Chapter 2:

Collection, Identification, Extraction, Chemical Screening and Characterization of Calotropis gigantea Linn.

Abstract

2.1 Introduction

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Abstract

To collect, identify, chemically screened and characterize the active component present in ethanol (EECGL) and water (WECGL) extract of *Calotropis gigantea (C. gigantea) Linn* latex extract.Th ephytochemical screening was done for alkaloids, amino acids, flavonoids, steroids and terpenoids, reducing sugars, gum, tanines, saponins, glycoside, cardiac glycoside and resins with standard screening methods. Characterization was done by spectroscopic methods like Infra-red (IR), Liquid chromatography-mass spectroscopy (LCMS) and Nuclear Magnetic Resonance (NMR). Chemical screening showed the presence of flavonoids, alkaloids, tri-terpinoides, Saponin and glycosidesis, cardiac glycoside present in the EECGL and WECGL.Analytical methods indicate the presence of some already reported phytochemicals like Calotoxin, Uscharin and Frugoside**,** Isorhamnetin-3-*O*-Glucopyranoside, 4-0 beta-D-glucopyranosyl frugoside from EECGL and Calotropogenin, Uscharin, Giganticine, Lupeolfrom WECGL and some other important components has been identified. It is evident from the study that *Calotropis gigantea* latex possessed majority of phytochemical classes of compounds and *C. gigantea* which may be responsible for potent anti-oxidant and anti-inflammatory and anti-cancer activity as well.

2.2. Introduction

Plant-derived substances have recently become of great interest owing to their versatile applications. Medicinal plants are the richest bio-resource of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs (Ncube et al., 2008).

Pharmaceutically, extraction is the process of separation of medicinally active portions of plant or animal tissues from the inactive or inert constituents by using selective solvents. These extracted products from plants are moderately impure liquids, semisolids or powders desired for only oral or external use. These preparations are classified as decoctions, infusions, fluid extracts, tinctures, pilular (semisolid) extracts and powdered extracts. Such preparations are termed as galenicals, named after the second century Greek physician Galen. The objectives of standardized extraction procedures for crude drug are to attain the portion of therapeutic interest and to eliminate the inert material by treatment with a selective solvent known as menstruum. Substantial effort has been made by researchers to find an efficient extraction method in order to acquire high efficiency and efficacy. Efficiency means the yield of extraction, whereas efficacy refers to the potency of the extract. Potency means the magnitude of bioactivity or the ability to produce an effect. For separation of biological components, extraction from plant is one of the most viable approaches (Jadhav et al., 2009).

Several factors make impact on quality of an extract. Such factors are - plant parts used as starting material, solvent used for extraction, extraction procedure and plant material: solvent ratio etc.

Extraction techniques isolate the soluble plant metabolites through selective use of solvents. The choice of appropriate solvent as well as application of a compatible extraction method has equal importance in extraction. '*Like dissolves like'* principle is applicable for solvent selection Thuspolar solvents can extract out polar substances and non-polar material will be extracted out by non-polar solvents. The most popular extraction method is solvent extraction.The choice of solvent is influenced by what is intended with the extract. Since the end product will contain traces of residual solvent, the solvent should be non-toxic and should not interfere with the bioassay. The choice will also depend on the targeted compounds to be extracted (Ncube et al., 2008; Das et al., 2008). The different types of solvents,such as water, alcohol, acetone, chloroform, ether, dichloromethane etc. can be used to in extraction process to have good yield.

A standard extraction process consists of following steps (Shah and Rohit, 2013):

- Collection, plant material authentication and shade drying
- Reduction of size
- Extraction
- Filtration
- Concentration
- Drying and reconstitution

2.2 Materials and methods

2.2.1 Chemicals

All the chemicals were used are of analytical grades. Chemicals were purchased from Himedia India, Ltd., Mumbai, India; and Merck India, Ltd., Mumbai, India for the experimentation.

NMR solvent: Methanol-d4 of Cambridge isotopes, NMR tubes are Wilmar® (diameter 5 mm).

2.2.2 Collection , identification and extraction of plant material

*C. gigantea*plant was identified from the out fields of Vidyasagar University, Midnapore,West Bengal, India and authenticated from Botanical Survey of India, Ministry of Environment & Forest, Govt. of India, Howrah (Identification No. CNH/2014/Tech.II/55).

For this study, the fresh latex of *C.gigantea*was collected from the aerial parts of mature plants. The collected latex was then air dried under shade up to four to five days at room temperature. Then 250 g of dried powdered latex was dissolved in 450 ml of ethanol and water separately and was incubated in room temperature again for 48 hours. Then filtered using Whatmann filter paper and the filtrates were concentrated using rotary evaporator (EYELA CCA 1110). The concentrated ethanolic filtrates were poured in petridishes and then were incubated at 37ºC for drying to produce crude ethanol (EECGL) extracts (yields 4.5% w/w). Water (WECGL) extract (yields 2.8% w/w) was obtained by lyophilizing the filtrate for the removal of water (Maiti et al, 2015).

2.2.3 Phytochemical screening of the extracts

Chemical tests for the screening and identification of bioactive chemical constituents of C.*gigantea*latex extract study were carried out in extracts using the standard procedures as described by Harborne, 1998.

