CHAPTER 3

PHYTOCHEMICAL STUDIES

3.1. Introduction

The phytochemical analysis require fresh plant tissues and the plant materials under investigation should be plunged into boiling alcohol within minutes of its collection. 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 [1]. The Flavonoids and Flavonols are also widely distributed in plants both as co-pigment to anthocyanins in petals and also in leaves of higher plants. The flavonols occur most frequently in glycosidic combination like anthocyanins. Alkaloids, steroids, triterpenoids, saponins are also present in plants [1].

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 crystallisation 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.

Flavonoids are a group of polyphenolic compounds, which are widely distributed through out the plant kingdom. They occur as aglycones, glycosides and methylated derivatives. The flavonoid aglycone consists of a benzene ring condensed with another six membered ring, in which the 2-position carries a phenyl ring as a substituent. Six membered ring condensed with the benzene ring is either a α-pyrone (flavonols and flavonones) or its dihydroderivative (flavanols and flavanones). The position of the benzoid substituent divides the flavonoid class into flavonoids (2-position) and isoflavonoids (3-position). Flavonols differ from flavonones by hydroxyl group at the 3-position and a C_2-C_3 double bond [2]. Flavonoids are often hydroxylated in position 3, 5, 7, 2', 3', 4', 5'. Methylethers and acetylesters of the alcohol group are known to occur in nature. When glycosides are formed, the glycosidic linkage is normally located in positions 3 or 7 and the carbohydrate can be L-rhamnose, D-glucose, glucorhamnose, galactose or arabinose [3].

Flavonoids are characteristic constituents of green plants with the exception of algae and hornworts. They virtually occur in all parts of plants including leaf, root, wood, bark, pollen, flowers, berries and seeds. In a few reported cases flavonoids are being found in animals. For examples in the beaver scent gland, propolis (bee secretion) and in butterfly wings, it is considered that the flavonoids originate from the plants upon which the animals feed rather than being biosynthesized *in situ*. The distribution of flavonoids in the plant kingdom is confined mainly in angiosperms. It is reported that flavonoids are being

restricted to plant groups with a degree of complexity of the Bryophyta or higher [1]. 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 root of the plants under investigation.

3.2. Materials and Methods

3.2.1. Plant material

The leaf and root of *Colebrookea oppositifolia* (*C.oppositifolia*) Smith and *Heracleum nepalense* (*H.nepalense*) D.Don were collected from the southern district of Sikkim. It was authenticated at Botanical survey of India, Gangtok, Sikkim. The voucher specimen was preserved in our laboratory for future reference. The leaf of *C.oppositifolia* and root of *H.nepalense* were dried in shade, pulverized in mechanical grinder and passed through 40-mesh sieve to get the powder of the plant parts.

3.2.2. Extraction procedure

Coarsely powered dry leaf of *Colebrookea oppositifolia* (1kg) as well as the powered dried root of *Heracleum nepalense* (1kg) were extracted separately by cold percolation with 3 liters of 70% methanol in a percolator for 72 h at room temperature [4]. The residue was removed by filtration. The solvent was then evaporated to dryness under reduced pressure in an Eyela Rotary Evaporator (Japan) at 42-45°C. The concentrated extract of the leave and root were kept in dessicator for further use.

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, ethyl acetate and acetone. The ethyl acetate soluble fraction was subjected to thin layer chromatographic analysis. The aqueous, acetone 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 Figure 3.1.

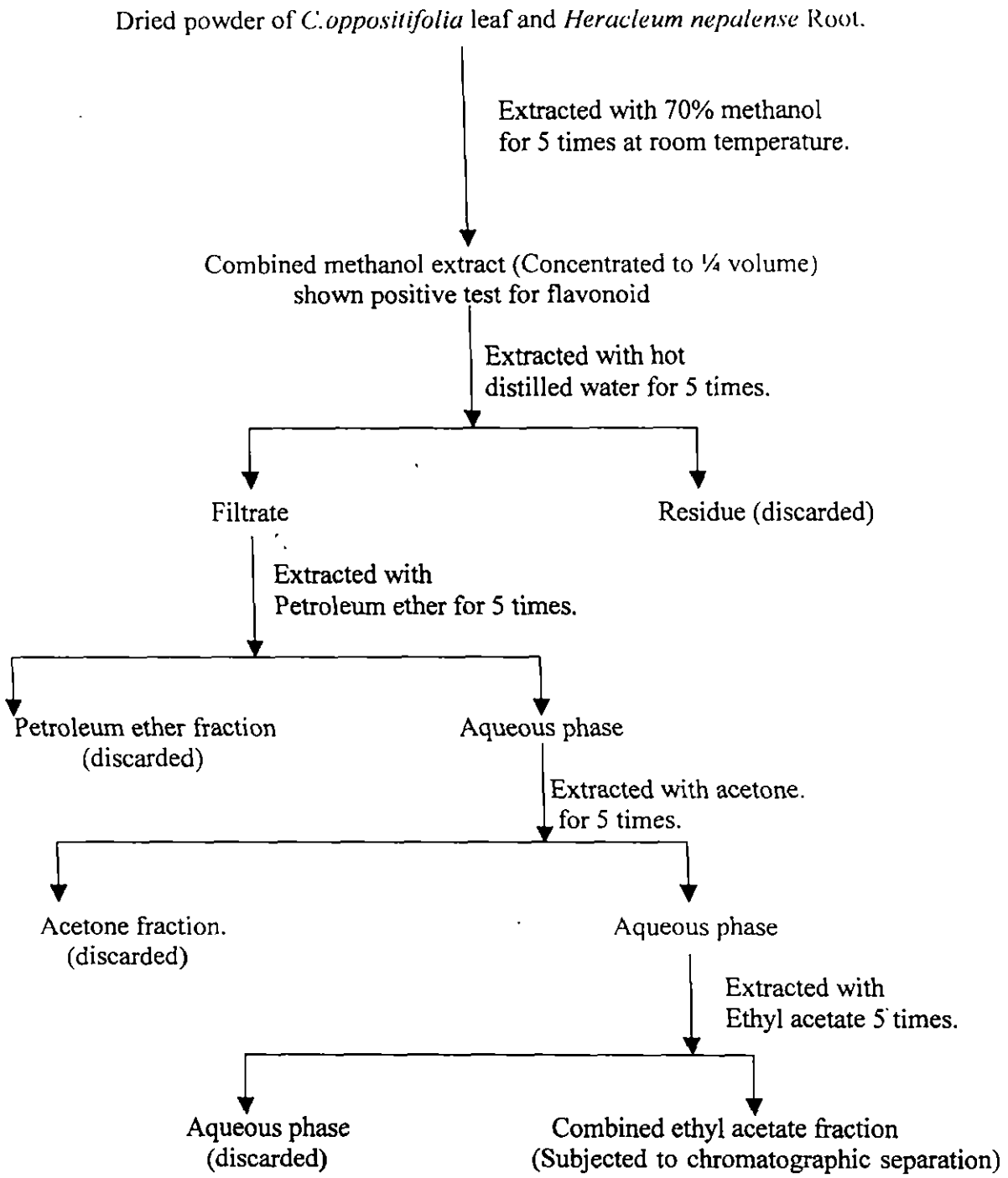


Figure 3.1. Flow chart showing the method of extraction of *Colebrookea oppositifolia* leaf and *Heracleum nepalense* root.

3.2.3. Isolation and purification of phytoconstituent from the leaf of *C. oppositifolia*

3.2.3.1. Preliminary phytochemical Test

The preliminary phytochemical group test of *C. oppositifolia* leaf extract was performed by the standard methods [5-7].

Tests for Alkaloids

- Small quantity of the leaf extract of *C.oppositifolia* 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 *C.oppositifolia* 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 *C.oppositifolia* 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 *C.oppositifolia* 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 *C.oppositifolia* was dissolved in a few ml of distilled water and treated with Ninhydrin reagent at pH 5.0. The absence of purple coloration suggested the negative test for amino acids.
- Small quantity of leaf extract of *C.oppositifolia* 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 *C.oppositifolia* was treated with 1.0 ml of concentrated nitric acid. The sample was boiled and cooled. Few drop of 40% sodium hydroxide was added drop by drop. Appearance of orange coloured solution indicated the presence of proteins.

- A small quantity of leaf extract of *C.oppositifolia* 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 *C.oppositifolia* was dissolved in minimum amount of distilled water and filtered. To the filtrate equal volume of Bendict'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 *C.oppositifolia* was dissolved in minimum amount of distilled water and filtered. To the filtrate equal volume of Fehling's A&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 *C.oppositifolia* 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 *C.oppositifolia* 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 *C.oppositifolia* 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. Development of yellow colour in all the tubes demonstrated the presence of flavonoids.

