CHAPTER 3

PHYTOCHEMICAL STUDIES

3.0 Phytochemical study

3.1 Introduction

It is very much essential that the freshly collected plant tissues and the plant materials under investigation should be plunged into boiling alcohol within minutes after collection for their phytochemical analysis. Alternatively, plants may be dried before extraction under controlled conditions or in shade to avoid any chemical changes occurring. It should be dried as quickly as possible, at low temperature, preferably in a good air draft. Indeed, analysis for flavonoids, steroids, alkaloids, quinines and terpenoids has been successfully carried out on herbarium plant tissues dating back many years (Harborne, 1983). Sterols and triterpenes are based on the cyclopentane per hydro-phenanthrene ring system. At one time, sterols were mainly considered to be animal substances, but in recent years, an increasing number of such compounds have been detected in plant tissue. These sterols occur both as free and simple glucosides.

Contamination of the plant tissue under study with other plants is an obvious point to watch. It is essential, to employ plants which are free from diseases, i.e., which are not affected by viral, bacterial or fungal infections, because, these may seriously alter plant metabolism and unexpected products could be formed, possibly in large amounts. The classical mode of extraction naturally depends on (i) the texture and water content of the plant material being extracted and (ii) the type of substance that is being isolated. The procedure for obtaining drug substances from dried plant tissue (whole plant, root, leaf and dried seeds) is the continuous hot percolation by soxhlet apparatus or cold percolation with a range of solvents like petroleum ether, methanol or rarely with diethyl ether. Methanol, in any case, is a good-purpose solvent for preliminary extraction and by extracting the same with different solvents; same or different compounds in varying proportions may be recovered in several fractions. The extract obtained is clarified by filtration through celite by a water pump and is then concentrated *in vacuo*. If the single component is present, it can be purified by crystallization and then the material should be used available for further analysis. In most cases, mixtures of components may be present and it is necessary to separate those compounds by chromatography techniques. As a standard precaution against loss of material, concentrated extract should be stored in the refrigerator and used for further investigations.

The isolation and purification of plant constituents is mainly carried out by using one or other, or a combination of four-chromatography technique viz. paper chromatography (PC), thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and gas-liquid chromatography (GLC). The choice of technique depends largely on the nature of the substances present. It is very important to note that, there is considerable overlap in the use of above techniques and often a combination of PC and TLC, and GLC, followed by HPLC may be

the best approach for separating a particular class of plant compounds. For preparative work, TLC is carried out and for isolation on large-scale coloumn chromatography is useful. This procedure yields very good quantity of purified components.

Triterpenoids are compounds with a carbon Skelton based on six isoprene units which are derived biosynthetically from the acyclic C_{30} hydrocarbon, squalene. They have relatively complex cyclic structures, most being either alcohols, aldehydes or carboxylic acids. Triterpenoids can be divided into four groups of compounds: true triterpenes, steroids, saponins and cardiac glycosides. The later two groups are essentially triterpenes or steroids which occur mainly as glycosides. These are also known as the steroidal alkaloids. Many triterpenes are known in plants and new ones are regularly being discovered and characterized (Connolly *et al.,* 1991). But only a few are known to be widely distributed. This is also true for the pentacyclic triterpenes α- and β-amyrin and the derived acids, ursolic acid and oleanolic acids. These and related compounds occur especially in the waxy coatings of leaves and fruits such as apple and pear and they may serve a protective function in repelling insect and microbial attack. Triterpene are also found in resins and barks of trees and in latex of many plants (Harborne, 1983).

3.2. Materials and Methods

3.2.1. Plant material

The leaf of *Urtica parviflora* (*U. parviflora*), leaf of *Callicarpa arborea* (*C. arborea*) and root bark of *Morinda citrifolia* (*M. citrifolia*) were collected from the southern and eastern district of Sikkim. They were authenticated at Botanical survey of India, Gangtok, Sikkim. The voucher specimens were preserved in our laboratory for future reference. The collected plant parts were dried in shade, pulverized in mechanical grinder and passed through 40-mesh sieve to get the powder.

3.2.2. Extraction procedure

The powdered plant materials were subjected to methanol extraction (70%) in a Soxhlet extractor fitted with a waterbath. The methanol extracts were concentrated, suspended in hot distilled water, cooled and the blast precipitate was filtered off. The water soluble component was fractionated by extracting it successively with petroleum ether, chloroform and acetone. The chloroform soluble fraction was subjected separately to chromatographic analysis in case of *U. parviflora* and *M. citrifolia.* Similarly, the acetone soluble fraction was taken for chromatographic analysis in *C. arborea.* The aqueous, and petroleum ether fraction did not show any positive pharmacological activities under perview of this investigation and was discarded. Flow chart of extraction has been shown in **Fig 3.0**.

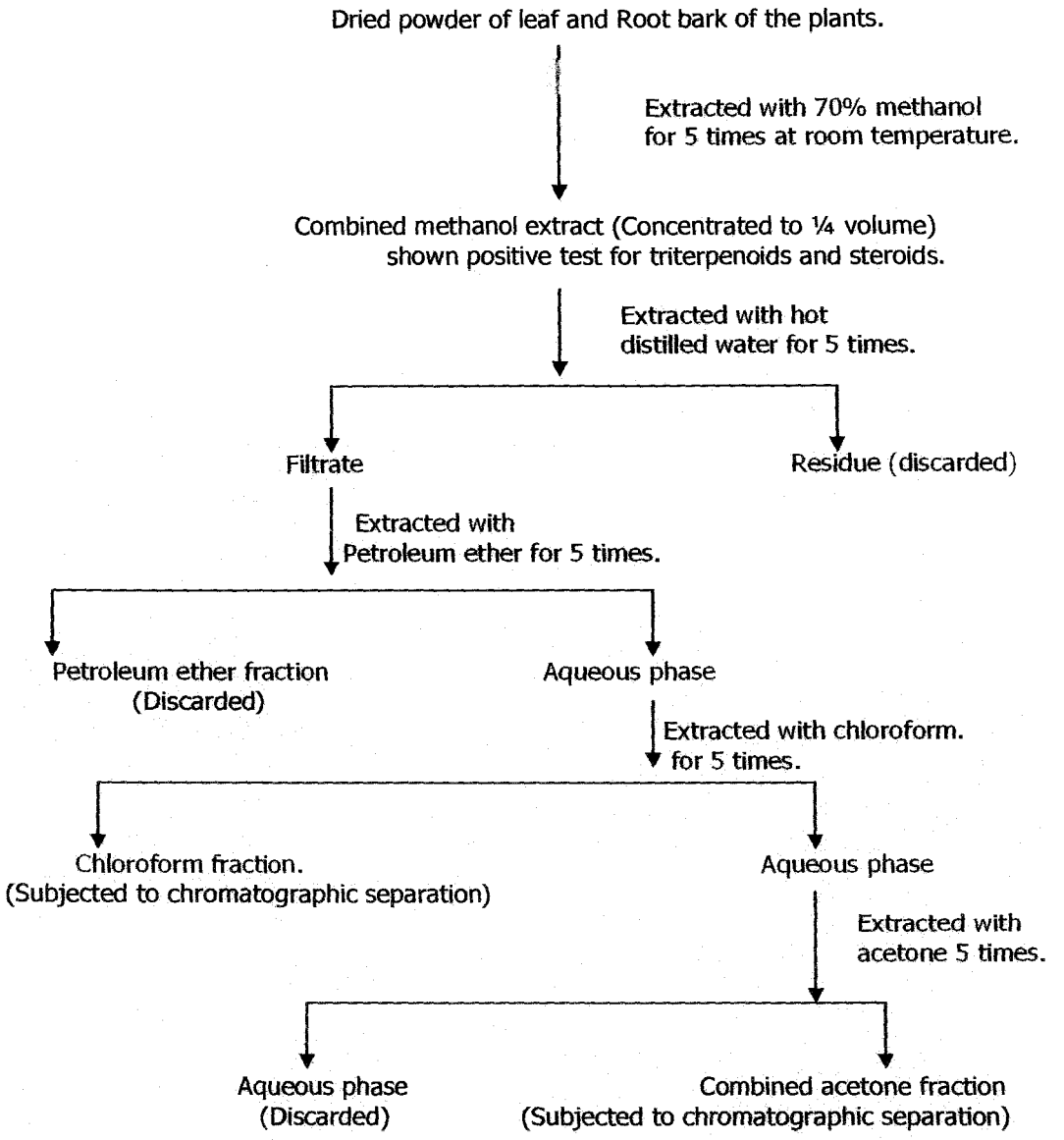

Fig 3.0 Flow chart showing the method of extraction of *Urtica parviflora* Roxb., *Callicarpa arborea* Roxb. and *Morinda citrifolia* Linn.

3.2.3. Isolation and purification of phytoconstituent from the leaf of *U. Parviflora.*

3.2.3.1. Preliminary phytochemical Test

The preliminary phytochemical group test of *leaf* extract was performed by the standard methods (Plummer, 1985; Pollock *et al.*, 1965 and Trease *et al.*, 1966).

Tests for Alkaloids

- Small quantity of the leaf extract of *U. parviflora* was treated with few drops of dilute hydrochloric acid and filtered. The filtrate was treated with Mayer's reagent. Absence of yellowish buff colored precipitate indicated the absence of alkaloids.
- A small quantity of leaf extract of *U. parviflora* was treated with few drop of dilute hydrochloric acid and filtered. The filtrate was then treated with Dragendroff"s reagent. Absence of orange brown precipitate indicated the absence of alkaloids.
- Small quantity of leaf extract of *U. parviflora* was treated with few drop of dilute hydrochloric acid and filtered. The filtrate was treated with Wagner's reagent. Failure to produce reddish brown precipitate suggested the absence of alkaloids.
- Small quantity of leaf extract of *U. parviflora* was treated with few drop of dilute hydrochloric acid and filtered. The filtrate was treated with Hager's reagent. Absence of yellowish precipitate demonstrated the negative test for alkaloids.

Test for amino acids and Proteins.

- Small quantity of leaf extract of *U. parviflora* was dissolved in a few ml of distilled water and treated with Ninhydrin reagent at pH 5.0. The presence of purple coloration suggested the positive test for amino acids.
- Small quantity of leaf extract of *U. parviflora* was treated with few drops of 10% sodium hydroxide solution. Few drops of 1% cupper sulphate solution was added and mixed. Formation of violet or purple colour demonstrated the presence of proteins.
- Small quantity of leaf extract of *U. parviflora* 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 colored solution indicated the presence of proteins.
- A small quantity of leaf extract of *U. parviflora* 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.

Test for reducing sugar

- A small quantity of leaf extract of *U. parviflora* was dissolved in minimum amount of distilled water and filtered. To the filtrate equal volume of Benedict's reagent was added and heated for few minutes. Formation of brick red precipitate confirmed the presence of reducing sugars.
- Small quantity of leaf extract of *U. parviflora* was dissolved in minimum amount of distilled water and filtered. To the filtrate equal volume of Fehling's A and B solution were added and heated for few minutes. Development of brick red colour demonstrated the presence of reducing sugars.

Test for steroids and triterponoids

- Libermann-Buchard test: 10mg of leaf extract of *U. parviflora* was dissolved in 1.0 ml of chloroform; 1.0 ml of acetic anhydride was then added to the mixture followed by 2.0 ml of concentrated sulphuric acid. A reddish violet ring at the junction of the two layers confirmed the presence of triterpenoids and steroids.
- Salkowski Test: 1.0 ml of concentrated sulphuric acid was added to 10 mg of leaf extract of *U. parviflora* and dissolved in 1.0 ml of chloroform. A reddish blue colour exhibited by chloroform layer and green fluorescence by the acid layer suggested the presence of steroids.

Test for flavonoids and their glycosides

- A small quantity of leaf extract of *U. parviflora* was dissolved in methanol and hydrolyzed with 10% sulphuric acid and cooled. Then, it was extracted with diethyl ether and divided into three portions in separate test tubes. 1.0 ml of diluted sodium carbonate solution, 1.0 ml of 0.1M sodium hydroxide and 1.0 ml of diluted ammonia solutions were added to the first, second and third test tubes respectively. Presence of yellow colour in all the tubes demonstrated the presence of flavonoids.
- A small quantity of leaf extract of *U. parviflora* was dissolved in methanol. One piece of magnesium followed by concentrated hydrochloric acid was added drop wise to the test sample and heated. Presence of magenta colour demonstrated the presence of flavonoids.

Test for Tannins

- Small quantity of leaf extract of *U. parviflora* was dissolved in minimum amount of distilled water and filtered. The filtrate was treated with 10% aqueous potassium dichromate solution. Presence of yellowish brown precipitate demonstrated the presence of tannins.
- Small quantity of leaf extract of *U. parviflora* was dissolved in minimum amount of distilled water and filtered. The filtrate was allowed to react with 10% lead acetate solution. Presence of yellow colour precipitate indicated the positive test for tannins.
- Small quantity of leaf extract of *U. parviflora* was dissolved in minimum amount of distilled water and filtered. The filtrates were then allowed to react with 1.0 ml of 5% ferric chloride solution. Presence of greenish black coloration demonstrated the presence of tannins.
- Small quantity of leaf extract of *U. parviflora* was dissolved in minimum amount of distilled water and filtered. The filtrates are then allowed to react with 1.0 ml of 1% gelatin and 1.0 ml of 10% sodium chloride solution. Presence of white buff coloured precipitate demonstrated the presence of tannins.

Test for Saponins

- Small quantities of leaf extract of *U. parviflora* was dissolved in minimum amount of distilled water and shaken in a graduated cylinder for 15 minutes. Absent of stable foam suggested the absence of saponins.
- A small quantity of leaf extract of *U. parviflora* was dissolved in methanol. 1.0 ml of extract solution was treated with 1% lead acetate solution. Absent of white precipitate indicated the absence of saponins.

