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Isolation and characterization of some natural compound from liquorice

Sejal Patel and Dr. Rakesh Jat

Abstract
The present research work is mainly concened with the natural compounds that are obtained from traditional medicinal plants. There are number of natural compounds have been available in the nature. Some natural compounds are very useful to human being and have life pontential to save human from many uncurable disease. Keeping view in the mind the research is focused to extract and isolate antioxidant, anti-inflammatory, anticancer, antihyperlipidmic, antidiabetic, antiulcer activites containing compounds are studied. The major natural compounds are liquorice, ginger, alliuam, triphala (harda, bahera, pipali, amla and guggulu). The glycyrrhyza glabra is obtained from rhizomes of liquorice and useful as expectorant and ulcer healing properties with carbenoloxolone as major constituents. All components are isolated from the concerned extracts. The extraction of all plants are based on successive solvent extraction method for all drugs. The constituents are confirmed by structure elucidation. The structure of each compounds are intrepereted by different spectral techniques like Infrared spectrum, nuclear magnetic spectrum (hydrogen anc carbon thirteen spectra) and mass spectroscopy for molecural formula and molecular weight of the unknown compounds.

Keywords: Liqurice, Isolation, Biological, assy

Introduction

Natural compounds

Natural compounds are obtained in the nature from different medicinal and herbal plants. These are generally secondary metabolites of the various plant parts or waste compound of plant. These are obtained various parts of the herbs, shrubs or plants like stem, root, leaves, fruits, seeds, barks, rhizomes, flowers and entire plants. The particular part of the plant is collected, extracted and isolated for the collection of various types of natural compounds. We include alkaloids, glycosides, terpenoids, resins, tannins, carbohydrates, gums and exudates, liganans. These obtained drugs have various types of pharmacological and therapeutic application.

Liquorice (Glycyrrhiza, Mulethi)

Liquorice consists of dried, peeled or unpleed, root & stolon of Glycyrhiza glabara belonging to family Leguminosae. It is also obtained from other species of glycyrrhiza, giving a drug with sweet taste. Liquorice have water soluble extractive not less than 20% w/w. On addision of 80% sulphirc acid, the thick secsion of drug or powder shows deep yellow color. Liquorice owes most of its sweet taste to glycyrhine, the potassium & calcium salts of glycyrhineic acid. The yellow color of liquorice is due to flavonoids. Carbenoloxone is used for ulcer healing as protective base.

Taxonomy of Plant (Liquorice)

Botanical Name	-	*Glycyrhiza glabara*
Kingdom	-	Planate – Plants
Subkingdom	-	Tracheobionta - Vascular plants
Super division	-	Spermatophyta - Seed plants

***Glycyrrhiza Glabra* Plant; Inset: Rhizome and roots**

Genus	-	*Glycyrhiza* - Liqorice
Division	-	Magnoliophyta - Flowering plants
Subclass	-	Rosidae
Order	-	Fabales
Family	-	Leguminosae/Fabaceae - Pea Family
Species-		*Glycyrhiza glabara* L. Cultivated Liqorice

Common Name – Liquorice, Liqorice (English), Lacrisse (German), Reglisse (French), Regolizia (Italian), Kanzoh (Japanese), Gancao (Chinese), Yasti-madhu in Ayurveda & Mulethi (Hindi).

Phytochemistry

Major Saponin

Glycyrrhizic acid is the major triterpenoid saponin (4–20%) in Liqorice rhizome & is used as a tool for recognizing the herb.

Minor Sapogenins

About fifty other sapogenins have been isolated from *Glycyrhiza* species. *G. glabra* have 13 minor sapogenins, liquoric acid is found in high amount as compare to other minor sapogenins, these are glabrolide, 11-deoxyglabrolide, liquiritic acid, isoglabrolide, 11-deoxoglycyrrhitinic acid, glycyrretol, 24-hydroxyglycyrrhetic acid, 24-hydroxy-11-deoxoglycyrrhitic acid, liquiridiolic acid etc.

Experimental

General

Pure marker compounds were isolated by using various chromatographic & their structures were determined based on various spectroscopic techniques as mentioned below.

Thin layer chromatography

Chromatographic reactions were monitored on analytical TLC (MERCK TLC Silica gel 60 F_{254}) precoated plates. TLC plates were developed in CAMAG glass twin trough chamber (20 × 10 cm). TLC chromatograms were visualized by: (a) UV Detecsion Chamber at 254nm, (b) UV Detection Chamber at 366 nm (CAMAG) & (c) derivatizing TLC plats with 0.5% anisaldehyde in 5% sulphuric acid & charring them at high temperature (80-100 ^{0}C) in a hot air oven.

Column chromatography

Column chromatography was performed by using silica gel (60-120 mesh), followed by purification with silica gel (100-200 or 200-400 mesh) column or with Sephadex LH-20 (size exclusion chromatography).

Spectroscopic techniques

For establishing the chemical structure of pure marker compounds & their derivatives, ^{1}H NMR, ^{13}C NMR, DEPT & COSY experiments were performed on the BRUKER AVANCE 200, 400, & 500 MHz instrument with tetra methyl silane (TMS) as an internal standard. Chemical shift was given in δ-ppm value. Electro spray mass (ES–MS) spectra were recorded on HP-1100 MSD instrument.

Dictionary of Natural Compounds

(CRC, Chapman & Hall, London, 2011) isolated compounds were dereplicated & identified based on their report available in DNP & some compounds were identified by comparison (TLC, CO-TLC, ^{1}H-NMR, MS) with authentic samples.

Plant material

The rhizome of *Glycyrhiza glabara* plant was collected from botany department of Indian Institute of Integrated Medicine (IIIM), Jammu. The plant was identified & authenticated by botanist, Dr. S.N. Sharma, Department of Taxonomy, IIIM, Jammu. A voucher specimen is held in the institutional Herbarium.

