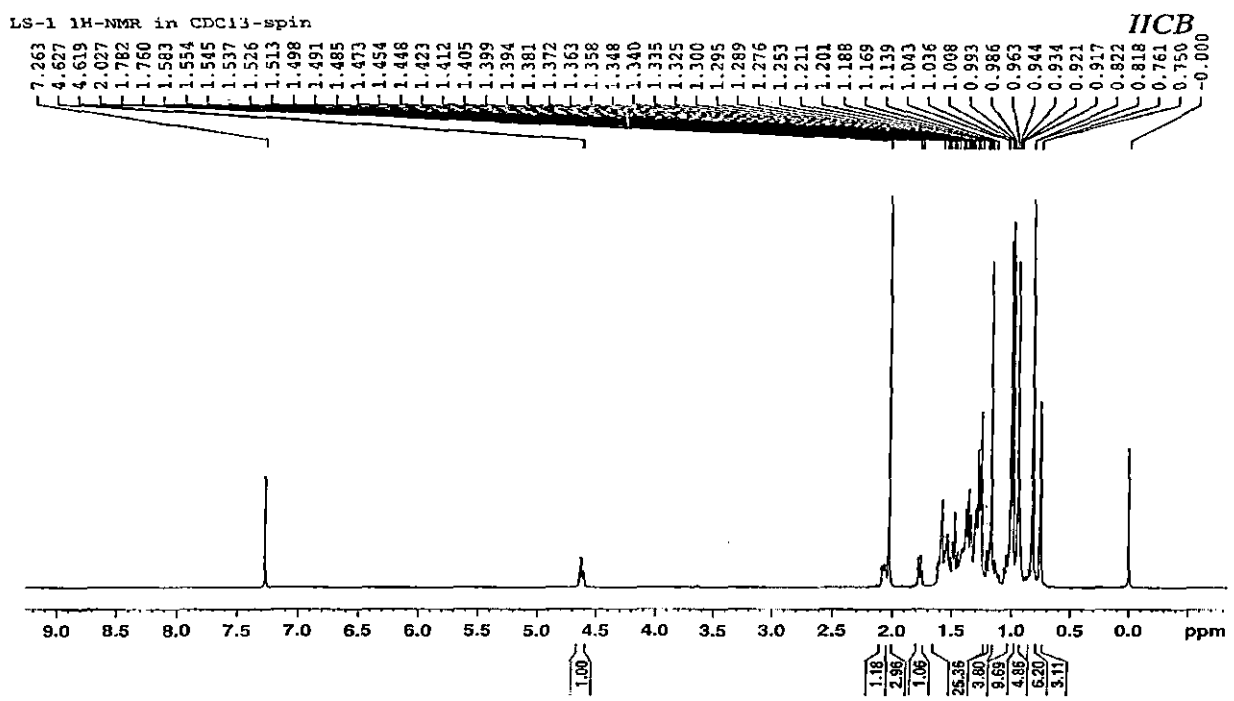


Figure 3.4 ^{1}H NMR spectrum of compound LS-1 isolated from the leaves of *Bischofia javanica*

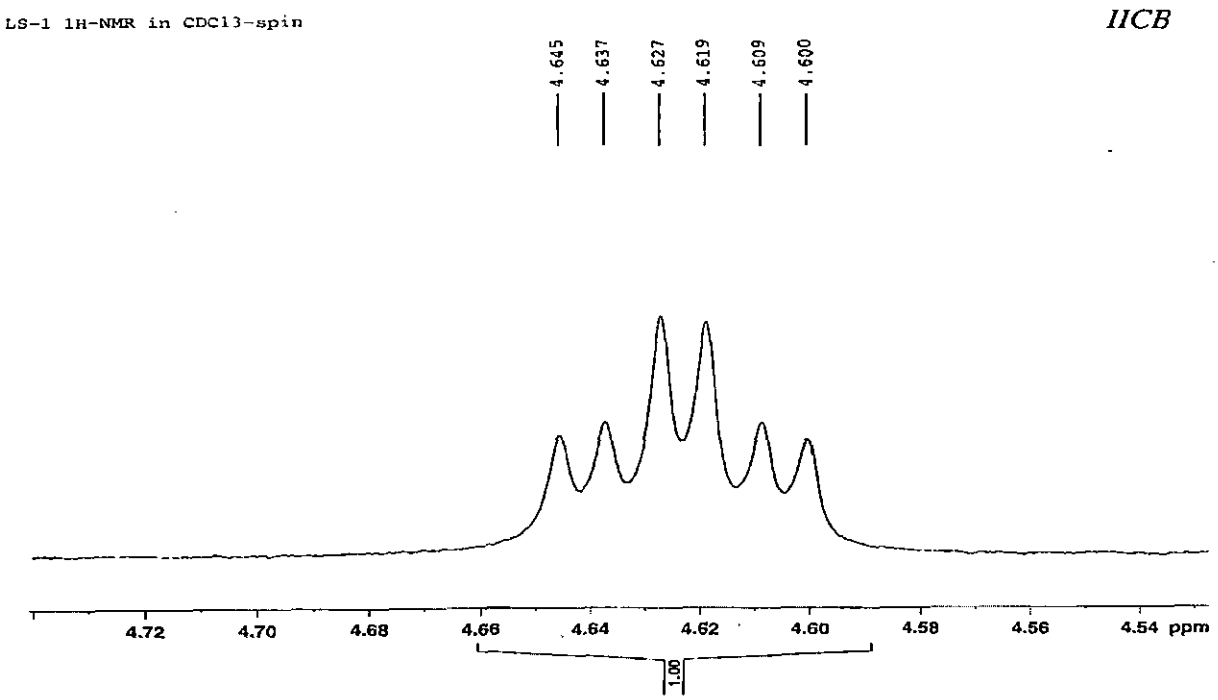


Figure 3.5 [1] H NMR spectrum of compound LS-1 isolated from the leaves of *Bischofia javanica*

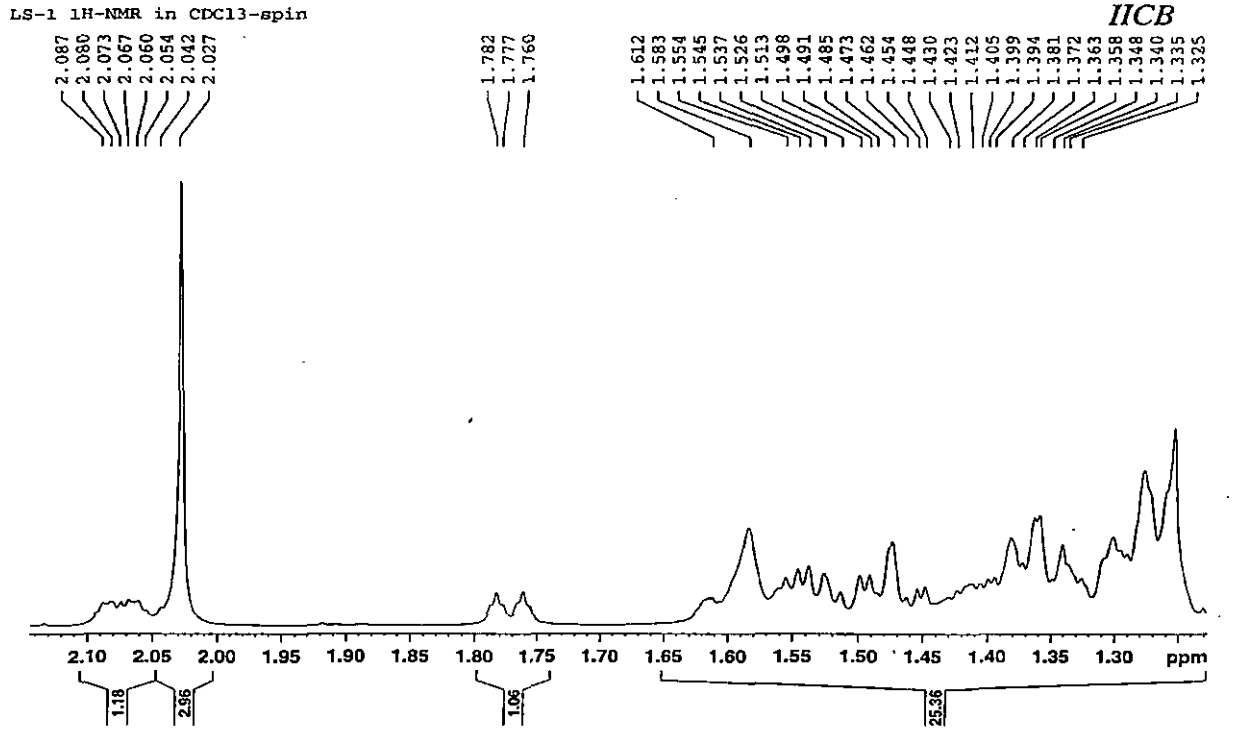


Figure 3.6 ^{1}H NMR spectrum of compound LS-1 isolated from the leaves of *Bischofia javanica*