2.2.3.1 Tests for flavonoids

(a) Alkaline reagent test

About 1 ml of ethanol and water extracts of *C.gigantea*latex (test solutions) was treated with few drops of sodium hydroxide solution. An intense yellow coloration was observed which disappeared after the addition of dilute Hydrochloric acid (HCl).

(b) Lead acetate Test

Few drops of lead acetate solution were added to 1 ml of each test extract and to produce a yellow colored precipitate.

2.2.3.2Tests for alkaloids

Briefly 0.5 to 0.6 g of each test extract was mixed in 8 ml of 1% HCl, warmed and filtered. 2 ml of the filtrate were treated separately with both reagents (Mayer's and Dragendorff's). The formation of yellowish buff and reddish brown color precipitate respectively indicates the presence of alkaloids.

2.2.3.3Tests for steroids (Libermann-Burchared test)

Two ml of acetic anhydride was mixed with each (0.5 g) of ethanol and water extract of *C.gigantea*latex followed by 2 ml of sulphuric acid (H_2SO_4). The colour was not changed from violet to blue or green which indicates the presence of steroids.

2.2.3.4 Test for triterpenoids (Salkowski test)

Five ml of each latex extract was mixed in 2 ml of chloroform followed by the careful addition of 3 ml of concentrated H_2SO_4. A layer of the reddish brown coloration was formed at the interface. It indicated a positive result for the presence of terpenoids.

2.2.3.5 Test for reducing sugar

A small amount of two extracts was dissolved individually in distilled water and filtered. To the filtrate equal amount of Fehling's reagent A and B were added and heated for few minutes. Brick red precipitate was not formed which indicates the absence of reducing sugar.

2.2.3.6 Test for gum (Molish's Test)

Small amount of both extracts was dissolved separately in small amount of distilled water and filtrate was taken. Equal volume of concentrated H_2SO_4 was added separately with filtrates. 15 % alcoholic solution of α-naphthol (Molish's reagent) was added in each test tube. Red-violet ring at the junction of sulfuric acid layer was not formed which indicates the absence of gums in the extract.

2.2.3.7 Test for tannins

Both extracts (0.25 g) was dissolved in 10 ml distilled water and filtered. 1% aqueous ferric chloride ($FeCl_3$) solution was added to the filtrate. The appearance of intense green, purple, blue or black colour was not seen that indicates the absence of tannins in the test samples.

2.2.3.8 Test for saponins

A small amount of ethanol and water extract of *C.gigantea*latex was individually dissolved in a small amountof distilled water and wasshaken in a graduated cylinder for 15 minutes. Formation of stable foam suggested the presence of saponins.

2.2.3.9 Tests for glycoside

(a) Legal test

Extract was dissolved in pyridine. Then sodium nitroprusside solution was added to it to make the solution alkaline. Formation of pink reddish color indicated the presence of glycoside.

(b) Borntrager's test

Few ml of dilute sulphuric acid was added to the extract solutions separately. After boiling for few minutes, filtrate was taken. Then the filtrate was extracted with ether or chloroform. An orange coloured ring was separated in which ammonia was added. Formation of pink red colour in organic layer indicated the presence of glycosides.

2.2.3.10 Tests for cardiac glycosides (Keller-Killani test)

5ml of extracts was treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution. A brown ring in the interface indicates a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer, a greenish ring may form just gradually throughout thin layer which shows the presence of cardiac glycosides.

2.2.3.11 Test for resins

Two g of the ethanolic extract was dissolved in 10ml of acetic anhydride. A drop of concentrated sulphuric acid was added. Appearance of purple colour, which rapidly changed to violet, is indicative of the presence of resins. Same procedure was repeated using the water extract of the plant material.

2.2.3.12 Tests for amino acid

Ninhydrin (1, 2, 3-Indantrione monohydrate, or triketohydrindene hydrate) is often used to detect α-amino acids and also free amino and carboxylic acid groups on proteins and peptides.

About 0.5 ml of a 0.1% solution of ninhydrin was boiled for one or two minutes with 10 mlof sample extract at pH4-8. Ninhydrin, which is originally yellow, reacts with amino acid and turns blue or purple pigment, sometimes called Ruhemann's purple.

2.2.2 Characterization by Analytical methods

2.2.2.1 IR spectroscopy

Methodology:

Semisolid part of EECGL and WECGL is dissolved in dichloromethane (DCM) and only one drop of sample taken to the highly polished KBr salt plate,then placed a second plate on top of the first plate to spread the liquid in a thin layer between the plates, and clamp the plates together; mount the sandwich plate onto the sample holder and performed IR spectra of %transmittance vs. Wavenumber ($cm^{-}1$) to get information about functional groups from the IR instrument (Perkin Elmer) (Ghosh et al., 2011).After finishing the experiment, clean the plates with isopropanol (No water as KBr is soluble in water).

2.2.4.2 LCMS

Methodology:

For analysis of EECGL, formic acid (HCOOH) acts as a buffer and was prepared in 0.05 % in water. ACN in GEMINI-NX (4.6 × 50 mm) 5µ column with 10% to 90% gradient method having a flow rate of 1 ml/min; run time of 5 minutes in API MASS instrument: Shimadzu is used for EECGL. For analysis of WECGL, ammonium acetate (NH_4OAc) acts as a buffer and was prepared in 0.1 % in water. ACN in YMC(4.6 × 50 mm)5µ column with 5% to 95% gradient method having a flow rate of 1 ml/min; run time 3 min in API MASS instrument: Waters is used for WECGL.