Test for Anthraquinones

- Small quantity of leaf extract of *U. parviflora* was dissolved in methanol. 5.0 ml of the extract solution was hydrolyzed with dilute sulphuric acid and extracted with benzene. 1.0 ml of dilute ammonia was then added to the samples. Development of rose pink colouration indicated the presence of anthraquinones.

3.2.3.2. Thin Layer Chromatography of the methanol leaf extract of *U. parviflora*.

A layer of silica gel G (Loba) was used all along in the present investigation. Chromatographic plates were prepared according to the general method. Glass plates of 20cm × 10 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 a 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 (Skoog, 1988; Fried *et al.*, 1994). 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 time required for the development of chromatograms differed from 45 to 60 min. The plates were removed from the chamber after completion of the run and were allowed to dry in air. The resulting bands were located using UV-light spraying with spray reagents followed by heating in the oven for 5-10 min at 120°C.

3.2.3.2.1 Solvent systems used

A. TLC 1. Methylene Chloride: acetone (85:15 v/v).

B. TLC 2. Benzene:acetone (95:5 v/v)

C. TLC 3. Cyclohexane: Ethyl acetate (80:20 v/v)

3.2.3.2.2 Spray reagents used

Anisaldehyde sulphuric acid reagent (Dequeker, 1964)

10% Sulphuric acid reagent (Wagner *et. al.*, 1997)

3.2.3.3 Coloumn Chromatography of leaf extract of *U. parviflora*.

3.2.3.3.1 Coloumn

A glass coloumn, 25 cm in length, 3.5 cm dia. The bottom of the coloumn was plugged with glass wool.

3.2.3.3.2 Adsorbent

Sephadex LH-20 (E. Merck) and Silica gel 60-120 (Loba).

3.2.3.3.3 Solvents used

Hexane: ethyl acetate (with increasing amount of ethyl acetate), Benzene: acetone (with increasing amount of acetone).

3.2.3.3.4 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 Sephadex LH-20 and Hexane. The solvent (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 Sephadex layer. The coloumn was recycled with Hexane for several times to prevent any shrinkage and air bubble. The final dimension of the Sephadex coloumn was 3.5 × 15 cm.

3.2.3.3.5 Separation of the compound isolated from chloroform fraction of *U. parviflora* leaf.

The excess solvent on the top of the coloumn was allowed to flow down and then the dried mixture of chloroform fraction of the leaf extract and Sephadex LH-20 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 Hexane: ethyl acetate with step increasing the polarity by increasing the ratio of ethyl acetate from 10% to 100%. The rate of elution was adjusted at 30 drops per minute and fractions of 25 ml each were collected in 100 ml of serially numbered conical flasks. TLC was done for each fraction with the same solvent system, which was used as the main eluent in the coloumn chromatography.

The eluted fraction number 41-54 having identical hR_f values were pooled together and evaporated to dryness. It was rechromatographed in a silica gel 60-120 (Loba) coloumn. Gradient elution was carried out using Benzene and increasing the polarity with acetone in 10% stepwise elutions to 100% acetone. Fraction number 16-28 were combined and evaporated to dryness to provide an amorphous powder, which was crystallized from methanol to give fine, needle shaped, white crystals. The isolated crystalline material was further examined by different physico-chemical techniques for its structure elucidation.

3.2.3.4 Qualitative analysis of the compound isolated from chloroform fraction of *U. parviflora* leaf.

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

3.2.3.5 Physical Nature of the compound isolated from chloroform fraction of *U. parviflora* leaf.

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

3.2.3.6 Thin Layer Chromatographic study of the compound isolated from chloroform fraction of *U. parviflora* 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 3.2.3.2 and used for the study. The chromatograms were run with three solvent systems mentioned below in a chromatography chamber.

A. TLC 1. Methylene Chloride: acetone (85:15 v/v).
B. TLC 2. Benzene:acetone (95:5 v/v)
C. TLC 3. Cyclohexane: Ethyl acetate (80:20 v/v)

The mobile phase was allowed to run up to 10 cm (solvent font) and the plates were dried. The spots were observed under UV light at 366 nm (long wave) before and after spraying the spraying reagents.

3.2.3.7 Ultraviolet-Visible absorption spectral analysis of the compound isolated from chloroform fraction of *U. parviflora* leaf.

Ultraviolet-visible absorption spectroscopy is the most useful technique available for triterpenoids and steroids structure analysis and is used to aid both identification of the type

and definition of the oxygenation pattern. The crystalline isolated compound was dissolved in spectroscopic grade methanol and the absorption spectra were taken in Shimadzu 1601 double beam UV-Visible spectrophotometer.

3.2.3.8 Infrared spectrum of the compound isolated from chloroform fraction of *U. parviflora* leaf.

The infrared (IR) absorption spectra of the isolated compound were taken with Perkin Elmer FTIR 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.

3.2.3.9 Nuclear Magnetic Resonance (NMR) spectra of the compound isolated from chloroform fraction of *U. parviflora* leaf.

The ^{1}H and ^{13}C NMR spectra of the isolated compound were undertaken in Brucker WM 400 Spectrophotometer in DMSO-d_6 (Dimethyl sulphoxide) solution. The spectra are recorded and tabulated.

3.2.3.10 Mass Spectrometry of the compound isolated from chloroform fraction of *U. parviflora* leaf.

GC-MS spectra were recorded using a Finnigan Matt GCQ Mass Spectrometer.

3.2.4 Isolation and purification of phytoconstituent from the leaf of *Callicarpa arborea.*

The methods used for isolation and purification of phytoconstituent from the leaf of *Callicarpa arborea* were as per the procedure followed for the isolation and purification of phytoconstituents from the leaf extract of *U. parviflora* described in 3.2.3.1 to 3.2.3.10 except the following points:

1. The identical eluted fractions number 32-46 in coloumn chromatographic separation in 3.2.3.3.5 were rechromatographed by using Benzene: methanol as eluent. Gradient elution was carried out by increasing the polarity with methanol in 10% stepwise elutions to 100% methanol.
2. The rechromatographed fractions numbers 18 to 26 were combined and evaporated to dryness followed by crystallization in hexane-ethyl acetate to result a triterpenoid.

3.2.5 Isolation and Purification of Phytoconstituent from the root bark of *Morinda citrifolia*

The methods used for isolation and purification of phytoconstituents from the root bark of *Morinda citrifolia* were as per the procedure followed for the isolation and purification of phytoconstituents from the leaf extract of *U. parviflora* described in 3.2.3.1 to 3.2.3.10 expect the following points:

1. The TLC study was carried out by using the spraying reagents: bromine water and dilute ammonia solution. (Rajendran *et.al.,* 2007)
2. The identical eluted fractions number 48-59 in coloumn chromatographic separation in 3.2.3.3.5 were rechromatographed by using Benzene: methanol as eluent. Gradient elution was carried out by increasing the polarity with methanol in 10% stepwise elutions to 100% methanol.
3. The rechromatographed fractions numbers 22 to 30 were combined and evaporated to dryness to result an anthraquinone derivative.

3.3 Results

3.3.1 Phytochemical Study of *U. parviflora* leaf.

The concentrated methanol extract obtained from the shade-dried leaf of *U. parviflora* was fractionated successively petroleum ether, chloroform and acetone. 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 amino acids, proteins, steroids and triterpenoids (**Table 3.1**). The thin layer chromatographic study of the chloroform fraction showed the presence of three components with selected mobile phase (**Table 3.2-3.3**). This fraction was subjected to coloumn chromatographic separation on Sephadex LH 20 coloumn in which fifty-eight fractions were collected. The fractions having identical results were mixed together (**Table 3.4**). They were purified with a silica gel (60-120) coloumn, which yielded a steroidal compound (compound I). It was further subjected to chemical tests and TLC studies to confirm the chemical nature of the compound I, which are depicted in **Table 3.5 and Table 3.6**. The chemical nature of the isolated compound was further characterized from its physical parameters and spectral (IR, GC-MS, ^{13}C and ^{1}H NMR) data (Faizi *et al.,* 2001; Peirs *et al.,* 2006).

Compound I was obtained as white shining, needle shaped crystal. It is insoluble in aqueous solvent and sparingly soluble in ethyl acetate. The compound was melted at 128.5^{0}- 129. 2^{0}C. The UV absorption spectrum of compound I showed strong absorption at 492 nm in its spectrum, which implied the presence of steroidal ring in its structure. The IR spectrum of the

compound is presented in **Fig 3.1**. The IR spectrum shows the presence of absorption bands at 3430, 2959, 2935, 2868, 1667, 1708 cm^{-1} The IR spectrum confirmed the presence of C=C (1667 cm^{-1}) and hydroxyl group (3430 cm^{-1}) in compound I.

The ^{1}H NMR spectrum of compound I is presented in **Fig 3.2**. Comparisons of ^{1}H and ^{13}C NMR spectra of the isolated compound facilitated the identification of the structure (**Table 3.7**). The ^{1}H NMR spectrum displayed H-6 at δ 5.4 as a multiplet. The same spectrum showed signals for H-3 at δ 3.5 (m, H-18 at δ 0.68 (s), H-19 at δ 0.98 (s), C-21 at δ 0.9 (d, J= 6.5 Hz). The ^{13}C NMR spectrum (**Fig 3.4**) showed C-5 and C-6 double bond carbons at δ 122.09 and 138.29 suggesting the sitosterol structure. The fragmentation ion at m/z 414 in its mass spectrum, which is presented in **Fig 3.3**, inferred the compound is corresponding to the molecular formula $C_{29}H_{50}O$. It was unambiguously identified as **β-sitosterol** on the basis of all the spectral data (**Fig 3.5**) (Faizi *et al.*, 2001).

Table 3.1 Preliminary phytochemical test of methanol extract of *Urtica parviflora* Roxb., *Callicarpa arborea* Roxb. and *Morinda citrifolia* Linn.

Phytoconstituents	*Urtica parviflora* (Leaf)	*Callicarpa arborea* (Leaf)	*Morinda citrifolia* (Root bark)
Alkaloids	—	—	+
Amino acids	+	—	+
Proteins	+	+	—
Reducing sugars	—	—	+
Steroids and Triterpenoids	+	+	+
Flavonoids	+	+	+
Tannins	+	—	—
Saponins	—	—	—
Anthraquinones	—	—	+
Gums and Mucilages.	—	—	—

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

Table 3.2 Thin Layer Chromatography study of chloroform fraction of *Urtica parviflora* Roxb.

No of spots	Colour of spots under long wave UV light			Colour of spots under long wave UV light after spraying the spraying reagent			hR_f values		
	TLC 1	TLC 2	TLC 3	TLC 1	TLC 2	TLC 3	TLC 1	TLC 2	TLC 3
1	Blue	Blue	Blue	Bright blue	Bright blue	Bright blue	83	59	58
2	Blue	Blue	Blue	Blue Florescence	Blue Florescence	Blue Florescence	64	29	52
3	–	–	Blue	–	–	Blue Florescence	–	–	41

Spray reagent: Anisaldehyde sulphuric acid reagent

A. TLC 1. Methylene Chloride: acetone (85:15 v/v).

B. TLC 2. Benzene:acetone (95:5 v/v)

C. TLC 3. Cyclohexane: Ethyl acetate (4:1 v/v)

Table 3.3 Thin Layer Chromatography study of chloroform fraction of *Urtica parviflora* Roxb.

No of spots	Colour of spots under long wave UV light			Colour of spots under long wave UV light after spraying the spraying reagents			hR_f values		
	TLC 1	TLC 2	TLC 3	TLC 1	TLC 2	TLC 3	TLC 1	TLC 2	TLC 3
1	Dark Violet	Dark Violet	Dark Violet	Violet florosce nce	Violet florosce nce	Violet florosce nce	83	59	58
2	Dark Violet	Dark Violet	Dark Violet	Violet florosce nce	Violet florosce nce	Violet florosce nce	64	29	52
3	–	–	Dark Violet	–	–	Violet florosce nce	–	–	41

Spray reagent: 10% sulphuric acid solution.

A. TLC 1. Methylene Chloride: acetone (85:15 v/v).

B. TLC 2. Benzene: acetone (95:5 v/v)

C. TLC 3. Cyclohexane: Ethyl acetate (4:1 v/v)

Table 3.4 Coloumn and Thin layer chromatography separation of chloroform fraction of *Urtica parviflora* Roxb.

Eluent Hexane: ethyl acetate	Fraction number	Residue of selective fraction (gm)	Colour of TLC spots with hR_f values.	Inference
100:0	1-12	5.2	NIL	Fatty oil
90:10	13-17	4.6	NIL	Fatty solid
80:20	18-25	3.0	NIL	Fatty solid
70:30	26-30	6.7	NIL	Fatty solid
60:40	31-34	16.1	NIL	Greenish semisolid
50:50	35-39	14.3	NIL	Dark green semisolid
40:60	41-43	6.5	Two blue spots. (59, 54)	Mixture of compounds
30:70	44-51	6.0	Two bright blue spots. (58, 54)	Mixture of compounds
20:80	52-54	4.5	Two blue spots (59, 54)	Mixture of compounds
10:90	55-56	3.5	Three bright blue spot (84, 57, 29)	Mixture of compounds
0:100	57-58	2.0	Nil	Colourless solid

Spray reagent: Anisaldehyde sulphuric acid reagent

Table 3.5 Qualitative analysis of the compound I isolated from chloroform fraction of *Urtica parviflora* Roxb.

S.no	Treatment	Observation	Inference
1	1 mg of the crystalline solid was dissolved in 0.5 ml of chloroform 1.0 ml of acetic anhydride was then added to the mixture followed by 2.0 ml of concentrated sulphuric acid.	A reddish violet ring at the junction of the two layers.	Presence of steroids.
2	1.0 ml of concentrated sulphuric acid was added to 1 mg of isolated crystalline solid and dissolved in 1.0 ml of chloroform.	A reddish blue colour exhibited by chloroform layer and green fluorescence by the acid layer.	Presence of steroids.

Table 3.6 Thin layer chromatography of the compound I isolated from chloroform fraction of *Urtica parviflora* Roxb.