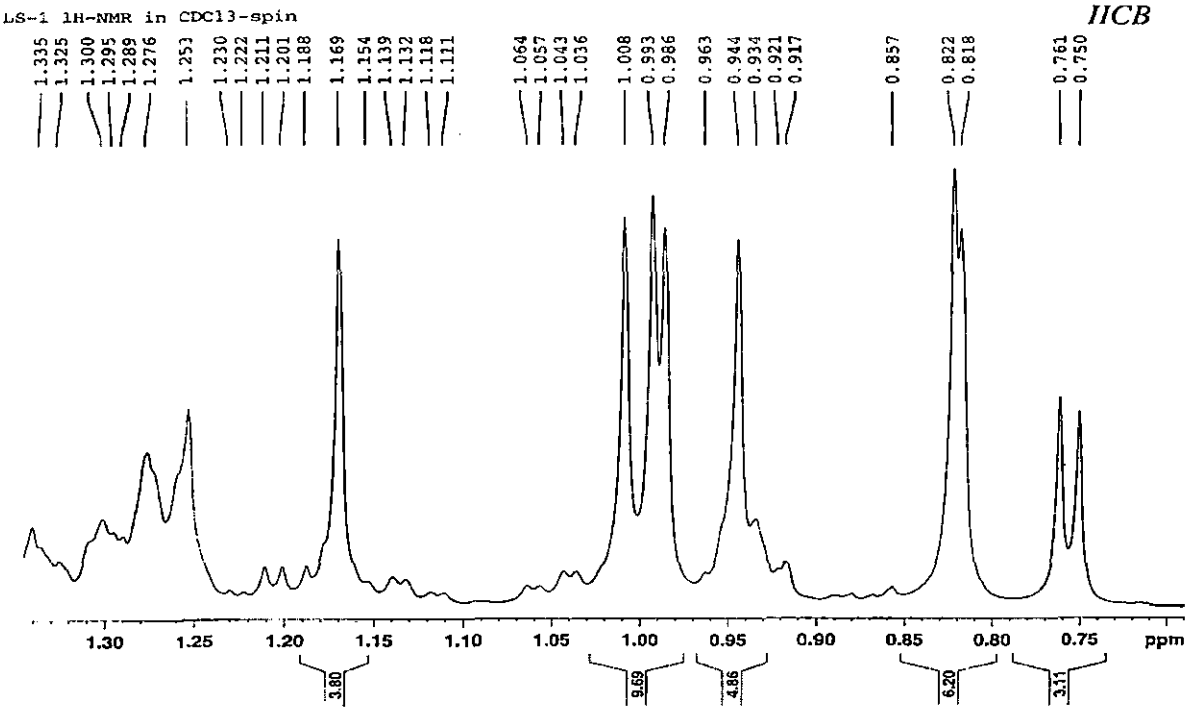


Figure 3.7 ^{1}HNMR spectrum of compound LS-1 isolated from the leaves of *Bischofia javanica*

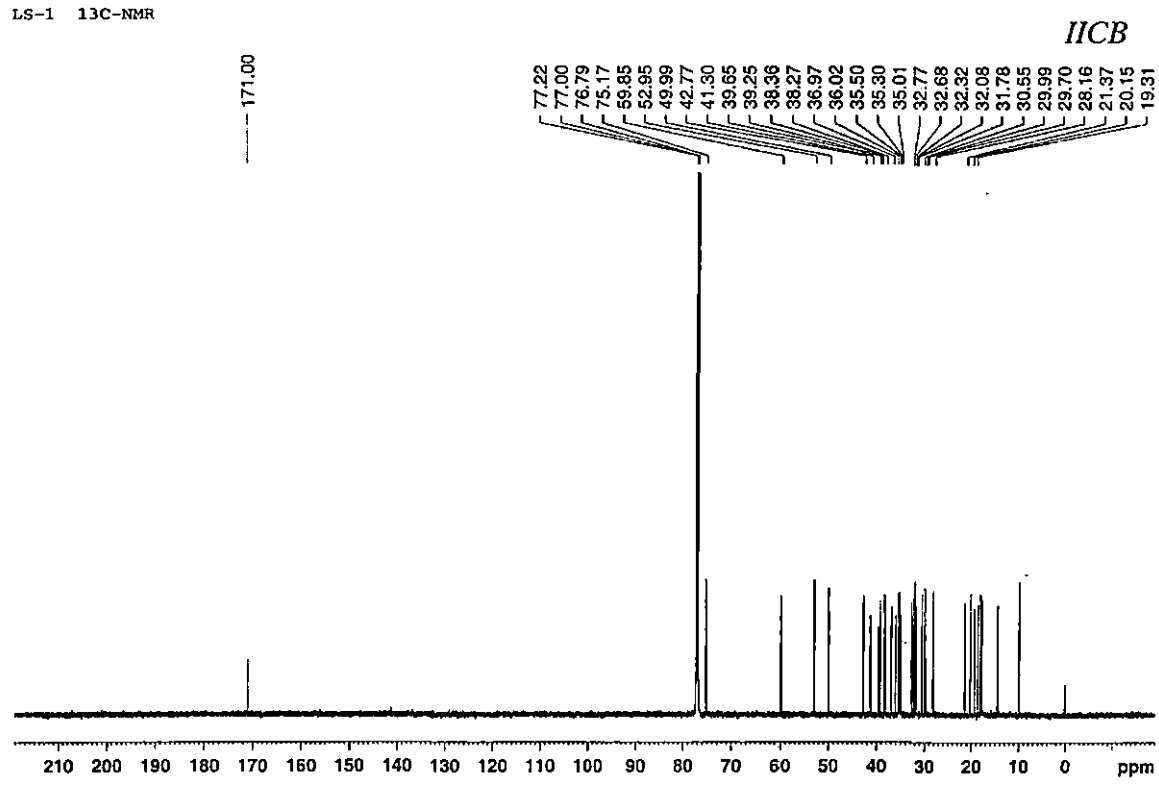


Figure 3.8 ^{13}CNMR spectrum of compound LS-1 isolated from the leaves of *Bischofia javanica*

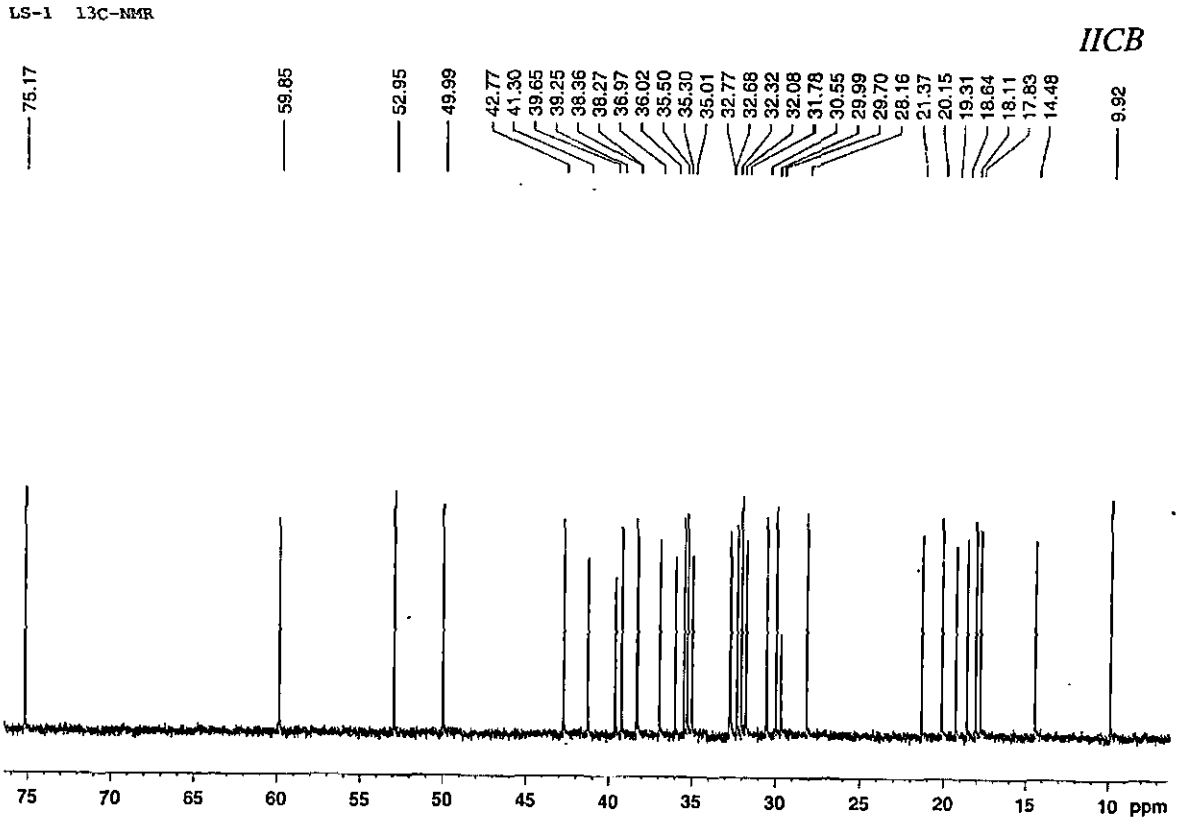


Figure 3.9 ^{13}C NMR spectrum of compound LS-1 isolated from the leaves of *Bischofia javanica*