Table 2.1 Gradient method for ACN/formic acid % for EECGL)

TIMING	***ACN %***	***HCOOH %***
0.01 min	*10*	*90*
1.5 min	*30*	*70*
3 min	*90*	*10*
4.0 min	*90*	*10*
5.0 min	*10*	*90*

Table 2.2: Gradient method for ACN/ NH4OAc %(EECGL)

TIMING	*ACN %*	*NH4OAc %*
0.01 min	*5*	*95*
1.00 min	*5*	*95*
1.5mn	*80*	*20*
2.0 min	*95*	*5*
3.0 min	*5*	*95*

2.2.4.3 NMR spectroscopy

Methodology:

About 50 mg of EECGL and WECGL was taken in two separate 5 mm original Wilmar® NMR tube (diameter 5 mm) and 1 ml of Methanol-d4(MeOD) was used to dissolve the sample and also for external locking system in NMR spectroscopy.

^{1}H NMR of EECGL was performed in MeOD solvent in a 400 MHz Bruker Ultra-shield magnet system and AVANCE spectrometer NMR instrument with zg30 pulse program having delay time (D1)=1sec; number of scan (NS)=72 using a 5 mm dual ^{13}C-1 probe. As WECGL was not totally soluble in MeOD; WECGL NMR spectra was recorded in MeOD solvent in a 400 MHz Bruker Ascend magnet system and AVANCE spectrometer NMR instrument with zg30 pulse program having delay time (D1) =5 sec; number of scan (NS)=64 using a 5 mm BBO probe head for enough relaxation of the nucleus and better resolution. Locking solvent signal from MeOD-d_4 was used as reference at 3.3 ppm (parts per million).

^{13}C-jmod APT (attached proton test; ^{13}C signal attached with CH and CH_3 gives positive and CH_2 and quaternary gives negative in phase as signal in spin echo concept) of both EECGL and WECGL was performed in a 400 MHz Bruker Ascend magnet system and AVANCE

spectrometer NMR instrument with jmod pulse program having delay time (D1) = 2 sec; number of scan (NS =72) using a 5 mm BBO probe.Locking solvent signal from MeOD-d4 was used as reference at ~ 49 ppm.

2.3 Results

2.3.1 Phytochemical analysis

Both extracts of latex part extracts were screened for the presence of various bioactive phytochemical compounds. The analysis which revealed the presence of different phytochemicals was documented in Table 2.3.

Table 2.3: Presence and absence of different phytochemicals in EECGL and WECGL latex extract. +Ve-Present, -Ve-Absent.

SL. NO.	***Experiment***	***Ethanolic extract of C. gigantea***	***Water extract of C. gigantea***
1.	*Flavonoids*		
1.1	*Alkaline reagent test*	*+Ve*	*+Ve*
1.2	*Lead acetate test*	*+Ve*	*+Ve*
2.	*Alkaloids*		
2.1	*Mayer's and Dragendorff'sreagent test*	*+Ve*	*+Ve*
3.	*Test for Steroids*		
3.1	*Libberman and Burchard's test*	*-Ve*	*-Ve*
4.	*Test for Triterpenoids*		
4.1	*Salwonski Test*	*+Ve*	*+Ve*
5	*Test for Reducing*		

	Sugar's		
5.1	*Fehling's test*	*-Ve*	*-Ve*
6.	*Test for gum*		
6.1	*Molish'sTest*	*-Ve*	*-Ve*
7	*Tannin*		
7.1	*Ferric chloride test*	*-Ve*	*-Ve*
8	*Saponin*		
8.1	*Foam Test*	*+Ve*	*+Ve*
9	*Glycoside*		
9.1	*Legal's test*	*+Ve*	*+Ve*
9.2	*Borntrager test*	*+Ve*	*+Ve*
10	*Cardiac Glycoside*		
10.1	*Keller-Killani test*	*+Ve*	*+Ve*
11	*Test for Resin*		
11.1	*Acetic anhydride test*	*-Ve*	*-Ve*
12	*Test for amino acid*		
12.1	*Ninhydrin test*	*-Ve*	*-Ve*