Extraction & Isolation of Marker compounds from *G. glabra*

Extraction

1 kg Powdered rhizomes were extracted with 1 L ethanol with mechanical stirring at room temperature this process repeated for 4 times for complete extraction. Then this extract was filtered & concentrated under vacuum (175 mbar) at 40 ^{0} C by using Rota vapor to provide crude extract (300 g).

Isolation of marker compounds

A neat & dried glass column was taken. A cotton plug was put at the base of the column & packed with silica gel (60-120 mesh, 1100 g).Then the extract (250 g) was dissolved in minimum quantity of chloroform-methanol & it was mixed with 500 g silica gel (60-120 mesh) for slurry & charged into the column.

Column Specificasions

Column Diameter	10 cm
Length of column	150 cm
Silica gel (60-120)	1100 g
Bed Length	35 cm

The column was eluted with step gradient solvent system of hexane-chloroform-ethyl acetate-methanol & water & 215 fractions were collected (100 ml each). All fractions were pooled on the basis of TLC & divided into 5 parts (Hexane fr-1 to 10, Chloroform fr-11 to 80, Ethyl acetate fr-81 to 180, Methanol fr-181 to 210 & Water fr-211 to 215) & then concentrated under high vacuum. All the fractions were then individually subjected to Column chromatography (Silica Gel 100-200 mesh) & Sephadex (LH-20) to isolate pure compounds as represented Identification of isolated compounds was carried out by comparison of spectral data & physical data with the reported data.

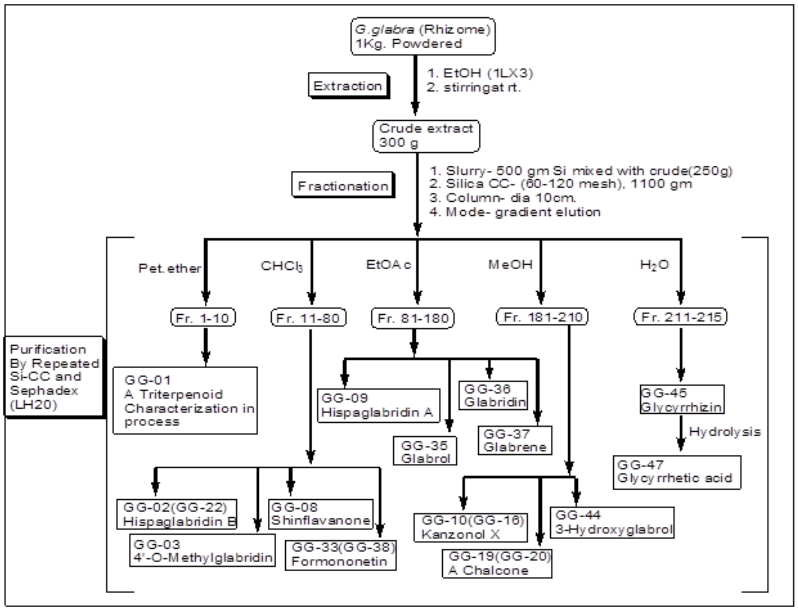
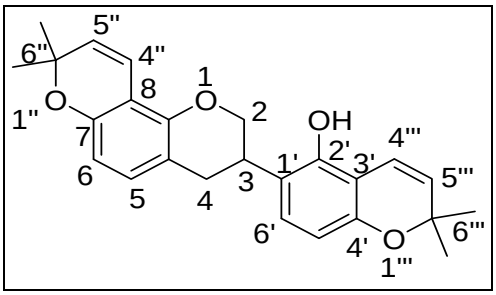


Fig 1: Extraction & Isolation Protocol.

Results & Discussion

Characterization of marker compound.

1. Compound GG-02 (Hispaglabridin B)



Light yellowish needle shaped crystalline compound (30 mg) isolated from chloroform fracsion using silica gel (100-200 mesh) column chrometography & Sephadex (LH-20). It was eluted in 15% of ethyl acetate in hexane from silica gel column & then purified by LH-20 using methanol as eluting solvent. It was then recrystallized using cyclohexane. The structure of compound GG-02 was characterized as Hispaglabridin B by comparison of its spectral data with reported data. [112]

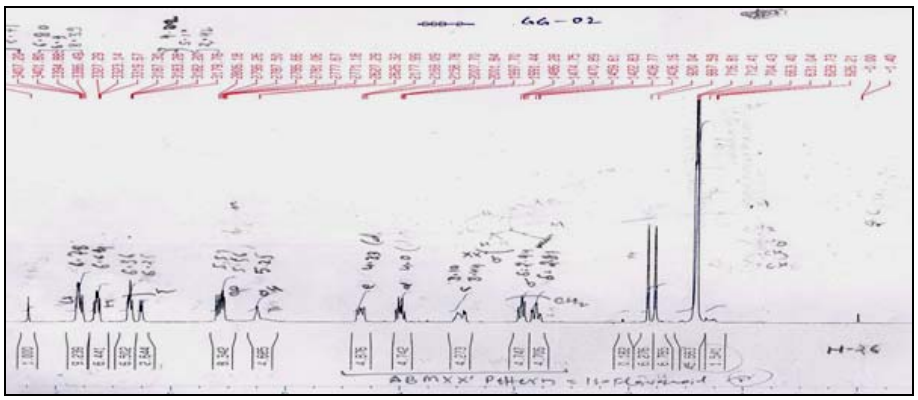
TLC: R_F = 0.55, Hexane: Ethyl acetate (80: 20), Visualizasion: Violet colour with Anisaldehyde-Sulphuric acid reagent (AS reagent).

