CHAPTER - 3

PHYTOCHEMICAL STUDIES

3.1. Introduction

Phytochemical analysis is intended to serve as a major source of information on analytical and instrumental methodology in the plant drug research. Medicinal plants are believed to be an important source of new chemical substances with potential therapeutic effects[1]. Searching for new drugs in plants implies the screening of plant extracts for the presence of novel compounds and investigation of their biological activities. The path that leads from an intact plant to its pure constituent is long and the work may last from weeks to years. This work includes collection of the plant material, identification of the species by a botanist, extraction using different solvents, followed by the analysis of the extracts by different chromatographic methods, fractionation and isolation steps using different preparative chromatographic techniques, structure elucidation of the constituents by a combination of various spectroscopic and chemical methods, pharmacological and toxicological testing[2].

When the whole sample *e.g.* a plant extract, has proved to be biologically active it is necessary to isolate the pure constituents that are responsible for the activity. The isolated constituents can be used for structure elucidation, further bioassays and structural modifications for studying the mode of action of the compounds, their side effects and toxicology[3]. Tracking and isolating the process of obtaining bioactive compounds from natural products is a challenge which needs new innovations in order to become more rapid, more effective and less expensive[4].

The plants extracted and submitted to the screening are selected on the basis of their use in traditional medicine, the available literature data and chemotaxonomic criteria. The extracts presenting the most interesting activities are then selected for activity-guided phytochemical investigation in order to identify the active compounds. Moreover, the isolation and characterization of further molecules presenting original chemical structures and a potential therapeutic interest is performed. The extract is screened for the presence of various constituents employing standard screening test. There is increasing scientific interest in the extraction and isolation of secondary metabolites from plants as part of biosynthetic biochemical, chemotaxonomic, ecological, phytochemical, pharmacological, and plant tissue culture. The most common plant secondary metabolites occur in the form

of alkaloid, anthraquinones, coumarins, essential oils, flavonoids, steroids, terpenoids etc[5].

Plant parts in fresh or dried form are used for extraction. Plants may be dried before extraction. The drying operations should be performed under controlled conditions to avoid to occur too many chemical changes. It should be dried as quickly as possible without using high temperatures preferably in a good air draft once thoroughly dried; plants can be stored before analysis for long period of time. The effective extraction of a plant compound of interest from a natural source depends largely on solubility stability and functional group considerations. Extraction methods to be employed to obtain a crude plant extract as well as precautions that must be taken to avoid compound decomposition, side reactions or rearrangements during extraction process. Mode of extraction naturally depends on the texture and water content of the plant material being extracted and on the type of substance that is being isolated.

The classical chemical procedure for obtaining organic constituents from dried plant tissue is to continuously extract powdered material in a Soxhlet apparatus with a range of solvents. The extract obtained is clarified by filtration through celite on a water pump and is then concentrated in vacuum. This is now usually carried out in a rotary evaporator, which will concentrate bulky solution down to small volume without bumping at temperature of 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 constituent from each other prior to chromatographic analysis.

Many plant extracts owe their potency to the presence of substances such as tannins, phenolic compounds and so on. These substances are usually found in various parts of the plants like roots, leaves, shoots and bark. Many plants have therefore become sources of important drugs and the pharmaceutical industries have come to consider traditional medicine as a source of bioactive agents that can be used in the preparation of synthetic medicine[6].

Recently, chromatographic fingerprint technique, as a more meaningful quality control method of herbal samples, has been attracting more and more people's attention because the fingerprint technique emphasizes on the integral characterization of compositions of samples with a quantitative degree of reliability and focus on identifying and assessing

the stability of the plants. Chromatographic fingerprint is a kind of method to show chemical information of medicinal constituents with chromatograms, spectrograms and other graphs by analytical techniques. Chromatographic techniques are used to isolate and purify the natural products. Varieties of chromatographic techniques involving fingerprint include TLC, gas chromatography, high performance liquid chromatography (HPLC), MPLC (medium pressure liquid chromatography) which play an important role in the isolation processes[7-12].

Identification of compounds usually involves a combination of different techniques including Ultra Violet (UV), Infrared (IR), Mass (MS) and Nuclear Magnetic Resonance (NMR) Spectrometry. Other ways of confirming the identification of the compounds include calculation of the R_f values in different solvent systems and determination of melting points.

Flavonoids are polyphenolic compounds that are ubiquitous in nature and are categorized according to chemical structure, into flavonols, flavones, flavanones, isoflavones, catechins, anthocyanidins and chalcones. Flavonoids are widely distributed in plants fulfilling many functions including producing yellow or red/blue pigmentation in flowers and protection from attack by microbes and insects. The widespread distribution of flavonoids, their variety and their relatively low toxicity compared to other active plant compounds (for instance alkaloids) means that many animals, including human, ingest significant quantities in their diet[13]. Over 4,000 flavonoids have been identified, many of which occur in fruits, vegetables and beverages (tea, coffee, beer, wine and fruit drinks). The flavonoids have aroused considerable interest recently because of their potential beneficial effects on human health. They have been reported to have antiviral, anti-allergic, antiplatelet, anti-inflammatory, antitumor, and antioxidant activities[14].

More than 1300 different flavonoid compounds have been isolated from plants. Individual flavonoids in a group differ from each other by the number and position of the hydroxy, methoxy, and sugar substituents.

Flavonoids, also referred to as bioflavonoids, are polyphenol antioxidants found naturally in plants. They are secondary metabolites, meaning thereby they are organic compounds that have no direct involvement with the growth or development of plants. More simply, flavonoids are plant nutrients that when consumed in the form of fruits and vegetables are

non-toxic as well as potentially beneficial to the human body. More importantly, the consumption of foods containing flavonoids has been linked to numerous health benefits. Though research shows flavonoids alone provide minimal antioxidant benefit due to slow absorption by the body, there is indication that they biologically trigger the production of natural enzymes that fight disease. Recent research indicates that flavonoids can be nutritionally helpful by triggering enzymes that reduce the risk of certain cancers, heart disease, and age-related degenerative diseases. Some research also indicates that flavonoids may help to prevent tooth decay and reduce the occurrence of common ailments such as the flu. These potential health benefits, many of which have been proven, have become of particular interest to consumers and food manufacturers. Foods that contain high amounts of flavonoids include blueberries, red beans, cranberries, and blackberries. Many other foods, including red and yellow fruits and vegetables and some nuts, also contain flavonoids. Red wine and certain teas also are rich in flavonoids.

Flavonoids are a large and important group of natural products derived from 'flavone'. Some flavonoids are intensely coloured, providing a spectrum of colours from red to blue in flowers, fruit and leaves. Other flavonoids are essentially colourless, producing the 'whiteness' of white flowers. Besides their contribution to plant colour, flavonoids have a variety of other roles in the growth and development of plants. Leaf flavonoids provide protection from the potential damage of UV radiation. Certain flavanones are formed as antifungal barriers in plant leaves in response to microbial infection and others play an important part in plant reproduction. Flavonoids also exhibit a wide range of biological properties including anti-microbial, insecticidal and oestrogenic activities[15]. This chapter mainly deals with the preliminary identification of phytochemical groups as well as the structural elucidation by using different spectroscopic methods of flavonoid, compounds isolated from the leaf and rhizome of the plants under investigation.

3.2. Materials and methods

3.2.1. Plant material

The rhizomes of *K. rotunda* Linn. and leaves of *E. cannabinum* Linn. were collected from different parts of Sikkim. They were authenticated at Botanical survey of India, Gangtok, Sikkim. A voucher specimen was retained in our laboratory for further reference. The rhizomes of *K. rotunda* and leaves of *E. cannabinum* were dried in shade, pulverized in a

mechanical grinder and passed through 40-mesh sieve to get the powder of the plant parts and kept under dry condition in the sterile container for further work.

3.2.2. Extraction procedure for *K. rotunda*

Coarsely powered dry rhizomes (1kg) were extracted separately by cold percolation with 4 liters of 80% methanol in a percolator for 72h at room temperature. The residue was removed by filtration and the methanol extract was concentrated and dried by using rotary flash evaporator, at 42-45°C to give a solid residue and kept in vacuum desiccators for complete removal of solvent and for use in different pharmacological experiments and to further fractionation with the help of different solvents.

3.2.3. Fractionation of the methanolic extract by different solvents

The above methanolic extract obtained was concentrated, subjected to fractionation by distilled water. The remaining hydro-alcoholic part was further fractionated with petroleum ether and ethyl acetate successively. The ethyl acetate soluble fraction was subjected to thin layer chromatographic analysis. The aqueous, petroleum ether fraction did not show any positive pharmacological activities of this investigation and was discarded. Flow chart of extraction has been shown in Fig 3.1.