2.3.2 IR Spectroscopy analysis

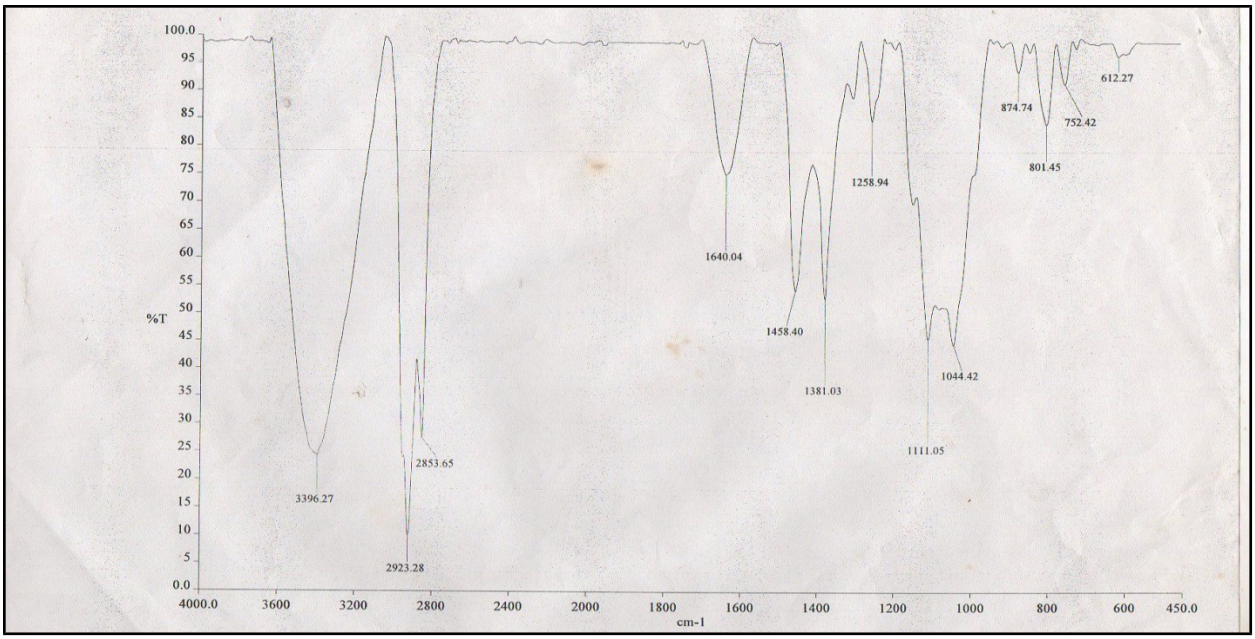


Figure 2.1 IR spectrum of EECGL

Table 2.4: Infra-red spectroscopic analysis of EECGL

Absorption	*Bond*	*Types of bond*	*Specific type of*	*Appearance*

peak(cm-1)			***bond***	
3155.50	*O-H*	*Alcohol/phenol*	*High concentration*	*Strong-Broad*
2932.27,2853.65	*Asymmetric stretch*	*H-Alkyl*	*Methylene*	*Strong*
1729.16 ; 1639.44	*CHO*	*Carbonyl*	*Any*	*Strong*
1400.50	*C-C*	*Ring C=C stretch*	*Any*	*Strong*
1246.26	*C-H*	*Ester- aromatic*	*Methyl*	*Moderate*
1094.45	*C-O*	*Alcohol*	*Primary*	*Moderate*
Peak from 880 to 610	*C-H*	*Aromatic*	*Meta/Para distributed benzene*	*Less intense*

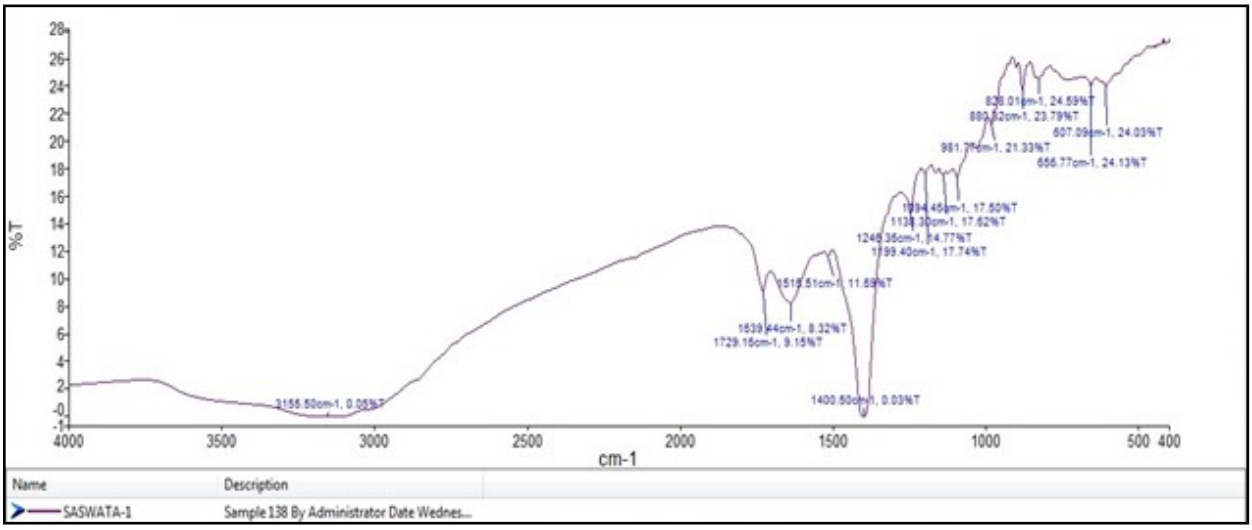


Figure 2.2 IR spectrum of WECGL

Table2.5: Infra-red spectroscopic analysis of WECG

Absorption peak(cm-1)	***Bond***	***Types of bond***	***Specific type of bond***	***Appearance***
3155.50	*O-H/Secondary amine*	*Alcohol/phenol*	*High concentration*	*Strong-Broad*
1729.16 ; 1639.44	*CHO*	*Carbonyl*	*Any*	*Strong*
1400.50	*C-C*	*Ring C=C stretch*	*Any*	*Strong*
1246.26	*C-H*	*Ester- aromatic*	*Methyl*	*Moderate*
1094.45	*C-O*	*Alcohol*	*Primary*	*Moderate*
Peak from 880 to 610	*C-H*	*Aromatic*	*Meta/Para distributed benzene*	*Less intense*

2.3.2 LCMS analysis of *C. gigantea* latex extract

From the LCMS data of EECGL below compound can be identified.