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

Test for Tannins

- Small quantity of leaf extract of *C.oppositifolia* 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 of leaf extract of *C.oppositifolia* 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 leaf extract of *C.oppositifolia* 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 leaf extract of *C.oppositifolia* 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 Saponins

- Small quantities of leaf extract of *C.oppositifolia* 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 leaf extract of *C.oppositifolia* 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 Anthraquinones

- Small quantity of leaf extract of *C.oppositifolia* 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. Formation of rose pink colouration indicated the presence of anthraquinones.

Test for Gums and Mucilage

- Small quantity of leaf extract of *C.oppositifolia* 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). Formation of red-violet ring at the junction of the two layers indicated the positive test for gums (Molish's test).
- Small quantity of leaf extract of *C.oppositifolia* was dissolved in minimum amount of distilled water and filtered. The filtrate was treated with 95% alcohol. Formation of precipitation indicated the presence of gums and mucilage.
- Small quantity of leaf extract of *C.oppositifolia* was dissolved in minimum amount of distilled water and filtered. The filtrate was treated with 0.008gm of ruthenium red in 10 ml of 10 % solution of lead acetate. Formation of red colour indicated the presence of gum and mucilage.

3.2.3.2. Thin Layer Chromatography of the methanol leaf extract of *C.oppositifolia*.

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

3.2.3.2.1. Solvent systems used

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

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

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

3.2.3.2.2. Spray reagents used

1% Aluminium chloride in ethanol.

Ammonia solution.

5% solution of 2-aminoethyl diphenyl borinate in methanol.

3.2.3.3. Coloumn Chromatography of leaf extract of *C.oppositifolia*.

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

Benzene: Ethyl acetate (with increasing amount of ethyl acetate), Benzene: Methanol (with increasing amount of methanol).

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 benzene. The solvent (benzene) 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 benzene 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 ethyl acetate fraction of *C.oppositifolia* leaf

The excess solvent on the top of the coloumn was allowed to flow down and then the dried mixture of ethyl acetate 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 benzene and 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 ethyl acetate and increasing the polarity with methanol in 10% stepwise elutions to 100% methanol. 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, yellowish 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 ethyl acetate fraction of *C.oppositifolia* 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 ethyl acetate fraction of *C.oppositifolia* 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 ethyl acetate fraction of *C.oppositifolia* 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. Glacial acetic acid: Water (15:85).

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

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 366 nm (long wave) before and after spraying the spraying reagents or exposing with Ammonia.

3.2.3.7. Ultraviolet-Visible absorption spectral analysis of the compound isolated from ethyl acetate fraction of *C.oppositifolia* leaf

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. In addition, the siting on 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 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 500 nm. The spectra was also recorded with the addition of Sodium methoxide (NaOMe), 5% Aluminium chloride ($AlCl_3$), sodium acetate (NaOAc) powder and (NaOAc) powder with boric acid (H_3BO_3).

3.2.3.8. Infrared spectrum of the compound isolated from ethyl acetate fraction of *C.oppositifolia* 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 ethyl acetate fraction of *C.oppositifolia* leaf

The ^{1}H and ^{13}C NMR spectra of the isolated compound from ethyl acetate fraction of *Colebrookea oppositifolia* leaves 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 spectra of the compound isolated from ethyl acetate fraction of *Colebrookea oppositifolia* leaf

Mass spectrum ($FAB^{+}MS$) of the isolated compound from the leaves of *Colebrookea oppositifolia* was recorded on a JEOL JMS 600 Spectrophotometer

3.2.3.11. Elemental analysis of the compound isolated from ethyl acetate fraction of *C.oppositifolia* leaf

The isolated compound was dried at 60°C in a high vacuum for 8 hr and the elemental analysis was performed with Perkin Elmer 2400 elemental analyzer. In the analysis, the percentage of carbon, hydrogen and nitrogen was determined.

3.2.3.12. Sugar analysis of the compound isolated from ethyl acetate fraction of *C.oppositifolia* leaf

1 mg of the isolated compound was hydrolyzed by addition of 1% hydrochloric acid, boiled under reflux for 2 hr. The hydrosylate was analyzed for sugars (co-TLC) with standard sugars by using Ethyl alcohol: Ammonium hydroxide: Water (20:1: 4) as mobile phase [14]. They were visualized by spraying with aniline phthalate and heating at 105°C.

3.2.4. Isolation and Purification of Phytoconstituent from the root of *H.nepalense*

The methods used for isolation and purification of phytoconstituents from the root of *H.nepalense* were as per the procedure followed for the isolation and purification of phytoconstituents from the leaf extract of *C.oppositifolia* described in 3.2.3.1 to 3.2.3.12 expect 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 chloroform: 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 to result an isolated flavonoidal glycoside.

3.3. Results

3.3.1. Phytochemical Study of *C.oppositifolia* leaf

The concentrated methanol extract obtained from the shade-dried leaf of *C.oppositifolia* was fractionated successively with petroleum ether, acetone and ethyl acetate. It was observed that only the ethyl acetate fraction exhibited significant pharmacological

activities under perview of this investigation. So this fraction was subjected to phytochemical analysis.

The preliminary phytochemical group tests indicated the presence of steroids, flavonoids, proteins, tannins, saponins and reducing sugar (Table 3.1). The thin layer chromatographic study of the ethyl acetate fraction showed the presence of three components with selected mobile phase and spraying reagents (Table 3.2-3.4). 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 3.5). They were purified with a silica gel (60-120) coloumn, which yielded a flavonoidal glycoside (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 3.6 & Table 3.7. The chemical nature of the isolated compound was further characterized from its physical parameters and spectral (UV, IR, Mass, ^{13}C and ^{1}H NMR) data [8-12].

Compound I was obtained as yellow needle shaped crystal, soluble in water & methanol. The compound was melted at 181-185°C. The complete acid hydrolysis of compound I gave Catechin and Rhamnose. The compound showed strong absorption at 281 nm in its spectrum, which implied the presence of phenolic aromatic rings. The UV absorption spectrum of compound I is shown in Figure 3.1. The UV spectrum showed absorption bands reagents shifts of the compound to be a 7-substituted derivatives. The absence of free 7-hydroxyl group in the compound was observed in lack of shift of Band II in the presence of NaOAc (Table 3.8). The IR spectrum of the compound is presented in Figure 3.2. The IR spectrum shows the presence of absorption bands at 3480, 1029 and 1078 cm^{-1}. Other prominent peaks were 1866, 1771, 1632, 1606, 1519, 1456, 1286, 1146, 1113, 1029, 963, 731, 578 cm^{-1}. The IR spectrum confirmed the presence of aromatic ring (1632 cm^{-1}) and hydroxyl group (3480 cm^{-1}) in compound I.

The ^{1}H NMR spectrum of compound I is presented in Figure 3.3. In the ^{1}H NMR spectra in DMSO-d_6, the signals for aglycone were the same as for 7-O substituted catechin

(Table 3.9). The doublets at 6.212 ppm (J=2.0 MHz) and 6.226 ppm (J=2.0 MHz) in its ^{1}H NMR spectrum were suggestive of two aromatic protons existing at the *meta* positions[11-13]. The signals at 6.591 ppm (J=2.0 Hz, J=7.6 Hz), 6.681 ppm (J=7.6Hz) and 6.731 ppm (J=2.0 Hz) inferred the presence of another set of aromatic protons existing at 1,3,4 positions. The signals at 2.506 ppm and 2.699 ppm, and the double double doublet at 3.476 ppm suggested that the compound should be a flavan-3-ol derivative. Existence of a sugar in its structure was evidenced by the anomeric proton signal, which appeared at a 5.698 ppm (J= 3.0 Hz). The coupling constant (J= 3.0 Hz) of the anomeric proton sugar suggested that the phenoxy group be attached to the anomeric carbon atom by β-configuration. The fragmentation ion at m/z 291 in its mass spectrum, which is presented in Figure 3.5, inferred that the compound should have (+) – Catechin as an aglycone. The structure of the compound was further confirmed by ^{13}C NMR and elemental analysis of Catechin from the available literature [9, 13]. The signals of C-5, C-7 and C-9 were observed at higher value than 150 ppm, the signals at 156.75 ppm only showed correlations with these of H-6 and H-8 (at 6.212 ppm and 6.226 ppm respectively), we assigned it to C-7 (Figure 3.4). The signals at 155.68 ppm and 156.52 ppm showed correlations with those of H-6 and H-8 respectively, so we assigned them to C-5 and C-9 respectively. The correlation of the anomeric proton signals at 5.698 ppm with that of C-7 at 156.75 ppm suggested that the sugar should be attached at the C-7 position. In the elemental analysis of compound I, the percentage of carbon, hydrogen and nitrogen was found to be 54.12, 4.24 and nil respectively. The result is corresponding to the molecular formula $C_{24}H_{22}O_8$ (confirmed by elemental analysis). From these data we concluded that the structure of the isolated flavonoid compound I is (+)–Catechin-7-O-β-rhamnopyranoside.