Solvent system	**hR_f values**	**Colour of fluorescent produced**		
		UV_{366nm}	UV_{366nm} +Ansl	UV_{366nm} +10% H_2SO_4
TLC 1	64	Blue	Bright blue	Bright blue
TLC 2	59	Blue	Bright blue	Bright blue
TLC 3	41	Blue	Bright blue	Bright blue

Ansl: Anisaldehyde sulphuric acid reagent

A. TLC 1. Methylene Chloride: acetone (85:15 v/v).

B. TLC 2. Benzene:acetone (95:5 v/v)

C. TLC 3. Cyclohexane: Ethyl acetate (80:20 v/v)

Table 3.7 ^{1}H and ^{13}C data for compound I, isolated from chloroform fraction of *Urtica parviflora* Roxb.

Position	δ_H, 400 MH_Z, $CDCl_3$	δ_C, 400 MH_Z, $CDCl_3$
1	–	37.11
2	–	30.10
3	3.5, m	16.12
4	–	41.48
5	–	139.29
6	5.4, m	122.69
7	–	32.86
8	–	32.86
9	–	51.08
10	–	46.79
11	–	21.1
12	–	39.7
13	–	42.5
14	–	57.1
15	–	24.2
16	–	28.1
17	–	57.06
18	0.68, s	12.1
19	0.98, s	–
20	–	36.3
21	0.9, d (6.5)	21.2
22	–	33.9
23	–	26.0
24	–	–
25	–	29.8
26	0.81, d (6.6)	19.0
27	–	–
28	–	22.1
29	0.82, t (6.4)	

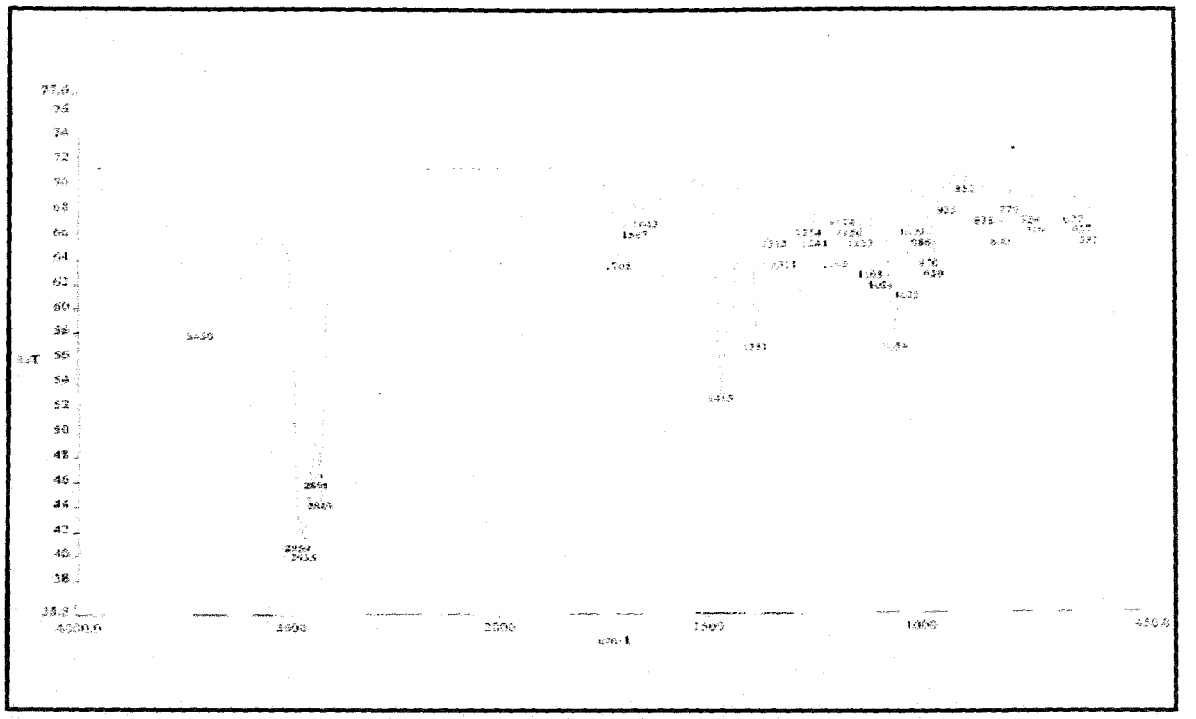

Fig 3.1 IR Spectrum of Compound I isolated from *Urtica parviflora* leaf extract.

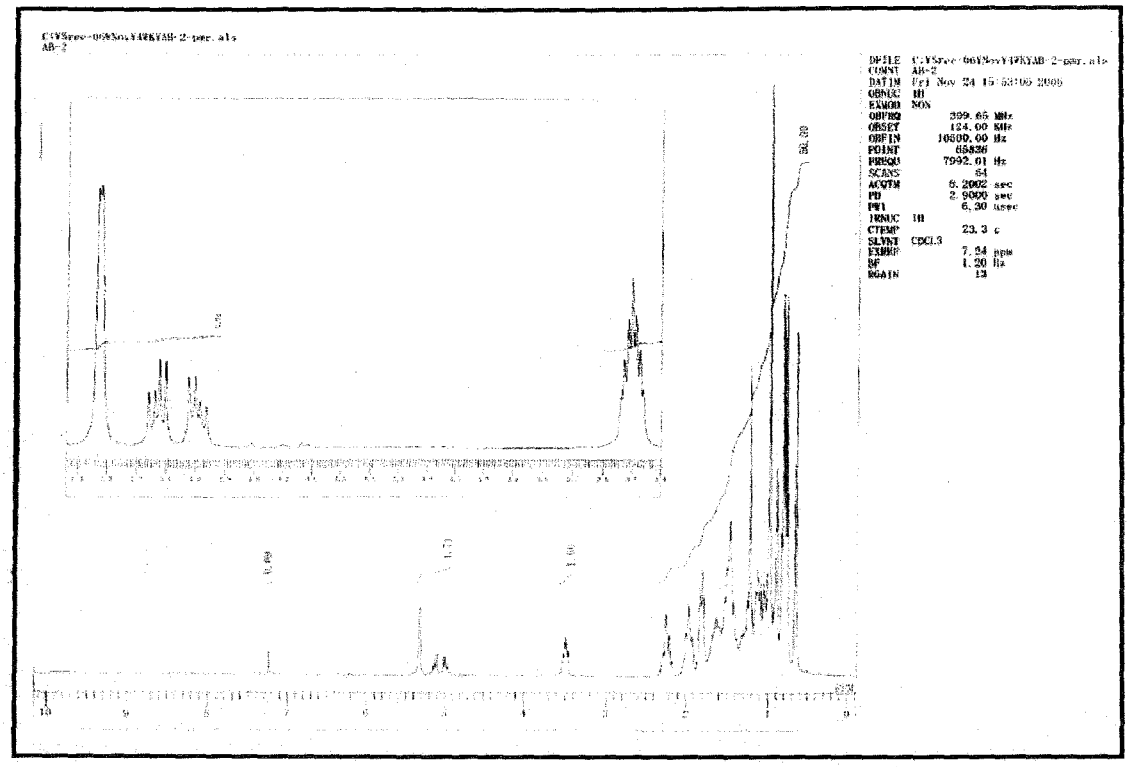

Fig 3.2 ^{1}H NMR Spectrum of Compound I isolated from *Urtica parviflora* leaf extract.

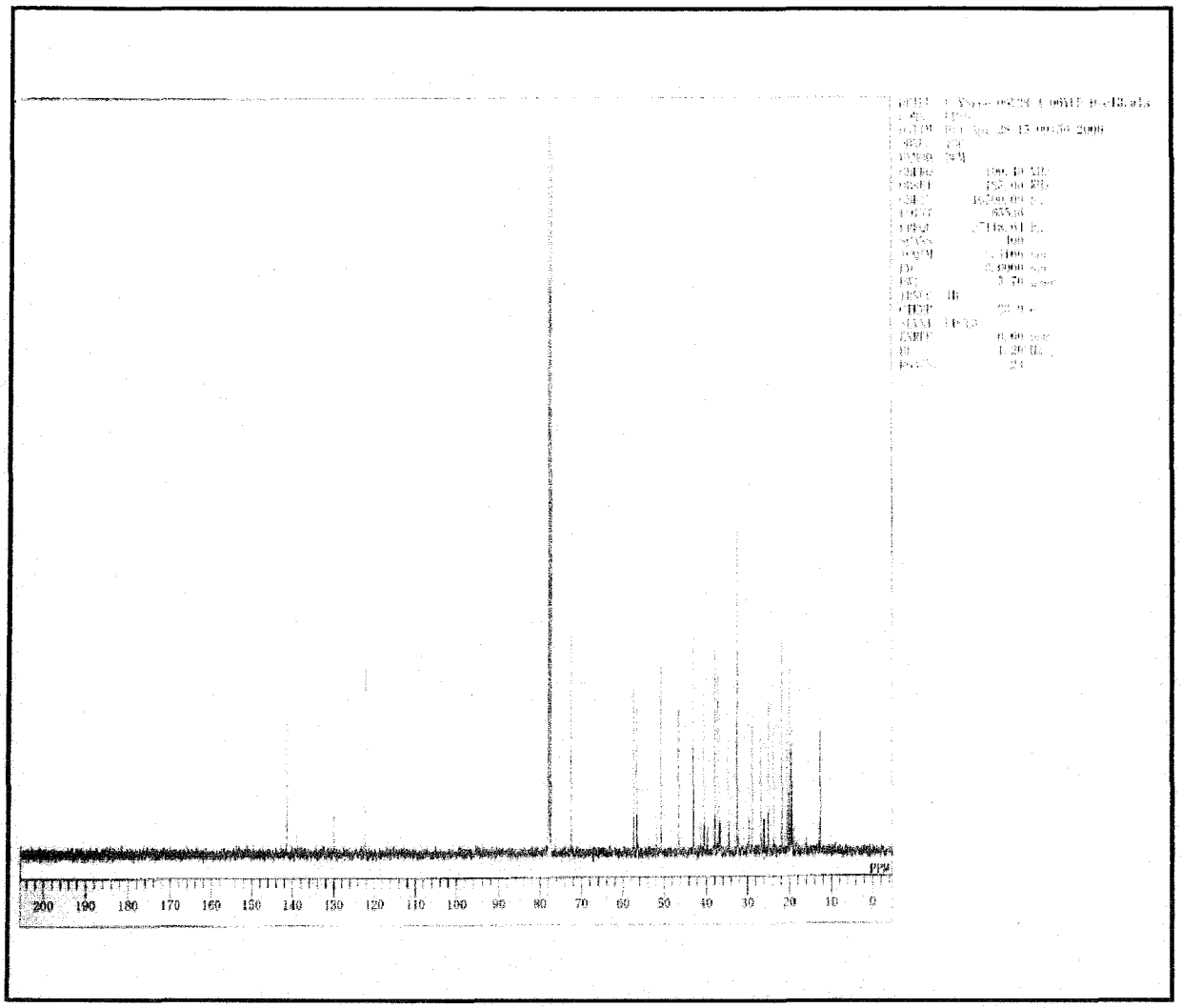

Fig 3.3 ^{13}C NMR Spectrum of Compound I isolated from *Urtica parviflora* leaf extract.

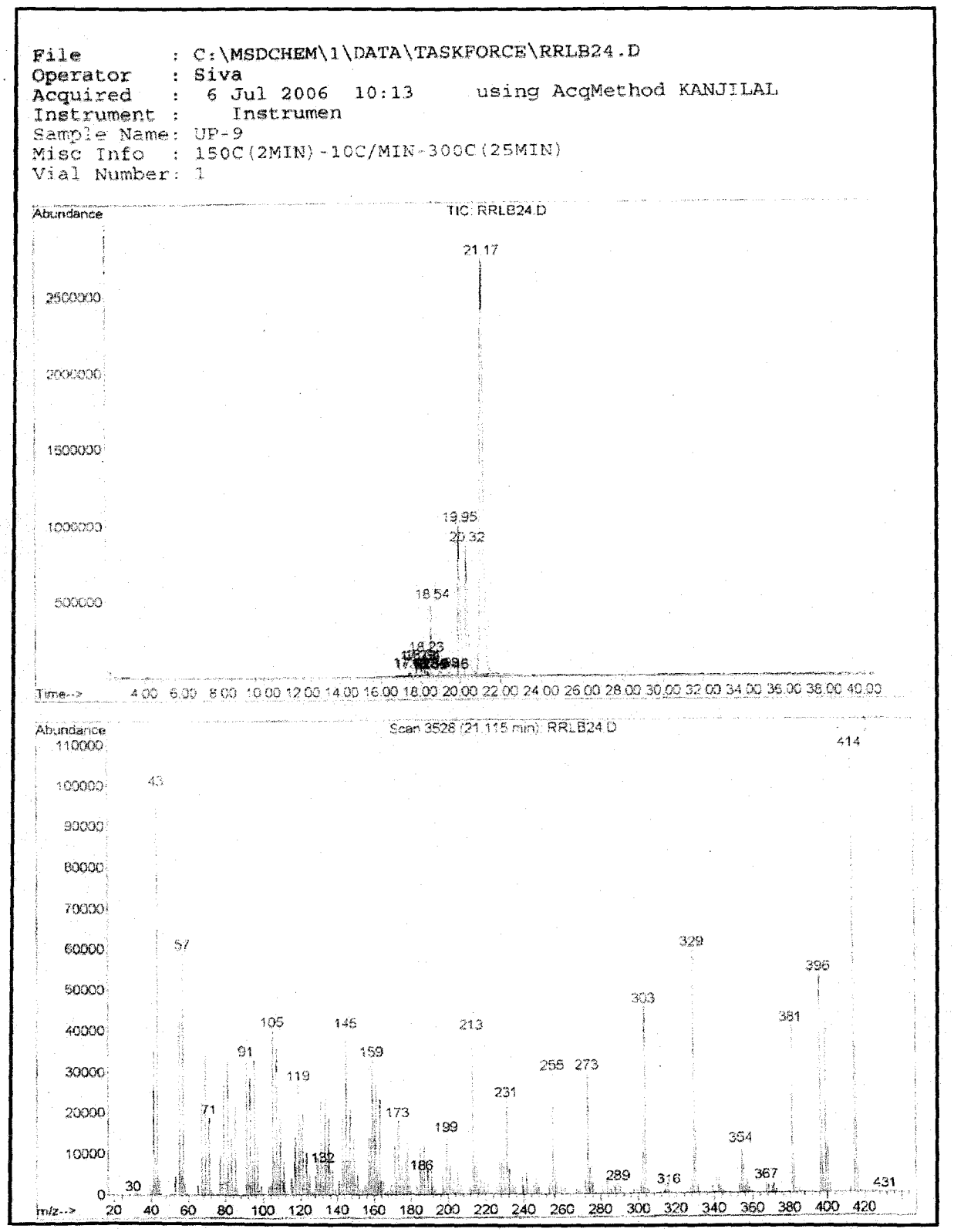

Fig 3.5 GCMS Spectrum of Compound I isolated from *Urtica parviflora* leaf extract.