Calotoxin(Molecular formula: $C_{29}H_{40}O_{10;}$ exact mass:548.26).

Fragment at m/z 547.2 [M-H](260nm and retention time 2.658); 547.2 [M-H](220 nm and retention time 2.772) indicates the presence of Calotoxin in EECGL.

Uscharin: (Molecular formula: $C_{31}H_{41}NO_8S;$ exact mass:587.26).

Fragment at m/z 586.2 [M-H], 632.4(Parhira et al2014) [M+$HCOO^-$](260nm and retention time 3.229) indicates the presence of Uscharin in EECGL.

Frugoside: (Molecular formula: $C_{29}H_{44}O_{9;}$ exact mass:536.3).

Fragment at m/z 535.2 [M-H] (260nm and retention time 2.316) indicates the presence of Frugoside in EECGL.

4-0 beta-D-glucopyranosylfrugoside: (Molecular formula: $C_{35}H_{54}O_{14;}$ exact mass: 698.35)

Fragment at m/z 700.2[M+H] (220nm and retention time 2.316) indicates the presence of 4-0beta-D-glucopyranosylfrugoside in EECGL.

Isorhamnetin-3-*O*-Glucopyranoside: (Molecular formula: $C_{22}H_{22}O_{12;}$ exact mass: 478.11)

Fragment at m/z 477.0[M+H] (260nm and retention time 2.658) indicates the presence of Isorhamnetin-3-*O*-Glucopyranoside in EECGL.

In EECGL one major peaks is identified and showed molecular ion m/z at 451.2 at 260 nm UV range at channel 2 with retention time of 2.316 are close to Taraxasteryl acetate $[M+H]^+$ peak at m/z 468.4 which is a flavonol present in aerial part of EECGL. Some other small and less intense peak is also visible in EECGL. So these peaks may be the derivatives of already reported compound present in latex or totally unknown compound.

From the LCMS data of WECGL below compound can be identified.

Calotropogenin: (Molecular formula: $C_{22}H_{32}O_{5;}$ exact mass:388.22).

Fragment at m/z 387.2 [M-H] (retention time 1.665) indicates the presence of Calotropogenin in WECGL.

Uscharin: (Molecular formula: $C_{31}H_{41}NO_8S_{;}$ exact mass:587.26).

Fragment at m/z 646.4 [M+$CHCOO^-$](retention time 1.665) indicates the presence of Uscharin in WECGL.

Giganticine: (Molecular formula: $C_{13}H_{16}N_2O_{5;}$ exact mass:280.11).

Fragment at m/z 279.3[M-H] and 280.3(retention time 1.665) indicates the presence of Frugoside in WECGL.

Lupeol: (Molecular formula: $C_{30}H_{50}O$, exact mass:426.39).

Fragment at m/z 427.2[M+H] and 428.2(retention time 1.665) indicates the presence of Lupeol in WECGL.

In WECGL the major peak at the retention time 1.128 , m/z 229.1; and at retention times 1.665, m/z 194.1, 196.3, 225.3, 242.2, 357.2, 369.1, 570.3, 710.4 and some other small less intense peaks may be the fragment or daughter peak of some already isolated compound or coming from some completely unknown compounds.

So, they may be some derivative or completely unknown new compound which is yet to be studied. Some other intense and non-instance peaks are present in EECGL and WECGL LCMS spectrum which may be coming from the daughter fragments of these compounds or some other already important compound and their derivatives or some totally undiscovered present in EECGL and IN WECGL. Detail is in table no: 2.6.