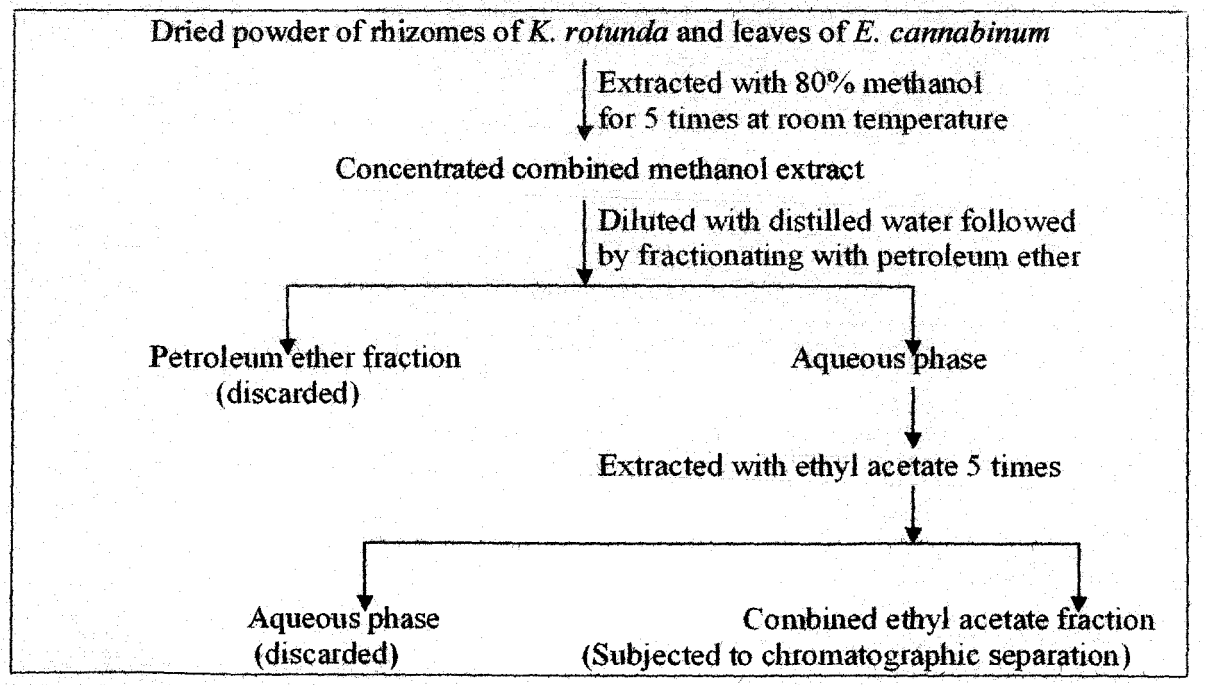


Figure 3.1. Flow chart showing the method of extraction of rhizomes of *K. rotunda* and leaves of *E. cannabinum*

3.2.4. Isolation and purification of phytoconstituent from the extract of *K. rotunda*

3.2.4.1. Preliminary phytochemical tests

The preliminary phytochemical group test of extract of *K. rotunda* rhizome was performed by the standard methods[16-18].

Tests for alkaloids

Small quantity of the extract was treated with few drops of dilute hydrochloric acid and filtered. The filtrate was treated with Mayer's reagent. There was absence of yellowish buff colored precipitate which indicated the negative test for alkaloids.

A small quantity of extract was treated with few drop of dilute hydrochloric acid and filtered and the filtrate was then treated with Dragendroff's reagent. There was no orange brown precipitate which indicated the negative test for alkaloids.

Small quantity extract treated with few drop of dilute hydrochloric acid and filtered. The filtrate was treated with Wagner's reagent. There was no reddish brown precipitate which indicated negative test for alkaloids.

Small quantity extract was treated with few drop of dilute hydrochloric acid and filtered. The filtrate was treated with Hager's reagent. There was no yellowish precipitate which indicated the negative test of alkaloid.

Test for steroids and triterponoids

Libermann-Buchard test: 10mg of extract 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 and dissolved in 1.0 ml of chloroform. A reddish blue colour exhibited by chloroform layer and green fluorescence by the acid layer indicated the presence of steroids.

Test for flavonoids

A small quantity of extract of was dissolved in methanol. One piece of magnesium was given followed by concentrated hydrochloric acid drop wise to the test sample and heated. Appearance of magenta colour demonstrated the presence of flavonoids.

A small quantity of extract 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 solution and 1.0 ml of diluted ammonia solutions were added to the first, second and third test tubes respectively. Development of yellow colour in all the tubes demonstrated the presence of flavonoids.

Test for saponins

Small quantities of extract was dissolved in minimum amount of distilled water and shaken in a graduated cylinder for 15 minutes. Formation of stable foam suggested the presence of saponins.

A small quantity of extract was dissolved in methanol. 1.0 ml of extract solution was treated with 1% lead acetate solution. Formation of white precipitate indicated the presence of saponins.

Test for tannins

Small quantity of extract was dissolved in minimum amount of distilled water and filtered. The filtrate was treated with 10% aqueous potassium dichromate solution. Development of yellowish brown precipitate demonstrated the presence of tannins.

Small quantity extract of was dissolved in minimum amount of distilled water and filtered. The filtrate was allowed to react with 10% lead acetate solution. Formation of yellow colour precipitate indicated the positive test for tannins.

Small quantity of extract 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. Formation of greenish black coloration demonstrated the presence of tannins. Small quantity of extract 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. Formation of white buff coloured precipitate demonstrated the presence of tannins.

Test for gums and mucilage

Small quantity of extract was dissolved in minimum amount of distilled water and filtered. The filtrate was treated with equal volume of concentrated sulphuric acid. Then,

it was treated 15% alcoholic solution of α-napthol (Molish's reagent), absence of red-violet ring at the junction of the two layers indicated the negative test for gums (Molish's test).

Small quantity of extract was dissolved in minimum amount of distilled water and filtered. The filtrate was treated with 95% alcohol. Absence of precipitation indicated the absence of gums and mucilage.

Small quantity of extract was dissolved in minimum amount of distilled water and filtered. The filtrate was treated with 0.008gm of ruthenium red in 10ml of 10% solution of lead acetate. Absence of red colour indicated the absence of gum and mucilage.

Test for reducing sugar

A small quantity of extract 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. No brick red precipitate was found which confirmed the absence of reducing sugars.

Small quantity of extract 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. No development of brick red colour demonstrated the absence of reducing sugars.

Test for volatile oil

50gm of powdered material was taken in volatile oil estimation apparatus and subjected it to hydrodistillation, for the detection of volatile oil, the distillate collected in a graduated tube, which indicates the presence of volatile oil.

3.2.4.2. TLC of the methanolic extract and its ethylacetate fraction of *K. rotunda*

Thin layer chromatography (TLC) is a technique used to separate chemical compounds by following processes.

3.2.4.2.1. Preparation of TLC plates

Chromatographic plates having the size of 20cm x10cm were prepared by coating of the slurry of 20% suspension of Silicagel G with the help of conventional spreader to a layer thickness of 0.25mm. After spreading the resultant plates were allowed to dry at room

temperature and then activated by heating in an oven for 30 minutes at 110°C. After cooling the plates were kept in a dessicator until required for further use.

3.2.4.2.2. Sampling on plates and development of Chromatogram

With the help of microcapillary tubes the methanolic extract and its ethylacetate fraction of *K. rotunda* was spotted at 2cm from the edge of the plate. On the plates the chromatogram was developed in chromatographic chambers using selected solvent systems at a room temperature (28°C) and at an angle of 70°. The loaded TLC plate was carefully placed in the TLC chamber with the spot of solution containing the methanolic extract and ethylacetate fraction toward the bottom. The plate whose top was leaned against the jar wall was allowed to sit on the bottom of the chamber and was in contact with the developing solvent (solvent surface was well below the extract line). The TLC chamber was covered. The TLC plate was allowed to remain undisturbed. When the solvent front has reached three quarters of the length of the plate, the plate was removed from the developing chamber and the position of the solvent front was immediately marked. The solvent moved up the plate by capillary action and met the sample mixture, which was dissolved and was carried up the plate by the solvent. Different compounds in the sample mixture travel at different rates due to differences in solubility in the solvent, and due to differences in their attraction to the stationary phase. 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 plates were observed under UV light for the appearance of spots before and after spraying with spray reagents. Alternatively the developed TLC plates were placed in iodine chamber. Further the R_f values of the spots were calculated.

3.2.4.2.3. Solvent systems used for *K. rotunda*

i) Hexane: Ethyacetate (1:1) ii) Chloroform: Methanol: Water (64:50:10)

iii) Tolune: Ethyacetate (93:7) iv) n-Butanol: Glacial acetic acid: Water (4:1:5)

v) Ethyacetate: Formic acid: Glacial acetic acid: Water (100:11:11:2)

3.2.4.2.4. Spray reagents used

i) NPPEG- Natural product polyethylene glycol reagent

ii) VS: Vaniline sulphuric acid reagent iii) AS: Anisaldehyde sulphuric acid reagent

iv) $SbCl_3$: Antimony trichloride reagent v) ADB: 2-aminoethyl diphenyl borinate

3.2.5. Column Chromatography of extract of *K. rotunda*

The isolation and purification of different components from ethyl acetate fractions of total methanolic extract of *K. rotunda* rhizome by column chromatography[22] involved the following steps.

3.2.5.1. Column

A glass column (25cm in length, 3.5cm dia.) fitted with a stop cock and the bottom of the column was plugged with glass wool was used for separation.

3.2.5.2. Adsorbent: Silica gel 60-120.

Silica gel slurry: Using a beaker of an appropriate size, silica gel was made into thin slurry with the eluting solvent and the slurry was poured into the glass column.

3.2.5.3. Eluting solvent (mobile phase): Hexane-Ethylacetate

Hexane and ethylacetate was used as eluting solvent with increasing polarity of ethylacetate.

3.2.5.4. Preparation of column and separation of the compound isolated from ethyl acetate fraction of extract of *K. rotunda*

The column 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 (60-120mesh) and solvent system. The solvent system was allowed to drip at the rate of 50 drops per minute and a layer of the solvent was maintained on the top of the silica gel surface.