Table 3.1. Preliminary phytochemical test of methanol extract of *C.oppositifolia* leaf and *H.nepalense* root.

	C.oppositifolia		*H.nepalense*	
Phytoconstituents	Methanol Extract	Ethyl acetate Fraction	Methanol Extract	Ethyl acetate Fraction
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 ethyl acetate fraction of the methanol extract of *C.oppositifolia* leaf.

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	Bright yellow	Bright yellow	Bright yellow	Bright yellow	Bright yellow	Bright yellow	83	59	58
2	Yellow	Yellow	Yellow	Yellow Florescence	Yellow Florescence	Yellow Florescence	64	29	52
3	–	–	Green	–	–	Yellowish green.	–	–	41

Spray reagent: 1% Aluminium chloride in ethanol.

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

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

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

Table 3.3. Thin Layer Chromatography study of ethyl acetate fraction of the methanol extract of *C.oppositifolia* leaf.

No of Spots	Colour of spots under long wave UV light			Colour of spots under long wave UV light after exposure to NH_3			hR_f values		
	TLC 1	TLC 2	TLC 3	TLC 1	TLC 2	TLC 3	TLC 1	TLC 2	TLC 3
1	Bright yellow	Bright yellow	Bright yellow	Bright yellow	Bright yellow	Bright yellow	83	59	58
2	Yellow	Yellow	Yellow	Yellow Florescence	Yellow Florescence	Yellow Florescence	64	29	52
3	–	–	Green	–	–	Yellowish green.	–	–	41

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

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

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

Table 3.4. Thin Layer Chromatography study of ethyl acetate fraction of the methanol extract of *C.oppositifolia* leaf.

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	Bright yellow	Bright yellow	Bright yellow	Bright yellow	Bright yellow	Bright yellow	83	59	58
2	Yellow	Yellow	Yellow	Orange	Orange	Orange	64	29	52
3	–	–	Green	–	–	Yellowish green.	–	–	41

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

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

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

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

Table 3.5. Coloumn and Thin layer chromatography separation of ethyl acetate fraction of *C.oppositifolia* leaf.

Eluent Benzene: 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 yellowish spots. (59, 54)	Mixture of compounds
30:70	44-51	6.0	Two bright yellow spots. (58, 54)	Mixture of compounds
20:80	52-54	4.5	Two yellowish spots (59, 54)	Mixture of compounds
10:90	55-56	3.5	Three red spot (84, 57, 29)	Mixture of compounds
0:100	57-58	2.0	Nil	Colourless solid

Spray reagent: 1% Aluminium chloride in ethanol.

Table 3.6. Qualitative analysis of the compound I isolated from ethyl acetate fraction of *C. oppositifolia* leaf.

S.no	Treatment	Observation	Inference
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.	Olive green colour.	Presence of flavonoid.
5	1 mg of the crystalline solid was treated with 1 ml of concentrated hydrochloric acid.	Intense yellow with green florescence.	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

All of the above mentioned colour reactions are identical with the reference literature for flavonoid [1, 8, 9].

Table 3.7. Thin layer chromatography of the compound I isolated from ethyl acetate fraction of *C.oppositifolia* leaf.

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	Yellow	Bright Yellow	Bright Yellow	Bright Yellow
TLC 2	59	Yellow	Bright Yellow	Bright Yellow	Bright Yellow
TLC 3	41	Yellow	Bright Yellow	Bright Yellow	Bright Yellow

$AlCl_3$: Aluminium chloride in ethanol; NH_3: Ammonia; ADB: 5% solution of 2-aminoethyl diphenyl borinate in methanol.

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

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

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

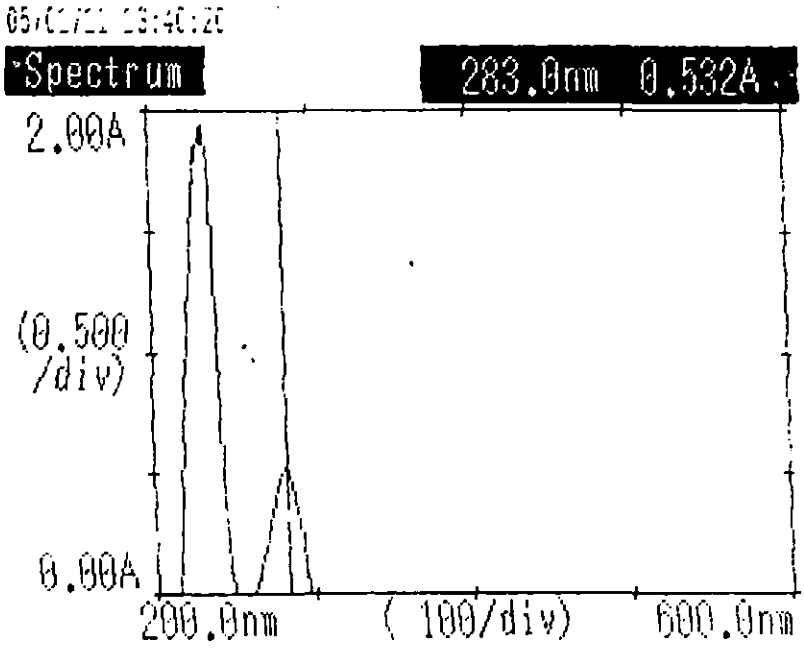
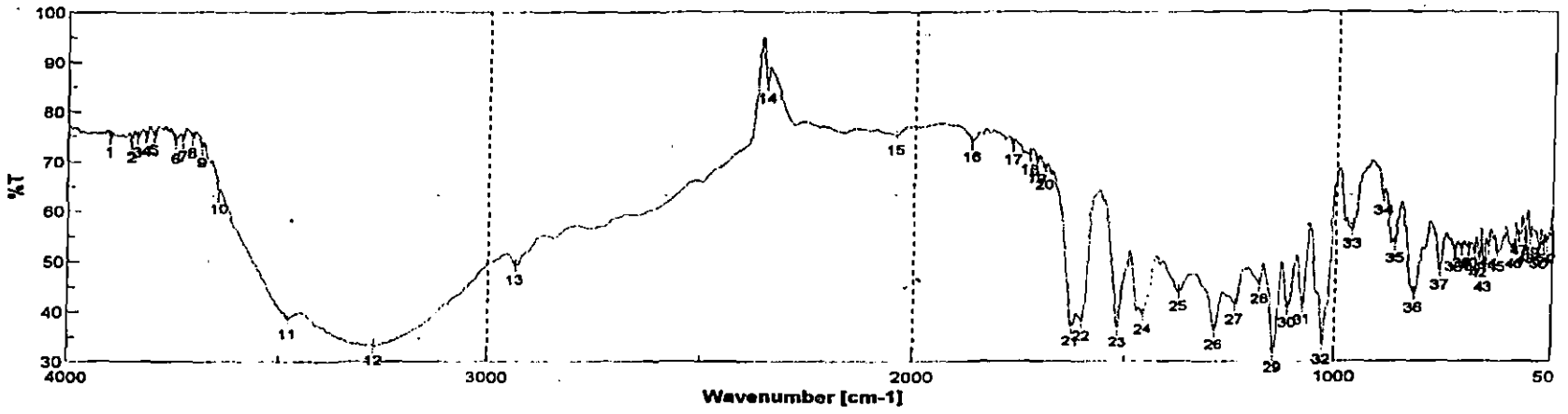


Figure 3.1. UV-Visible Spectro photometric analysis of compound I from *C.oppositifolia* leaf extract.