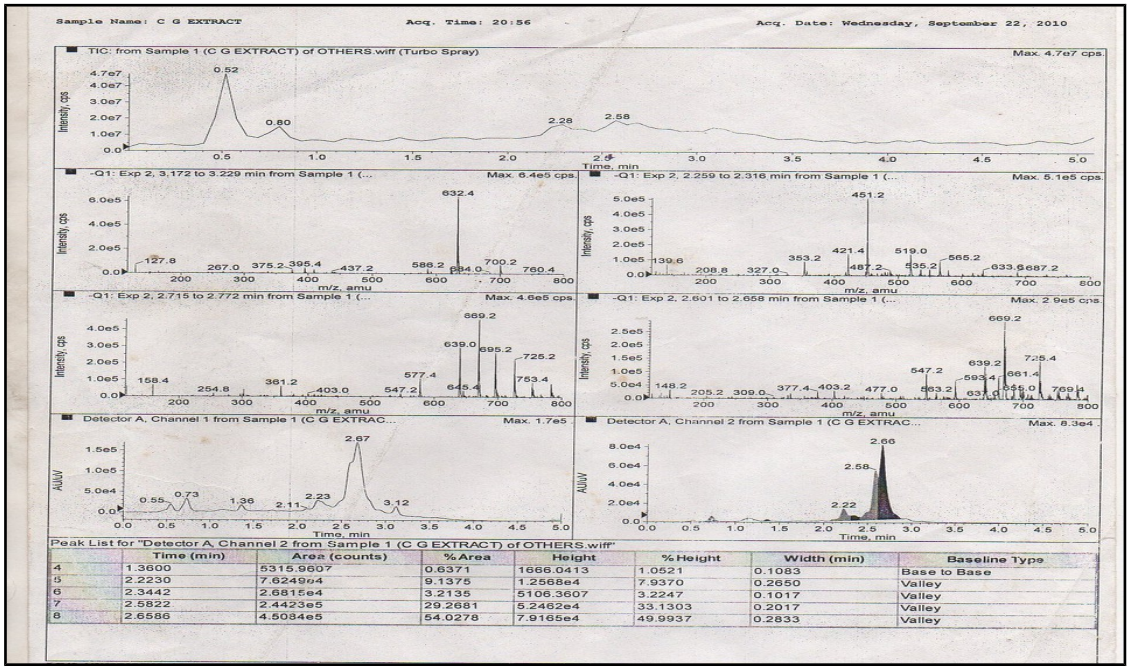


Figure 2.3 LCMS analysis of EECGL.

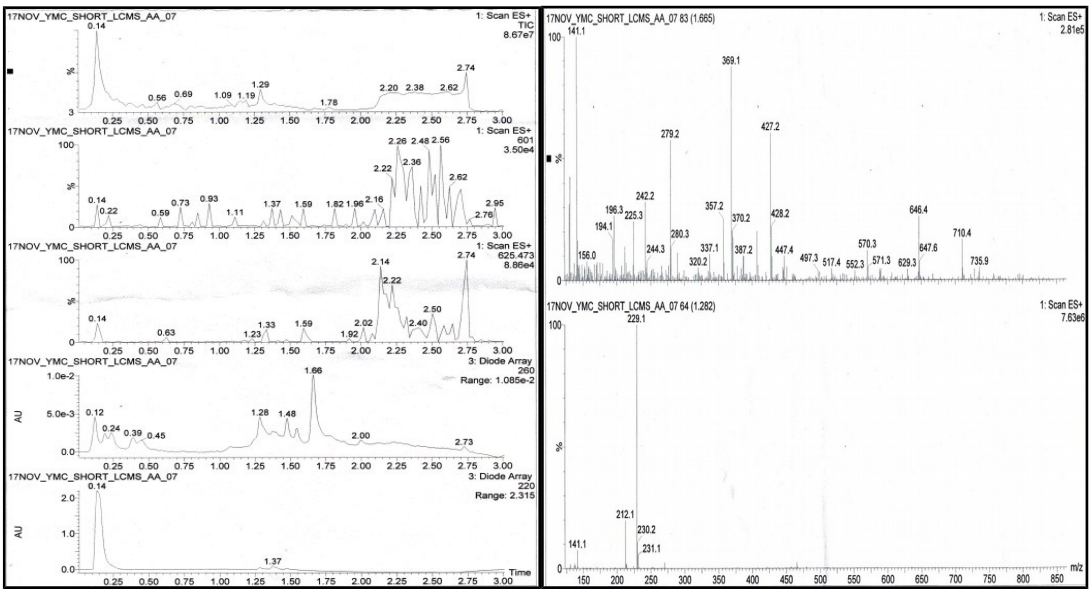


Figure 2.4. LCMS analysis of WECGL

2.3.4 NMR analysis of *C. gigantea* latex extracts

^{1}H NMR (400 MHz) (MeOD) : Peak at δ_H (ppm) ~ 10.04 (1H,s) indicates the presence of aldehyde (CHO) group which is a characteristic peak of cardiac glycoside i.e. Calotropogenin (H_{24}), Calotoxin(H_{35}), Calotropin (H_{25}), Uscharin (H_{30}), Calactin (H_5) which are already reported to be present in latex part of C. *gigantea.* In the aromatic region peak at δ_H (ppm) ~ 5.91 (1H,s) is the characteristic peak of Calotoxin (H_{30}), Calotropin (H_{20}), Uscharin (H_{25}), Calactin (H_{32}), reported to be present in latex (Tiwari *et al* 1978; Chaudhary et al 1966,Parhira et al2014) and

other cardenolide glycoside like Coroglaucigenin (H_{23}), Frugoside (H_{23}), 4-0 beta-D-glucopyranosylfrugoside (H_{23}) which are reported to be present in root part (Kiuchi et al 1998). Peak at δ_H (ppm) ~ 7.56 (1H, t) in EECGL and WECGL comes from Uscharin (H_{32}; triplet peak of H_{32} is broad due to presence of quadrupole nitrogen atom attached to it). There are the common component may be present in both in EECGL and WECGL.