M.P. = 120-121 ^{0}C

^{1}H-NMR (500 MHz, $CDCl_3$): δ(ppm) 1.49 (12H, s, 2x(CH_3)$_2$), 2.81 (1H, m, H-2), 2.94 (1H, m, H-2), 3.3-3.56 (1H, m, H-3), 4.0 (1H, m, H-4), 4.33 (1H, m, H-4), 5.25 (1H, br s, H-2'), 5.55 (1H, d, *J* = 9.9 Hz, H-4'), 5.67 (1H, d, *J* = 9.9 Hz, H-9'), 6.26 (1H, d, *J* = 8.2 Hz, H-6), 6.36 (1H, d, *J* = 8.2 Hz, H-5), 6.64 (1H, d, *J* = 9.9 Hz, H-4"), 6.67 (1H, d, *J* = 9.9 Hz, H-5'"), 6.80 (1H, d, *J* = 8.2 Hz, H-5'), 6.83 (1H, d, *J* = 8.2 Hz, H-6').

MS-ESI: (Negative): m/z 389 $[M-H]^+$ so the m/z 390 $[M]^+$ corresponded with molecular formula $C_{25}H_{26}O_4$ (MW.-390.13).

^{1}H NMR



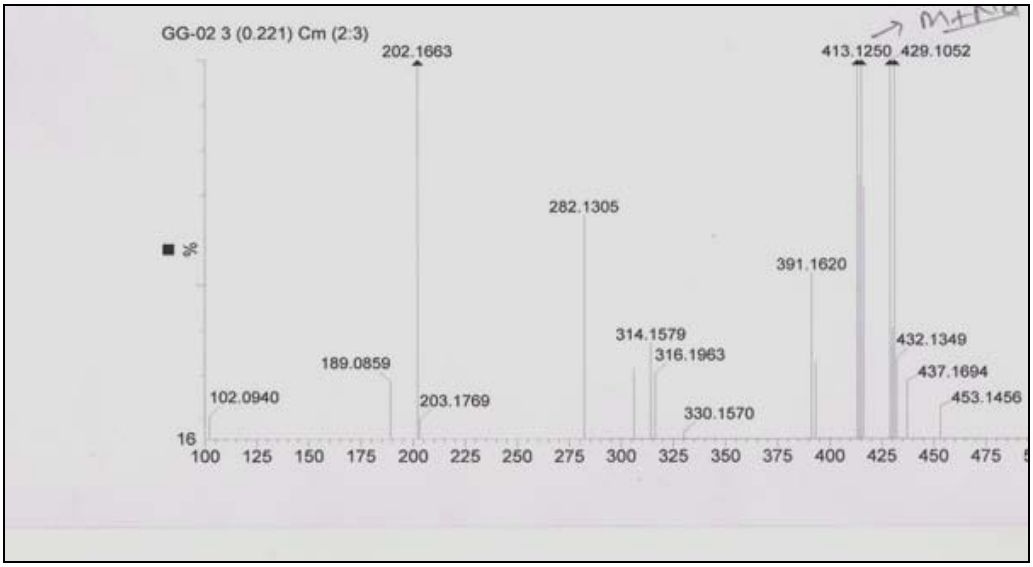
1.1 ^{1}H-NMR of Hispaglabridin-B

Name of atom	Chemical shift Interpretation
Aromatic hydrogen	6-8.5 ppm neighboring group effect
Aliphatic hydrogen	2-3.2 ppm splitting of signal
Vinyl group hydrogen	3.5-4 ppm intensity is fast

Hydroxyl group hydrogen	2.1-2.5 ppm number of signal
Epoxide hydrogen	2.3-2.4 ppm nil
Methyl group	1.2-1.5 ppm primary hydrogen

On the basis of hydrogen nuclear magnetic spectra the compound has aromatic and aliphatic hydrogen that are identified on the basis of chemical shift value in parts per million. The vinyl hydrogen has different chemical shift value. The methyl group contains primary hydrogen with minimum chemical shift value in parts per million. The epoxide shows different part per million value of chemical shift.

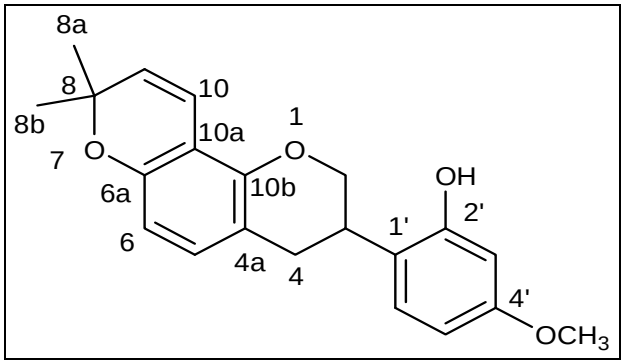
ES-MS



1.2 ES-MS of Hispaglabridin-B

On the basis of molecular mass we can determine the molecular weight of the compound. We know that the molecular ion peak gives the mass or molecular weight of the uknown compounds. In the given figure the base peak shows the peak at 429.678 that is the mass of the product. The parent peak is known as mass or molecular weight of the compound. The fragementations of the compounds shows breaking points and metabolites of the compounds that is very helpful for structure elucidation of the compounds. The m+1, m+2, m+3 3tc. Peaks are known as isotopic peak of the compound. These peaks are very helpful to determine the molecular fornula of the unknown compounds. These two things molecular weight and molecular formular are very important determinant of the structural elucidation of the unkonw compound. We represents relative intensity or abundance on the y-axis and molecular mass on the x-axis for interpretation.