The excess solvent on the top of the column was allowed to flow down and then the concentrated solution of ethyl acetate fraction of the methanolic extract with required quantity of silicagel (60-120mesh) was layered on the top of the column. A thin layer of cotton was placed over it. The eluting solvent was allowed to flow down slowly till the mixture was adsorbed on the top of the column. Gradient elution was carried out using

hexane and ethylacetate and with step increasing the polarity by increasing the ratio of ethylacetate from 10% to 100%. The rate of elution was adjusted at 50 drops per minute and fractions of 20 ml each were collected in 100 ml of serially numbered conical flasks. After collection of fractions from the column, TLC was done for each fraction with the same solvent system, which was used as the main eluent in the column chromatography.

The eluted fraction number 42-62 having identical hR_f values were pooled together and evaporated to dryness. It was re-chromatographed in a silicagel 60-120 column. Gradient elution was carried out using ethyl acetate and increasing the polarity with methanol in 10% stepwise elutions to 100% methanol. Fraction number 19-26 were combined and evaporated to dryness to provide a crystalline powder, which was crystallized to give fine, needle shaped yellowish crystals. The isolated crystalline material was further examined by different physico-chemical techniques for its structure elucidation.

3.2.6. Qualitative analysis of the compound isolated from ethyl acetate fraction of *K. rotunda*

The isolated compound was further examined by chemical tests to confirm its chemical nature.

3.2.6.1. Physical nature of the compound isolated from ethyl acetate fraction of *K. rotunda*

The isolated compound was examined by various physicochemical parameters i.e. physical appearance, solubility and melting point.

3.2.6.2. TLC study of the compound isolated from ethyl acetate fraction of *K. rotunda*

Thin layer chromatographic study of the isolated compound was carried out on silicagel G plates with different solvent systems. The plates were prepared as described in 3.2.4 and used for the study. The chromatograms were run with three solvent systems mentioned below in a chromatography chamber.

A) TLC 1- Glacial acetic acid: Water (15:85).

B) TLC 2- n-Butanol: Glacial acetic acid: Water (4:1:5).

C) TLC 3- Ethyl acetate: Formic acid: Water (10:2:3).

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 366nm before and after spraying the spray reagents i.e 5% solution of 2-aminoethyl diphenyl borinate in methanol.

3.2.7. UV absorption spectral analysis of the compound isolated from ethyl acetate fraction of *K. rotunda*

Ultraviolet-visible absorption spectroscopy is the single most useful technique available for flavonoid structure analysis and is used to aid both identification of the flavonoid type and definition of the oxygenation pattern. The flavonoid nucleus of unsubstituted phenolic hydroxyl groups may be established by adding shift reagents to the sample solution and observing the resultant shifts in the absorption peaks. Thus, indirectly, the technique may be useful in determining the location of a sugar or methyl group attached to one of the phenolic hydroxyl groups.

The purified crystalline isolated compound was dissolved in spectroscopic grade methanol and the absorption spectra was taken in Shimadzu 1601 double beam UV-Visible spectrophotometer from 200 to 800nm.

3.2.8. Infrared spectrum of the compound isolated from ethyl acetate fraction of *K. rotunda*

The IR spectra of the isolated compound were taken with Perkin-Elmer FTIR spectrophotometer in KBr discs. The spectra were recorded in the region of 4000 cm^{-1} to 400 cm^{-1}, which is the typical region of absorption for isolated compound.

3.2.9. Nuclear Magnetic Resonance (NMR) spectra of the compound isolated from ethyl acetate fraction of *K. rotunda*

The ^1H NMR spectra of the isolated compound from crystallization process was undertaken in Brucker WM 400 Spectrophotometer in DMSO-d_6 (Dimethyl sulphoxide) solution. The NMR data chemical shifts are expressed in δ (ppm) from tetramethylsilane as an internal standard and coupling constants (J) are given in Hz. The spectra are recorded and tabulated.

3.2.10. Mass spectra of the compound isolated from ethyl acetate fraction of *K. rotunda*

Mass spectrum (FAB^+MS) of the isolated compound was recorded on a JEOL JMS 600 Spectrophotometer.

3.2.11. Isolation and purification of phytoconstituent from the leaf extract of *Eupatorium cannabinum*

The methods used for isolation and purification of phytoconstituents from the leaf of *E. cannabinum* were as per the procedure followed for the isolation and purification of phytoconstituents from extract of *K. rotunda* rhizome described in **3.2.3** to **3.2.10** except the following points. The phytochemical studies show the presence of alkaloids in both the methanol extract and ethylacetate fraction of methanolic extract *E. cannabinum*. Alkaloid showed the presence in the solvent system of tolune: thylacetate: di-ethylamine by spraying Dragendorff's reagent.

The identical eluted fractions number 49-94 in column chromatographic separation were re-chromatographed by using chloroform and methanol as eluent. Gradient elution was carried out by increasing the polarity with methanol in 10% stepwise to 100% methanol. The re-chromatographed fractions numbers 15 to 25 were combined and evaporated to dryness to result a pale yellow amorphous powder. The isolated material was further examined by different physico-chemical techniques for its structure elucidation.

3.3. Results

3.3.1. Phytochemical study of *Kaempferia rotunda*

The concentrated methanol extract obtained from the shade-dried rhizome was fractionated successively with distilled water, petroleum ether, and ethyl acetate. It was observed that methanolic extract and the ethyl acetate fraction exhibited significant pharmacological activities under this investigation, so they were subjected to phytochemical analysis.

The preliminary phytochemical group tests indicated the presence of steroids, flavonoids, tannins, triterpenoid, volatile oil and saponins (Table 3.1). The thin layer chromatographic study of the methanol extract and ethyl acetate fraction showed the presence of components with selected mobile phase and spraying reagents (Table 3.2 and

3.3). Ethylacetate fraction was subjected to column chromatographic separation on Silicagel (60-120mesh) column in which sixty-eight fractions were collected. The fractions having identical results were mixed together (Table 3.4). They were purified, which yielded 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 & 3.6. The chemical nature of the isolated compound was further characterized from its physical parameters and spectral (UV, IR, Mass, and ^{1}H NMR) data[19,20].

Compound I was obtained as yellow needle shaped crystal, soluble in methanol. The compound was melted at 138-139°C. The compound showed strong absorption at 242nm in its spectrum, which implied the presence of phenolic aromatic rings. The UV absorption spectrum of compound I is shown in figure 3.2. The IR spectrum of the compound is presented in figure 3.3 The IR spectrum shows the presence of absorption bands at 3424, 2852 and 1070 cm^{-1}. Other prominent peaks were 1272, 1373, 1452, 1178, 1070, 756, 712 cm^{-1}. The IR spectrum confirmed the presence of aromatic ring (2923 cm^{-1}) and hydroxyl group (3424 cm^{-1}) in compound I. The ^{1}H NMR spectrum of compound I is presented in figure 3.4. In the ^{1}H NMR spectra in DMSO-d_6, the signals for aromatic proton were observed at δ6 (J=2MHZ) δ 7.7 (J=4MHZ). The signals at δ 5.75 (J=1.8MHZ) and δ 5.79 (J=1.8MHZ) signified the presence of aromatic two protons. The signals at 2x6.96 (J=2MHZ) indicates the presence of two aromatic protons and δ 7.70 (J=3MHZ) signified the presence of another two aromatic protons. The signals at δ 3.73 (J=3MHZ) signify the presence of three methoxy groups. The signals at δ 8.17 (J=6MHZ) and δ 7.39 (J=3MHZ) indicated the presence chalcone i.e. HC=CH–C=O. The signal at δ 6.69 (J=3MHZ) indicates the presence of free hydroxyl groups. The mass spectrum in fig. 3.5. of the compound I is in agreement with the assigned structure. The molecular ion peak (base peak, M^+) at m/z 312.2670 (calculated for $C_{18}O_5H_{18}$, 314.1154) and the fragmentation peaks at m/z 153, m/z 165 revealed the empirical formula of $C_{18}O_5H_{18}$. The result is corresponding to the molecular formula $C_{18}O_5H_{18}$. It has been concluded that the structure of the isolated compound I was established according to combined spectral data i.e. 2- hydroxy, 4, 4′, 6- trimethoxy chalcone.

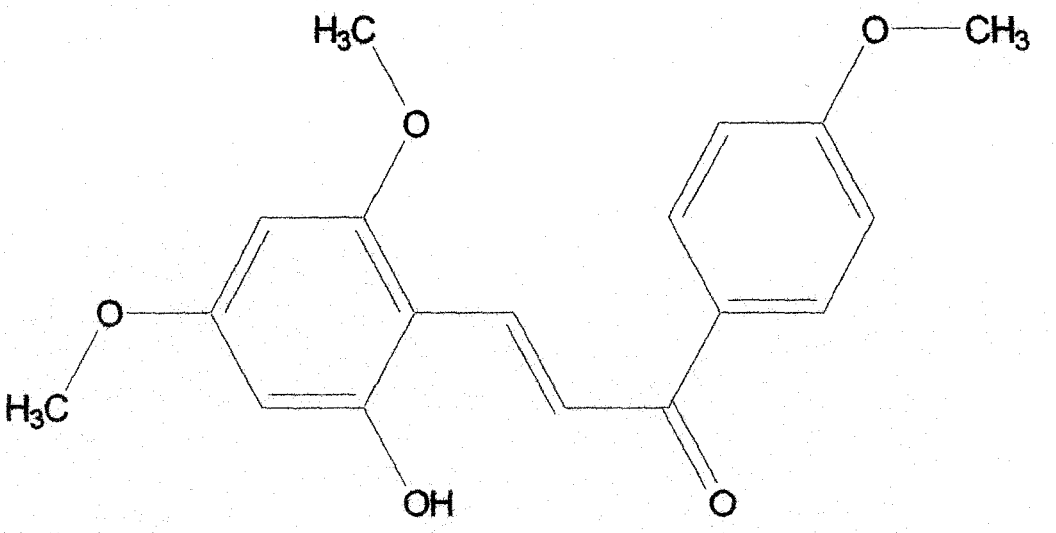
3.3.2. Phytochemical study of *Eupatorium cannabinum*