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Gain	Auto (32)	Scanning Speed	Auto (2 mm/sec)
Date/Time	1/7/2005 5:49PM	Update	1/11/2005 6:16PM
Operator	msn		
File Name	sidash-c.oppositifolia.jws		
Sample Name	pymtf-1		
Comment			

No.	cm-1	%T	No.	cm-1	%T	No.	cm-1	%T	No.	cm-1	%T	No.	cm-1	%T
1	3900.32	74.6083	2	3852.11	73.7005	3	3838.61	74.6846	4	3818.36	74.9047	5	3799.08	75.1178
6	3748.94	73.5257	7	3732.55	73.8894	8	3710.37	74.5506	9	3688.19	72.6248	10	3647.7	63.4221
11	3480.88	38.5811	12	3278.47	33.1736	13	2937.06	49.2171	14	2348.87	85.4135	15	2046.1	75.0259
16	1867.72	73.5728	17	1771.3	73.0351	18	1732.73	71.0013	19	1716.34	69.4906	20	1697.05	68.1782
21	1632.45	36.9995	22	1606.41	38.0989	23	1519.83	36.812	24	1456.96	39.4063	25	1368.25	43.9113
26	1286.29	36.2951	27	1237.11	41.2983	28	1181.19	45.4316	29	1146.47	31.6571	30	1113.69	40.6449
31	1078.01	41.2623	32	1029.8	33.0952	33	963.269	56.3393	34	892.88	63.0292	35	867.81	53.4416
36	823.455	43.6056	37	764.637	48.1111	38	731.853	51.6932	39	715.461	52.5072	40	699.069	52.4487
41	685.57	51.4253	42	674.963	50.5806	43	663.393	47.8805	44	651.822	52.5055	45	631.573	51.772
46	589.147	52.3518	47	578.54	55.0131	48	562.148	53.2168	49	551.542	54.5801	50	531.293	52.317
51	521.65	52.8595	52	513.936	53.3909									

Figure 3.2. IR spectrum of compound I isolated from *C.oppositifolia* leaf extract.

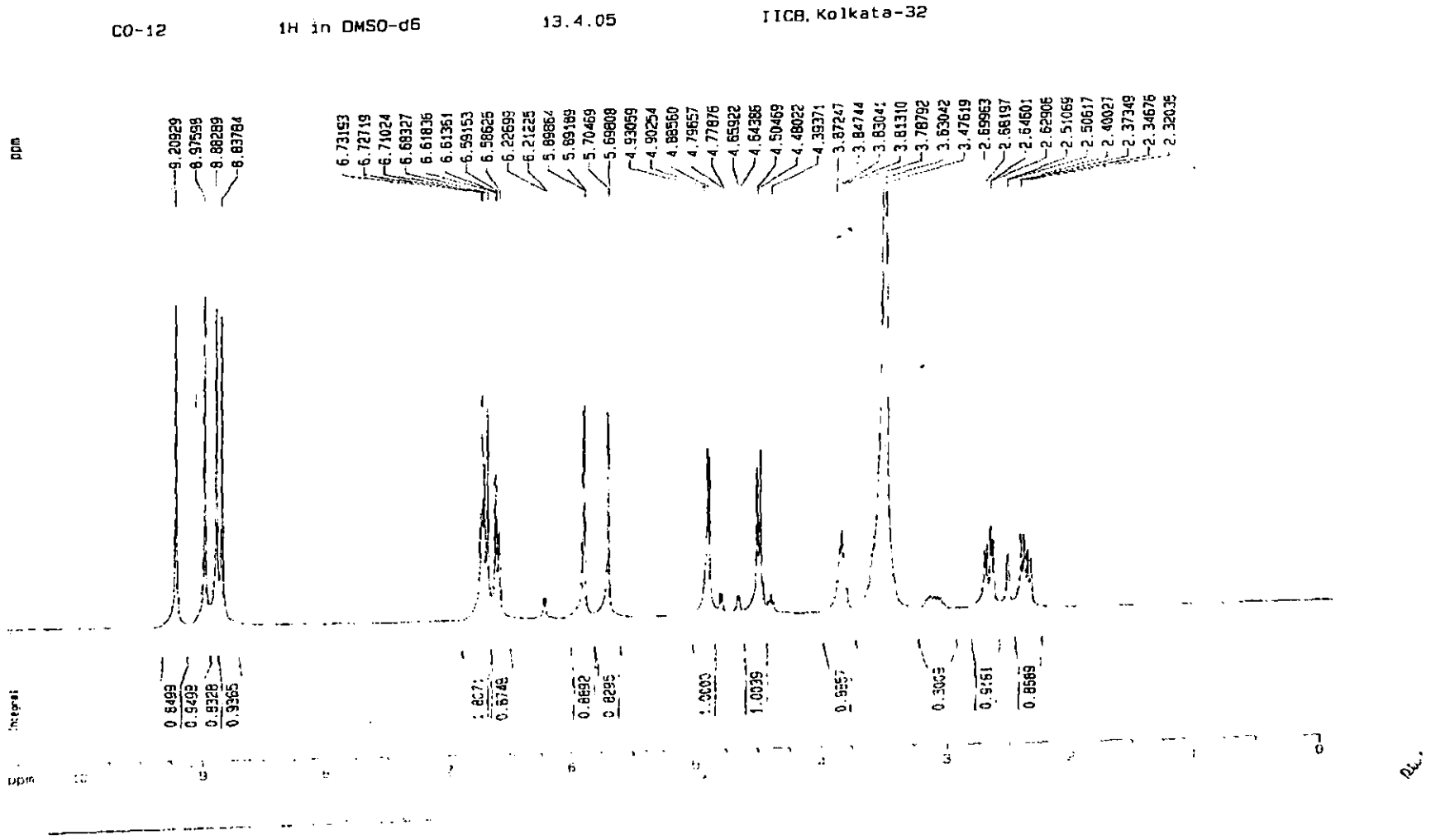


Figure 3.3. ^{1}HNMR spectrum of compound I isolated from *C.oppositifolia* leaf extract.

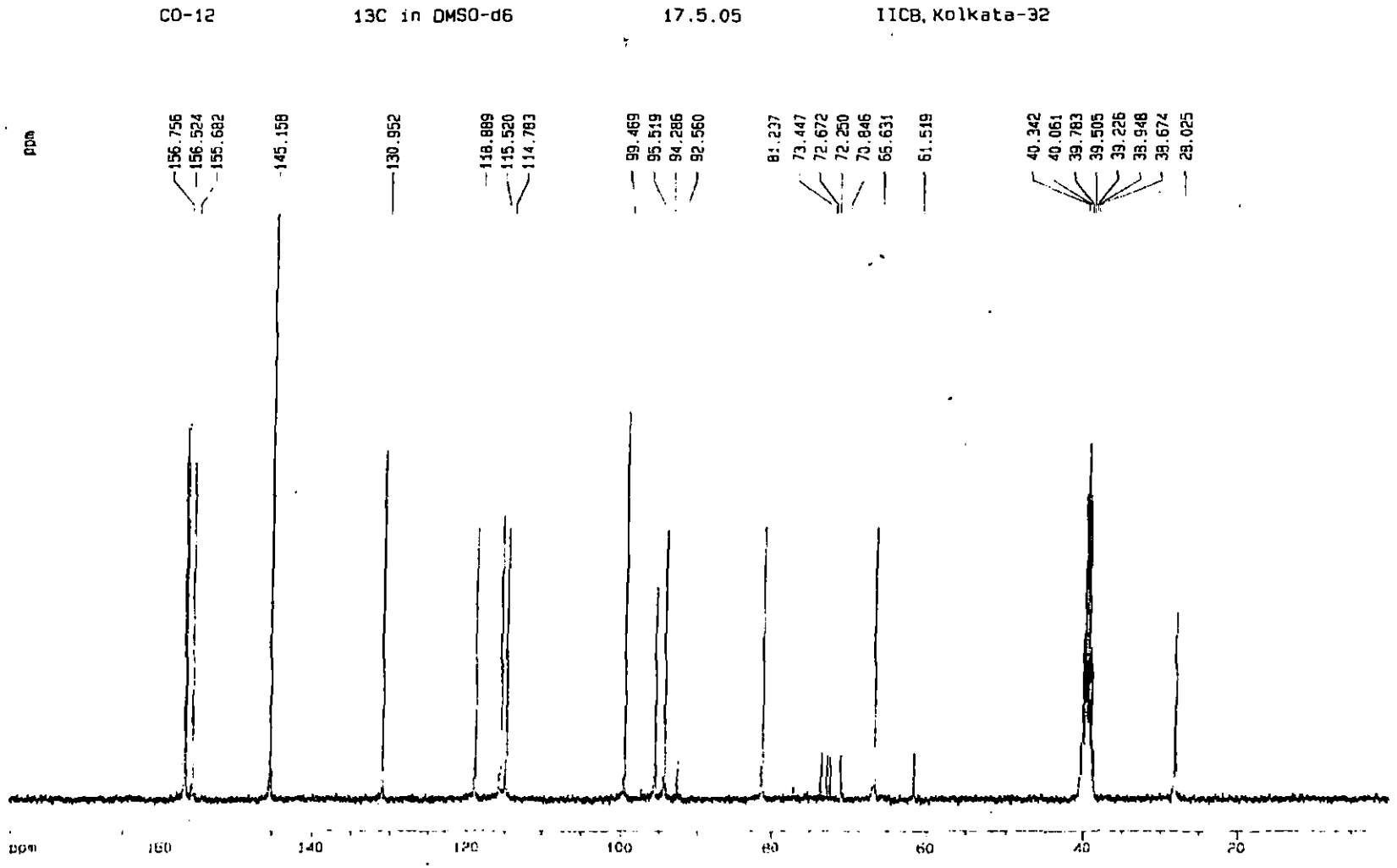


Figure 3.4. ^{13}CNMR spectrum of compound I isolated from *C.oppositifolia* leaf extract.