2. Compound GG-03 (4-O-Methylglabridin)



White needle shaped crystalline compound (50 mg) isolated from chloroform fracsion using silica gel (100-200 mesh) column chrometography & Sephadex (LH-20). It was eluted in 15% of ethyl acetate in hexane solvent. It was then recrystallized using cyclohexane. The structure of compound GG-03 was characterized as 4-O-Metylglabridin by comparison of its spectral data with reported data. [112]

TLC: R_F = 0.65, Hexane: Ethyl acetate (80: 20), Visualizasion: Violet colour with Anisaldehyde-Sulphuric acid reagent (AS reagent).

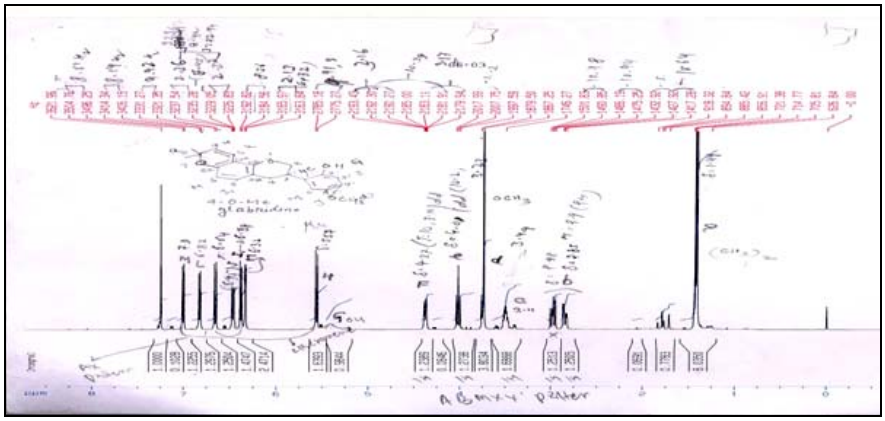
M.P. = 120-121 ^{0}C

^{1}H-NMR (500 MHz, $CDCl_3$): δ(ppm) 1.44 (6H, s, H-8a,8b), 2.85 (2H, m, H-2), 3.49 (1H, m, H-3), 3.73 (3H, s, H-4'), 4.01 (1H, dd, *J* =10.2 Hz, H-4), 4.37 (1H, dd, *J* =10.3 Hz, H-4), 5.55 (1H, s, H-2'), 5.57 (1H, d, *J* =9.91Hz, H-9), 6.32 (1H, s, *J* =2.13, H-3), 6.38 (1H, d, *J* =8.26, H-5), 6.45 (1H, d, *J* =2.22 Hz, H-5'), 6.64 (1H, d, *J* =9.92 Hz, H-10), 6.82 (1H, d, *J* =8.19, H-6'), 7.01 (1H, d, *J* =8.51 Hz, H-6).

^{13}C-NMR (500 MHz, $CDCl_3$): δ(ppm) 27.4, 27.69, 30.50, 31.75, 55.29, 70.02, 75.73, 102.04, 105.91, 108.69, 109.93, 114.54, 116.95, 119.94, 128.13, 128.97, 129.22, 149.70, 151.70, 154.43, 159.20

MS-ESI: (Positive): m/z 338 $[M+H]^+$ corresponded with molecular formula $C_{21}H_{22}O_4$ (MW.-338.46). from silica gel column & then purified by LH-20 using methanol as eluting

^{1}H NMR

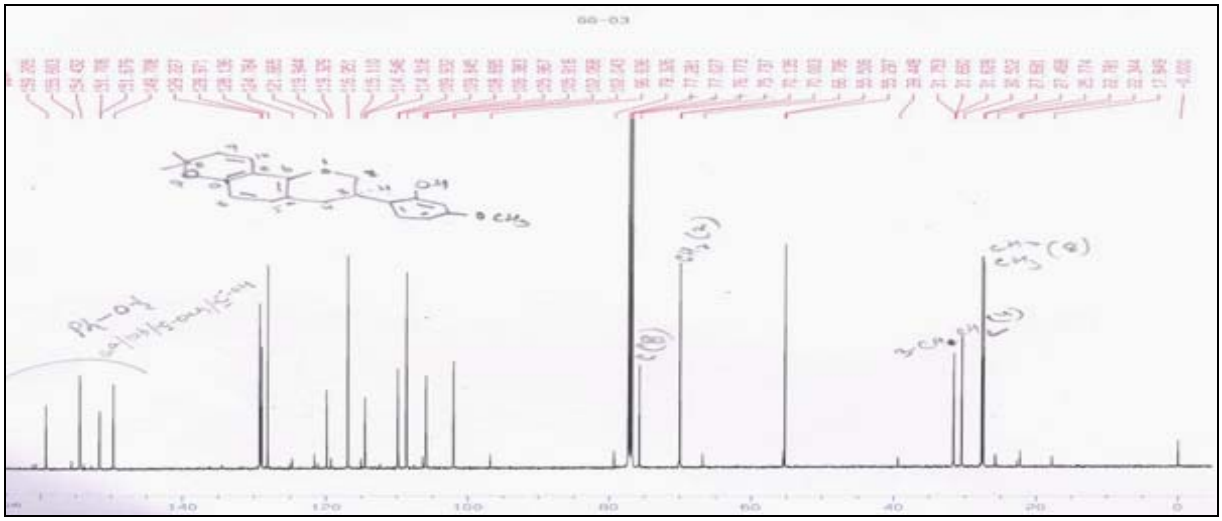


1.3[1] H-NMR of 4-O methyl glabridin

Name of atoms	Chemical shift (δ) Interpretation
Aromatic hydrogens	6-8.3 parts per million neighboring group effect
Aliphatic hydrogens	2-3.2 ppm splitting of signal
Vinyl group hydrogen (-CH=CH2)	3.5-4 ppm intensity is fast
Hydroxyl group hydrogen	2.1-2.5 ppm number of signal
Epoxide hydrogen 2.3-2.4 ppm nil	
Methyl group 1.2-1.5 ppm primary hydrogen	

On the basis of hydrogen nuclear magnetic spectra (NMR) the compound has aromatic and aliphatic hydrogen that are identified on the basis of chemical shift value in parts per million. The vinyl hydrogen has different chemical shift value. The methyl group contains primary hydrogen with minimum chemical shift value in parts per million. The epoxide shows different part per million value of chemical shift.