The concentrated methanol extract prepared from the shade-dried leaves of *E. cannabinum* was fractionated successively with distilled water, petroleum ether and

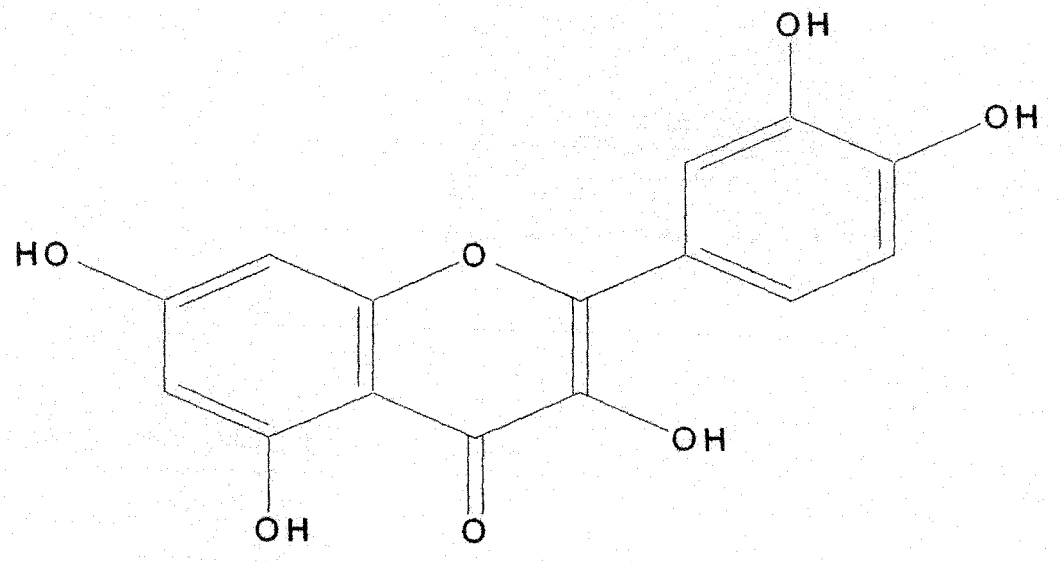
ethyl acetate. Different extract were undertaken for preliminary pharmacological studies. It was observed that the methanol extract and ethyl acetate fraction exhibited significant pharmacological activities. So they were subjected to phytochemical analysis.

The preliminary phytochemical group tests indicated the presence of flavonoids, tannins, saponins and alkaloids (Table 3.7). The TLC study of methanol extracts and ethyl acetate fraction showed the components with selected mobile phase and spraying reagents (Table 3.8 and 3.9). Ethylacetate fraction from methanolic extract was subjected to column chromatography, which yielded 103 fractions (Table 3.10). The fractions having identical hRf values were mixed together (Table 3.9). They were purified with a silica gel column, by using chloroform and methanol as mobile phase, which yielded (compound II). It was further subjected to chemical tests and TLC to confirm the chemical nature and was depicted in (Table 3.10 & 3.11). The chemical nature of the isolated compound was further characterized by physical parameters and spectral (UV, IR, Mass, ^{13}C and ^{1}H NMR) data[24, 25].

Compound II was obtained as pale-yellow amorphous powder, partially soluble in water & soluble in methanol. The compound was melted at 210-214°C. The UV spectrum showed two major absorption bands at 256 and 372nm which are typical for flavonols (Fig. 3.6). The IR spectrum shows the presence of absorption bands at 3648- 3611 cm^{-1} ν (OH), 3406 cm^{-1} ν (C=O) in flavone, 1610-1507 cm^{-1} ν (aromatic rings), 1382 cm^{-1}, 1263 cm^{-1}, 1132 cm^{-1}, 941 cm^{-1} and 700cm^{-1} (Fig. 3.7). In the ^{1}HNMR spectra (Fig. 3.8) in DMSO-d_6, showed two distinctive resonance groups. It displayed two doublets at δ7.78 (1H, J=1.8) and δ6.95 (1H, J=9HJ) and one doublet of doublets at δ at 7.65 (1H,J=9,1.8HZ) and δ, characteristics of a 1, 2, 4 trisubstituted benzene ring and two doublets at δ6.41 (1H, J=2HZ) and δ 6.26 (1H, J=2HZ) characteristics of a 1, 2, 3, 5-tetrasubstituted benzene ring. The isolated compound II showed significant peak in its mass spectra in fig. 3.9. The molecular ion peak (base peak, M^+) at m/z 302 and the fragmentation peaks at m/z 153, m/z 285 revealed the empirical formula of $C_{15}H_{10}O_7$. The UV, ^{1}H NMR and EI-MS data led to the identification of the compound II corresponds to be molecular formula $C_{15}H_{10}O_7$ and the structure of the isolated compound II was 3′,4′,5,7- tetrahydroxy flavonol or quercetin.



Structure of compound I



Structure of compound II

Table 3.1. Preliminary phytochemical tests of extract of *K. rotunda* rhizome

Kaempferia rotunda		
Phytoconstituents	**Methanolic extract**	**Ethyl acetate fraction**
Alkaloids	-	-
Amino acids	-	-
Proteins	-	-
Reducing sugars	-	-
Steroids and Triterpenoids	+	+
Flavonoids	+	+
Tannins	-	-
Saponins	+	-
Anthraquinones	-	-
Gums and Mucilages.	-	-
Volatile oil	+	-

'+ve' indicates presence and '-ve' indicates absence of the phytoconstituents.

Table 3.2. TLC study of methanolic extract of *K. rotunda* rhizome

Solvent system	Colour of the spot under UV light	Colour of the spot after spraying	hRf values	Constituent present
Ethyl acetate : Formic acid : Glacial-acetic acid : Water (100:11:11:2)	Yellow	Deep brown (NP-PEG reagent)	87	Flavonoids
Chloroform : Methanol : Water (64:50:10)	Nil	Blue (VS reagent)	65	Saponin
Toluene : Ethyl acetate (93:7)	Blue	Light green (AS reagent)	44	Essential oil
Hexane : Ethyl acetate (1:1)	Red	Violet ($SbCl_3$)	75	Triterpenoids
Butanol : Acetic acid : Water (4:1:5)	Dark purple	Pink (VS reagent)	52	Tannins

NP-PEG reagent : Natural product polyethylene glycol reagent

VS reagent : Vanillin- sulphuric acid reagent

AS reagent : Anisaldehyde- sulphuric acid reagent

$SbCl_3$: Antimony trichloride reagent

Table 3.3. TLC study of ethylacetate fraction of the methanolic extract of *K. rotunda*

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	Dark brown	Dark brown	Dark brown	Dark brown	Dark brown	Dark brown	87	89	90
2	Yellow	Yellow	Yellow	Yellow florescence	Yellow florescence	Yellow florescence	68	39	51
3	Brown	Brown	Brown	Brown	Yellow	Brown	59	78	45

TLC 1 : Glacial acetic acid: Water (15:85).

TLC 2 : n-Butanol: Glacial acetic acid: Water (4:1:5)

TLC 3 : Benzene-ethyl acetate-formic acid (9:7:4)

Spray reagent : 5% solution of 2-aminoethyl diphenyl borinate in methanol

Table 3.4. Column and Thin layer chromatographic separation of ethyl acetate fraction of *K. rotunda*.

Eluent Hexane:Ethyl acetate	Fraction number	Residue of selective fraction (gm)	Colour of TLC spots with hR_f values	Inference
100:0	1-10	4.2	NIL	Fatty solid
90:10	11-18	3.6	NIL	Fatty solid
80:20	19-26	3.1	NIL	Fatty solid
70:30	27-31	3.7	NIL	Fatty solid
60:40	32-35	10.1	NIL	Greenish semisolid
50:50	36-41	11.3	NIL	Dark green semisolid
40:60	42-46	5.5	Two yellowish spots (88, 82)	Mixture of compounds
30:70	47-52	6.0	Two bright yellow spots (77, 88)	Mixture of compounds
20:80	53-56	3.5	Two yellowish spots (79, 84)	Mixture of compounds
10:90	57-62	3.5	Three brown spots (87, 69)	Mixture of compounds
0:100	63-68	2.0	Nil	Colourless solids

Spray reagent : 1% Aluminium chloride in ethanol

Table 3.5. Qualitative analysis of the compound I isolated from ethyl acetate fraction of *K. rotunda*[21-23]

Sl. No.	Treatments	Observations	Inferences
1	1 mg of the crystalline solid was dissolved in 0.5 ml of methanol and treated with 1 ml of dilute ammonia solution	A bright yellow colour	Presence of flavonoid
2	1 mg of the crystalline solid was dissolved in 1 ml of methanol and treated with 1 ml of 0.1 N sodium hydroxide solutions	A bright yellow colour	Presence of flavonoid
3	1 mg of the crystalline solid was dissolved in 1 ml of methanol and treated with 1 ml of dilute sodium carbonate solution	A bright yellow colour	Presence of flavonoid
4	1 mg of the crystalline solid was dissolved in 1 ml of methanol and allowed to react with 5% alcoholic ferric chloride solution	Yellowish green colour	Presence of flavonoid
5	1 mg of the crystalline solid was treated with 1 ml of concentrated hydrochloric acid	Yellowish green colour	Presence of flavonoid
6	1 mg of the crystalline solid was treated with magnesium hydrochloride solution (Shinoid's test)	Red to magenta colour	Presence of flavonoid

Table 3.6. TLC study of the compound I isolated from ethylacetate fraction of *K. rotunda*.