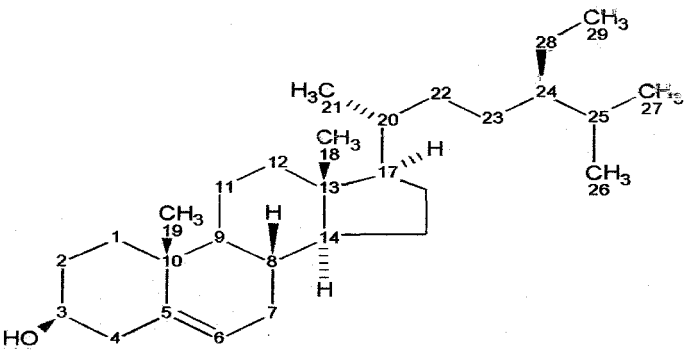

Fig 3.6 Compound I (β-sitosterol)

3.3.2 Phytochemical Study of *C. arborea* leaf.

The concentrated methanol extract prepared from the shade-dried root of *C. arborea* was fractionated successively with petroleum ether, chloroform and acetone. Different fractions of the extract were undertaken for preliminary pharmacological studies. It was observed that the acetone 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, (**Table 3.1**). The thin layer chromatographic study of acetone fraction of methanol extract showed the mixture of four components with selected mobile phase and spraying reagents (**Table 3.8-3.9**). This fraction was subjected to column chromatography, which yielded sixty-two fractions. The fractions having identical hRf values were mixed together (**Table 3.10**). They were purified with a silica gel column, by using benzene: methanol as mobile phase, which yielded a triterpenoid glycoside (compound II). It was further subjected to chemical tests and TLC to confirm the chemical nature and was depicted in **Table 3.11** and **3.12**. The chemical nature of the isolated compound was further characterized by comparison of its physical parameters and spectral (UV, IR, Mass, ^{13}C and ^{1}H NMR) data with that of the reported values of triterpenoid glycoside (Alvarez *et al.,* 2003; Abe *et al.,* 2002 and Yoshida *et al.,* 2005).

Compound II was obtained as colorless amorphous powder, partially soluble in water and soluble in organic solvent. The compound was melted at $139^0 - 143^0C$. The UV analysis (**Fig 3.6 a, b**) showed a significant absorption at 242 nm. It indicates that the compound is an isoprene derivative (Yu Ping Lin *et al.,* 2003). The IR spectrum (**Fig 3.7**) shows the presence of absorption bands for the groups hydroxyl (2928 cm^{-1}), carbonyl (1691 cm^{-1}), double bond (1459 cm^{-1}) and ester (1030 cm^{-1}). The ^{1}H and ^{13}C NMR spectra of compound II which are presented in **Table 3.13** showed that most of the signals of the aglycone were in good agreement with literature data of oleanolic acid (Kubota *et al.,* 1968). The ^{13}C NMR (**Fig 3.8**) showed the presence of cabonyls at δ C 144.17 (C), δ C (122.92) and δ H 5.25 (1 H, C=12). The spectra also showed the presence of hydroxyl at δ C 78.76 to 47.37 and δ H 5.236 to 3.13 (**Fig 3.9 a, b, c, d**). The fragmentation ion at 248 in its mass spectrum (**Fig 3.10**) inferred the compound having the molecular formula $C_{30}H_{48}O_3$. From these data it is concluded that the structure of the isolated triterpenoid is **Oleanolic acid**.

Table 3.8 Thin Layer Chromatography study of acetone fraction of *C. arborea.*

No of Spots	Colour of spots under long wave UV light			Colour of spots under long wave UV light after spraying the spray reagent			hR_f values		
	TLC 1	TLC 2	TLC 3	TLC 1	TLC 2	TLC 3	TLC 1	TLC 2	TLC 3
1	Blue	Blue	Blue	Bright blue	Bright blue	Bright blue	68	54	69
2	Blue	Blue	Blue	Blue Florescence	Blue Florescence	Blue Florescence	64	28	49
3	–	Blue	Blue	–	Blue Florescence	Blue Florescence	–	29	41
4	–	–	Blue	–	–	Blue Florescence	–	–	53

Spray reagent: Anisaldehyde sulphuric acid reagent
A. TLC 1. Methylene Chloride: acetone (85:15 v/v).
B. TLC 2. Benzene:acetone (95:5 v/v)
C. TLC 3. Cyclohexane: Ethyl acetate (80:20 v/v)

Table 3.9 Thin Layer Chromatography study of acetone fraction of *C. arborea.*

No of Spots	Colour of spots under long wave UV light			Colour of spots under long wave UV light after spraying the spraying reagents			hR_f values		
	TLC 1	TLC 2	TLC 3	TLC 1	TLC 2	TLC 3	TLC 1	TLC 2	TLC 3
1	Dark Violet	Dark Violet	Dark Violet	Violet florosce nce	Violet florosce nce	Violet florosce nce	68	54	69
2	Dark Violet	Dark Violet	Dark Violet	Violet florosce nce	Violet florosce nce	Violet florosce nce	64	28	49
3	–	–	Dark Violet	–	–	Violet florosce nce	–	–	41
4	–	–	Dark Violet	–	–	Violet florosce nce	–	–	53

Spray reagent: 10% sulphuric acid solution.
A. TLC 1. Methylene Chloride: acetone (85:15 v/v).
B. TLC 2. Benzene: acetone (95:5 v/v)
C. TLC 3. Cyclohexane: Ethyl acetate (80:20 v/v)

Table 3.10 Coloumn and Thin layer chromatography separation of acetone fraction of *C. arborea.*

Eluent Benzene: methanol	Fraction number	Residue of selected fractions (gm)	Colour of TLC spots with hR_f values	Inference
100:0	1-14	4.6	NIL	Fatty oil
90:10	15-20	4.2	NIL	Fatty solid
80:20	21-24	2.7	NIL	Fatty solid
70:30	25-28	6.3	NIL	Fatty solid
60:40	29-30	14.5	NIL	Greenish semisolid
50:50	31-32	12.3	NIL	Dark green semisolid
40:60	32-38	6.3	Two yellowish spots. (68, 41)	Mixture of compounds
30:70	38-42	6.5	Two bright yellow spots. (68, 41)	Mixture of compounds
20:80	42-46	4.6	Two yellowish spots (69, 42)	Mixture of compounds
10:90	47-56	3.2	Three red spot (67, 52, 27)	Mixture of compounds
0:100	57-62	3.0	Nil	Colourless solid

Spray reagent: Anisaldehyde sulphuric acid reagent

Table 3.11 Qualitative analysis of the compound II isolated from acetone fraction of *C. arborea.*

S.no	Treatment	Observation	Inference
1	1 mg of the crystalline solid was dissolved in 0.5 ml of chloroform 1.0 ml of acetic anhydride was then added to the mixture followed by 2.0 ml of concentrated sulphuric acid.	A reddish violet ring at the junction of the two layers	Presence of triterpenoids
2	1.0 ml of concentrated sulphuric acid was added to 1 mg of isolated crystalline solid and dissolved in 1.0 ml of chloroform.	A reddish blue colour exhibited by chloroform layer and green fluorescence by the acid layer.	Presence of triterpenoids

Table 3.12 Thin layer chromatography of the compound II isolated from triterpenoids.

Solvent system	hR_f values	Colour of fluorescent produced		
		UV_{366nm}	UV_{366nm} +Ansl	UV_{366nm} +10% H_2SO_4
TLC 1	64	Blue	Bright blue	Bright blue
TLC 2	28	Blue	Bright blue	Bright blue
TLC 3	49	Blue	Bright blue	Bright blue

Ansl: Anisaldehyde sulphuric acid reagent

A. TLC 1. Methylene Chloride: acetone (85:15 v/v).
B. TLC 2. Benzene:acetone (95:5 v/v)
C. TLC 3. Cyclohexane: Ethyl acetate (80:20 v/v)

Table 3.13 ^{1}H and ^{13}C data for compound isolated from acetone fraction of *C. arborea*.

Position	δ_H, 400 MHz, $CDCl_3$	δ_C, 400 MHz, $CDCl_3$
1	0.55, 1.59	38.89
2	1.10, 1.70	27.69
3	2.21, dd	78.76
4	–	37.15
5	0.64	55.74
6	1.32, 1.43	18.53
7	1.29, 1.61	37.11
8	–	39.37
9	1.49	47.65
10	–	26.91
11	1.89	23.43
12	5.25 t (3)	122.92
13	–	144.171
14	–	39.43
15	1.30, 1.71	23.32
16	1.32, 1.54	20.76
17	–	47.37
18	2.21	47.93
19	1.34, 162	48.04
20	–	48.22
21	1.32, 1.40	48.50
22	1.33, 1.89	48.79
23	0.91	27.92
24	0.74	16.81
25	0.88	15.51
26	0.76	16.90
27	1.12	26.91
28	–	180.70
29	0.89	49.07
30	0.88	15.21

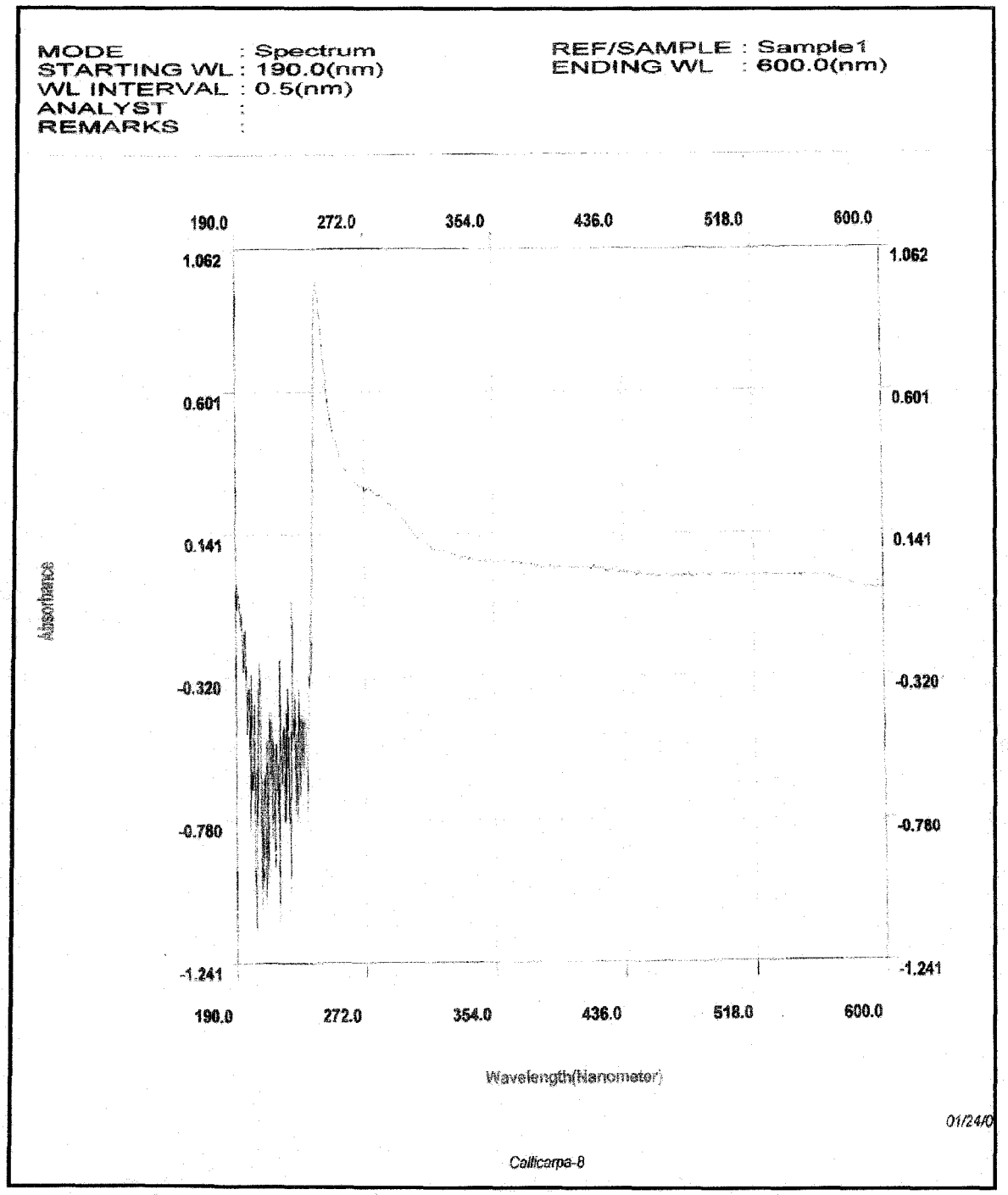

Fig 3.6 (a) UV Spectrum of Compound II isolated from *C. arborea* leaf extract.