^{13}C NMR

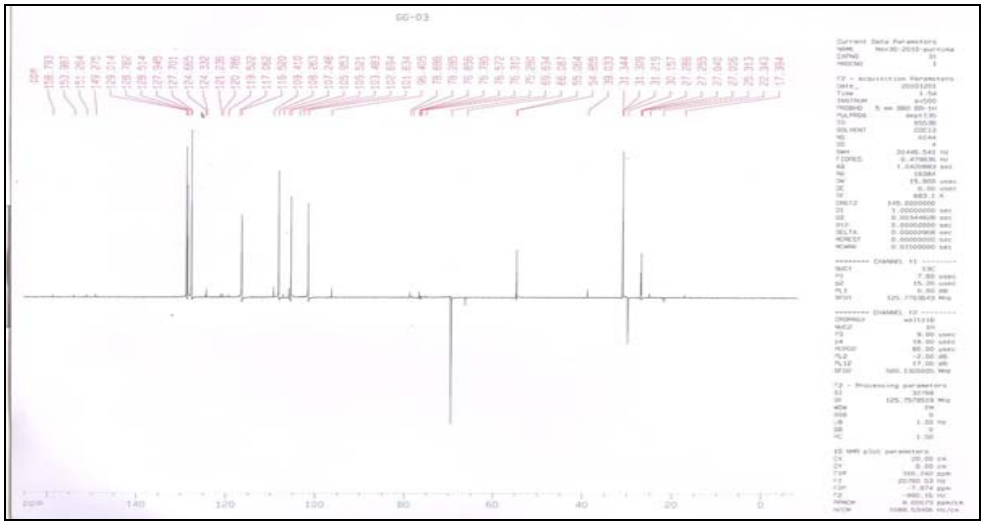


1.4 ^{13}C-NMR of 4-O methyl glabridin

Name of atoms	Chemical shift (δ) Interpretation
Aromatic carbons	26-28.3 parts per million neighboring group effect
Aliphatic carbons	22-23.2 ppm splitting of signal
Vinyl group carbon (-CH=CH2)	2 3.5-4 ppm intensity is fast
Hydroxyl group hydrogen	20.1-22.5 ppm number of signal
Epoxide carbon	20.3-20.4 ppm nil
Methyl group	21.2-21.5 ppm primary carbon

On the basis of hydrogen nuclear magnetic spectra (NMR) the compound has aromatic and aliphatic carbon that are identified on the basis of chemical shift value in parts per million. The vinyl hydrogen has different chemical shift value. The methyl group contains primary carbon with minimum chemical shift value in parts per million. The epoxide shows different part per million value of chemical shift.

DEPT (135)

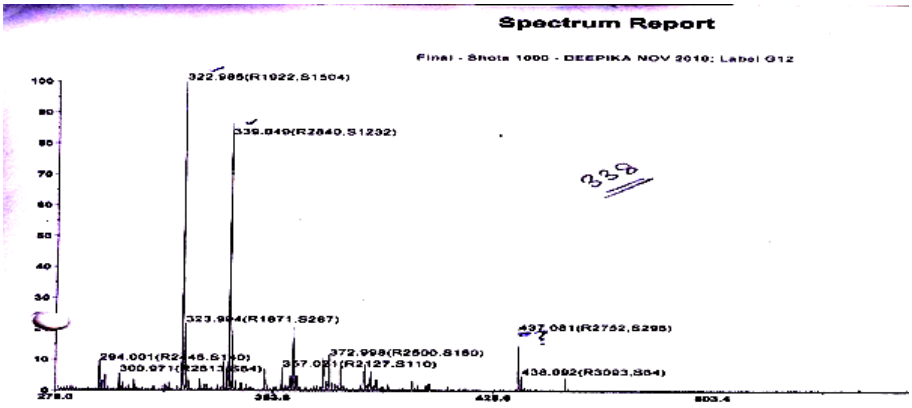


1.5 DEPT of 4-O methyl glabridin

Name of atom	Chemical shift Interpretation
Aromaticdeuterium	6-8.5 ppm neighboring group effect
Aliphaticdeuterium	2-3.2 ppm splitting of signal
Vinyl groupdeuterium	3.5-4 ppm intensity is fast
Hydroxyl groupdeuterium	2.1-2.5 ppm number of signal
Epoxidedeuterium	2.3-2.4 ppm nil
Methyl group	1.2-1.5 ppm primarydeuterium

On the basis of deuterium nuclear magnetic spectra the compound has aromatic and aliphaticdeuterium that are identified on the basis of chemical shift value in parts per million. The vinyldeuterium has different chemical shift value. The methyl group contains primarydeuterium with minimum chemical shift value in parts per million. The epoxide shows different part per million value of chemical shift.