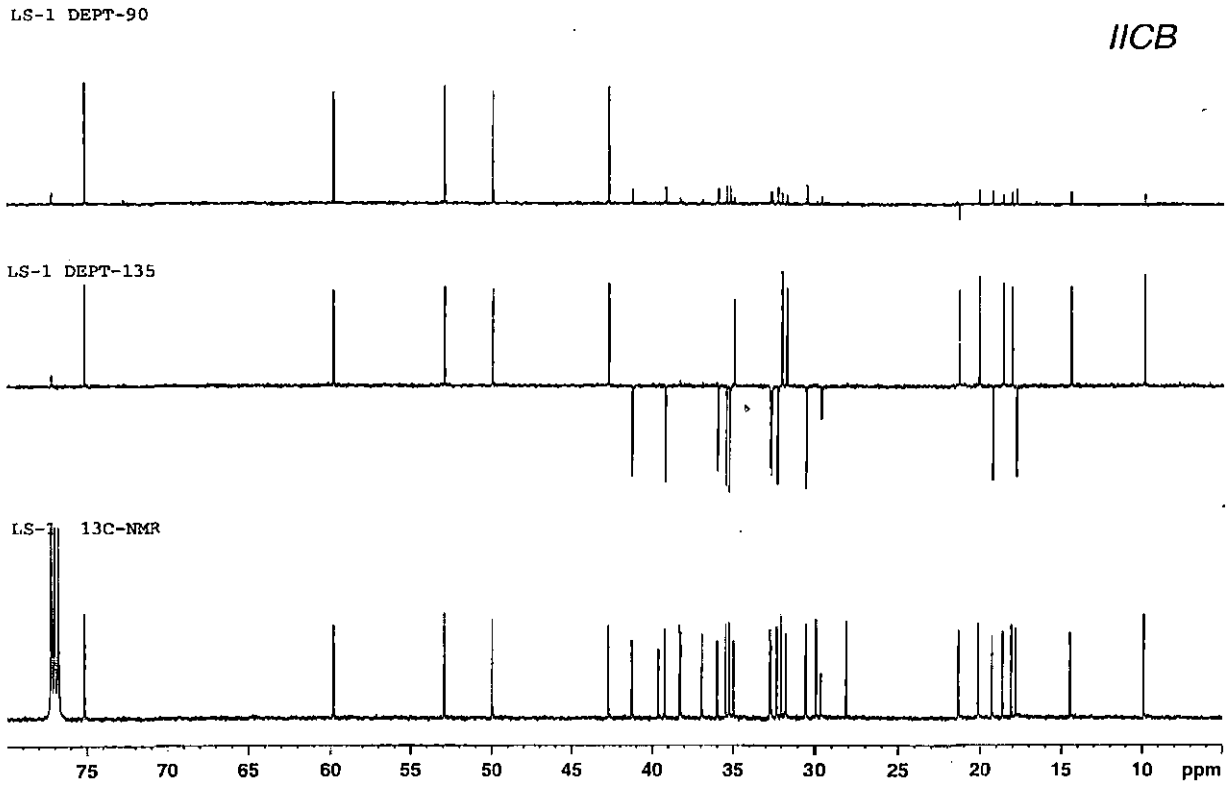


Figure 3.10 DEPT-90, DEPT-135 and ^{13}C NMR spectra of compound LS-1 isolated from the leaves of *Bischofia javanica*

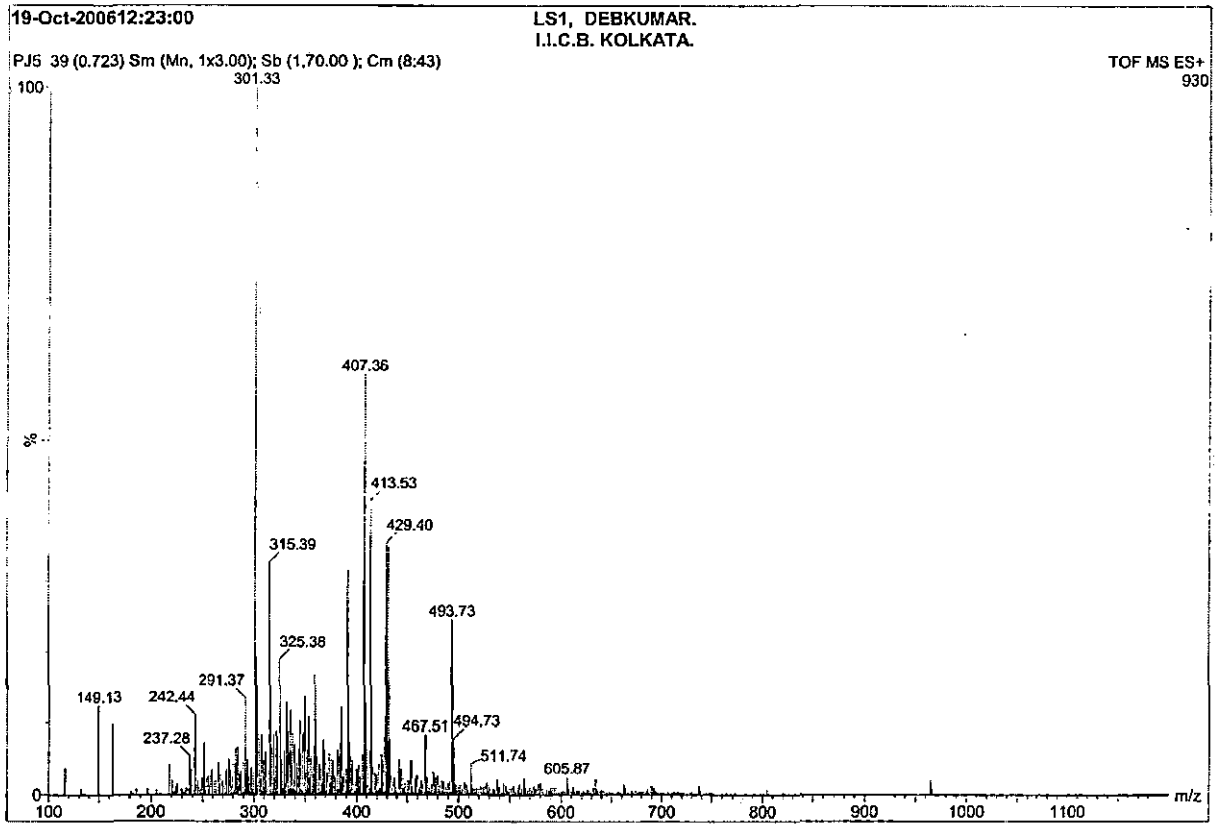


Figure 3.11 Mass spectrum of isolated compound LS-1 from the leaves of *Bischofia javanica*

Table 3.8 Hydrogen and carbon chemical shifts of compound LS-1 isolated from the leaves of *Bischofia javanica*

Position	δ_H	Position	δ_C		Position	δ_H	Position	δ_C
H-1	1.59(α)	C-1	17.83		H-16	1.46(α)	C-18	42.77
	1.36(β)	C-2	32.68			1.54(β)	C-19	35.55
H-2	1.58(α)	C-3	75.17		H-17	1.47	C-20	28.16
	1.38(β)	C-4	49.99		H-18	1.51	C-21	32.77
H-3	4.63	C-5	38.27		H-19	1.78(α)	C-22	39.25
H-4	1.55	C-6	41.3			1.29(β)	C-23	9.92
H-6	1.25(α)	C-7	19.31		H-21	1.76(α)	C-24	14.48
	1.48(β)	C-8	52.95			1.28(β)	C-25	18.11
H-7	1.35(α)	C-9	36.97		H-22	1.58(α)	C-26	18.21
	1.49(β)	C-10	59.85			1.25(β)	C-27	20.15
H-9	1.51	C-11	35.3		CH_3-23	0.98	C-28	31.78
H-10	1.53	C-12	30.55		CH_3-24	0.76	C-29	35.01
H-11	1.34(α)	C-13	39.65		CH_3-25	0.82	C-30	32.08
	1.42(β)	C-14	38.36		CH_3-26	0.99	C-31	171.0
H-12	1.37(α)	C-15	32.32		CH_3-27	1.04	C-32	21.37
	1.27(β)	C-16	36.02		CH_3-28	1.13		
H-15	1.50	C-17	39.99		CH_3-29	1.18		
					CH_3-30	1.16		
					$COOCH_3$	2.03		

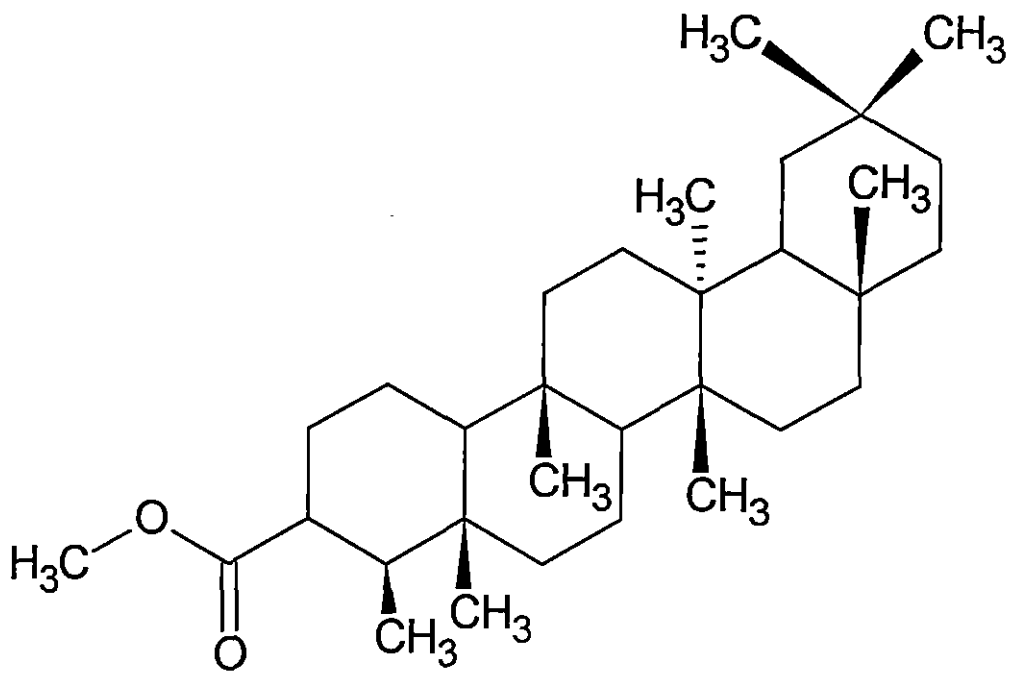


Figure 3.12 Structure of Friedelin-3α-acetate (Compound LS-1)

3.6 Extraction and Isolation of Phytoconstituents from *Fraxinus Floribunda*:

Fraxinus floribunda Wallich is a tree, grown in eastern Himalayas and Khasi hills. Leaves are pinnate, leaflets 7-9, opposite, stalled, ovate-oblong. Manna is obtained by incision from the stem of the tree and it is used as laxative. [27] The barks and leaves of the plant have been traditionally used for the treatment of bone fracture and dislocation. [28] The leaves are employed as diuretic and for the treatment of gout. [29] Some coumarins have been isolated from the leaves. [30]

3.7 Extraction of Phytoconstituents from the Leaves of *Fraxinus floribunda*:

3.7.1 Collection of Plant Material:

The leaves of *Fraxinus floribunda* were collected from Pakyong region of Sikkim, India in the month of September.The collected plant material was identified and authenticated at Botanical Survey of India (BSI) Gangtok. A herbarium numbered as (LS/FF /O4/RPS) was also kept at the parent institute for future reference.