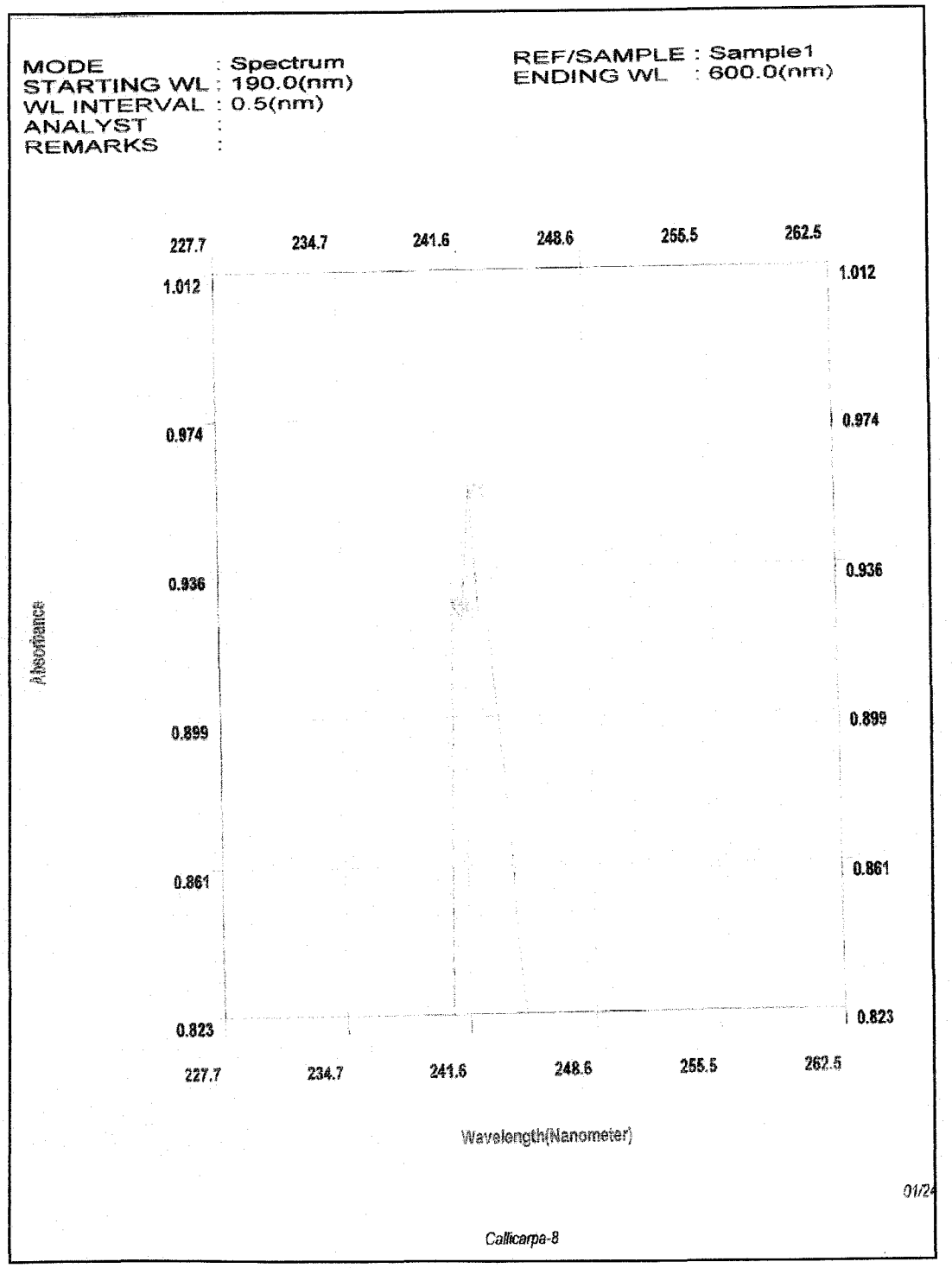

Fig 3.6 (b) UV Spectrum of Compound II isolated from *C. arborea* leaf extract.

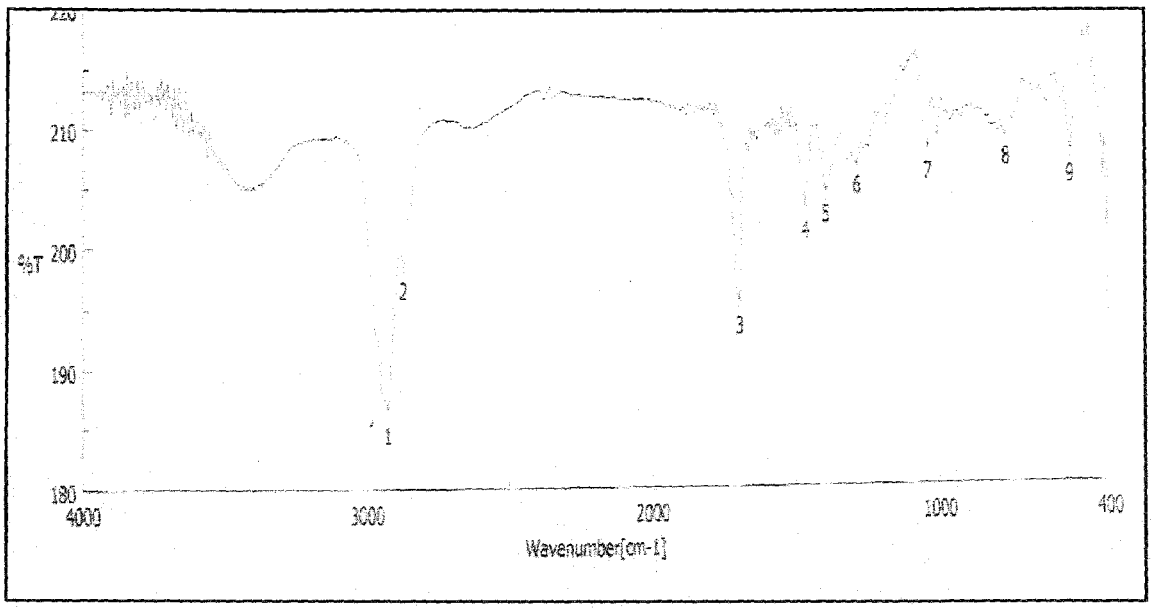

Fig 3.7 IR Spectrum of Compound II isolated from *Callicarpa arborea* leaf extract.

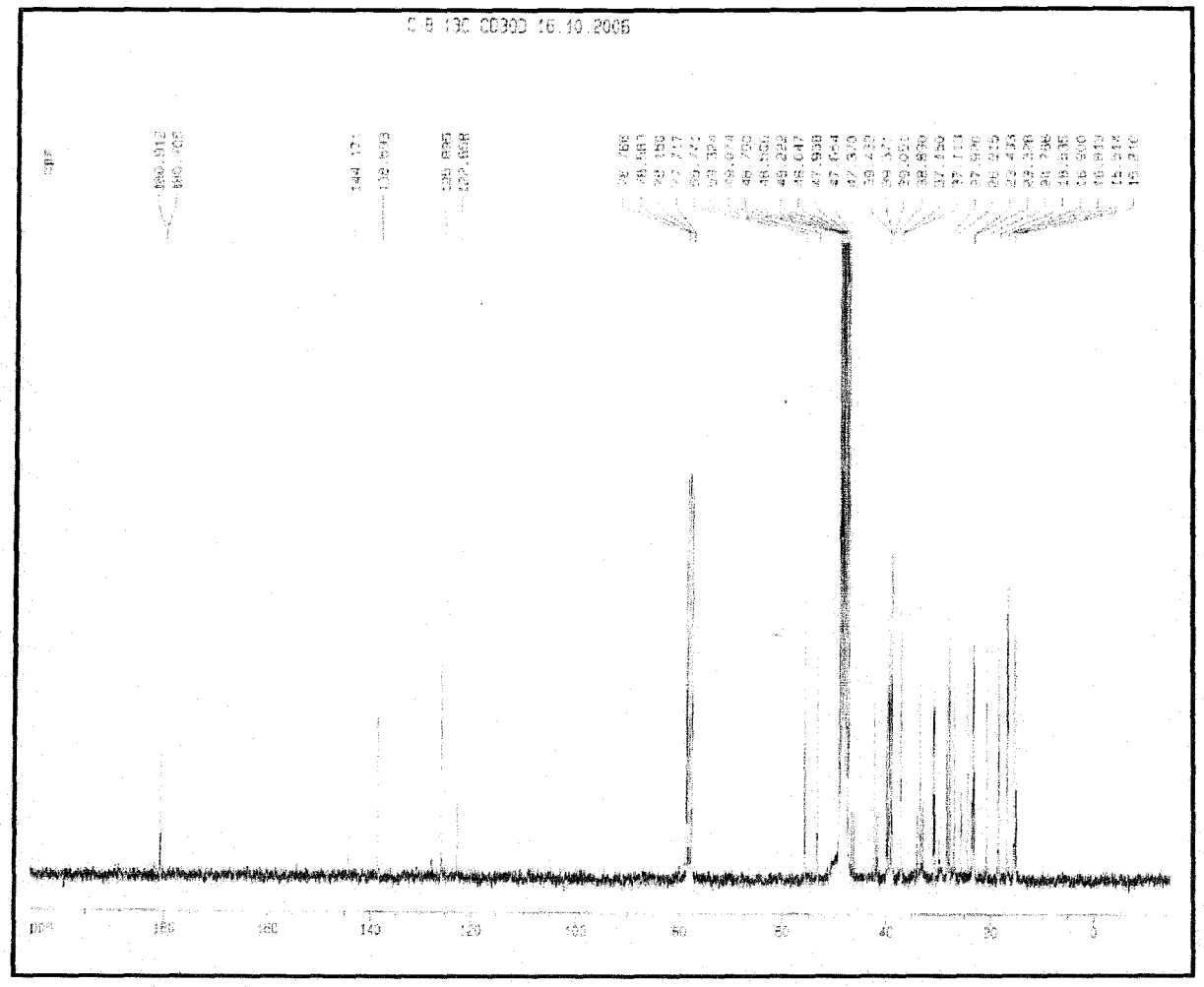

Fig 3.8 ^{13}C NMR Spectrum of Compound II isolated from *C. arborea* leaf extract.

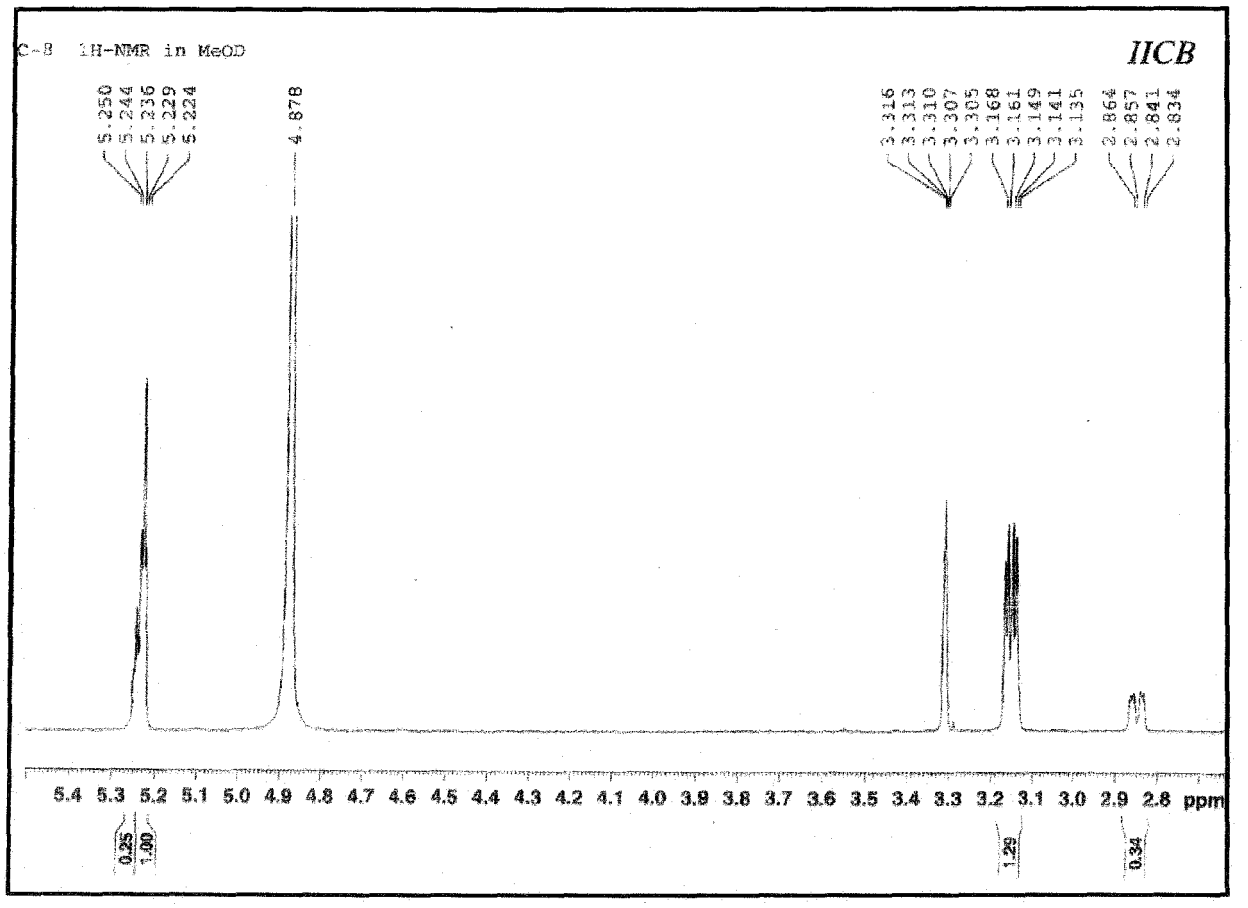

Fig 3.9 (a) ^{1}H NMR Spectrum of Compound II isolated from *C. arborea* leaf extract.