Solvent system	hR_f values	Colour of fluorescent produced		
		UV_{366nm}	UV_{366nm} +$AlCl_3$	UV_{366nm} +NH_3
TLC 1	88	Dark Brown	Dark Brown	Dark Brown
TLC 2	81	Yellow	Yellow	Yellow
TLC 3	69	Brown	Brown	Brown

TLC 1 : Glacial acetic acid: Water (15:85)

TLC 2 : n-Butanol: Glacial acetic acid: Water (4:1:5)

TLC 3 : Ethyl acetate: Formic acid: Water (10:2:3)

$AlCl_3$: Aluminium chloride in ethanol

NH_3 : Ammonia

Table 3.7. Preliminary phytochemical tests of extract of of *E. cannabinum*

Eupatorium cannabinum		
Phytoconstituents	**Methanol Extract**	**Ethyl acetate fraction**
Alkaloids	+	-
Amino acids	+	-
Proteins	-	-
Reducing sugars	-	-
Steroids and Triterpenoids	+	+
Flavonoids	+	+
Tannins	+	-
Saponins	+	-
Anthraquinones	–	-
Gums and Mucilages.	–	-
Volatile oil	+	-

'+ve' indicates presence and '-ve' indicates absence of the phytoconstituents

Table 3.8. TLC study of methanolic leaf extract *E. cannabinum*

Solvent system	Colour of the spot under UV light	Colour of the spot after spraying	hRf values	Constituent present
Ethyl acetate : Formic acid : Glacial acetic acid : Water (100:11:11:2)	Yellow	Deep Brown (NP-PEG reagent)	85	Flavonoids
Chloroform : Methanol : Water (64:50:10)	Nil	Blue (VS reagent)	60	Saponin
Toluene : Ethyl acetate (93:7)	Blue	Light green (AS reagent)	44	Essential oil
Hexane : Ethyl acetate (1:1)	Red	Violet ($SbCl_3$)	73	Triterpenoids
Butanol : Acetic acid : Water (14:1:5)	Dark purple	Pink (VS reagent)	51	Tannins
Toluene: Ethyl acetate: Diethylamine (70:20:10)	Nil	Orange (DRG)	25	Alkaloid

NP-PEG reagent: Natural product polyethylene glycol reagent

VS reagent : Vanillin- sulphuric acid reagent

AS reagent : Anisaldehyde- sulphuric acid reagent

$SbCl_3$: Antimony trichloride reagent

DRG : Dragendorff reagent

Table 3.9. TLC study of ethyl acetate fraction of *E. cannabinum.*

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	Orange	Orange	Orange	Bright yellow	Bright yellow	Bright yellow	64	51	66
2	Orange	Orange	Orange	Orange florescence	Orange florescence	Orange florescence	58	31	39
3	Yellow	–	Yellowish green	Yellow	Yellow	Yellowish green	42	47	38

TLC 1 : Chloroform : Ethyl acetate (6:4)

TLC 2 : n-Butanol : Glacial acetic acid : Water (4:1:5)

TLC 3 : Ethyl acetate : Formic acid : Water (10:2:3)

Spray reagent : 5% solution of 2-aminoethyl diphenyl borinate in methanol

Table 3.10. Column and Thin layer chromatographic separation of ethylacetate fraction of *E. cannabinum*

Eluent Hexane: Ethyl acetate	Fraction number	Residue of selected fractions (gm)	Colour of TLC spots with hR_f values	Inference
100:0	1-12	4.1	NIL	Fatty solid
90:10	14-20	3.2	NIL	Fatty solid
80:20	21-28	3.7	NIL	Fatty solid
70:30	29-32	7.3	NIL	Greenish semisolid
60:40	33-35	8.5	NIL	Greenish semisolid
50:50	36-40	9.3	NIL	Dark yellow semisolid
40:60	41-48	5.3	Two yellowish spots (70, 45)	Mixture of compounds
30:70	49-60	4.5	Two bright yellow spots (70, 44)	Mixture of compounds
20:80	61-65	3.6	Two yellowish spots (69, 43)	Mixture of compounds
10:90	65-94	4.2	Three red spots (67, 55, 29)	Mixture of compounds
0:100	94-103	2.0	Nil	Colourless solid

Spray reagent: 1% Aluminium chloride in ethanol

Table 3.11. Qualitative analysis of compound II from ethylacetate fraction of *E. cannabinum*

Sl.No	Treatment	Observation	Inference
1	1mg of the amorphous power was dissolved in 0.5 ml of methanol and treated with 1 ml of diluted ammonia solution	A bright yellow colour	Presence of flavonoid
2	1 mg of the amorphous power was dissolved in 1 ml of methanol and treated with 1 ml of 0.1 N sodium hydroxide solutions	A bright yellow colour	Presence of flavonoid
3	1 mg of the amorphous power was dissolved in 1 ml of methanol and treated with 1 ml of diluted sodium carbonate solution	A bright yellow colour	Presence of flavonoid
4	1 mg of the amorphous power was dissolved in 1 ml of methanol and allowed to react with 5% alcoholic ferric chloride solution	Yellowish green colour	Presence of flavonoid
5	1 mg of the amorphous power was treated with 1 ml of concentrated hydrochloric acid	Yellowish with green florescence	Presence of flavonoid
6	1 mg of the amorphous power was treated with magnesium hydrochloride solution (Shinoid's test)	Red to magenta red colour	Presence of flavonoid

Table 3.12. TLC of the compound II isolated from ethyl acetate fraction of *E. cannabinum*.

Solvent system	hR_f values	Colour of fluorescent produced			
		UV_{366nm}	UV_{366nm} +$AlCl_3$	UV_{366nm} +NH_3	UV_{366nm} +ADB
TLC 1	64	Orange	Orange	Orange	Orange
TLC 2	59	Yellow	Bright Yellow	Bright Yellow	Bright Yellow
TLC 3	41	Yellow	Yellow	Yellow	Yellow

TLC 1 : Chloroform: Ethyl acetate (6:4)

TLC 2 : n-Butanol: Glacial acetic acid: Water (4:1:5)

TLC 3 : Ethyl acetate: Formic acid: Water (10:2:3)

$AlCl_3$: Aluminium chloride in ethanol

NH_3 : Ammonia

ADB : 5% solution of 2- aminoethyl diphenyl borinate in methanol

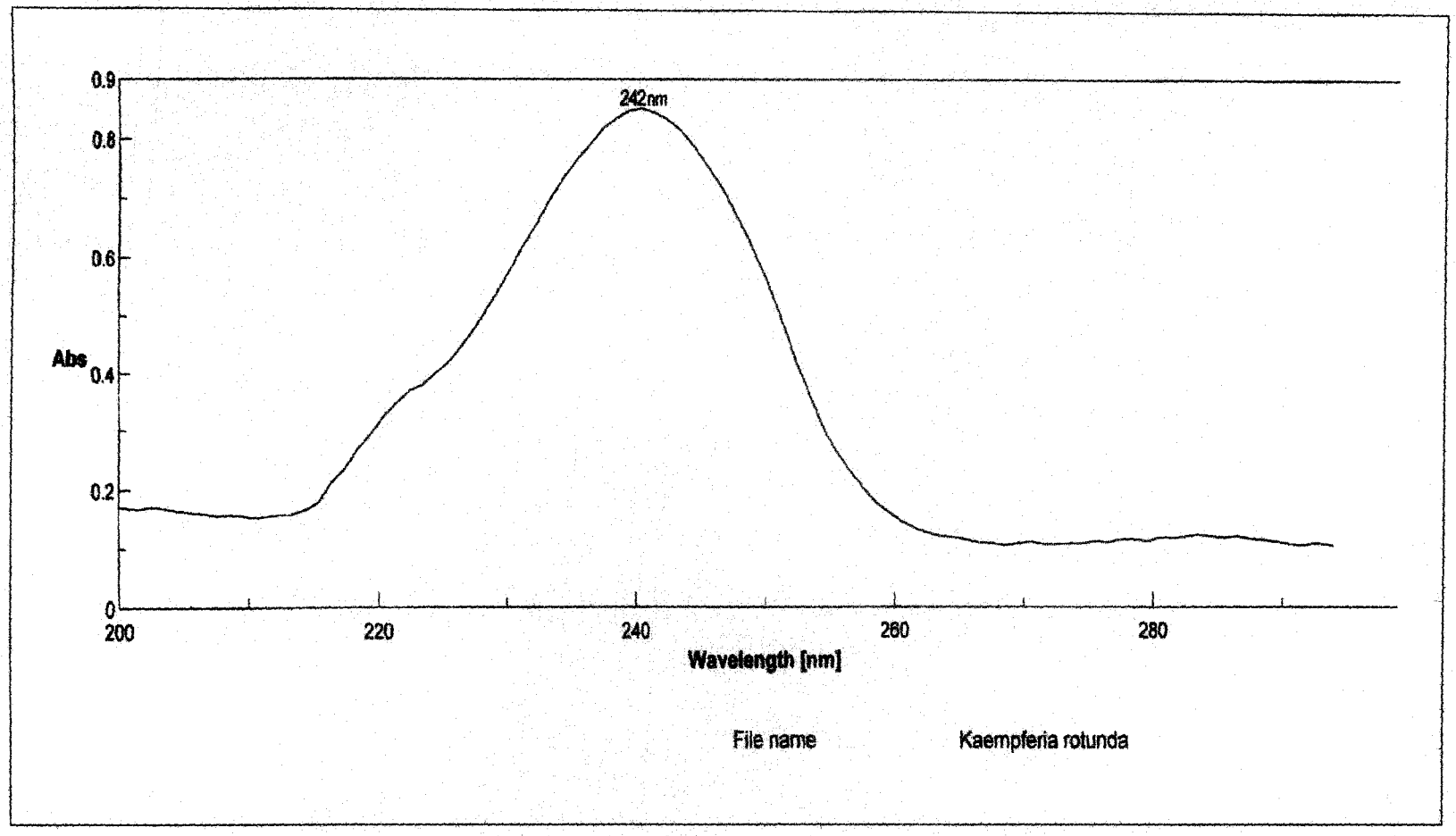


Figure 3.2. UV-Visible Spectro photometric analysis of compound I from *K. rotunda*.