3.7.2 Extraction Procedure:

The collected leaves of *F. floribunda* (6kg) was shade dried for 15 days and reduced to coarse powder using laboratory grinder. It was stored in a well-closed container to protect from light and moisture till used. The powdered leaves (2.5 kg) were extracted with methanol in soxhlet apparatus. After exhaustive extraction, the extract was concentrated *in vaccuo* and freezes dried to yield a solid extract (75g) which was approximately 3.0 %w/w of original powdered plant material. The procedure indicated in the **(Figure 3.2)** was exactly followed for the extraction. The methanol extract was suspended in distilled water and partitioned with petroleum ether and chloroform [14-15]. The chloroform soluble fraction was subjected to thin layer chromatographic analysis. The aqueous and petroleum ether fraction did not show any positive pharmacological activities under preview of this investigation and was discarded.

3.8 Isolation and Purification of Phytoconstituents from the Leaf of *F. floribunda*:

3.8.1 Preliminary Phytochemical Test:

The preliminary phytochemical group test of *F. floribunda* extract was performed by the standard methods. [16-17]

Test for Flavanoids:

- 5ml of extract solution was hydrolyzed with 10%v/v sulphuric acid and cooled. Then it was extracted with diethyl ether and divided into three portions in three separate test tubes.1ml of dilute ammonia, 1ml of dilute sodium bicarbonate and 1ml of 0.1N sodium hydroxide were added to the first, second and third test tubes respectively. In each test tube, development of yellow colour indicated the presence of flavanoids.
- The extract was dissolved in alcohol. One piece of magnesium followed by concentrated hydrochloric acid was added to the alcoholic solution drop wise and heated. Appearance of magenta color demonstrated the presence of flavanoids.

Test for Steroids and Triterpenoids:

Liebermann-Burchard Test:

- 10mg of extract was dissolved in 1ml of choloroform.1ml of acetic anhydride was added following by addition of 2ml of concentrated sulphuric acid. Formation of blue-green colour indicated the presence of steroids and triterpenoids.

Salkowski Test:

- 1ml of concentrated sulphuric acid was added to 10 mg of extract dissolved in chloroform. A reddish-blue colour produced in the chloroform layer and green fluorescence in the acid layer indicated the presence of steroids and triterpenoids

Test for Alkaloids:

- To 1ml of solution of the extract in a test tube, 0.2ml of dilute hydrochloric acid and 0.1ml of Mayer's reagent were added. Formation of yellowish-buff precipitate confirmed positive test for alkaloids

- 1ml of dilute hydrochloric acid and 0.1ml of Dragendroff's reagent were added to 2ml solution of the extract in a test tube. Development of orange-brown colour precipitate revealed the presence of alkaloid.
- 2ml of the extract solution was treated with 1 ml of dilute hydrochloric acid and 0.1ml of Hager's reagent. A yellowish precipitate confirmed the presence of alkaloid.

Test for Reducing Sugar:

- To 5ml of the extract solution, 5ml of Fehling's solution was added and boiled for 5 minutes. Formation of brick red coloured precipitate demonstrated the positive test for reducing sugars
- To 5ml of the extract solution, 5ml of Benedict's solution was added in a test tube and boiled for few minutes. Development of brick-red colour precipitate confirmed the presence of reducing sugars

Test for Tannins:

- 5ml of the extract solution was allowed to react with 1ml of 5% ferric chloride solution. Greenish-black coloration indicated the presence of tannins.
- 5ml of the extract solution was treated with 1ml of aqueous potassium dichromate solution. Formation of yellowish-brown precipitate suggested the presence of tannins.
- 5ml of the extract solution was treated with 1ml of aqueous 10% lead acetate solution. Yellow coloured precipitation gave the test for tannins.

Test for Saponins:

- 1ml solution of the extract was diluted with distilled water to 20 ml and shaken in a graduated cylinder for 15 min. No development of stable foam suggested the absence of saponins
- 1ml of the extract solution was treated with 1.0% lead acetate solution. Absence of formation of white precipitate indicated the absence of saponins

Test for Anthraquinones:

- 5ml of the extract solution was hydrolyzed with dilute sulphuric acid and then extracted with benzene.1ml of dilute ammonia was added to the extract. No colour change was observed indicating the absence of anthraquinones.

Test for Amino acids and Proteins:

- Small quantity of leaf extract of *F. floribunda* was dissolved in a few ml of distilled water and treated with ninhydrin reagent at pH 5.0. The absence of purple coloration suggested the negative test for amino acids.
- Small quantity of leaf extract of *F. floribunda* was treated with few drops of 10% sodium hydroxide solution. Few drops of 1% copper sulphate solution was added and mixed. Formation of violet or purple colour demonstrated the presence of proteins.
- Small quantity of leaf extract of *F. floribunda* was treated with 1.0 ml of concentrated nitric acid. The sample was boiled and cooled. Few drop of 40% sodium hydroxide was added drop by drop. Appearance of orange coloured solution indicated the presence of proteins.
- A small quantity of leaf extract of *F. floribunda* was treated with few drops of Millon's reagent. The samples were boiled and cooled. Few drop of 40% sodium nitrate solution was added to the sample drop by drop. Appearance of red precipitate confirmed the presence of proteins.

3.8.2 Thin Layer Chromatographic Study of the Chloroform Fraction of Methanol Leaf Extract of the *F. floribunda*:

Thin layer chromatographic study of the chloroform fraction was carried out on silica gel G (Merck) plates with different solvent systems. Glass plates of 15cm × 5 cm were coated with slurry of silica gel prepared by mixing 6 gm of silica gel G with 12 ml of distilled water. The layer was allowed to set for 30 min at room temperature and was then activated at 110°C for 30 min.

The sample of methanol extract was spotted on the plates and chromatogram was developed in chromatographic chambers using selected solvent systems at room temperature (28°C) and at an angle of 70°. Since the rate of migration of a compound on a given adsorbent depends upon the solvent used, the solvent system can be arranged in order of elutive power. Mixtures of two or three solvents of different polarities give better separation than the chemically homogeneous solvents. In the present investigation, several solvent systems were studied for effective separation of the components, but the under mentioned solvent systems showed better result. The mobile phase (solvent system) was allowed to run up to a distance of 10 cm from the origin. The plates were removed from the chamber after completion of the run and were allowed to dry in air. The plates were observed under UV light for the appearance of spots before and after spraying with spray reagents.

The chromatograms were run with three solvent systems mentioned below in a chromatography chamber. [18]

3.8.2.1 Solvent Systems Used:

TLC 1. n-hexane: Ethyl acetate (6:4).

TLC 2. n-hexane: Ethyl acetate (5:5)

TLC 3. n-hexane: Chloroform: Ethyl acetate (8:1:1)

3.8.2.2 Spray Reagents Used:

Liebermann Burchard (LB) reagent

3.8.3 Coloumn Chromatography of Leaf Extract of *F. floribunda*:

3.8.3.1 Coloumn:

A glass coloumn, 80 cm in length, 3.5 cm diameter. The bottom of the coloumn was plugged with glass wool.

3.8.3.2 Adsorbent:

Silica gel 60-120 (E.Merck).

3.8.3.3 Solvents Used:

n-hexane: Ethyl acetate (with increasing concentration of ethyl acetate).

n-hexane: chloroform: ethylacetate (8:1:1)

3.8.3.4 Preparation of Coloumn:

The coloumn was washed with distilled water and finally rinsed with acetone to remove the impurities. It was fixed with a stand and packed with slurry of Silica gel and n-hexane. The solvent n-hexane was adjusted to drip at the rate of 100 drops per minute and a level of 5 cm of the solvent was maintained on the top of the silica gel layer.

3.8.4 Isolation of Compound from Chloroform Fraction of *F. floribunda* Leaf:

The dried solid chloroform fraction of the leaf extract and silica gel was layered on the top of the coloumn. A thin layer of cotton was placed over it. The solvent was allowed to flow down slowly till the mixture was adsorbed on the top of the coloumn. Gradient elution was carried out using n-hexane and ethyl acetate as solvent by increasing the polarity of n-hexane by adding increments of ethyl acetate [19-20]. The fractions obtained from the coloumn chromatography using different ratios of n-hexane: ethyl acetate were numbered as F-1 to F-5 for the ratio 10:0, F-6 to F-14 for the ratio 9:1, F-15 to F-22 for 8:2, F-23 to F-32 for 7:3, F-33 to F-43 for 6:4, F-44 to 50 for 5:5 and F-51 to57 for 4:6. TLC study was performed on the fractions and the fraction F-44 to 50 revealed the elution of the mixture of triterpenoid amongst all the fractions. So the fractions were combined together and was further purified in coloumn chromatography using n-hexane-chloroform-ethyl acetate (8:1:1) solvent system. The sub fraction (SF-4-8) of F-44 to F-50 in the eluting solvent system of n-hexane-chloroform-ethyl acetate in the ratio of 8:1:1 gave the pure compound LS-2, which was confirmed by TLC studies. The isolated compound of LS-2 was further examined by different physico-chemical techniques for its structure elucidation.