Peak at δ_H (ppm) ~ 6.9 (1H, s) in EECGL may be the merging peaks come from the Calotropogenin (H_{19}) and of Isorhamnetin-3-*O*-rutinoside (H_7) and Isorhamnetin-3-*O*-Glucopyranoside (H_7). Peak at δ_H (ppm) ~ 5.93 (2H,s) of EECGL may be from Isorhamnetin-3-*O*-rutinoside and Isorhamnetin-3-*O*-Glucopyranoside (H_{15}) , (H_{22}) merging with peak atδ_H (ppm) ~ 5.91 ppm (1H,s) ppm of previously assigned peak of cardiac glycoside present in EECGL and WECGL reported to be present in the areal part of C *gigantea*(Sen*et al*., 1992). Peak at δ_H (ppm) ~ 6.7 (2H, d) can be assigned the merged peak from Isorhamnetin-3-*O*-rutinoside and Isorhamnetin-3-*O*-Glucopyranoside (H_8 and H_{14}).

Peak at δ_H (ppm) ~ 7.73, (2H, d) and ~ 7.70 (2H, d) in WECGL may come from Giganticine (H_1, H_5, H_2, H_4) reported to be present in root bark part, Pari K et al., 1998.

Peaks at aliphatic region from 5 to 0 ppm are very clumsy and difficult to interpret. Peak at δ_H (ppm) ~3.8 (1H, s) may be the merged peak coming from H_{18}, H_{24}, H_{25}, H_{30}& H_{35}, H_{36}, H_{38}, and H_{40} present in isorhamnetin-3-*O*-rutinoside and Isorhamnetin-3-*O*-Glucopyranoside. Other aliphatic peaks between δ_H (ppm) ~ 3.7 to 0.5 are coming from the aliphatic region of above mentioned components and may be other components present in the latex part from both EECGL and EECGL.

^{13}C (jmod APT) (100MHz) (MeOD): EECGL shows aldehyde peak at δ_C (ppm) ~ 208.05, carbonyl peaks at δ_C (ppm) ~ 176.85 and 175.86 ppm. Peaks at δ_C (ppm) and ~ 161.69 is the characteristic peak of Uscharin (C_{32}). Peak at δ_C (ppm) ~ 118.80, 118.67, 116.51, 114.70, 109.58, 110.26, 101.03, 95.38, 91.67 indicate the presence of phenyl groups and the characteristic peaks of Isorhamnetin-3-*O*-rutinoside, Isorhamnetin-3-*O*-Glucopyranoside, cardiac glycosides and other important compound present in latex extract. Quaternary peak at δ_C (ppm) ~ 98.28 may come from Taraxasteryl acetate (C_{30}).

WECGL shows carbonyl peaks at δ_C (ppm) ~ 177.73. Aromatic CH peaks at δ_C (ppm) ~131.16 ppm and 128.50 ppm is the characteristic peaks of Giganticine (C_1, C_5, C_2, C_4). Peak at ~ 106.56 (C_{31}) is the Olefinic CH2 ofLupeol. Quaternary carbon peak and other many peaks did not develop properly due to low concentration and less solubility. Other carbon signal at the aliphatic region from 100 ppm to 10 ppm indicates the presence of other important components and previously assigned components present in latex part.

As the % yield of EECGL (4.5%) and WECGL (2.8%) is very low and are partly soluble in MeOD and mostly insoluble in other deuterated solvent and specially mixture of many components, hence 2D NMR like COSY (Homonuclear correlation spectroscopy, HSQC (Heteronuclear Single Quantum Coherence) and HMBC (Heteronuclear Multiple Bond Correlation) was not able to be done for further structure determination.

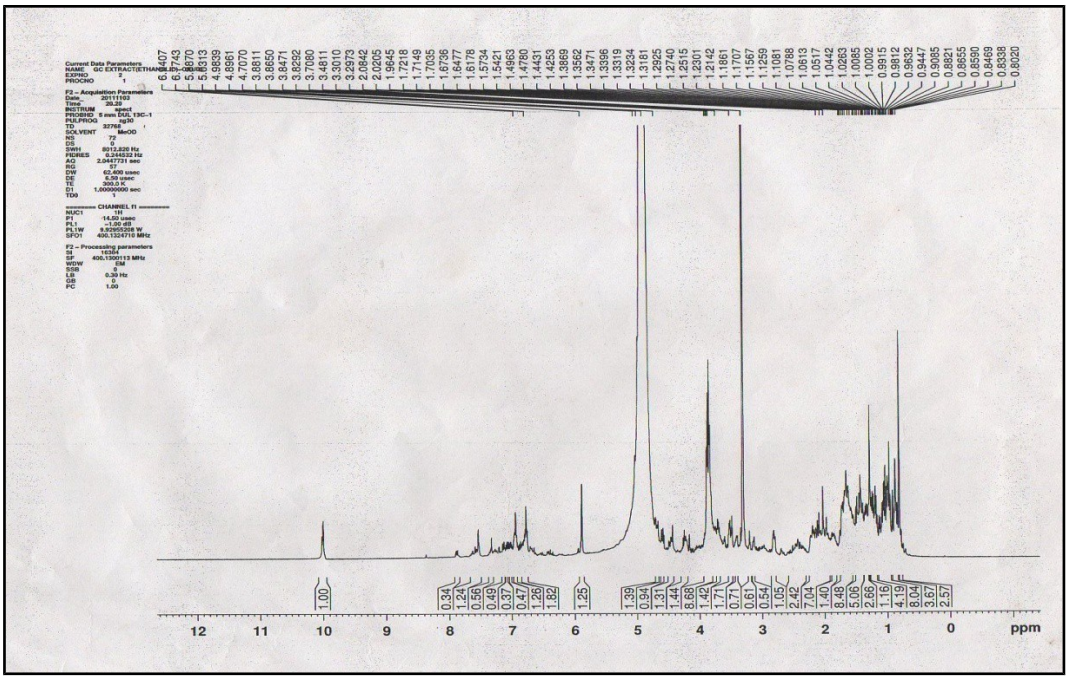


Figure 2.5.[1]H NMR analysis of EECGL. (Chemical shift is according to MeOD NMR solvent).