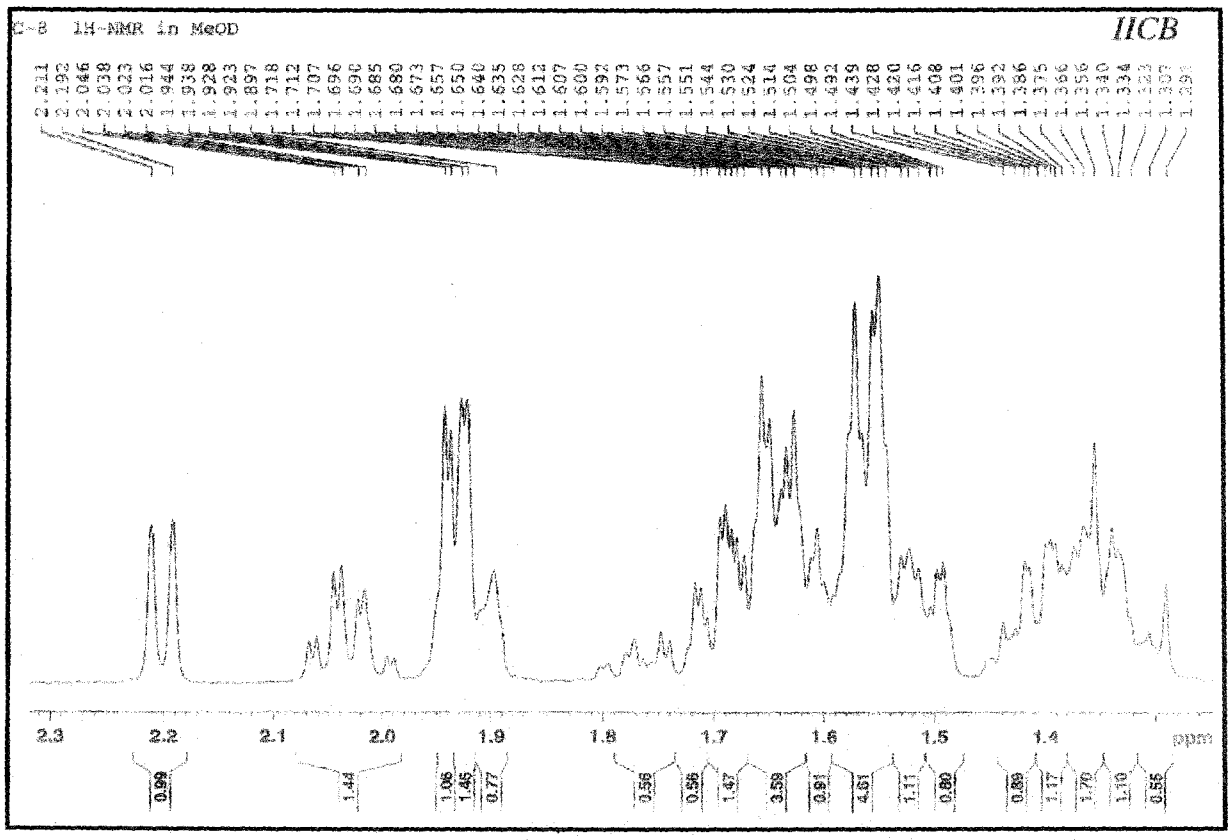

Fig 3.9 (b) ^{1}H NMR Spectrum of Compound II isolated from *C. arborea* leaf extract.

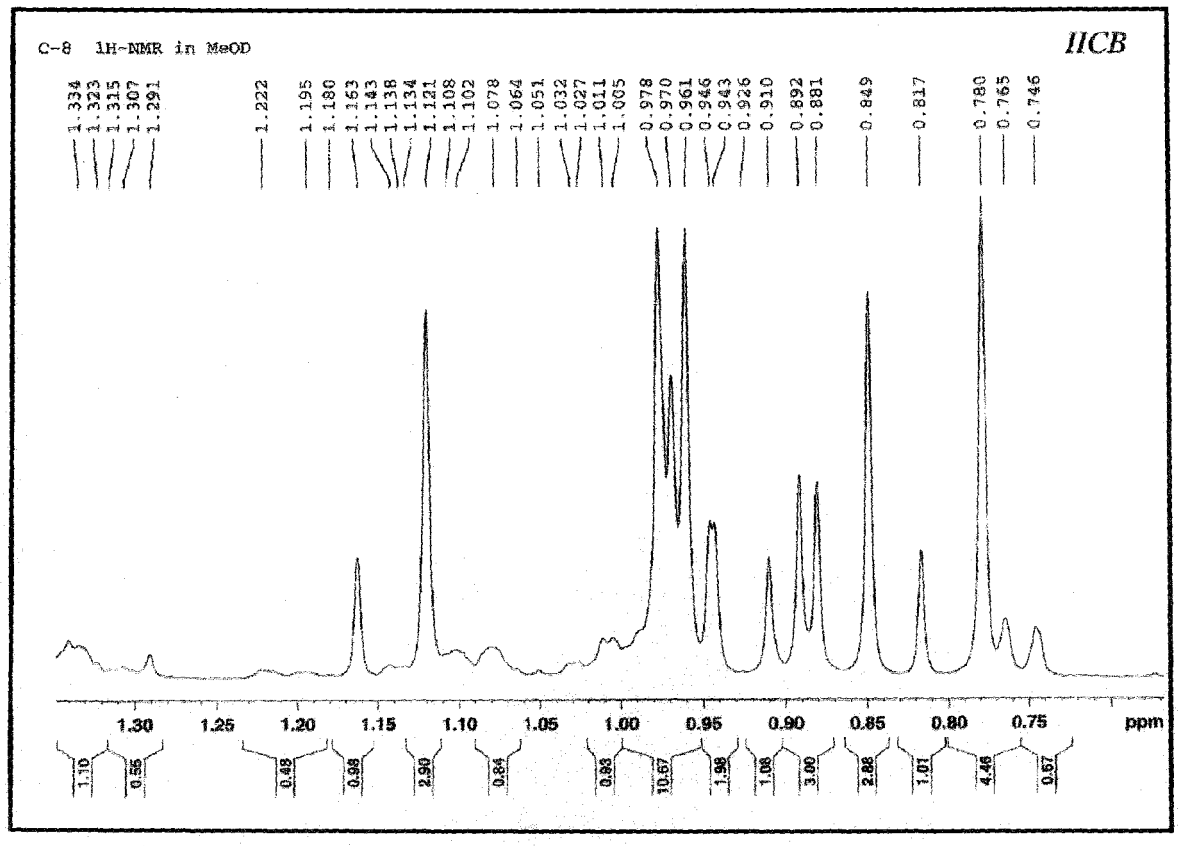

Fig 3.8 (c) ^{1}H NMR Spectrum of Compound II isolated from *C. arborea* leaf extract.

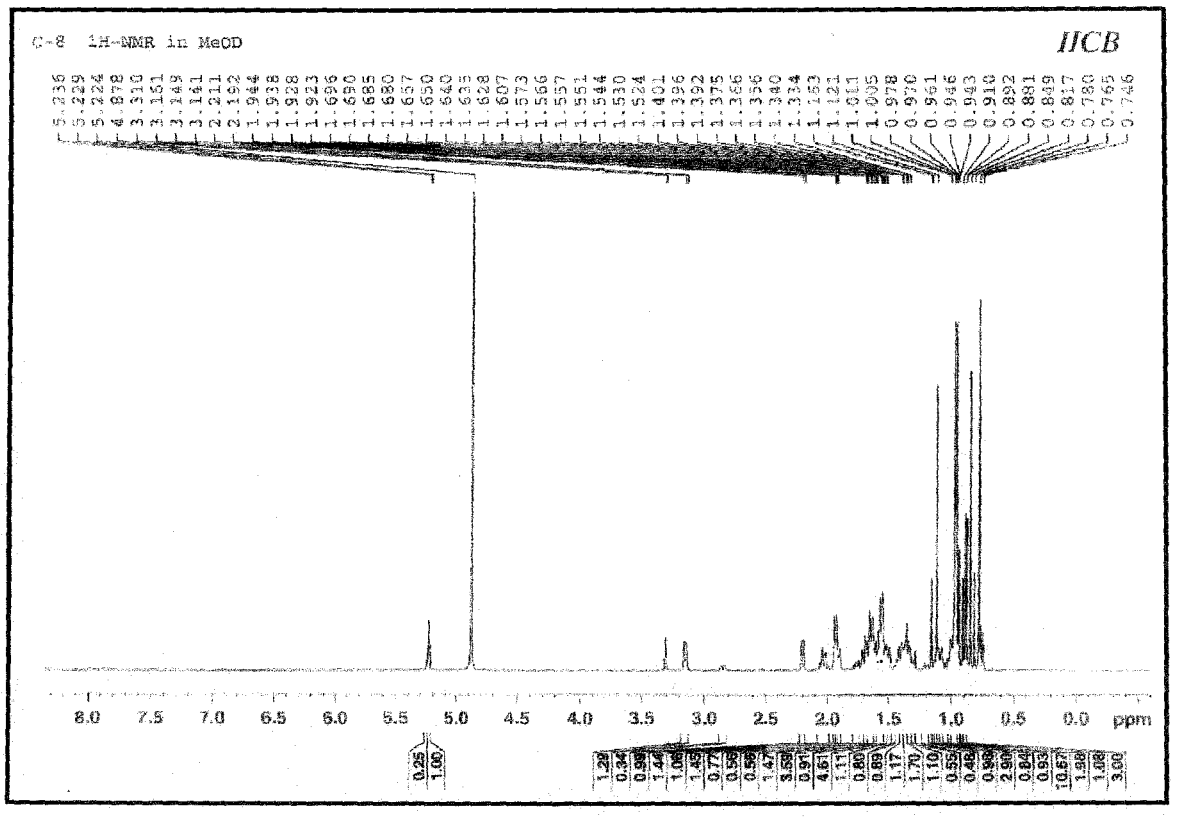

Fig 3.8 (d) ^{1}H NMR Spectrum of Compound II isolated from *C. arborea* leaf extract.

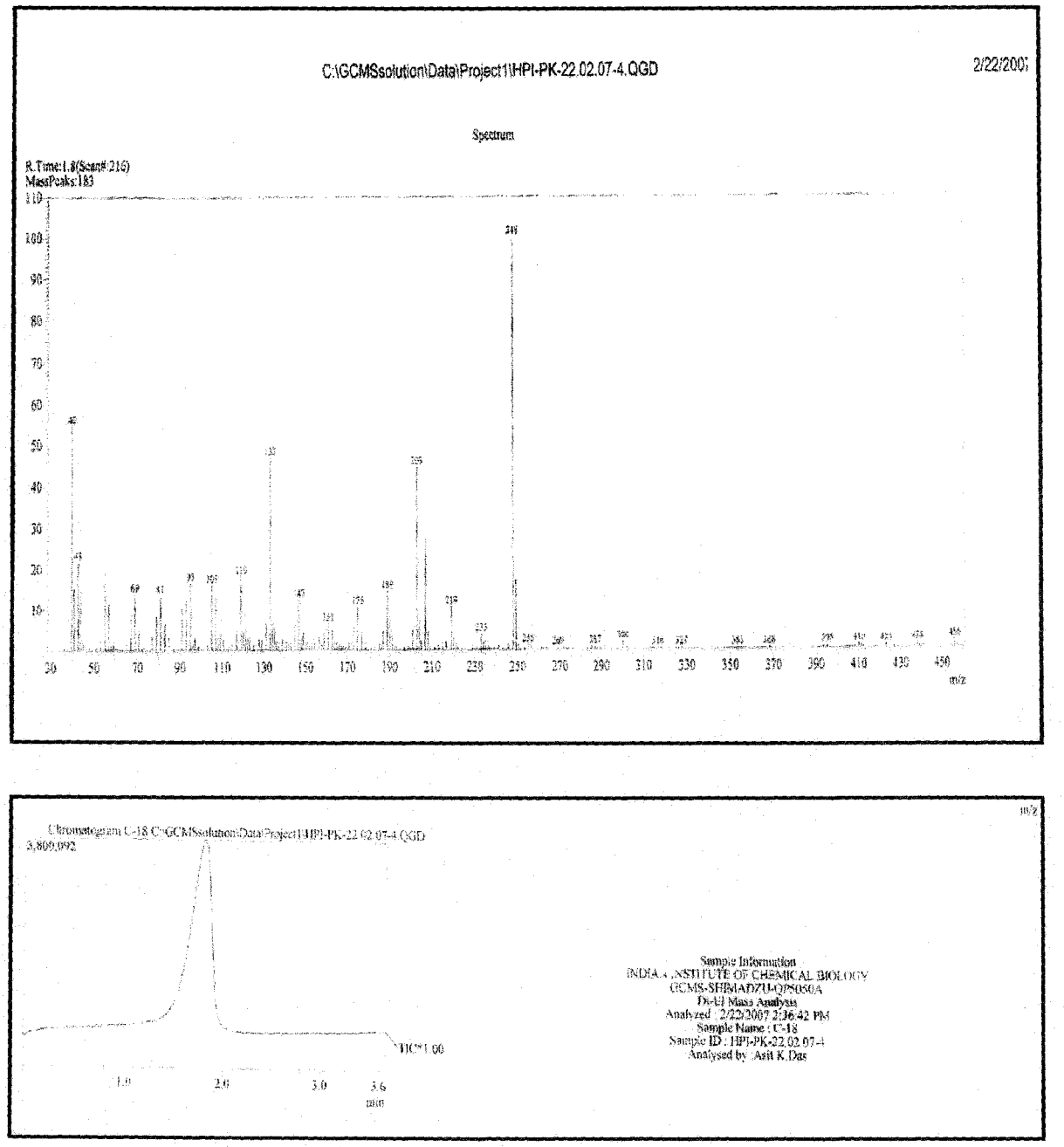

Fig 3.10 GCMS Spectrum of Compound II isolated from *C. arborea* leaf extract.