3.8.5 Qualitative Study of the Compound Isolated from Chloroform Fraction of *F. floribunda* Leaf:

The isolated compound LS-2 was further subjected to chemical tests for confirmation of its chemical nature.

3.8.6 Physical Nature of the Compound Isolated from Chloroform Fraction of *F. floribunda* Leaf:

The isolated compound LS-2 was subjected to various physicochemical parameters viz: physical appearance, solubility and melting point.

3.8.7 Thin Layer Chromatographic Study of the Compound Isolated From Chloroform Fraction *F. floribunda* Leaf:

Thin layer chromatographic study of the isolated compound was carried out on silica gel G plates with different solvent systems. The plates were prepared as described in 3.8.2 and used for the study. The chromatograms were run with three solvent systems mentioned below in a chromatography chamber.

3.8.7.1 Solvent Systems Used:

TLC 1. n-hexane: Ethyl acetate (6:4).

TLC 2. n-hexane: Ethyl acetate (5:5)

TLC 3. n-hexane: Chloroform: Ethyl acetate (8:1:1)

3.8.7.2 Spray Reagents Used:

Lieberman-Burchard (LB) reagent

The plates were observed under UV light for the appearance of spots before and after spraying with spray reagents.

3.8.8 Infrared Spectrum of the Compound Isolated from Chloroform Fraction of *F. floribunda* Leaf:

The infrared (IR) absorption spectra of the isolated compound were taken with Perkin Elmer FTIR-1750 spectrophotometer, in potassium bromide discs. The spectra were

recorded in the region of 4000 cm^{-1} to 400 cm^{-1}. The spectra of the isolated compound with absorption bands were recorded and tabulated.

3.8.9 Nuclear Magnetic Resonance (NMR) Spectra of the Compound Isolated from Chloroform Fraction of *F. floribunda* Leaf:

The ^{1}H and ^{13}C NMR spectra of the isolated compound from chloroform fraction of *F. floribunda* leaves were undertaken in Brucker DRX 600 Spectrometer in $CDCl_3$ (Duterated chloroform) solution. The spectra are recorded and tabulated.

3.8.10 Mass Spectra of the Compound Isolated from Chloroform Fraction of *F. floribunda* Leaf:

Mass spectrum (ES-MS) of the isolated compound from the leaves of *F. floribunda* was recorded on a Micromass Quattro II instrument.

3.9 Results:

3.9.1 Phytochemical Study of *F. floribunda* Leaf:

The concentrated methanol extract obtained from the shade-dried leaf of *F. floribunda* was fractionated successively with petroleum ether, chloroform and water. It was observed that only the chloroform fraction exhibited significant pharmacological activities under perview of this investigation. So this fraction was subjected to phytochemical analysis.

The preliminary phytochemical group tests indicated the presence of steroids, flavonoids, proteins, tannins, and reducing sugar **(Table 3.9)**. The thin layer chromatographic study of the chloroform fraction of methanol extract of *F. floribunda* showed the presence of about four components with selected mobile phases and spraying reagent **(Table 3.10)**. This fraction was subjected to coloumn chromatographic separation on Silica gel coloumn in which fifty seven fractions were collected. The fractions having identical Rf values (F- 44 - 50) from TLC studies were mixed together **(Table 3.11)**. They were again purified with a silica gel (60-120) coloumn, which yielded a pure compound (LS-2)

which was again confirmed by TLC studies **(Table 3.12).** On evaporation of the solvent amorphous powder was obtained whose melting point is 179-181°C. The powder is insoluble in water and soluble in organic solvents.

It was further subjected to chemical tests and TLC studies to confirm the chemical nature of the compound LS-2, which are presented in **Table 3.13 & Table 3.14.** The chemical nature of the isolated compound was further characterized from its physical parameters and spectral (IR, Mass, ^{13}C and ^{1}H NMR) data. [21-22]

The IR spectrum of the compound is presented in **Figure 3.13.** The IR spectrum shows the presence of absorption bands at 3424, 2936, 2872,1465,1379,1054 and 524 cm^{-1}. The IR spectrum of the compound LS-2 revealed the presence of hydroxyl group (1465 cm^{-1}), -CH=CH- (Alkene) (1054cm^{-1}) and C-H stretching (3424 cm^{-1}). The ^{1}H NMR spectrum of compound LS-2 is presented in **(Figure 3.14-3.15).** The ^{1}H NMR spectrum revealed the presence of eight CH_3 proton and these signals are at 0.80, 0.82, 0.91, 1.18, 1.01, 0.97, 0.85 and 0.87ppm. H-3 gave signal at 5.36 ppm, it inferred the OH group attached produced steric hinderance at C-3 carbon. The proton in alkene bond at H-12 provided the chemical shift at 2.27ppm. Pentacyclic skeleton of the compound was confirmed by the CH_2 protons in the cyclohexane nucleus showing the chemical shift in between 1.2-1.5 ppm depends on steric hinderance.

The ^{13}C NMR spectrum of compound LS-2 showed signals at 72.2 ppm for C-3 in which the OH group is attached. The alkene bridge between C-12 and C-13 showed the signals at 122 and 141ppm **(Table 3.15).**^{13}C NMR spectra confirmed the hydroxyl group and alkene bridge of the compound LS-2 **(Figure 3.16).** The CH_3 group of the compound provided the chemical shift between 19-42 ppm. The signals for C-30 carbons confirmed the total molecular structure belongs the pentacyclic triterpenoids [31-32]. DEPT-90 and DEPT-135 studies have provided the supportive evidence for the Singlet and Doublet in the carbon skeleton of the compound LS-2 **(Figure 3.17 and 3.18).** It gave the clear indication of the C-3 (δ_C 72.21) CH (s),C-12(δ_C122.11) =CH(s) and

methylene doublet between the range of δ_C between 19-42 ppm. Mass spectra of the compound revealed the molecular weight of the compound LS-2 as 427.23 and m/z relative intensity for the fragmented ions are $\{M^+Na\}$ 450.27 (10),397.4(5) **(Figure 3.19).** The spectral data of the compound LS-2 was similar to the known pentacyclic triterpenoid compound β-amyrin.[31-34] Therefore it was concluded that the compound LS-2 is **β-amyrin** with the molecular formula $C_{30}H_{51}O$,whose structure is presented in the **Figure 3.20**.

Table 3.9 Preliminary phytochemical test of methanol extract of *F. floribunda* leaf

Phytoconstituents	***B.javanica***	
	Methanol Extract	**Chloroform Fraction**
Alkaloids	+	+
Aminoacids	–	–
Proteins	+	–
Reducing sugar	+	–
Steroids and triterpenoids	+	+
Flavanoids	+	+
Tannins	+	–
Saponins	–	–
Anthraquinones	–	–

+ ***ve*** indicates presence and – ***ve*** indicates absence of the phytoconstituents.

Table 3.10 Thin layer chromatography study of the chloroform fraction of methanol extract of *F. floribunda*

No of Spots	Colour of spots under UV light			Colour of spots under UV light after spraying the spraying reagent			R_f values		
	TLC 1	TLC 2	TLC 3	TLC 1	TLC 2	TLC 3	TLC 1	TLC 2	TLC 3
1	Blue-Green	Blue-Green	Blue-Green	Bright Blue-Green	Bright Blue-Green	Bright Blue-Green	0.53	0.49	0.63
2	Blue-Green	Blue-Green	Blue-Green	Bright Blue-Green	Bright Blue-Green	Bright Blue-Green	0.61	0.57	0.68
3	Green	Green	Green	Green fluorescence	Green fluorescence	Green fluorescence	0.84	0.87	0.86
4	–	Green	Green	–	Green fluorescence	Green fluorescence	–	0.94	0.87

Spray reagent: Liebermann Burchard reagent

Solvent system

TLC1 ----- n-hexane: Ethyl acetate (6:4).

TLC2 ----- n-hexane: Ethyl acetate (5:5)

TLC3 ----- n-hexane: Chloroform: Ethyl acetate (8:1:1)

Table 3.11 Coloumn and thin layer chromatographic separation of chloroform fraction of *F. floribunda* leaf.

Eluent n-hexane: ethyl acetate	Fraction Number	Colour of TLC spots	Inference
10:0	1-5	NIL	Fatty oil
9:1	6-14	NIL	Fatty oil
8:2	15-22	NIL	Fatty oil
7:3	23-32	Green	Fatty oil with chlorophyll
6:4	33-43	Green to Blue green (Mixture of spots)	Greenish semisolid
5:5	44-50	Blue green (Mixture of spot)	Colourless powder
4:6	51-57	Green to Blue green (Mixture of spots)	Greenish semisolid

Spray reagent: Liebermann Burchard reagent

Table 3.12 Coloumn and thin layer chromatography of Sub-fraction of chloroform fraction of leaves of *F.floribunda*

Fraction Number	Colour of TLC spots	Inference
1-3	Blue-Green (mixture of spots)	Colourless solid (mixture of compounds)
4-8	Blue-Green (single spot)	Colourless amorphous powder (pure compound)
9-13	Blue-Green (mixture of spots)	Colourless solid (mixture of compounds)

Eluent : n-hexane: chloroform: ethyl acetate(8:1:1)

Spray reagent: Liebermann Burchard reagent

Table 3.13 Qualitative analysis of the compound LS-2 isolated from chloroform fraction of *F.floribunda* leaf.

S.No	Test	Observation	Inference
1	*Liebermann-Burchard test:* 1mg of the isolated compound LS-2 is dissolved in chloroform and 1ml of acetic anhydride was added followed by 0.5ml of concentrated sulphuric acid was added by side of the test tube	Formation of blue-green colour	Presence of triterpenoid.
2	*Salkowskis Test:* 0.5ml of concentrated sulphuric acid was added to 1mg of the isolated compound LS-2 and dissolved in chloroform.	A reddish-blue colour produced in the chloroform layer and green fluorescence in the acid layer.	Presence of triterpenoid.