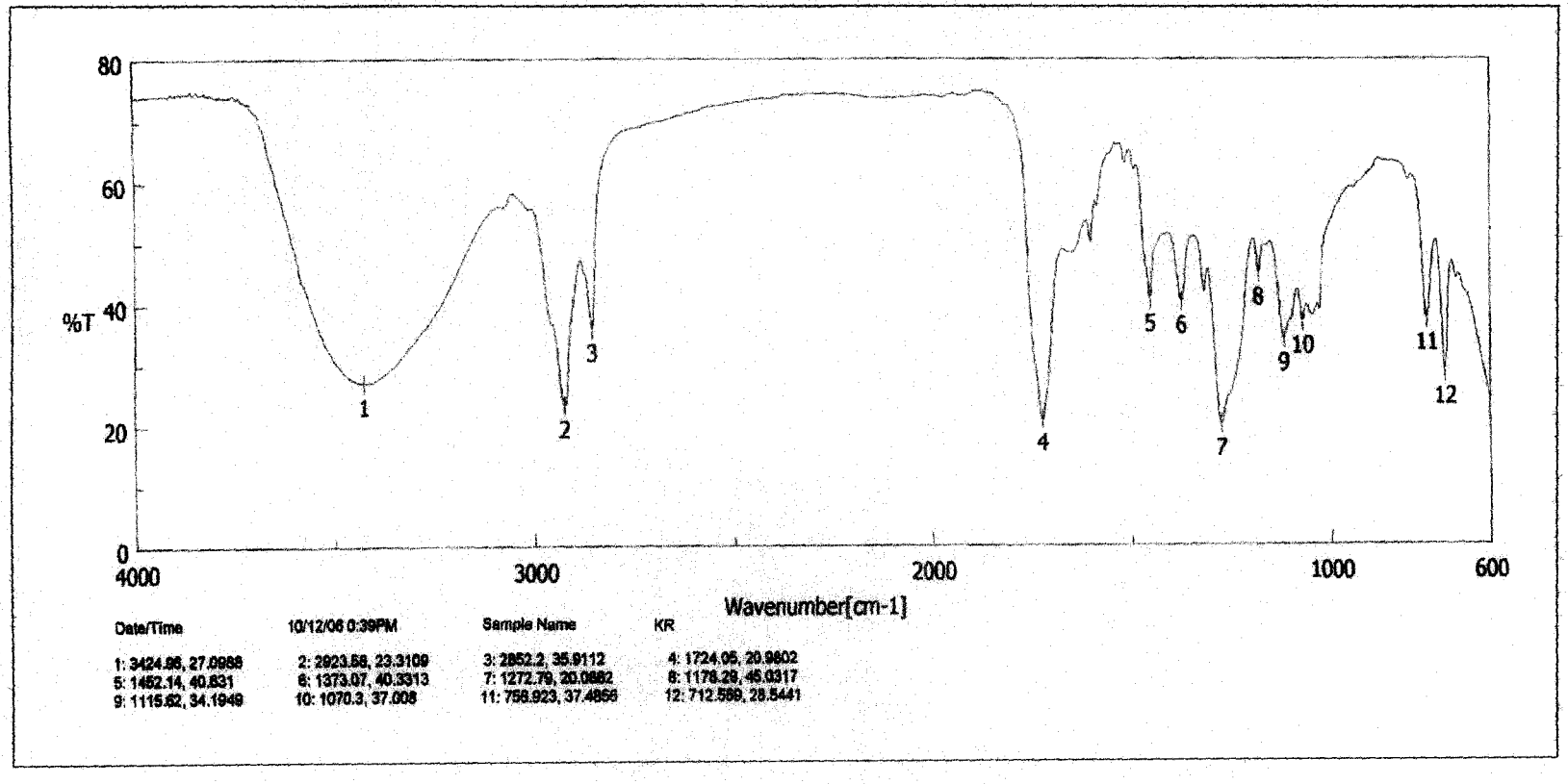


Figure 3.3. IR spectrum of compound I isolated from *K. rotunda*.

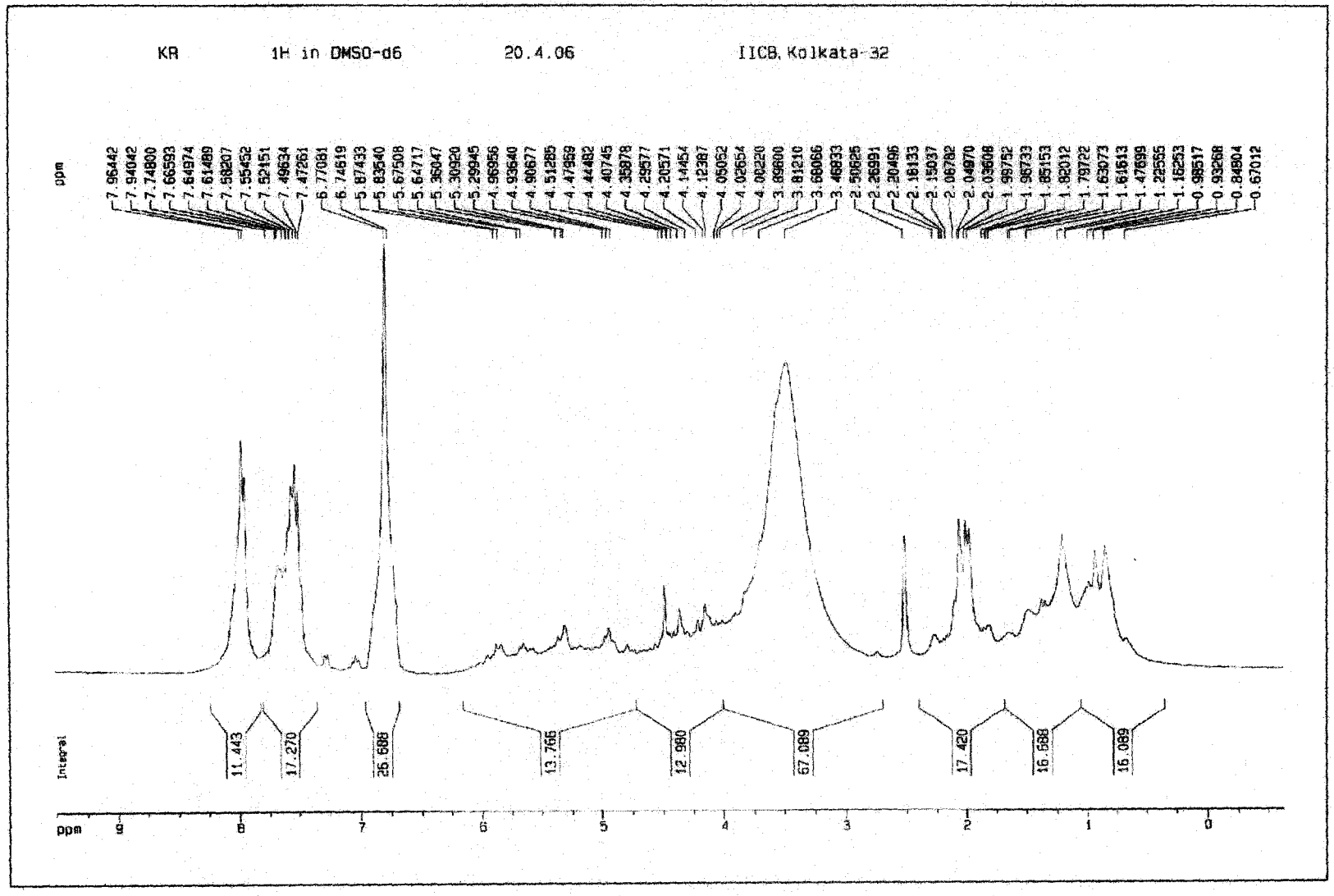


Figure 3.4. ^{1}HNMR spectrum of compound I isolated from *K. rotunda*.