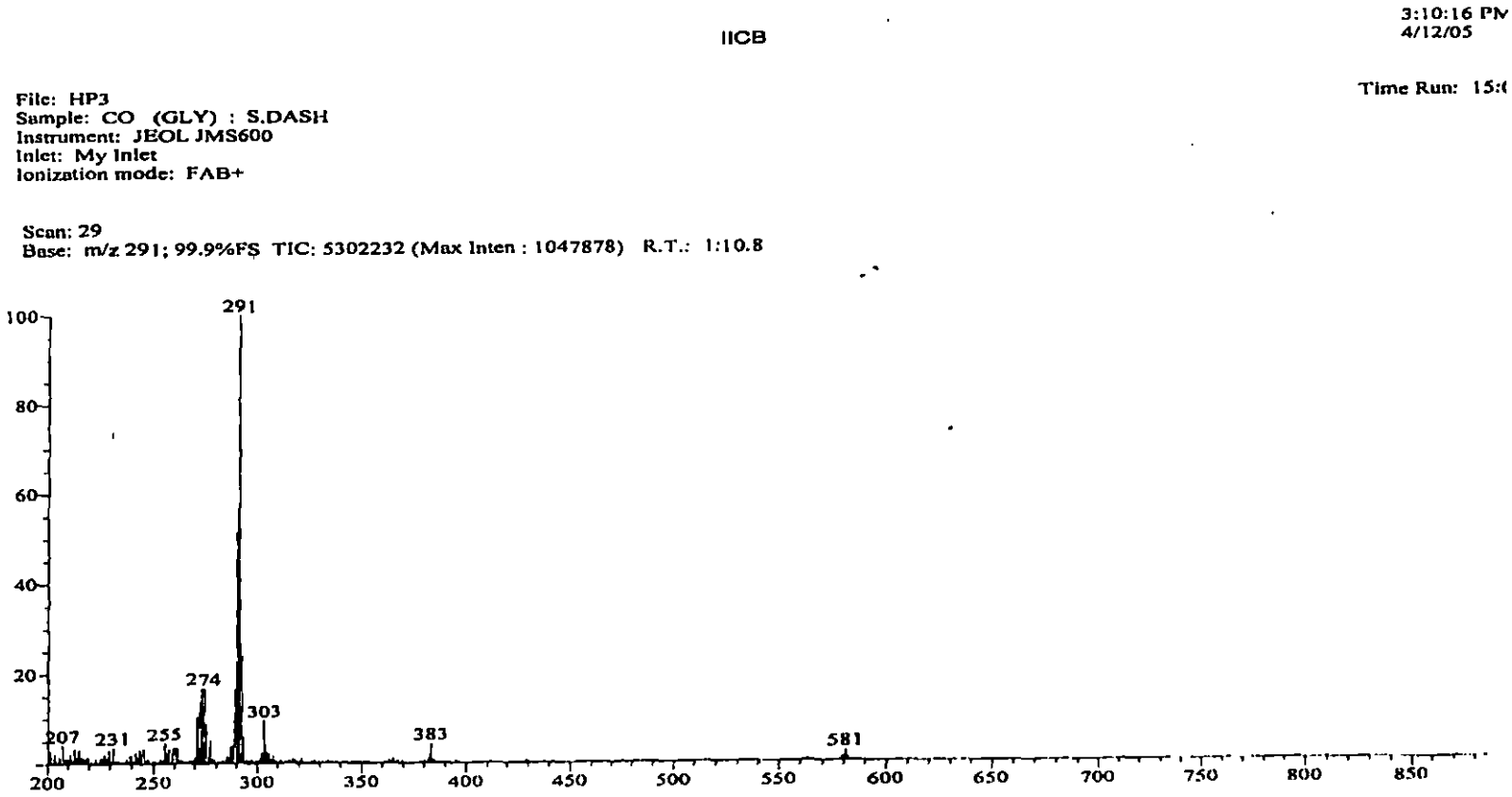


Figure 3.5. Mass spectrum of compound I isolated from *C.oppositifolia* leaf extract.

Table 3.8. UV spectral data of compound I isolated from ethyl acetate fraction of *C.oppositifolia* leaf.

Sl. No	Preparation	λ_{max} (nm) of compound I	Spectral effect	Structural diagnosis
1	Methanol solution of compound I.	241, 281		Flavan-3-ol
2	Methanol solution of compound I + 3 drops of sodium methoxide solution	250,293	69 nm Bathochromic shift in Band I	4'-OH free
3	Methanol solution of compound I + 6 drops of Aluminium chloride	248,282	25 nm Shift in Band I	5-OH Free
4	Methanol solution of compound I + 6 drops of Aluminium chloride and 3drops of Hydrochloric acid.	280,314,361	30 nm shift in Band I	Presence of di-OH in B ring
5	Methanol solution of compound I + powdered NaOAc	243, 283	Lack of shift in Band II	Absence of free 7-hydroxyl group
6	Methanol solution of compound I + powdered NaOAc and H_3BO_3	281	12 nm shift in Band I relative to methanol.	Presence of di-OH in B ring

[Note – Structural analysis was done on basis of reference literature for flavonoid.]

Table 3.9 ^{1}H and ^{13}C NMR spectral data of compound I isolated from ethyl acetate fraction of *C.oppositifolia* leaf.

Position of C/H	Department	Value of δ H	Value of δ C
2	C		145.1
3	C		130.95
4	CH	2.506, 2.699	81.23
5	C		155.68
6	CH	6.226	94.28
7	C		156.75
8	CH	6.212	92.56
9	C		156.52
10	C		99.46
1'	C		118.88
2'	CH	6.731	95.51
3'	C		115.52
4'	C		114.78
5'	CH	6.681	92.56
6'	CH	6.591	91.23
7-rha			
1"	CH	5.698	72.67
2"	CH	4.393	72.25
3"	CH		70.84
4"	CH	3.787-3.872	61.51
5"	CH	3.476	66.63
6"	CH_3	2.320	28.51

3.3.2. Phytochemical study of *H.nepalense* root.

The concentrated methanol extract prepared from the shade-dried root of *H.nepalense* was fractionated successively with petroleum ether, acetone and ethyl acetate. Different fractions of the extract were undertaken for preliminary pharmacological studies. It was observed that the ethyl acetate fraction exhibited significant pharmacological activities under perview of this investigation. So this fraction was subjected to phytochemical analysis.

The preliminary phytochemical group tests indicated the presence of steroids, flavonoids, proteins, tannins, saponins and reducing sugar (Table 3.1). The thin layer chromatographic study of ethyl acetate fraction of methanol extract showed the mixture of three components with selected mobile phase and spraying reagents (Table 3.10-3.12). This fraction was subjected to coloumn chromatography, which yielded sixty-two fractions. The fractions having identical hRf values were mixed together (Table 3.13). They were purified with a silica gel coloumn, by using chloroform: methanol as mobile phase, which yielded a flavonoidal glycoside (compound II). It was further subjected to chemical tests and TLC to confirm the chemical nature and was depicted in Table 3.14 & 3.15. 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 flavonoidal glycoside [8-20].