Table 3.14 Thin layer chromatography of the compound LS-2 isolated from chloroform fraction of *F.floribunda* leaf.

Solvent system	R_f values	Colouration	
TLC-1	0.53	UV_{366nm}	UV_{366nm}+LB Reagent
TLC 1	64	Blue-Green	Bright Blue-Green
TLC 2	0.57	Blue-Green	Bright Blue-Green
TLC 3	0.63	Blue-Green	Bright Blue-Green

LB Reagent - Liebermann Burchard Reagent

Solvent system

TLC1------ n-hexane: Ethyl acetate (6:4).

TLC2------ n-hexane: Ethyl acetate (5:5)

TLC3------ n- hexane: Chloroform: Ethyl acetate (8:1:1)

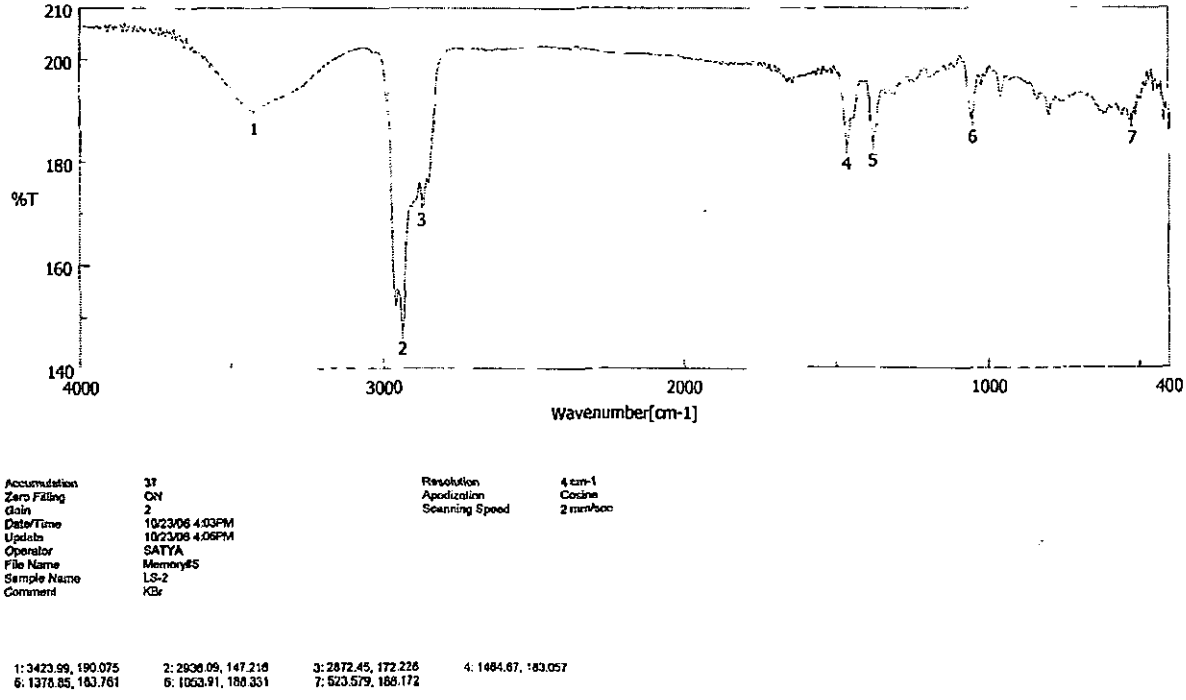


Figure 3.13 IRspectrum of compound LS-2 isolated from the leaves of *F.floribunda*

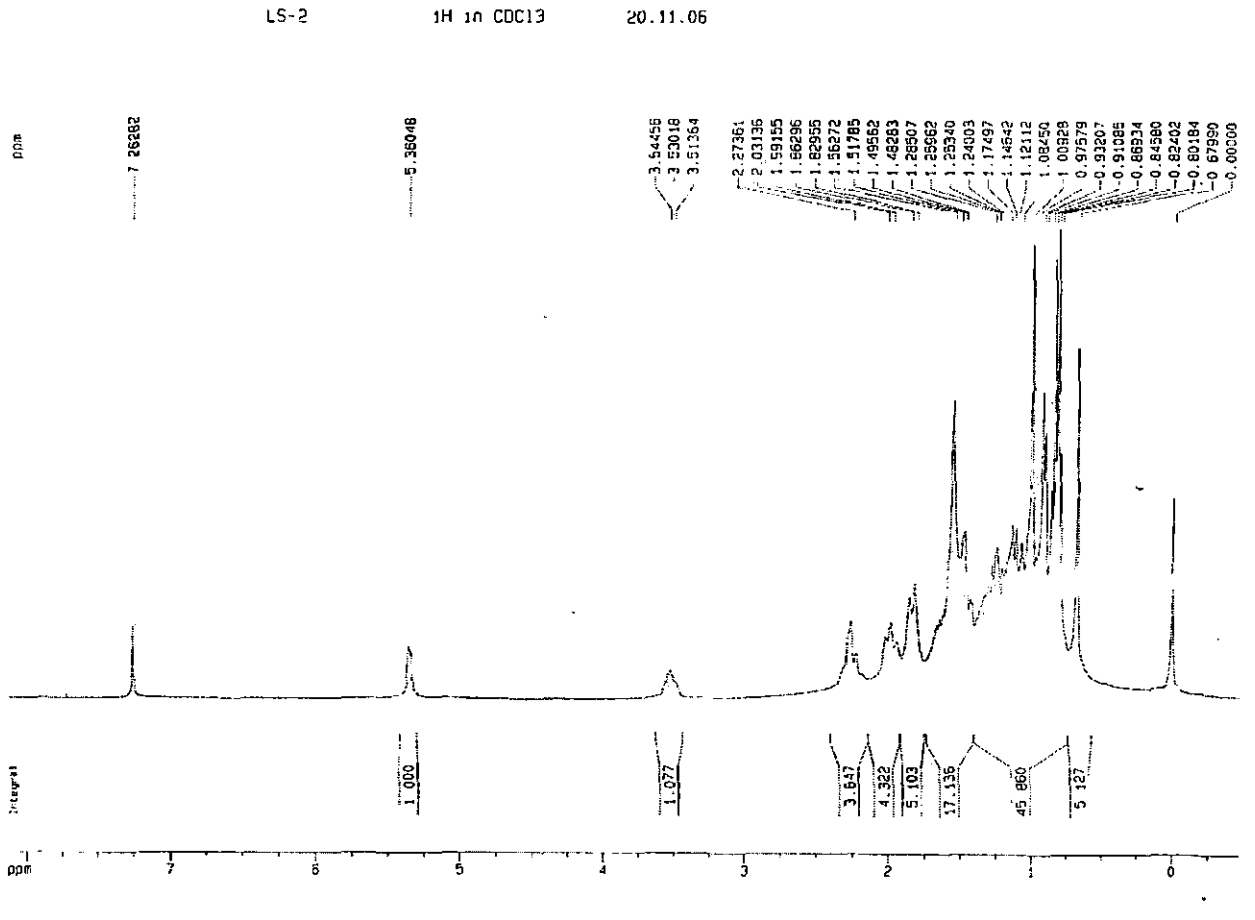


Figure 3.14 ^{1}H NMR spectrum of compound LS-2 isolated from the leaves of *F.floribunda*