MALDI- MS

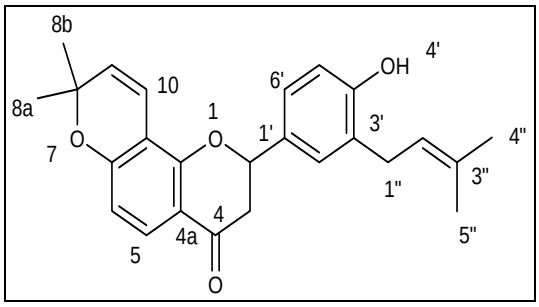


1.6 MALDI-MS of 4-O methyl glabridin

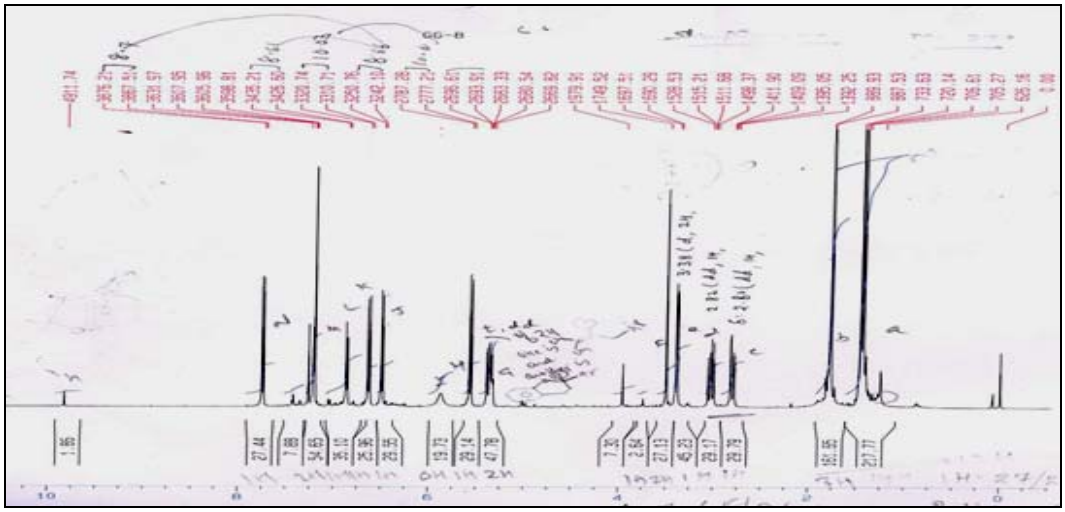
Mass Spectra of 4-O-Methyl Glabridin

On the basis of molecular mass we can determine the molecular wt of the compound. We know that the molecular ion peak gives the mass or molecular weight of the uknown compounds. In the given figure the base peak shows the peak at 338.678 that is the mass of the product. The parent peak is known as mass or molecular weight of the compound. The fragementations of the compounds shows breaking points and metabolites of the compounds that is very helpful for structure elucidation of the compounds. The m+1, m+2, m+3 3tc. Peaks are known as isotopic peak of the compound. These peaks are very helpful to determine the molecular fornula of the unknown compounds. These two things molecular weight and molecular formular are very important determinant of the structural elucidation of the unkonw compound. We represents relative intensity or abundance on the y-axis and molecular mass on the x-axis for interpretation.

GG-08 (Shinflavonone)



[1] H NMR

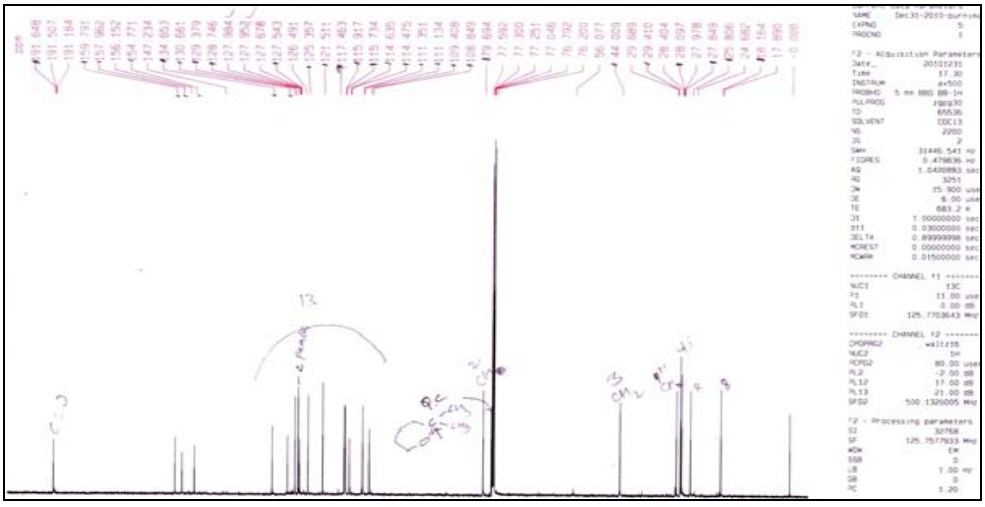


1.7 [1] H-NMR of Shinflavanone

Name of atom	**Chem. shift Interpretation**
Aromat. Hydrogen	7-8.5 ppm neighboring group's effect
Aliphatic hydrogen	2-3.2 ppm splitting of signal
Vinyl group hydrogen	3.5-4 ppm intensity is fast
Hydroxyl group hydrogen	2.1-2.5 ppm number of signal
Epoxide hydrogen	2.3-2.4 ppm nil
Isopropyl group	1.-1.2 ppm primary hydrogen

On the premise of hydrogen atomic attractive spectra the compound has fragrant and aliphatic hydrogen that are distinguished on the premise of concoction move an incentive in parts for every million. The vinyl hydrogen has diverse concoction move esteem. The methyl amass contains essential hydrogen with least synthetic move an incentive in parts for every million. The epoxide demonstrates diverse part per million estimation of substance move.