Compound II was obtained as yellow needle shaped crystal, partially soluble in water & soluble in methanol. The compound was melted at 210-214°C. The complete acid hydrolysis of compound II gave Quercetin and glucose. The UV analysis (Table 3.16) proved the absence of 3-hydroxyl group in the compound [47 nm bathochromic shift of Band I (in MeOH) in Aluminium chloride and hydrochloric acid] (Figure 3.6). Therefore, it was indicated that the compound was monodesmoside [15]. The IR spectrum shows the presence of absorption bands at 3648- 3611 cm^{-1} ν (OH), 2904 cm^{-1} ν (C=O) in flavone, 1615- 1507 cm^{-1} ν (aromatic rings), 1304 cm^{-1}, 1263 cm^{-1}, 1132 cm^{-1}, 942 cm^{-1} and 768 cm^{-1} (Figure 3.7). In the ^{1}HNMR spectra (Table 3.17) in DMSO- d_6, the signals for aglycone were the same as for 3-O substituted quercetin [16]. The doublet of the glucose

anomeric proton appeared (Figure 3.8) at 4.92 ppm (J= 7.5 Hz) and assigned to β-glucopyransone linked to quercetin at C-3 [17]. The fragmentation ion at 303 (98.8) in its mass spectrum inferred that the compound contains quercetin as aglycone (Figure 3.10). The structure of the compound was further confirmed by ^{13}C NMR and elemental analysis [9, 10, 15, 16, 17, 18, 20]. The observed chemical shifts for carbon atoms of aglycone and sugar at C-3 (Figure 3.9) are consistent with those for the quercetin-3-glucopyranoside at C-3 (C"-98.4 ppm) [17]. The isolated compound II showed significant peaks in its mass spectra (Figure 3.10) at m/z 495 (10.2), 415 (15.8), 371 (20.0), 327 (19.2), 303 (98.8), 301 (29.7), 287 (10.2), 277 (12.0), 239 (8.2), 207 (8.0). This data showed a molecular ion at 415 (M^+); which corresponds to be molecular formula $C_{19}H_{22}O_{12}$ as confirmed by elemental analysis. The percentage of carbon, hydrogen and nitrogen in the compound was found to be 48.70, 5.29 and nil respectively. From these data it is concluded that the structure of the isolated flavonoid was quercetin-3-O-β-D-glucopyranoside.

Table 3.10 Thin Layer Chromatography study of ethyl acetate fraction of the methanol extract of *H.nepalense* root.

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	Yellow	Yellow	Yellow	Bright yellow	Bright yellow	Bright yellow	68	54	69
2	Yellow	Yellow	Yellow	Yellow Florescence	Yellow Florescence	Yellow Florescence	64	28	49
3	–	–	Green	–	–	Yellowish green.	–	–	41

Spray reagent: 1% Aluminium chloride in ethanol

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

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

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

Table 3.11 Thin Layer Chromatography study of ethyl acetate fraction of the methanol extract of *H.nepalense* root

No of Spots	Colour of spots under long wave UV light			Colour of spots under long wave UV light after exposure to NH_3			hR_f values		
	TLC 1	TLC 2	TLC 3	TLC 1	TLC 2	TLC 3	TLC 1	TLC 2	TLC 3
1	Yellow	Yellow	Yellow	Bright yellow	Bright yellow	Bright yellow	68	54	69
2	Yellow	Yellow	Yellow	Yellow Florescence	Yellow Florescence	Yellow Florescence	64	28	49
3	–	–	Green	–	–	Yellowish green.	–	–	41

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

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

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

Table 3.12. Thin Layer Chromatography study of ethyl acetate fraction of the methanol extract of *H.nepalense* root.

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	Yellow	Yellow	Yellow	Bright yellow	Bright yellow	Bright yellow	68	54	69
2	Yellow	Yellow	Yellow	Orange	Orange	Orange	64	28	49
3	–	–	Green	–	–	Yellowish green.	–	–	41

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

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

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

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

Table 3.13. Coloumn and Thin layer chromatography separation of ethyl acetate fraction of *H.nepalense* root.

Eluent Benzene: ethyl acetate	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: 1% Aluminium chloride in ethanol

Table 3.14. Qualitative analysis of the compound II from ethyl acetate fraction of *H.nepalense root*.

Sl.No	Treatment	Observation	Inference
1	1 mg of the crystalline solid 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 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 diluted 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.	Olive green colour.	Presence of flavonoid.
5	1 mg of the crystalline solid was treated with 1 ml of concentrated hydrochloric acid.	Intense yellow with green florescence.	Presence of flavonoid.
6	1 mg of the crystalline solid was treated with magnesium hydrochloride solution (Shinoid's test)	Red to magenta red colour.	Presence of flavonoid.

All of the above mentioned colour reactions are identical with the reference literature for flavonoid [1, 8, 9].

Table 3.15. Thin layer chromatography of the compound II isolated from ethyl acetate fraction of *H.nepalense* root.

Solvent system	hR_f values	Colour of the fluorescent produced			
		UV_{366nm}	UV_{366nm} +$AlCl_3$	UV_{366nm} +NH_3	UV_{366nm} +ADB
TLC 1	69	Pale Yellow	Bright Yellow	Bright Yellow	Bright Yellow
TLC 2	55	Pale Yellow	Bright Yellow	Bright Yellow	Bright Yellow
TLC 3	41	Pale Yellow	Bright Yellow	Bright Yellow	Bright Yellow

$AlCl_3$: Aluminium chloride in ethanol; NH_3: Ammonia; ADB: 5% solution of 2-aminoethyl diphenyl borinate in methanol.

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

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

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

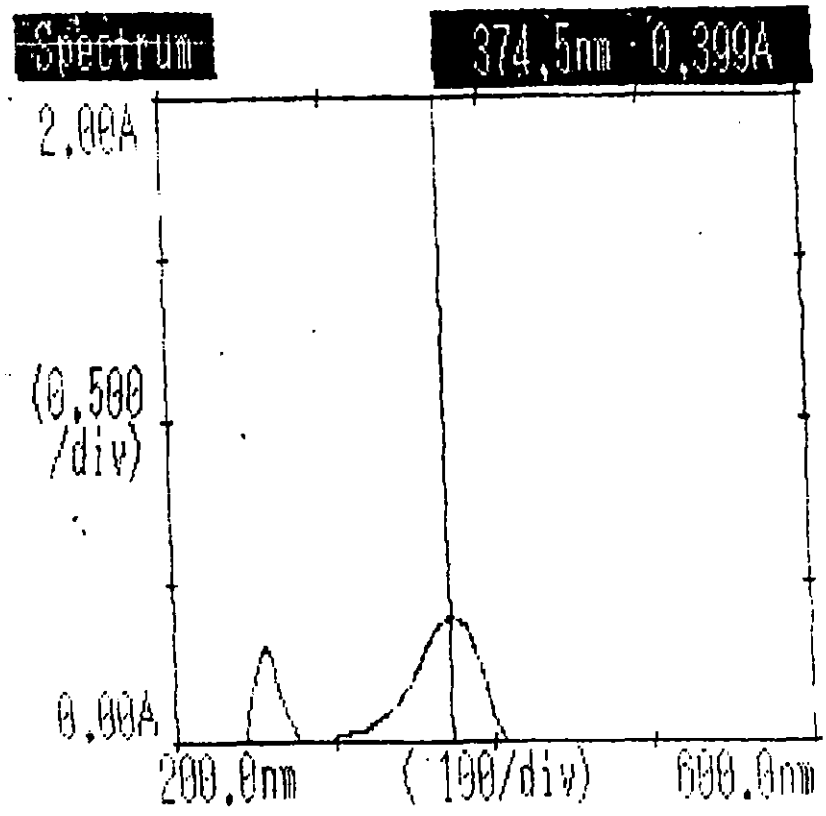
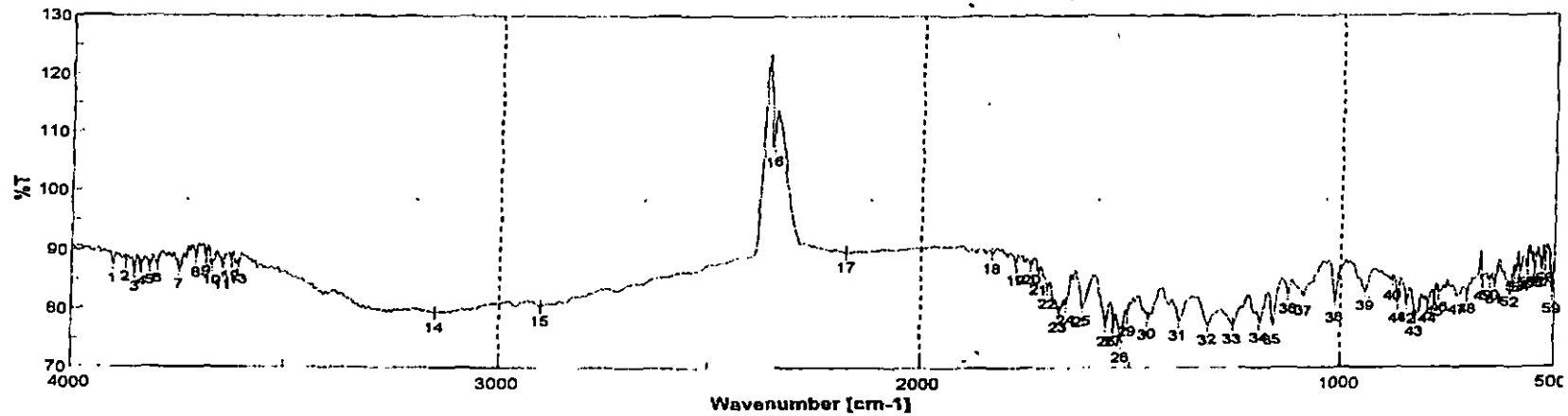


Figure 3.6. UV-Visible Spectro photometric analysis of compound II from *H.nepalense* root extract.