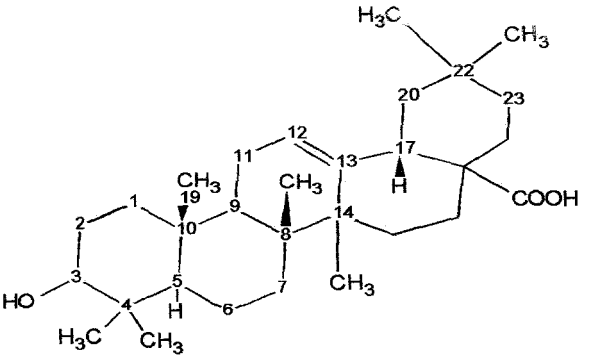

Fig 3.11 Compound II (Oleanolic acid)

3.3.3 Phytochemical Study of *M. citrifolia* root.

The concentrated methanol extract obtained from the root bark of *M. citrifolia* was fractionated successively petroleum ether, chloroform and acetone. 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 alkaloids, amino acids, reducing sugars, steroids and triterpenoids and anthraquinones (**Table 3.1**). The thin layer chromatographic study of the chloroform fraction showed the presence of five components with selected mobile phase (**Table 3.13-3.14**). This fraction was subjected to coloumn chromatographic separation on Sephadex LH 20 coloumn in which seventy-two fractions were collected. The fractions having identical results were mixed together (**Table 3.15**). They were purified with a silica gel (60-120) coloumn, which yielded a anthraquinone derivative (Compound III). It was further subjected to chemical tests and TLC studies to confirm the chemical nature of the compound III, which are depicted in **Table 3.16** and **Table 3.17**. The chemical nature of the isolated compound was further characterized from its physical parameters and spectral (UV, IR, MS, ^{13}C and ^{1}H NMR) data. (Rajendran *et al.,* 2007; Ling *et al.,* 2002)

Compound III was obtained as orange, needle shaped crystal with melting point at 200.5^{0}C. The UV absorption spectrum of compound III is shown in **Fig 3.12**. The compound showed strong absorption at 426 nm in its spectrum, which implied the presence of conjugated chromophoric group in its structure. The IR spectrum of the compound is presented in **Fig 3.13**. The IR spectrum shows the presence of absorption bands at 1633, 1614, 1035, 3572, 3636 and 2984 cm^{-1}. The IR spectrum confirmed the presence of hydroxyl group (3572, 3636 cm^{-1}), carbonyl group (1633 cm^{-1}) in compound III.

The ^{1}H NMR and ^{13}C NMR spectrum of compound I is presented in **Fig 3.14** and **Fig 3.15** respectively. Comparisons of ^{1}H and ^{13}C NMR spectra of the isolated compound facilitated the identification of the structure (**Table 3.13**). The ^{13}C NMR spectrum displayed C-3 at δ 26.69 and C-6 δ 61.27 respectively. Peaks of C-8. The rest of the aromatic carbons appears from δ 113.39 to 161.07. The fragmentation ion at m/z 291 in its mass spectrum, which is presented in **Fig 3.16**, inferred the compound is an anthraquinone derivative. By comparing the spectral data of the literature survey, it is identified as **1-8 dihydroxy, 3 methyl, 6 methoxy anthraquinone** (**Fig 3.17**) (Kamiya, *et al.,* 2005; Chan *et al.,* 2005 and Wab *et al.,* 2007).

Table 3.13 Thin Layer Chromatography study of chloroform fraction of the of *M. citrifolia.*

No of Spots	Colour of spots under long wave UV light			Colour of spots under long wave UV light after spraying the spraying reagent			hR_f values		
	TLC 1	TLC 2	TLC 3	TLC 1	TLC 2	TLC 3	TLC 1	TLC 2	TLC 3
1	Red	Red	Red	Bright red	Red Florescence	Red Florescence	74	43	25
2	Red	Red	–	Red Florescence	Red Florescence	–	52	32	–
3	–	Red	Red	–	Red Florescence	Red Florescence	–	24	41
4	–	Red	Red	–	Red Florescence	Red Florescence	–	18	33
5	Red	Red	Red	Red Florescence	Red Florescence	Red Florescence	28	12	25

Spray reagent: Dilute ammonia reagent

A. TLC 1. Methylene Chloride: acetone (85:15 v/v).

B. TLC 2. Benzene:acetone (95:5 v/v)

C. TLC 3. Cyclohexane: Ethyl acetate (80:20 v/v)

Table 3.14 Thin Layer Chromatography study of chloroform fraction of the of *M. citrifolia*

No of Spots	Colour of spots under long wave UV light			Colour of spots under long wave UV light after spraying the spraying reagents			hR_f values		
	TLC 1	TLC 2	TLC 3	TLC 1	TLC 2	TLC 3	TLC 1	TLC 2	TLC 3
1	Red	Red	Red	Bright red	Red Florescence	Red Florescence	73	43	25
2	Red	Red	–	Red Florescence	Red Florescence	–	52	32	–
3	–	Red	Red	–	Red Florescence	Red Florescence	–	23	41
4	–	Red	Red		Red Florescence	Red Florescence	–	18	31
5	Red	Red	Red	Red Florescence	Red Florescence	Red Florescence	28	12	25

Spray reagent: Bromine water.

A. TLC 1. Methylene Chloride: acetone (85:15 v/v).

B. TLC 2. Benzene: acetone (95:5 v/v)

C. TLC 3. Cyclohexane: Ethyl acetate (80:20 v/v)

Table 3.15 Coloumn and Thin layer chromatography separation of chloroform fraction of the of *M. citrifolia.*

Eluent Benzene: Methanol	Fraction number	Residue of selective fraction (gm)	Colour of TLC spots with hR_f values.	Inference
100:0	1-12	5.2	NIL	Fatty oil
90:10	13-17	4.6	NIL	Fatty solid
80:20	18-25	3.0	NIL	Fatty solid
70:30	26-30	6.7	NIL	Fatty solid
60:40	31-34	16.1	NIL	Greenish semisolid
50:50	35-39	14.3	NIL	Reddish green semisolid
40:60	40-47	6.5	Two blue spots. (58, 32)	Mixture of compounds
30:70	48-52	6.0	Two bright blue spots. (58, 24)	Mixture of compounds
20:80	53-55	4.5	Two blue spots (59, 24)	Mixture of compounds
10:90	56-59	3.5	Three bright blue spot (64, 57, 27)	Mixture of compounds
0:100	60-72	2.0	Nil	Colourless solid

Spray reagent: Bromine water.

Table 3.16 Qualitative analysis of the compound III isolated from chloroform fraction of the of *M. citrifolia.*

S.no	Treatment	Observation	Inference
1	Small quantity of isolated compound was dissolved in methanol. 5.0 ml of the solution was hydrolyzed with dilute sulphuric acid and extracted with benzene. 1.0 ml of dilute ammonia was then added to the samples.	Development of rose pink colouration.	Presence of anthraquinones.

Table 3.17 Thin layer chromatography of the compound III isolated from chloroform fraction of the of *M. citrifolia.*

Solvent system	hR_f values	Colour of fluorescent produced		
		UV_{366nm}	UV_{366nm} +BW	UV_{366nm} +DA
TLC 1	58	Red	Bright red	Bright red
TLC 2	42	Red	Bright red	Bright red
TLC 3	32	Red	Bright red	Bright red

BW: Bromine water, DA: Dilute ammonia

A. TLC 1. Methylene Chloride: acetone (85:15 v/v).

B. TLC 2. Benzene:acetone (95:5 v/v)

C. TLC 3. Cyclohexane: Ethyl acetate (80:20 v/v)

Table 3.18 ^{1}H and ^{13}C data for compound isolated from chloroform fraction of *M. citrifolia.*

Position	δ_H, 400 MH_Z, $CDCl_3$	δ_C, 400 MH_Z, $CDCl_3$
1	–	163.36
2	7.73 (d,d)	134.25
3	–	29.69
4	7.79 (d)	134.37
5	8.10 (d)	126.55
6	–	61.27
7	7.36 (d)	119.24
8	–	183.45
9	–	186.97
10	–	161.07
11	–	126.86
12	–	126.55
13	–	113.17
14	–	133.39
6- OMe	7.26	–

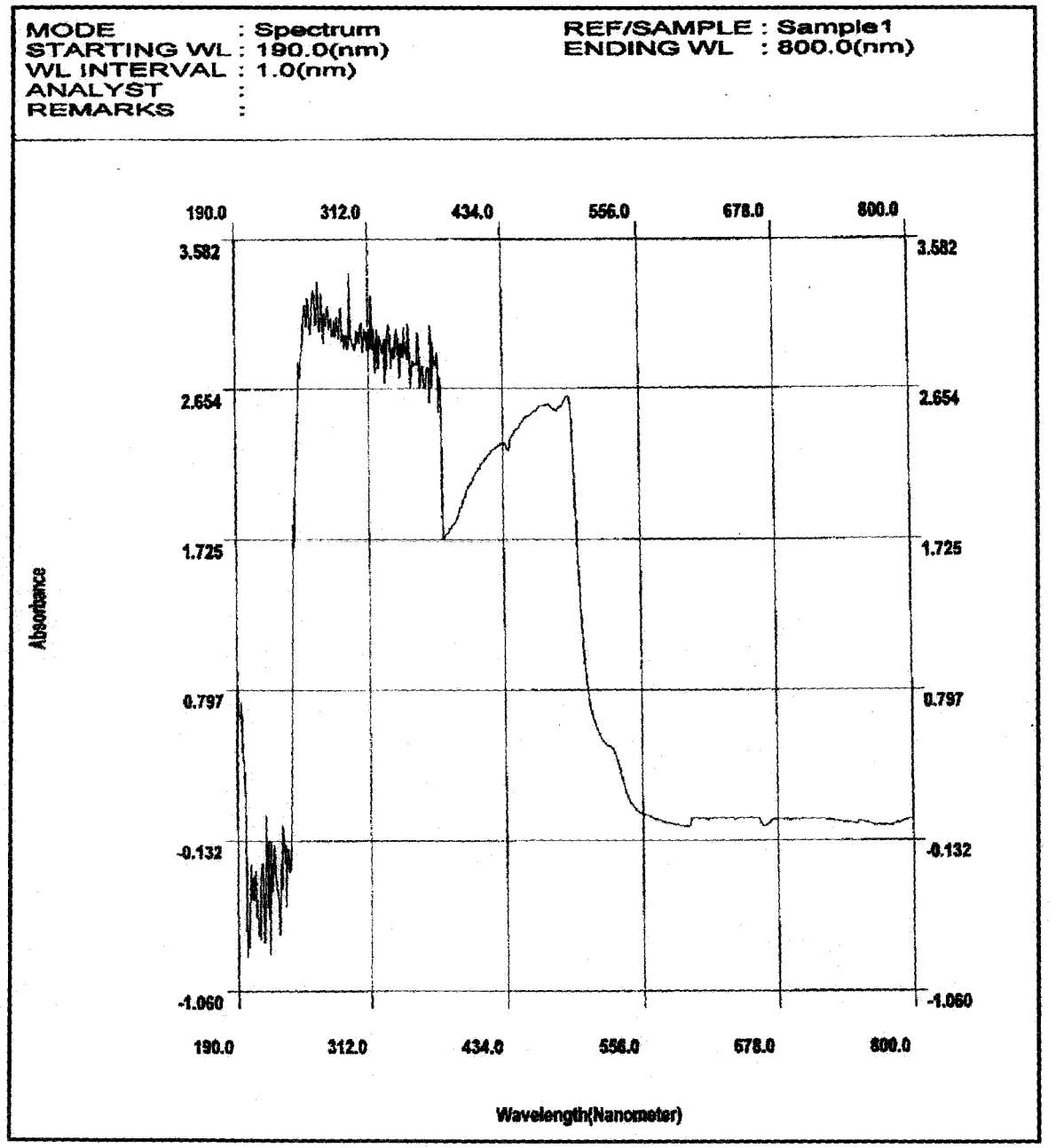

Fig 3.12 UV Spectrum of Compound III isolated from *Morinda citrifolia* root bark extract.