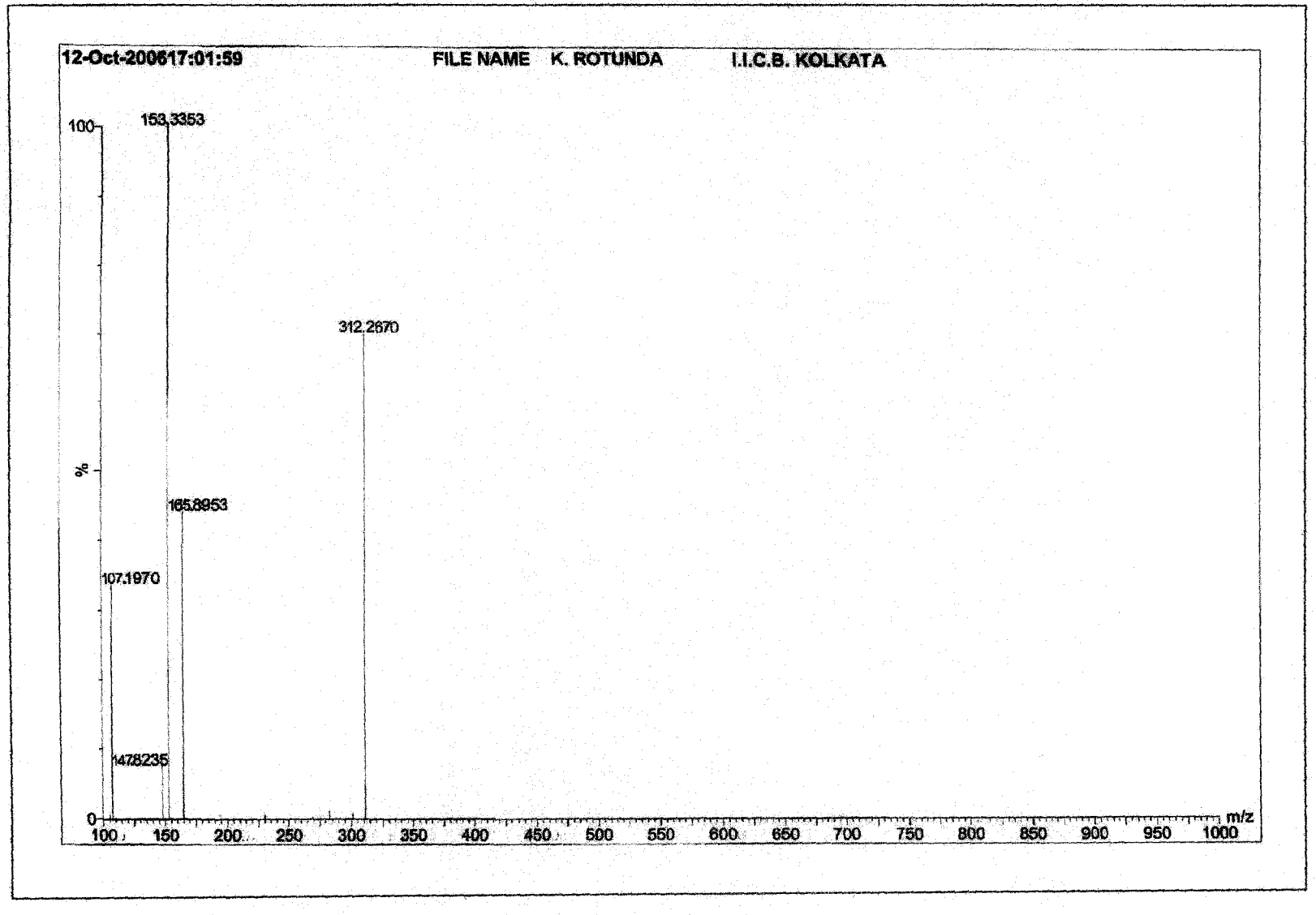


Figure 3.5. Mass spectrum of compound I isolated from *K. rotunda*.

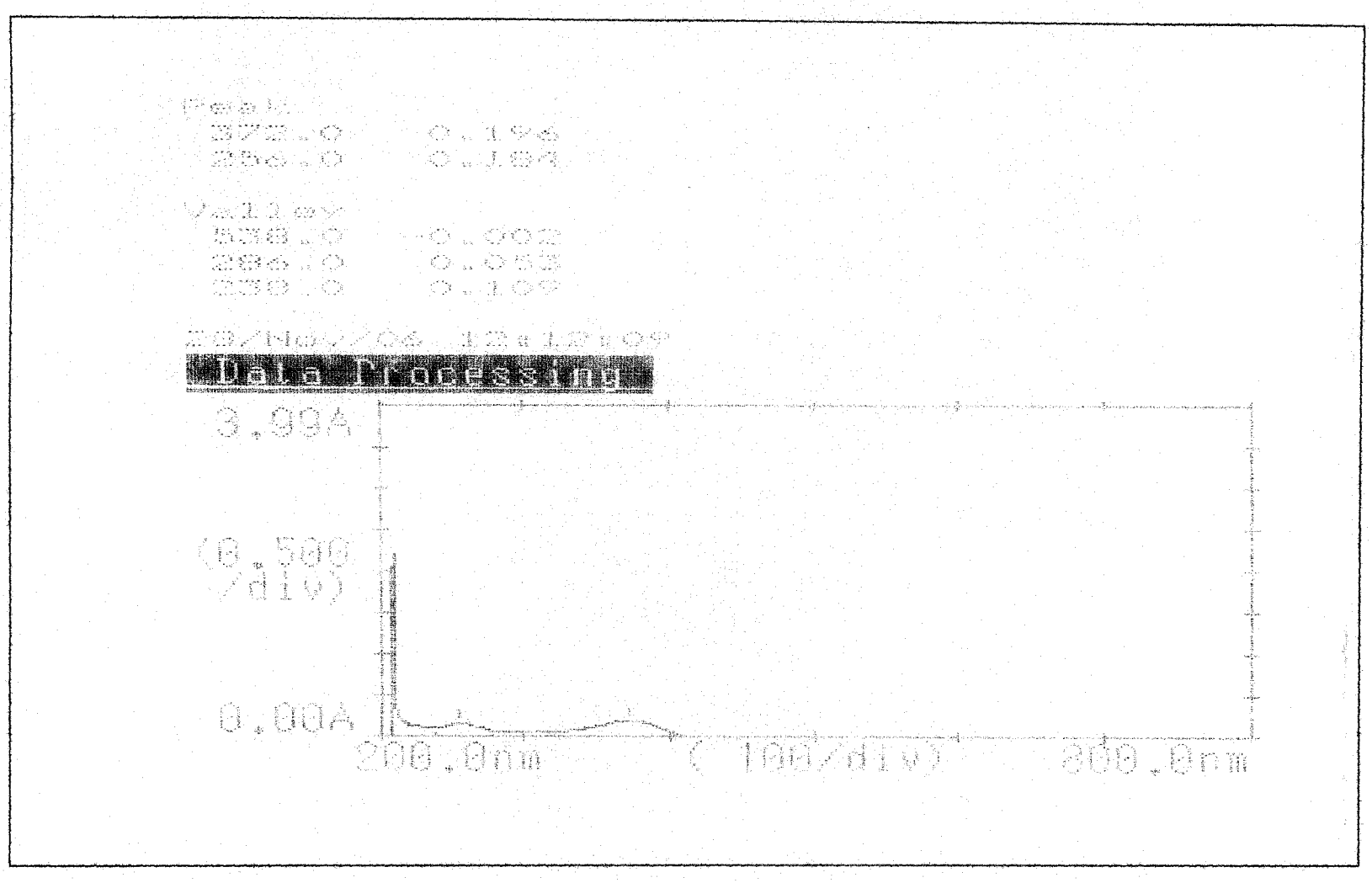


Figure 3.6. UV-Visible Spectro photometric analysis of compound II from *E. cannabinum*.