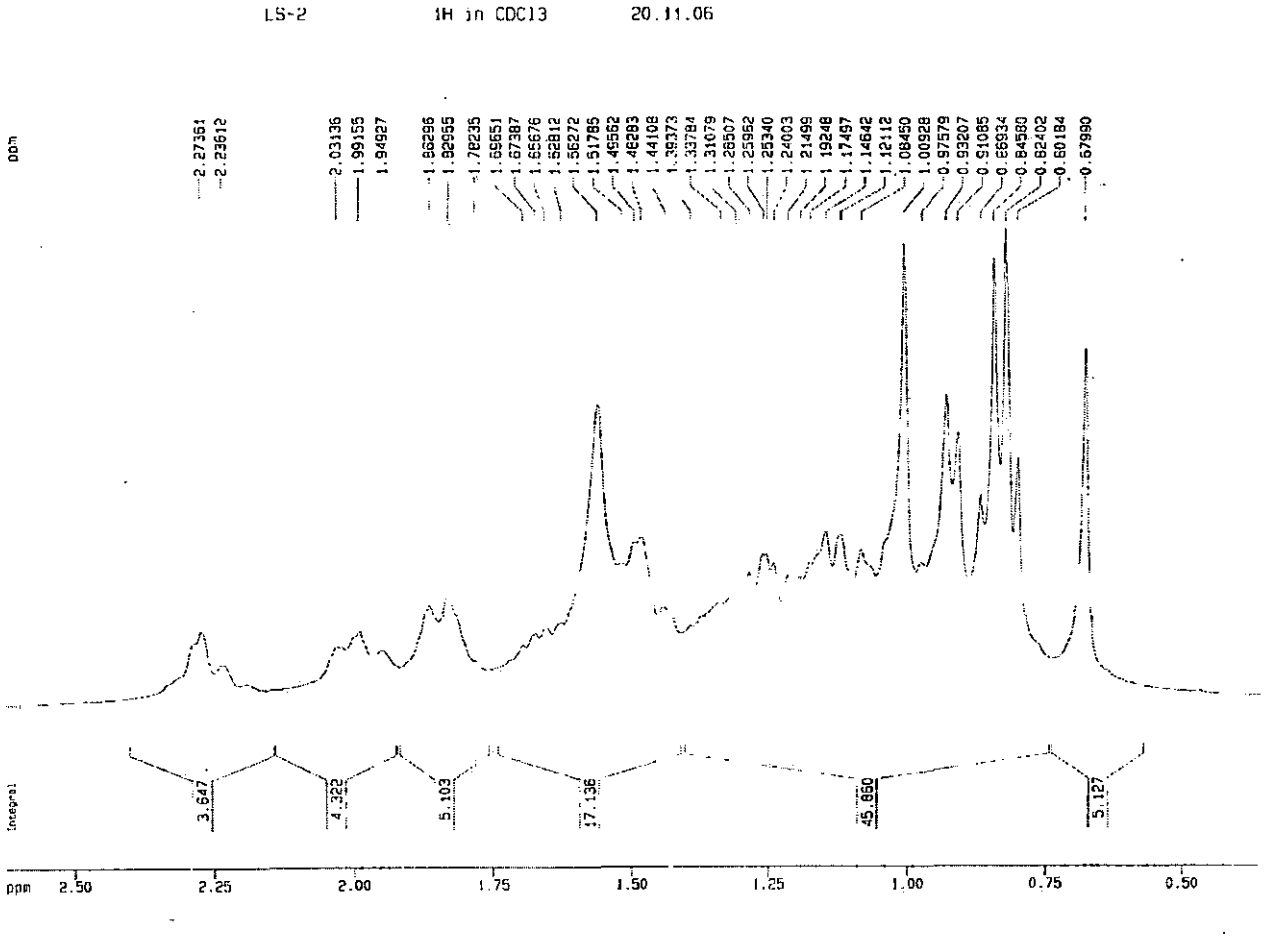


Figure 3.15 ^{1}H NMR spectrum of compound LS-2 isolated from the leaves of *F.floribunda*

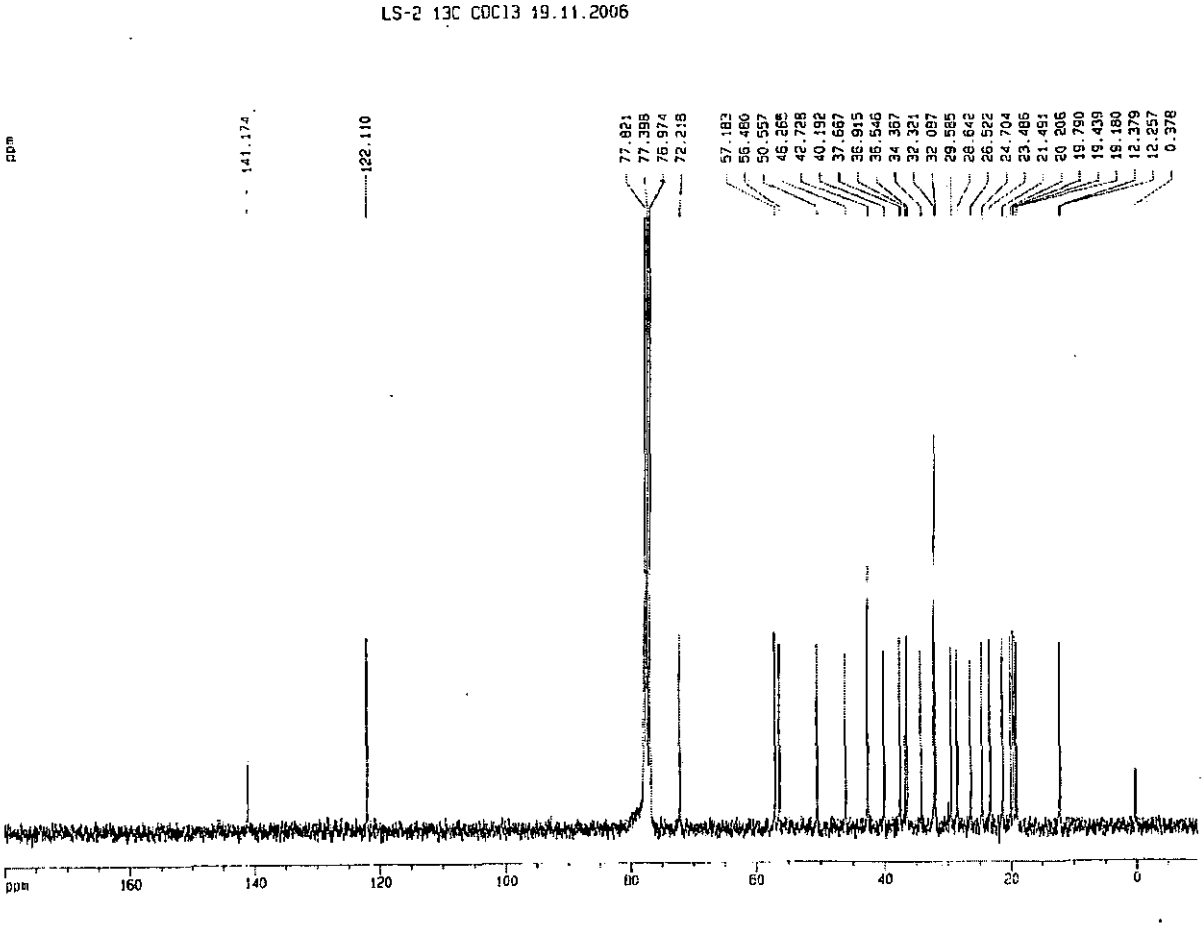


Figure 3.16 ^{13}**C NMR spectrum of compound LS-2 isolated from the leaves of *F.floribunda***

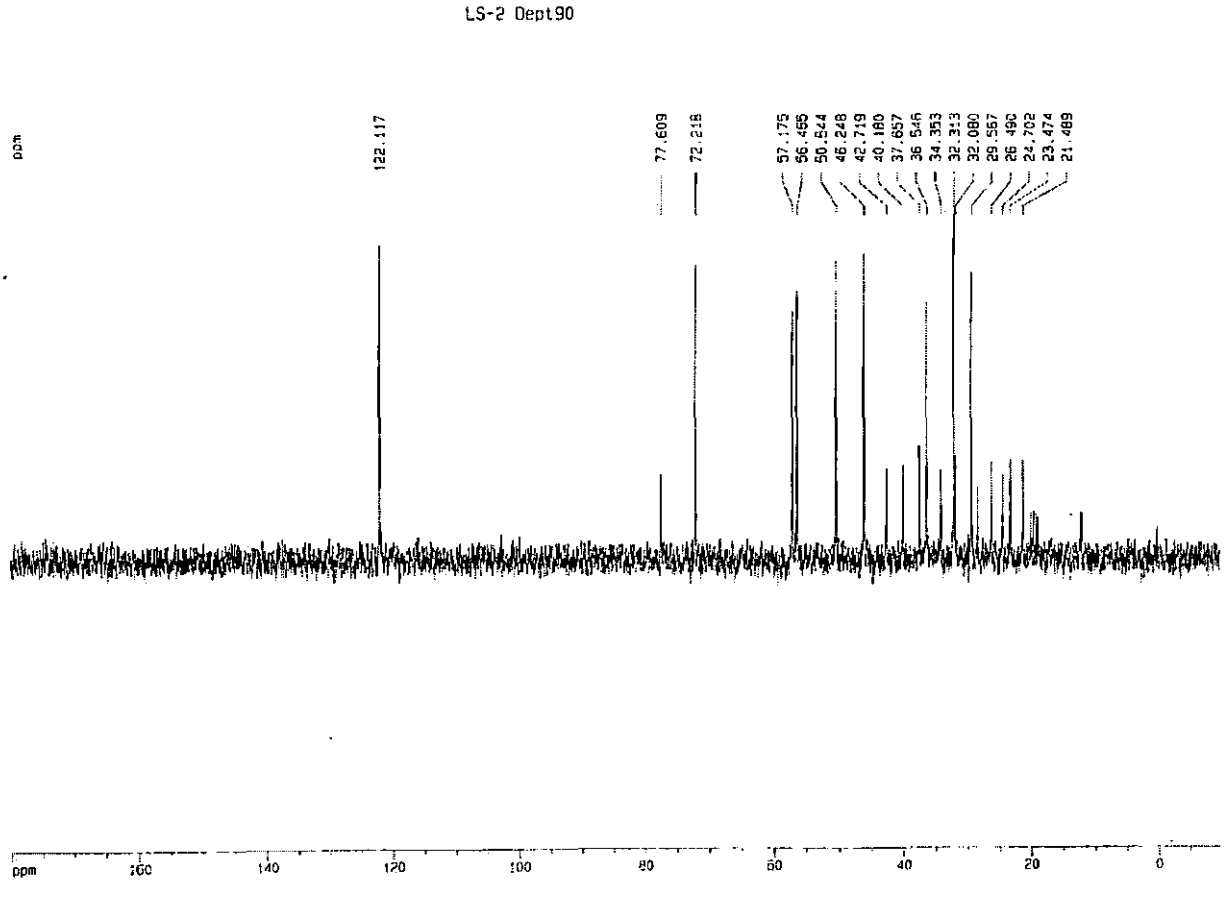


Figure 3.17 DEPT-90 spectrum of compound LS-2 isolated from the leaves of *F.floribunda*