[13]C NMR

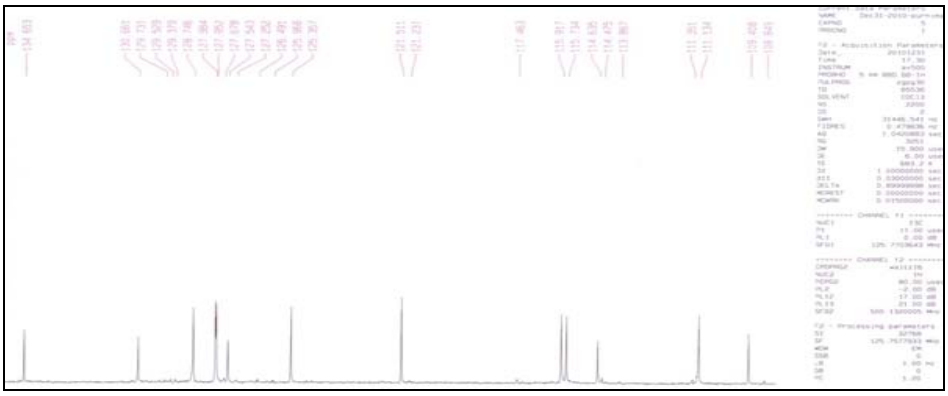


1.8 [13] C-NMR of Shinflavanone

Name of Carbon	Chemical shift (δ) Interpretation
Aromatic carbons	26-28.3 parts per million neighboring group effect
Aliphatic carbons	22-23.2 ppm splitting of signal
Vinyl carbon (-CH=CH2)	23.5-4 ppm intensity is fast
Hydroxyl group hydrogen	20.1-22.5 ppm number of signal
Epoxide carbon	20.3-20.4 ppm nearby
Methyl group	21.2-21.5 ppm primary carbon

On the basis of ^{13}C nuclear magnetic Resonnce spectra (NMR) the compound has aromatic and aliphatic carbon that are identified on the basis of chemical shift value in parts per million. The vinyl hydrogen has different chemical shift value. The methyl group contains primary carbon with minimum chemical shift value in parts per million(PPM). The epoxide shows different part per million value of chemical shift. Renforcing means increasing the value of chemical shift.

^{13}C NMR (Expantion)



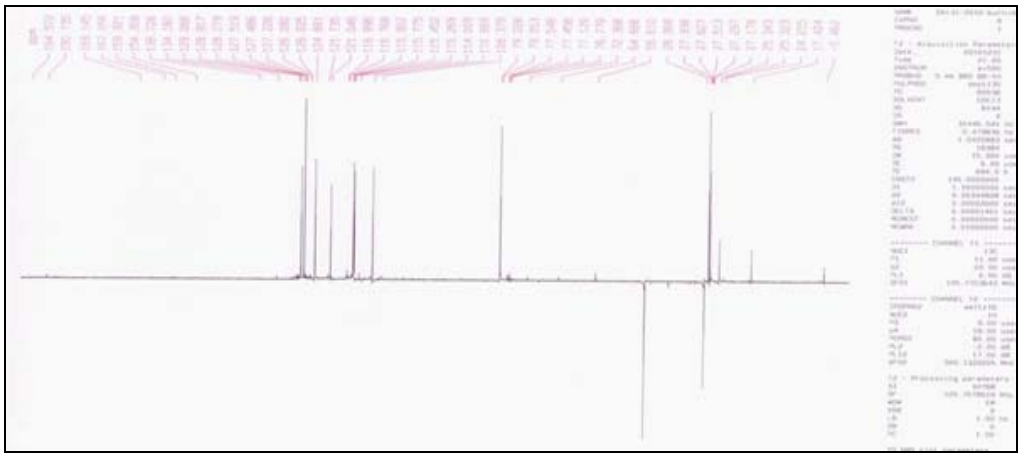
1.9 ^{13}C-NMR (Expation) of Shinflavanone

Name of atom	Chemical shift Interpretation
Aromatic carbons	26-28.3 parts per million neighboring group effect
Aliphatic carbons	22-23.2 ppm splitting of signal
Vinyl carbon (-CH=CH2)	2 3.5-4 ppm intensity is fast
Hydroxyl group hydrogen	20.1-22.5 ppm number of signal
Epoxide carbon	20.3-20.4 ppm nil
Methyl group	21.2-21.5 ppm primary carbon

On the basis of carbon^{13} nuclear magnetic spectra (NMR) the compound has aromatic and aliphatic carbon that are identified on the basis of chemical shift value in parts per million. The vinyl hydrogen has different chemical shift value. The methyl group contains primary carbon with minimum chemical shift value in parts per million. The epoxide shows different part per million value of chemical shift(ppm). The expantion of the C^{13} spctra is used to separate the peaks properly with effective results. The resolution is increased with the value of carbon chemical shift.

GG-08 (Shinflavonone)

DEPT (135)