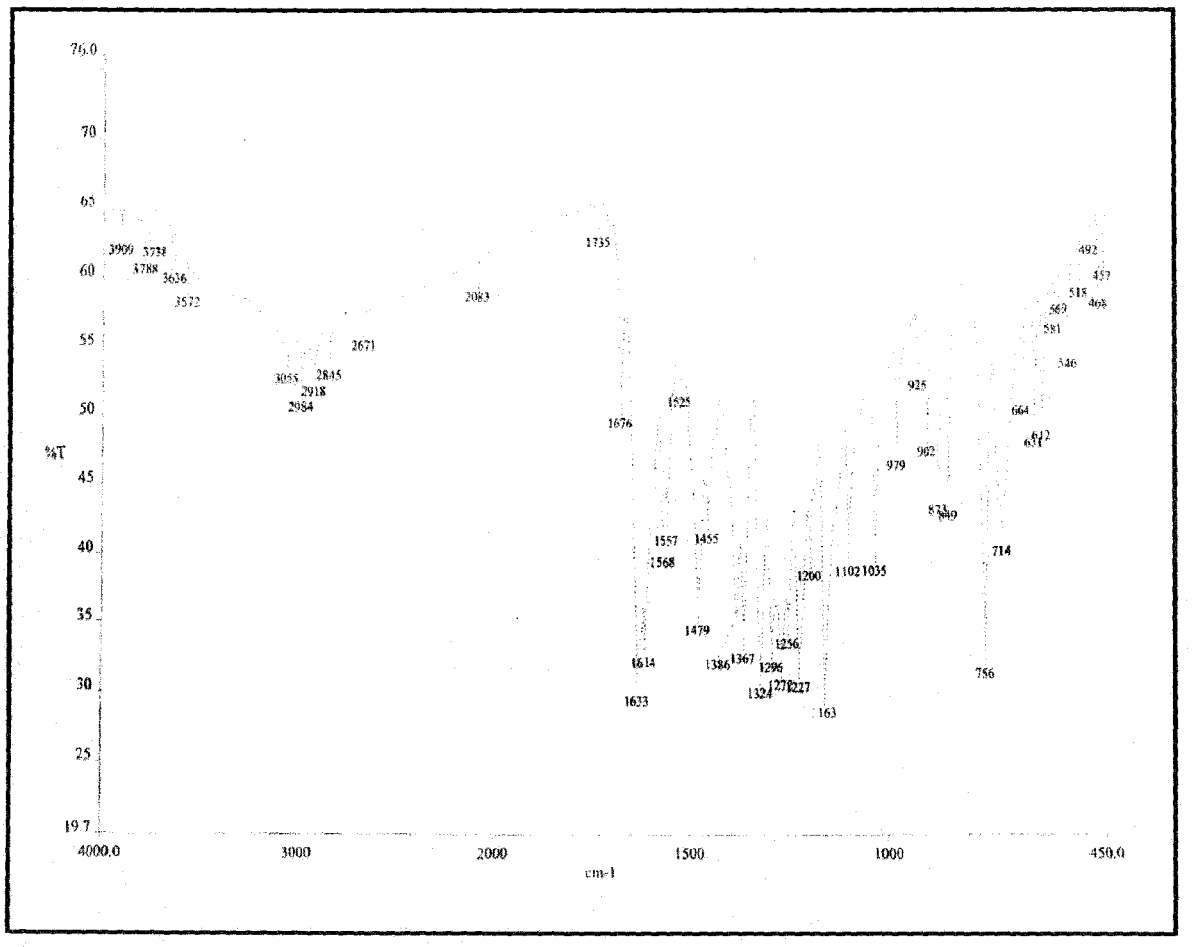

Fig 3.13 IR Spectum of Compound III isolated from *Morinda citrifolia* root bark extract.

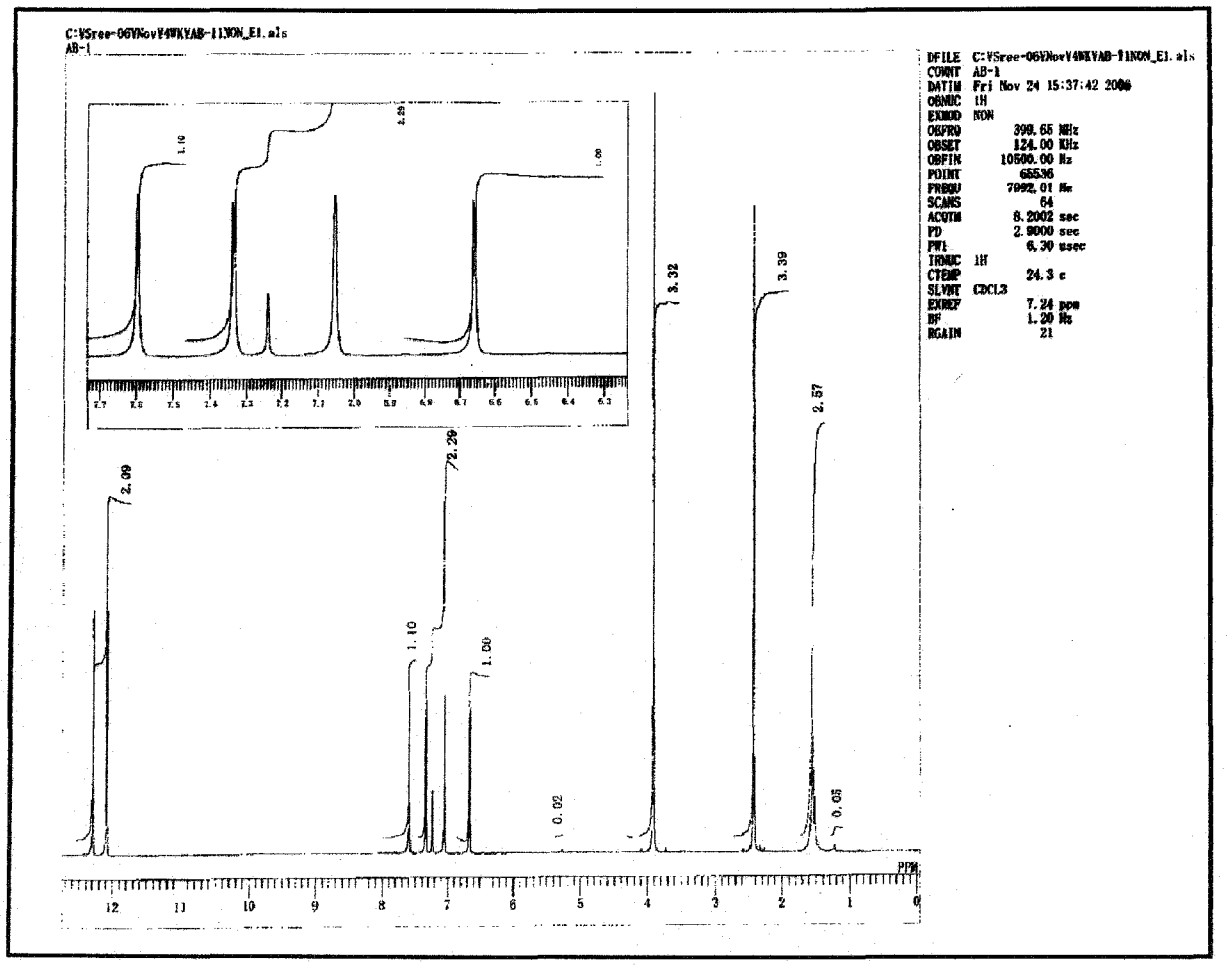

Fig 3.14 ^{1}H NMR Spectrum of Compound III isolated from *Morinda citrifolia* root bark extract.

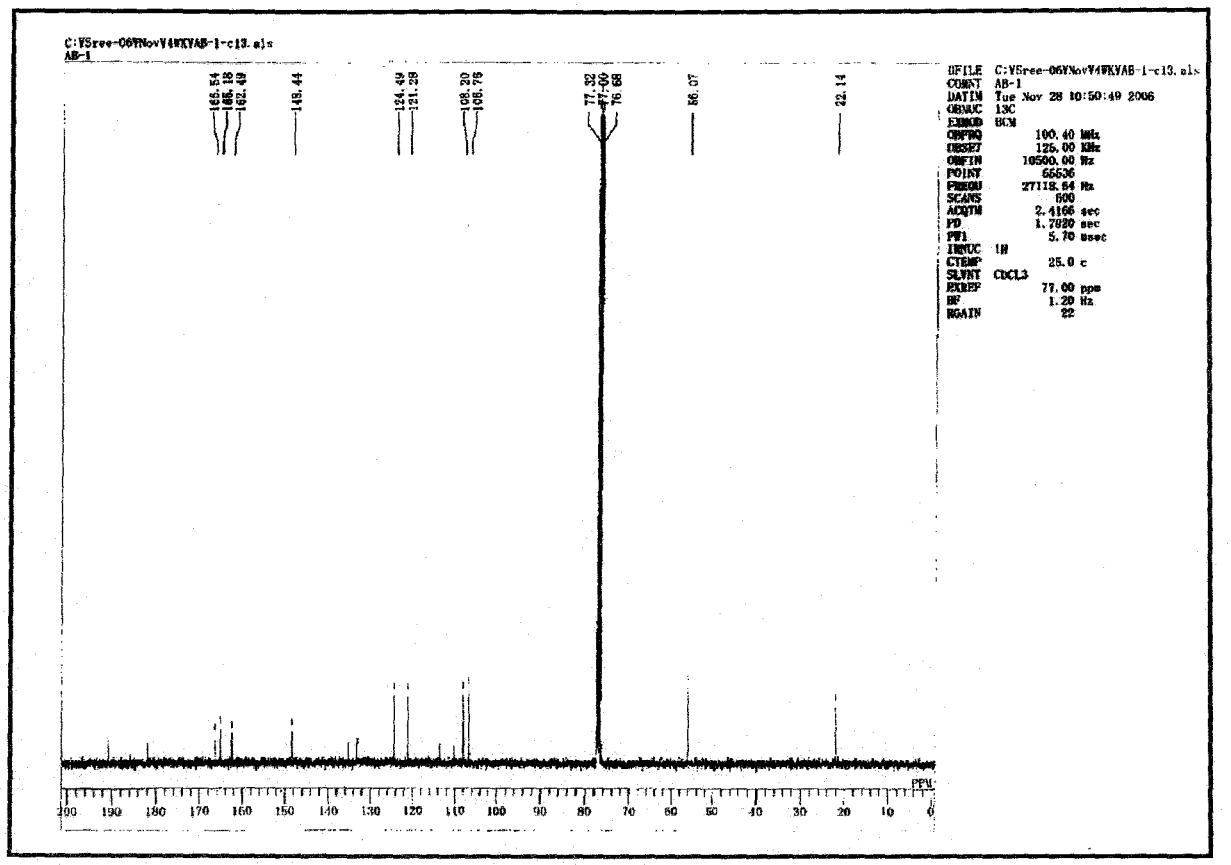

Fig 3.15 ^{13}C NMR Spectrum of Compound III isolated from *Morinda citrifolia* root bark extract.

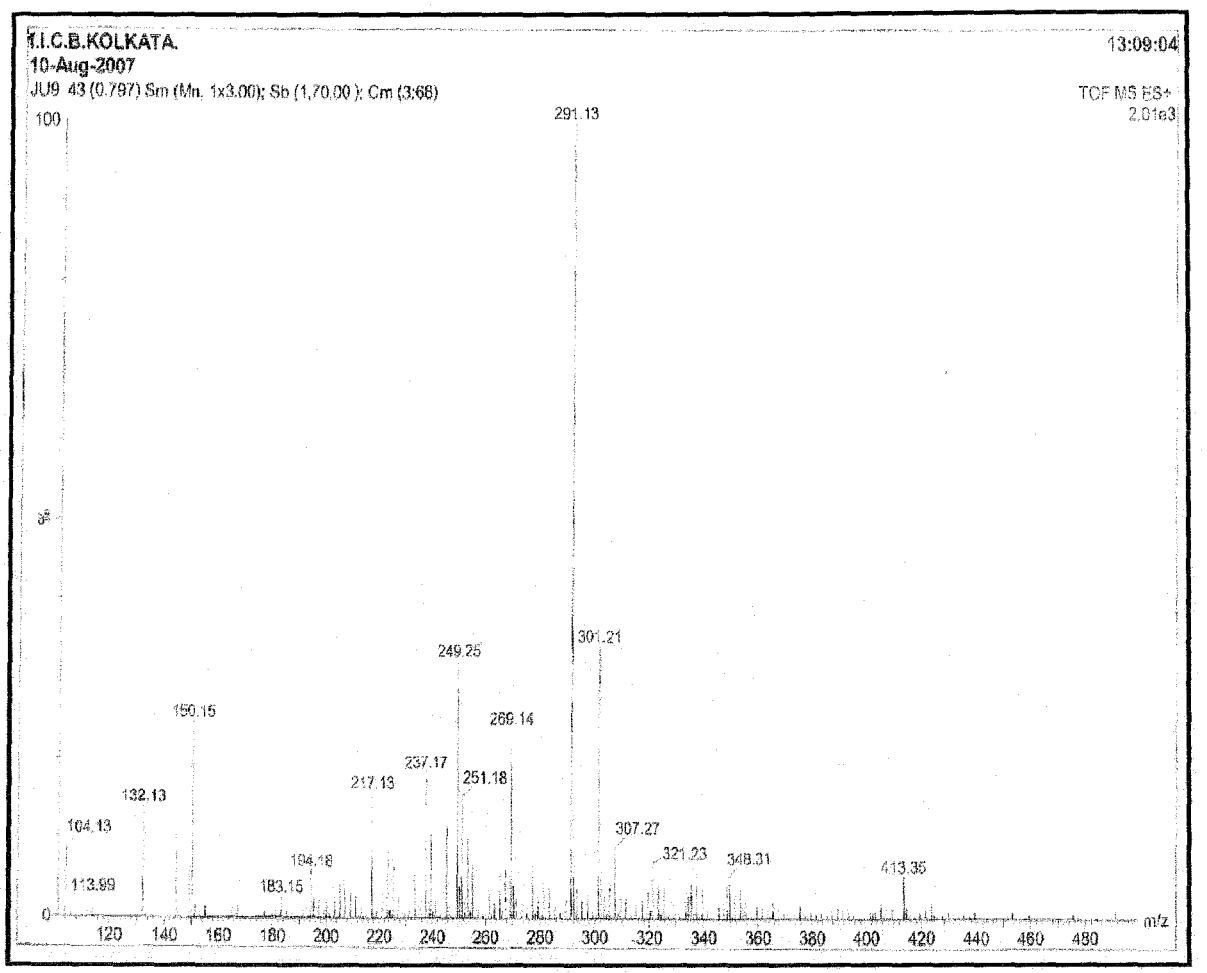

Fig 3.16 ESMS Spectrum of Compound III isolated from *Morinda citrifolia* root bark extract.

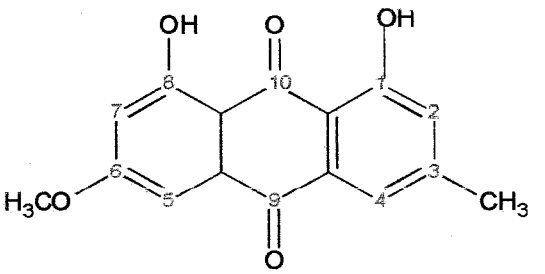

Fig 3.17 Compound III (1-8 dihydroxy, 3 methyl, 6 methoxy anthraquinone)

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