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Sample Name	pymtf-1		
Comment			

No.	cm-1	%T	No.	cm-1	%T	No.	cm-1	%T	No.	cm-1	%T	No.	cm-1	%T
1	3901.29	87.5754	2	3871.4	87.9453	3	3852.11	86.1638	4	3837.65	87.0306	5	3816.38	87.1426
6	3800.04	87.4405	7	3749.9	86.5231	8	3711.33	88.5096	9	3688.19	88.7485	10	3674.69	87.2105
11	3648.66	88.4514	12	3628.41	87.9507	13	3611.05	87.3781	14	3148.22	79.4085	15	2904.27	80.515
16	2347.91	107.41	17	2172.42	89.6264	18	1829.16	89.3982	19	1771.3	87.4239	20	1733.69	87.4787
21	1716.34	85.7855	22	1697.05	83.4544	23	1669.09	79.4463	24	1653.66	80.6909	25	1615.09	80.4333
26	1558.2	77.0754	27	1540.85	77.0906	28	1522.52	74.2389	29	1507.1	78.6935	30	1456.96	78.2279
31	1384.64	77.9328	32	1319.07	77.3197	33	1263.15	77.3191	34	1199.51	77.6034	35	1168.65	77.2709
36	1132.01	82.4384	37	1093.44	82.1123	38	1015.34	80.9954	39	942.056	82.9564	40	877.452	84.6844
41	864.917	80.8617	42	843.704	80.8026	43	823.455	78.5791	44	794.528	80.7525	45	779.101	81.9739
46	768.494	82.6587	47	725.104	82.2054	48	701.962	82.2845	49	685.321	84.4869	50	648.929	84.5789
51	637.358	83.9686	52	602.646	83.4754	53	589.147	86.1189	54	579.504	86.0268	55	562.148	86.8273
56	545.758	86.5099	57	528.4	86.699	58	520.686	87.426	59	504.294	82.2061			

Figure 3.7. IR spectrum of compound II isolated from *H.nepalense* root extract.

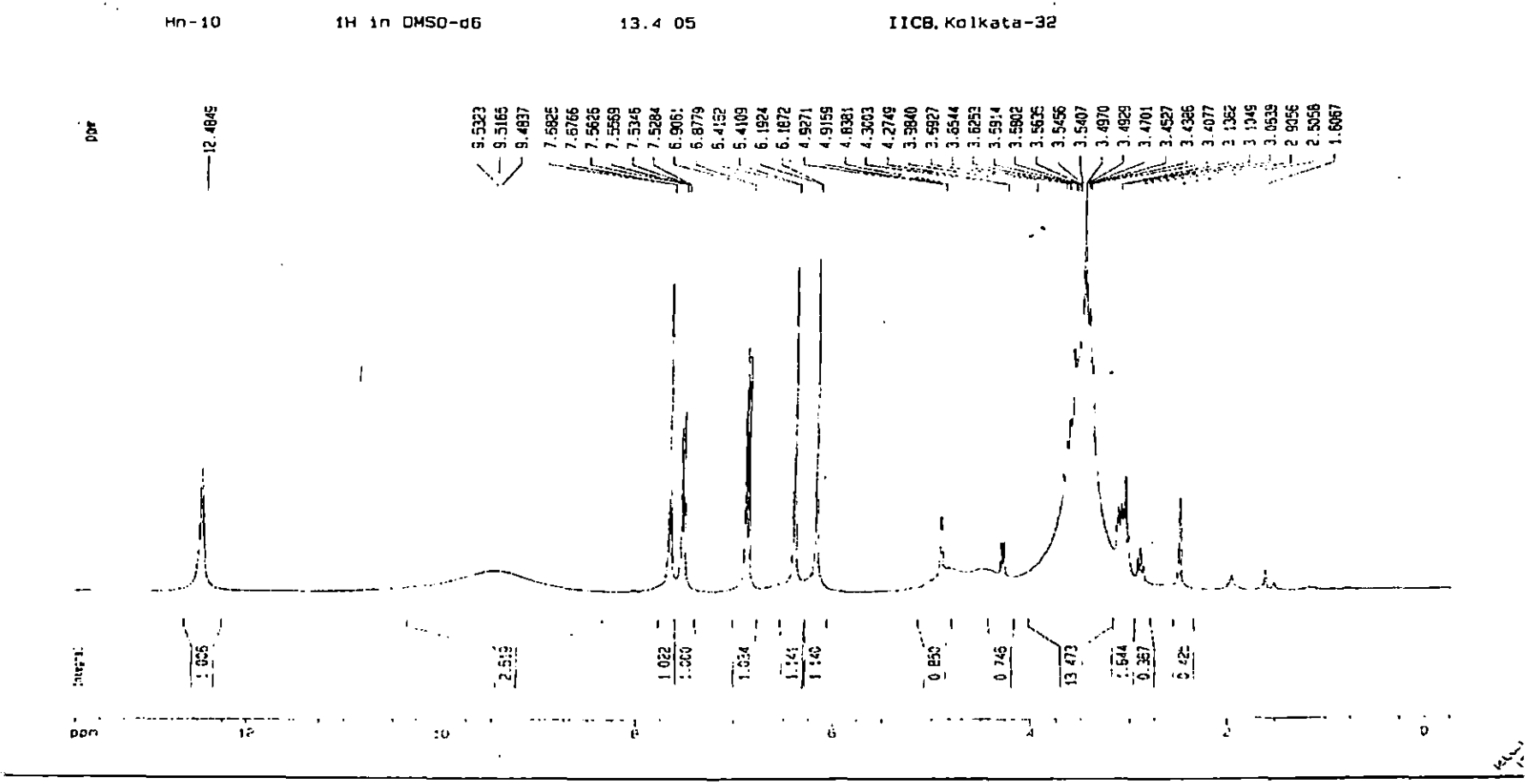

Figure 3.8. ^{1}H NMR spectrum of compound II isolated from *H.nepalense* root extract.