1.10 DEPT of Shinflavanone

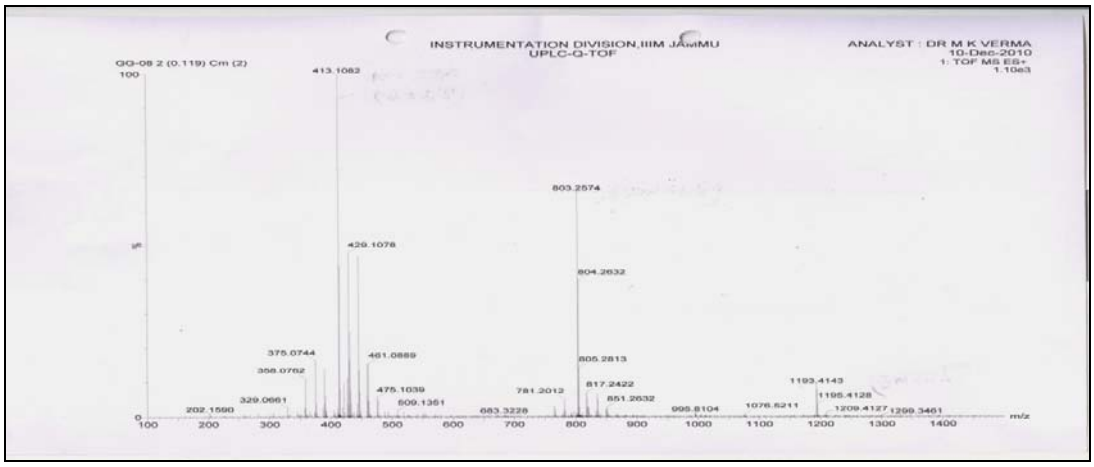
^{2}D NMR Spectrum

Name of atom	Chemical shift Interpretation
Aromatic deuterium	6-8.5 ppm neighboring group effect
Aliphatic deuterium	2-3.2 ppm splitting of signal
Vinyl group deuterium	3.5-4 ppm intensity is fast
Hydroxyl group deuterium	2.1-2.5 ppm number of signal
Epoxide deuterium	2.3-2.4 ppm nil
Methyl group	1.2-1.5 ppm primarydeuterium

On the basis of deuterium nuclear magnetic spectra (^{2}D NMR) the compound has aromatic and aliphaticdeuterium that are identified on the basis of chemical shift value in parts per million. The vinyldeuterium has different chemical shift value. The methyl group contains primarydeuterium with minimum chemical shift value in parts per million. The epoxide shows different part per million value of chemical shift.

GG-08 (Shinflavonone)

ES-MS



1.11 ES-MS of Shinflavanone

Mass Spectra of Shinfovonone

On the basis of molecule. Mass we could determine the molecular weight of the compound. We know that the molecular ion peak gives the mass or molecular weight of the uknown compounds. In the given figure above the base peak shows the peak at 387.678 that is the mass of the product. The parent peak is known as mass or mole. Weight of the unkown compound.The fragementations of the mixes indicates limits and metabolites of the intensifies that is exceptionally useful for structure explanation of the mixes. The m+1, m+2, m+3 and so forth. Pinnacles are known as isotopic pinnacle of the compound. These pinnacles are exceptionally useful to decide the sub-atomic fornula of the obscure mixes. These two things sub-atomic weight and sub-atomic formular are vital determinant of the basic clarification of the unkonw compound. We speaks to relative power or wealth on the y-pivot and atomic mass on the x-hub for elucidation.

Biological Assays

Pure Compounds GG-02, GG-03, GG-08, GG-09, GG-19, GG-33, GG-35, GG-36 & GG-44 isolated from *G. glabra* ethanolic extract were evaluated for their *in vitro* biological activities which include: - Antibacterial, Antifungal activity along with original crude.

Agar-Well Diffution Method for Antimicrobial Screening

Test organisms (Bacteria)

Methicillin Resistant *Staphylococcus aureus* (MRSA)
Vancomycin Resistant *Enterococcus faecalis* (VRE)
Pseudomonas aeruginosa ATCC 27583

Fungal

C&ida albicans (FCZ^{r})
Aspergillus fumigatus

Assay Media

Bacteria: Muller Hinton agar (Difco, USA)
Fungal: RPMI supplemented with 0.165M MOPS & 1.5% agar (Sigma)

Standard antimicrobial agents used

Ciprofloxacin - 5µg/50µl
Amphotericin B - 1 µg/50µl

Screening of extracts for antimicrobial activity

1. The inoculums were prepared in sterile normal saline of test organisms from the over night growth on trypticase soya broth (for bacteria) & sabouraud dextrose agar (for fungi). For *Aspergillus fumigatus*, used the conidial suspention.
2. Turbidity was adjusted to 0.5 Mc Farl& (equivalent to 1.5 x 10^{8} CFU/ml of *E.coli*& 1 x 10^{6} CFU/ml of *C. albicans*) using a densitometer. 3.500µl of McFarl& adjusted cultures was added to 50 ml of sterile molten agar (45 - 50 ^{0}C). Muller Hinton agar was used for bacterial cultural & RPMI media for fungal cultures. It was mixed & poured in to PD150 sterile plastic plates. Allowed it to set & then individual plates were marked with the organism inoculated.

- Punched the wells (6 mm diameter) & 50µl of sample (extract) was added to it. Appropriate standard antibiotic was used as a control.
- Plates was Incubated at 37^{0}C for 24 to 48hrs.

Zone of inhibition was measured & results were recorded.

Table 1: Antibacterial & Antifungal activities of Compounds

S. No.	Tested extracts	Sterility	*Tested organisms (Zone Diameter (in mm))*				
			Bacterial Pathogens			Fungal Pathogen	
			MRSA	VRE	*P. aeruginosa*	*C. albicans* (FCZ	*A. fumigatus*
1.	Crude	NG	0	0	0	0	0
2.	GG-02	NG	6	6	0	4	0
3.	GG – 03	NG	15	14	0	12	0
4.	GG-08	NG	7	5	0	4	3
5.	DMSO control	NG	0	0	0	11^{H}	0
6.	Drug control	NG	0	0	32	20	21

Here H = Hazy zone of inhibition, G = Growth & NG = No Growth. Ciprofloxacin (5µg/well) & Amphotericin-B (1µg/well) were used as a standard antibacterial & antifungal agent respectively in this study.

Results & Discussion of Antibacterial Activity

The Ethanol extract of rhizome of *G. glabra* was chromatographed over silica gel column & then final purification achieved by sephadex (LH-20). Known compounds, Hispaglabridin B (GG-02), 4-O-Methylglabridine (GG-03), Shinflavanone (GG-08), were isolated & their structure was determined by spectroscopic methods.

Compounds were screened for Antibacterial & Antifungal activity, resulted was tabulated in Table 4. However, the crude was not showing any activity (250µg/ml) but pure compounds have different activity pattern against tested organism. None of the compound was found to be active against *P. aeruginosa*.

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