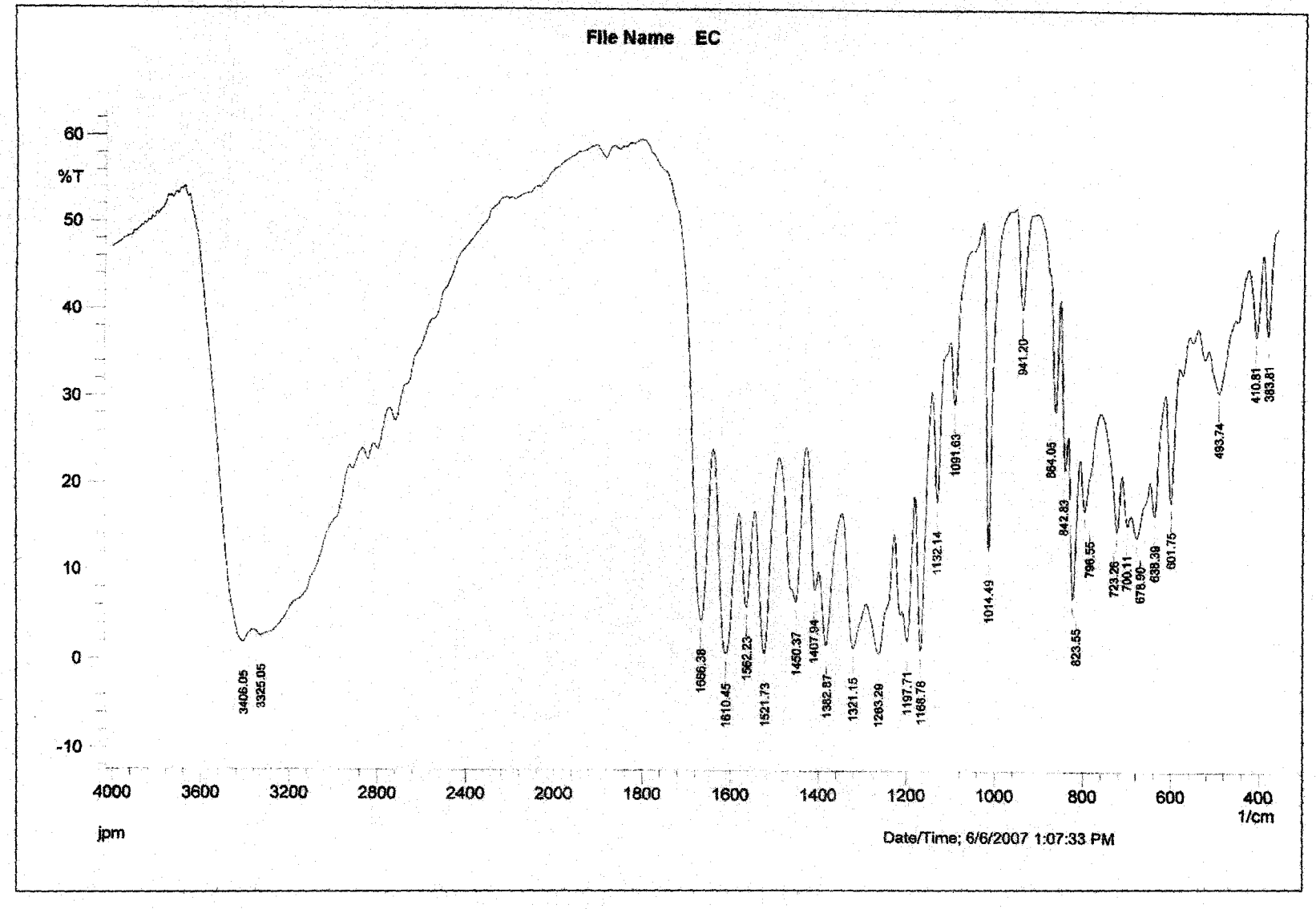


Figure 3.7. IR spectrum of compound II isolated from *E. cannabinum*.

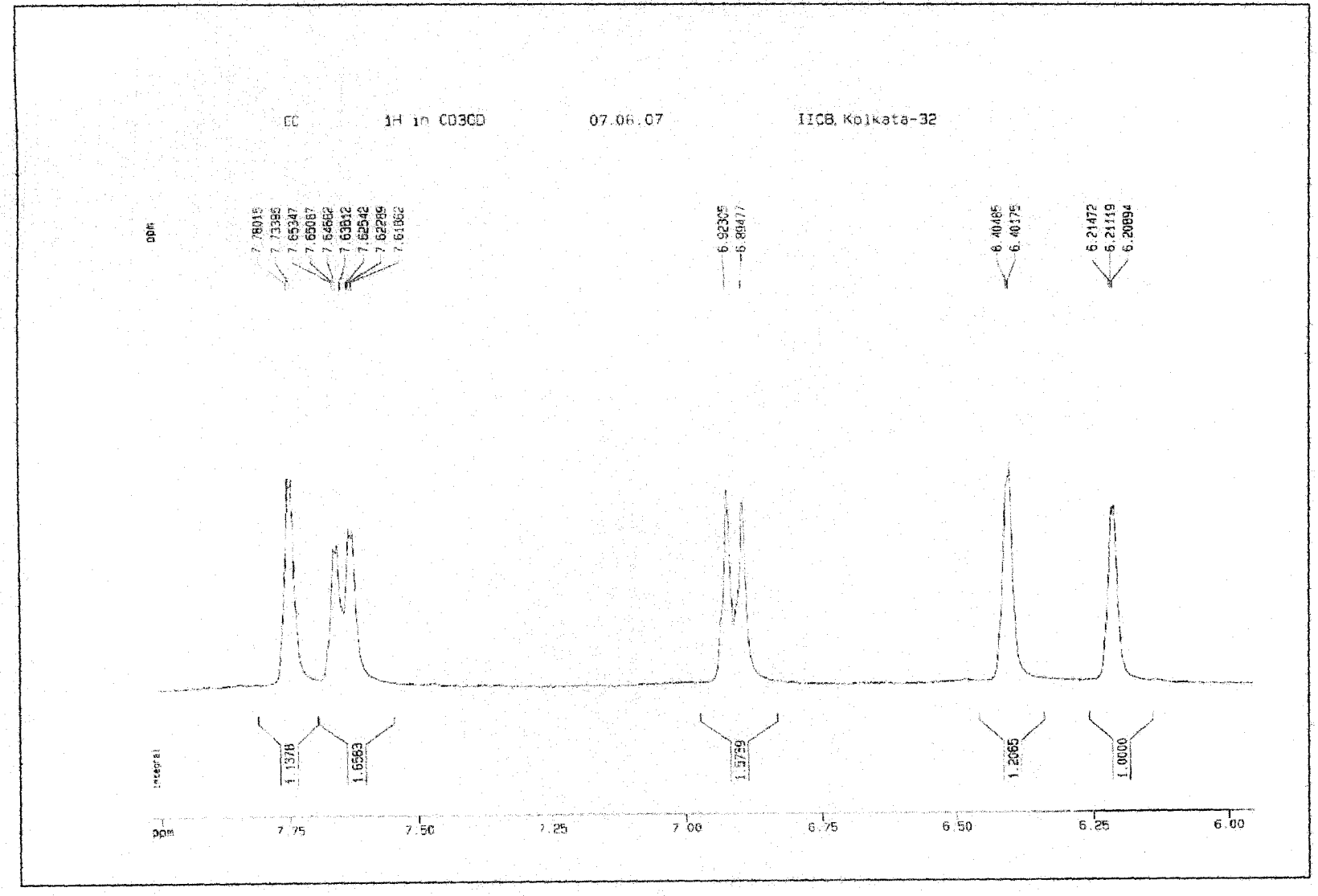


Figure 3.8. ^{1}H NMR spectrum of compound II isolated from *E. cannabinum*.

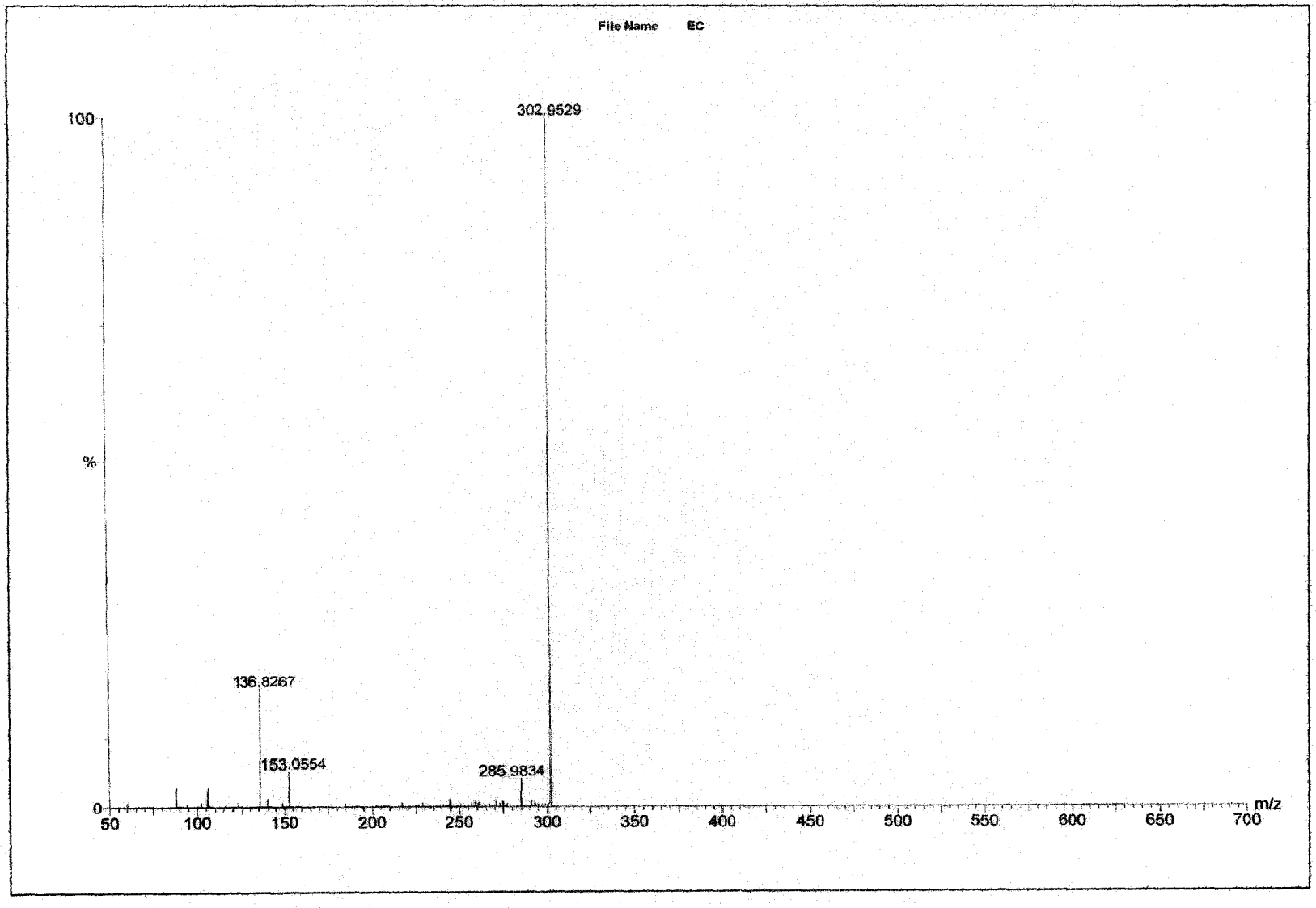


Figure 3.9. Mass spectrum of compound II isolated from *E. cannabinum*.

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