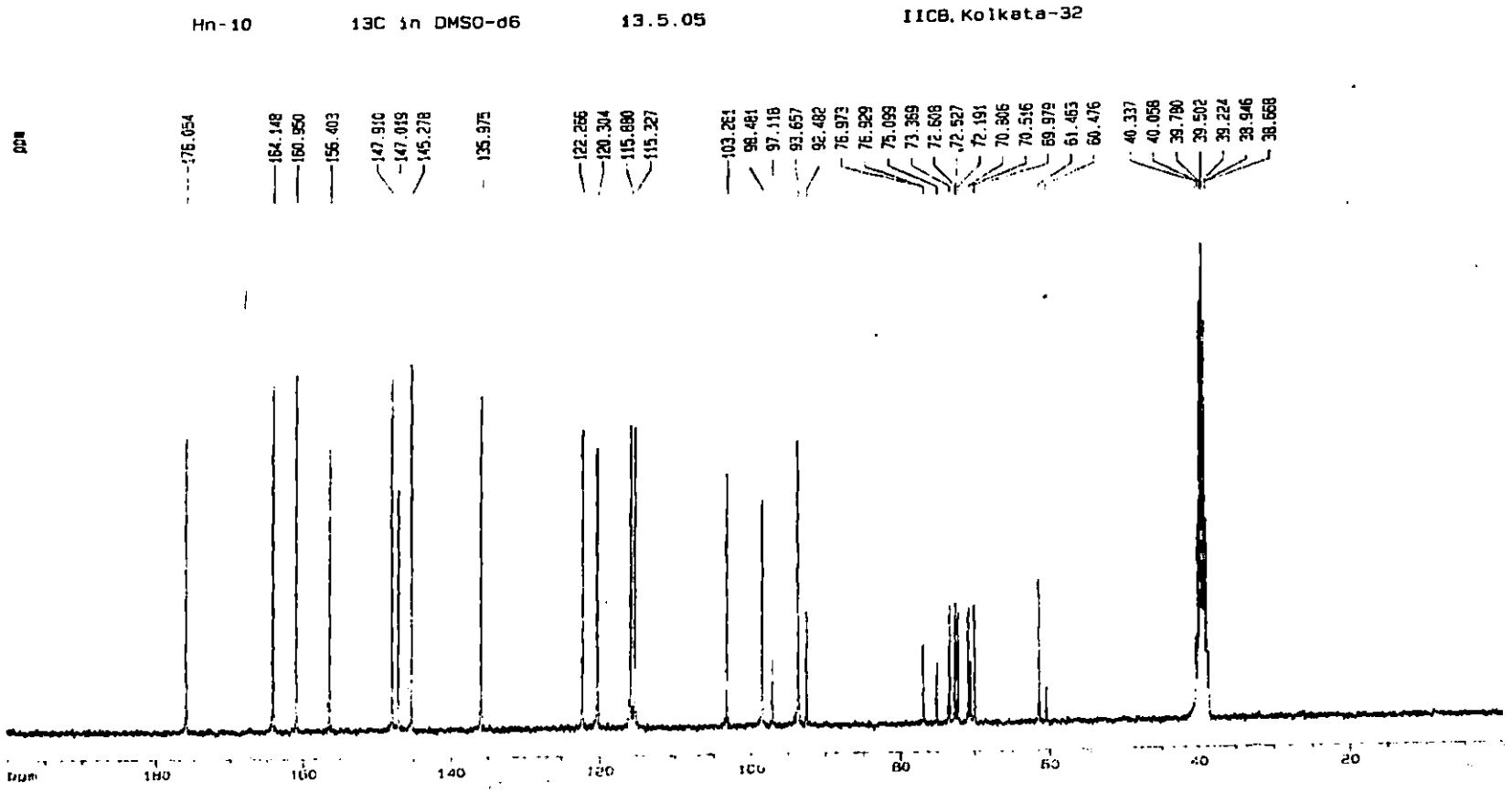

Figure 3.9. ^{13}C NMR spectrum of compound II isolated from *H.nepalense* root extract.

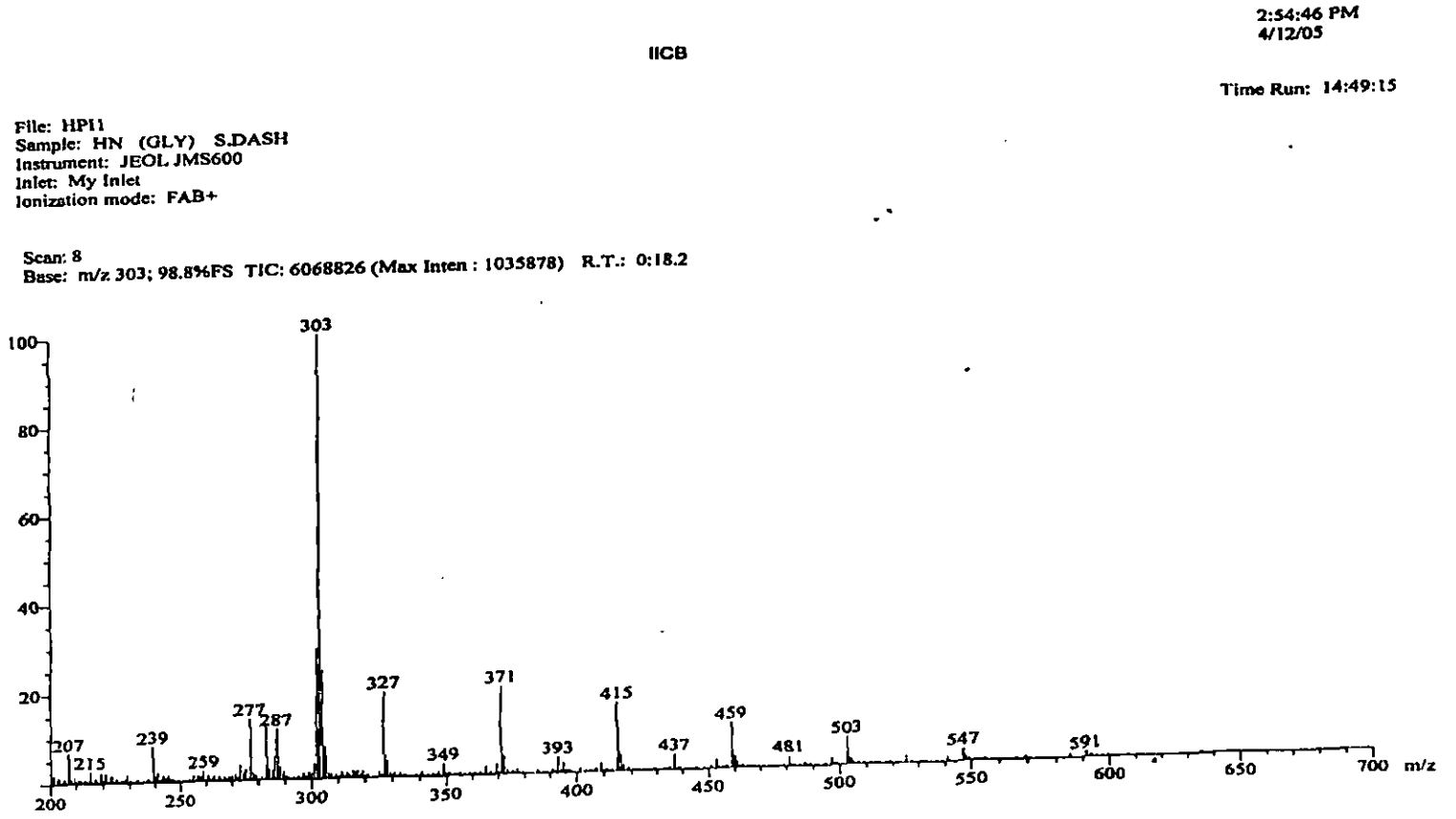

Figure 3.10. Mass spectrum of compound II isolated from *H.nepalense* root extract.

Table 3.16. UV spectral data of isolated compound II isolated from ethyl acetate fraction of *H.nepalense* root.

Sl.No	Preparation	λ_{max} (nm) of compound II	Spectral effect	Structural diagnosis
1	Methanol solution of compound II.	251, 374	–	Flavonol
2	Methanol solution of compound II + 3 drops of sodium methoxide solution	246, 328, 425	Rapid degradation of spectrum	3', 4'-OH free
3	Methanol solution of compound II + 6 drops of Aluminium chloride	272, 310	35 nm Shift in Band I	5-OH Free
4	Methanol solution of compound II + 6 drops of Aluminium chloride and 3drops of Hydrochloric acid.	268, 304, 361	47 nm shift in Band I	Lack of free 3-OH
5	Methanol solution of compound II + powdered NaOAc	269, 314	35 nm shift in Band II	7-OH
6	Methanol solution of compound II + powdered NaOAc and H_3BO_3	268, 300	12 nm shift in Band I relative to methanol.	Presence of di-OH in B ring

[Note – Structural analysis was done on basis of reference literature for flavonoid.]

Table 3.17. ^{1}H and ^{13}C NMR spectral data of compound II isolated from ethyl acetate fraction of *H.nepalense* root.

Position of C/H	Department	Value of δ H	Value of δ C
2	C		156.403
3	C		135.975
4	C		176.054
5	C	12.48 (OH)	160.950
6	CH	6.18	93.657
7	C		164.148
8	CH	6.41	92.482
9	C		147.910
10	C		103.261
1'	C		122.266
2'	CH	7.56	115.327
3'	C		145.278
4'	C		147.910
5'	CH	6.87	115.327
6'	CH	7.56	120.304
3-Glu			
1"	CH	4.92	98.481
2"	CH		75.099
3"	CH		76.929
4"	CH		69.979
5"	CH		76.973
6"	CH_2	1.60	60.472

References

1. Harborne, J. B. (1983). Phytochemical Methods (A Guide to Modern Techniques of Plant Analysis), 2nd ed., Chapman and Hall, London, pp. 1-80, 210, 256.
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