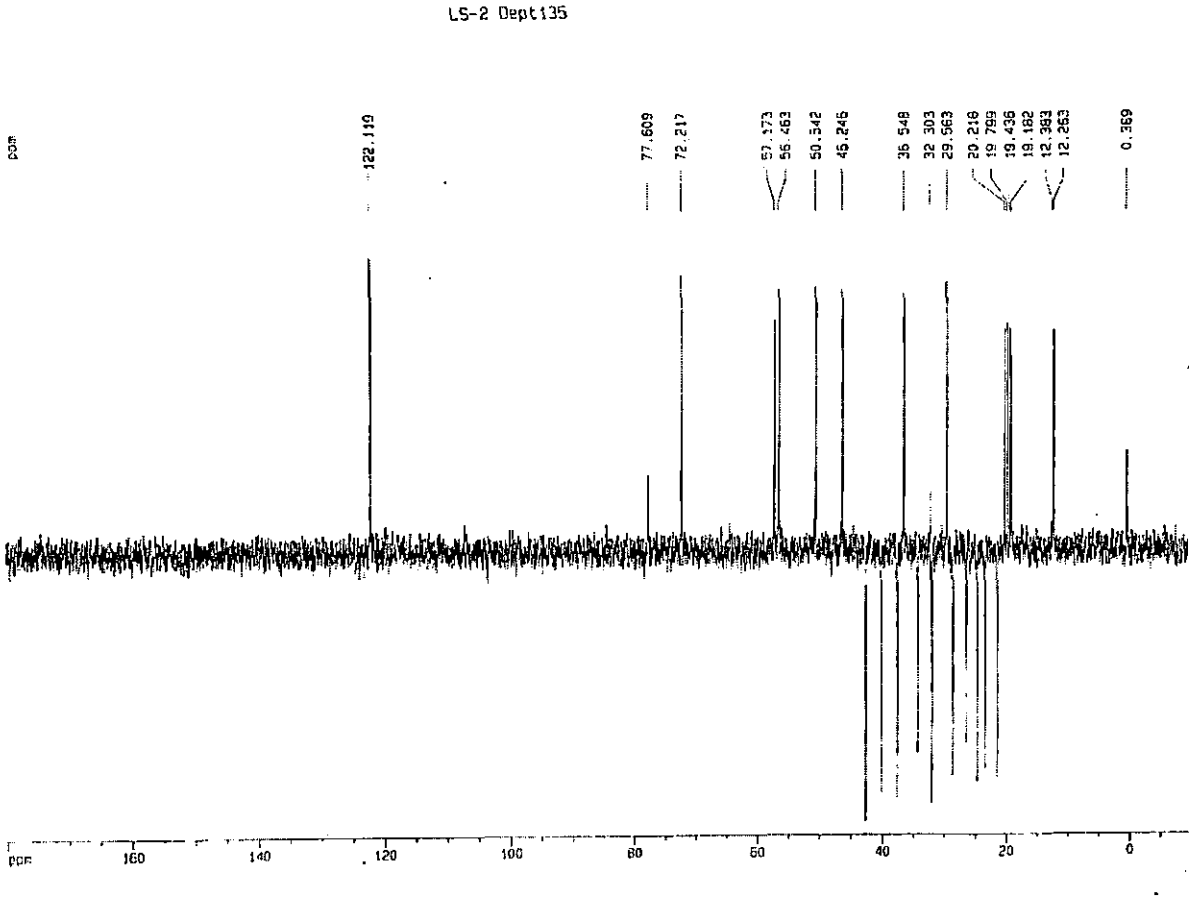


Figure 3.18 DEPT-135 spectrum of compound LS-2 isolated from the leaves of *F.floribunda*

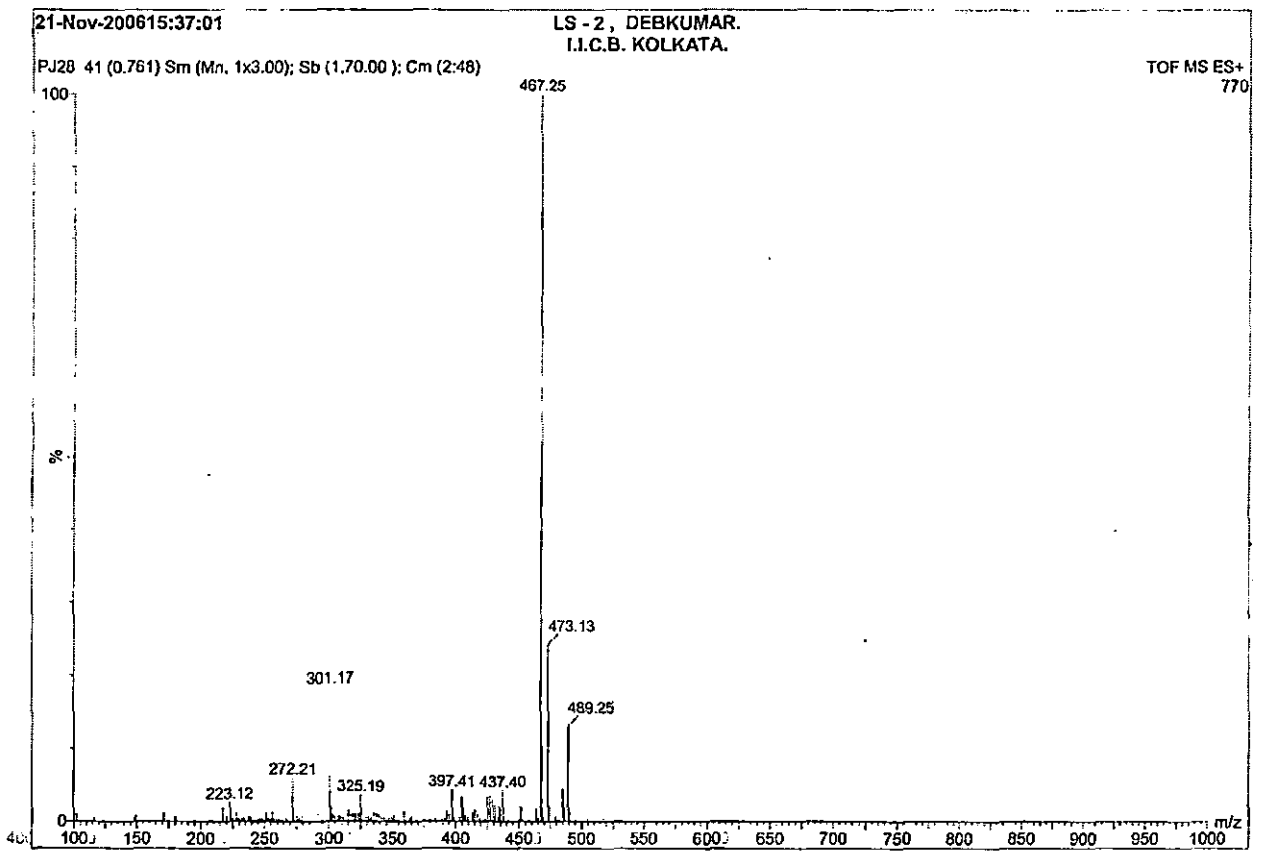


Figure 3.19 Mass spectrum of compound LS-2 isolated from the leaves of *F.floribunda*

Table 3.15 Hydrogen and carbon chemical shifts of compound LS-2 isolated from the leaves of *F.floribunda*

Position	δ_H	Position	δ_C		Position	δ_H	Position	δ_C
H-1	1.56(α)	C-1	37.67		H-18	2.23	C-19	46.73
	1.12(β)	C-2	26.52		H-19	1.44(α)	C-20	29.59
H-2	1.66(α)	C-3	72.22			1.14(β)	C-21	34.37
	1.62(β)	C-4	36.92		H-21	1.67(α)	C-22	37.66
H-3	5.36	C-5	56.48			1.39(β)	C-23	42.71
H-5	0.68	C-6	19.18		H-22	1.31(α)	C-24	19.18
H-6	1.49(α)	C-7	32.32			1.25(β)	C-25	19.43
	1.29(β)	C-8	40.19		CH3-23	0.87	C-26	19.79
H-7	1.24(α)	C-9	46.27		CH3-24	0.85	C-27	21.49
	1.17(β)	C-10	36.55		CH3-25	0.97	C-28	29.57
H-9	1.99	C-11	23.49		CH3-26	1.01	C-29	34.35
H-11	2.03(α)	C-12	122.10		CH3-27	1.18	C-30	23.47
	1.86(β)	C-13	141.17		CH3-28	0.91		
H-12	2.27	C-14	42.73		CH3-29	0.82		
H-15	1.78(α)	C-15	24.7		CH3-30	0.80		
	1.08(β)	C-16	28.64					
H-16	1.69(α)	C-17	32.09					
	0.94(β)	C-18	50.56					

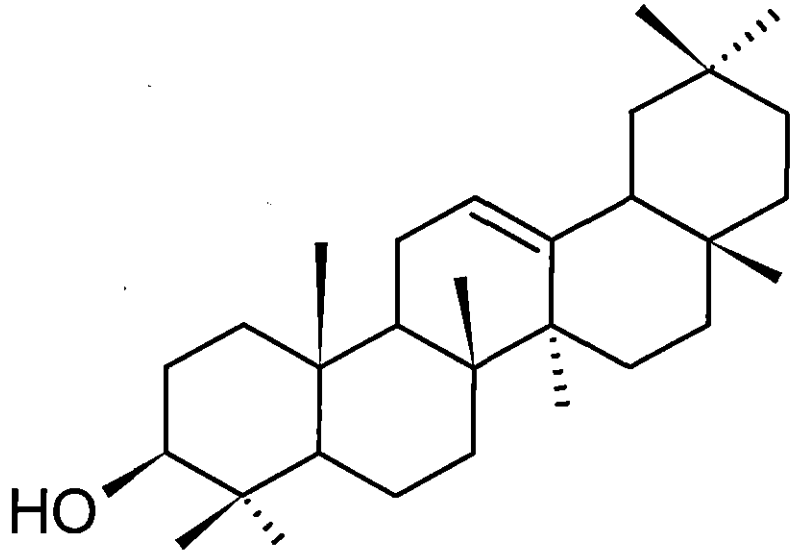


Figure 3.20 Structure of β-amyrin (Compound LS-2)

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