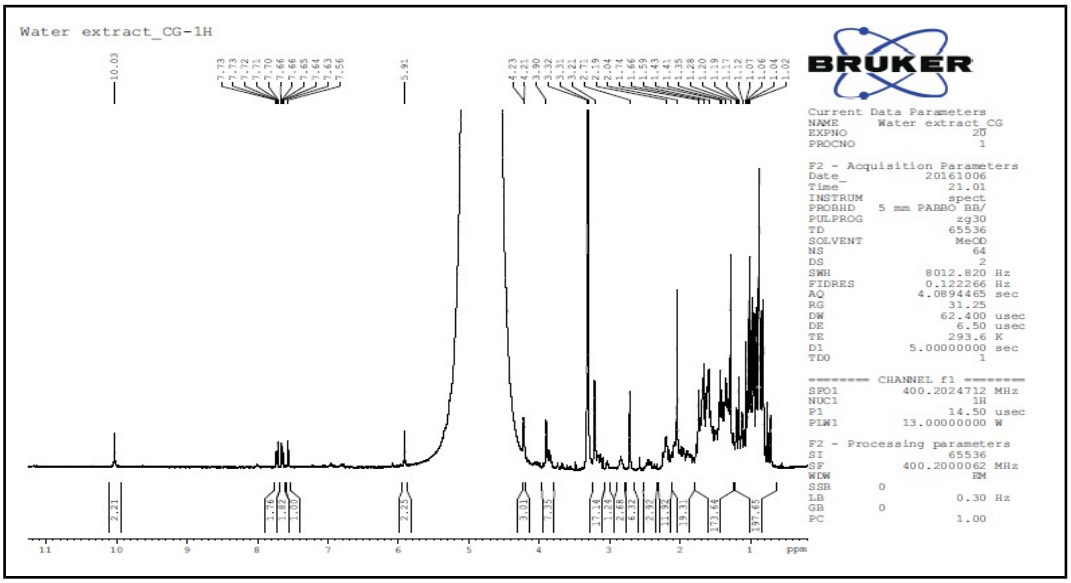


Figure 2.6[1]H NMR analysis of WECGL. (Chemical shift is according to MeOD NMR solvent)

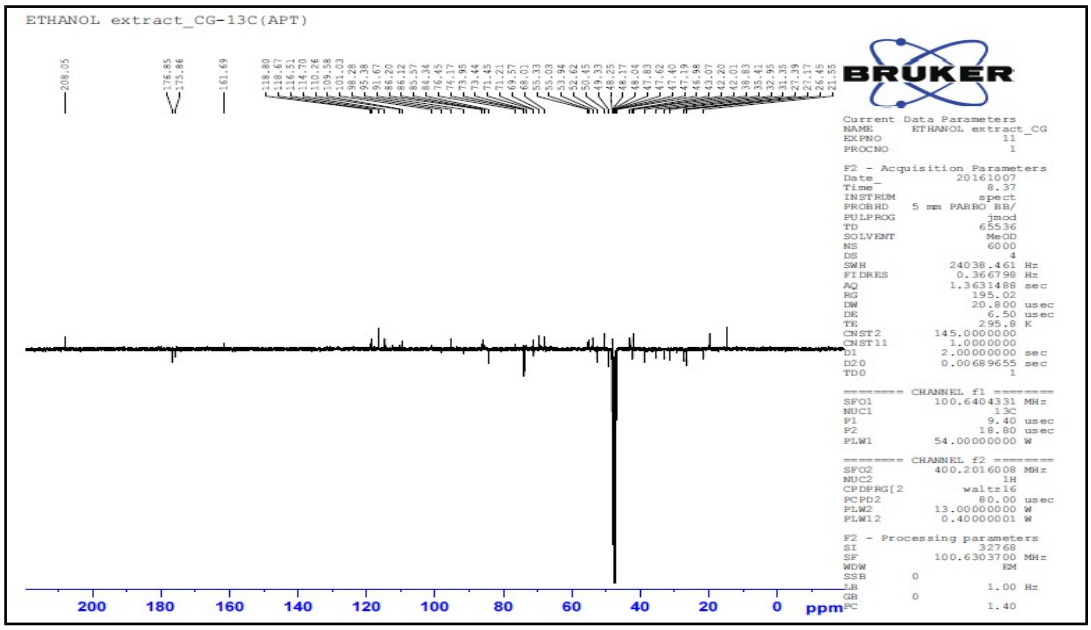


Figure 2.7. ^{13}C (jmod-APT) NMR analysis of EECGL. (Chemical shift is according to MeOD NMR solvent)

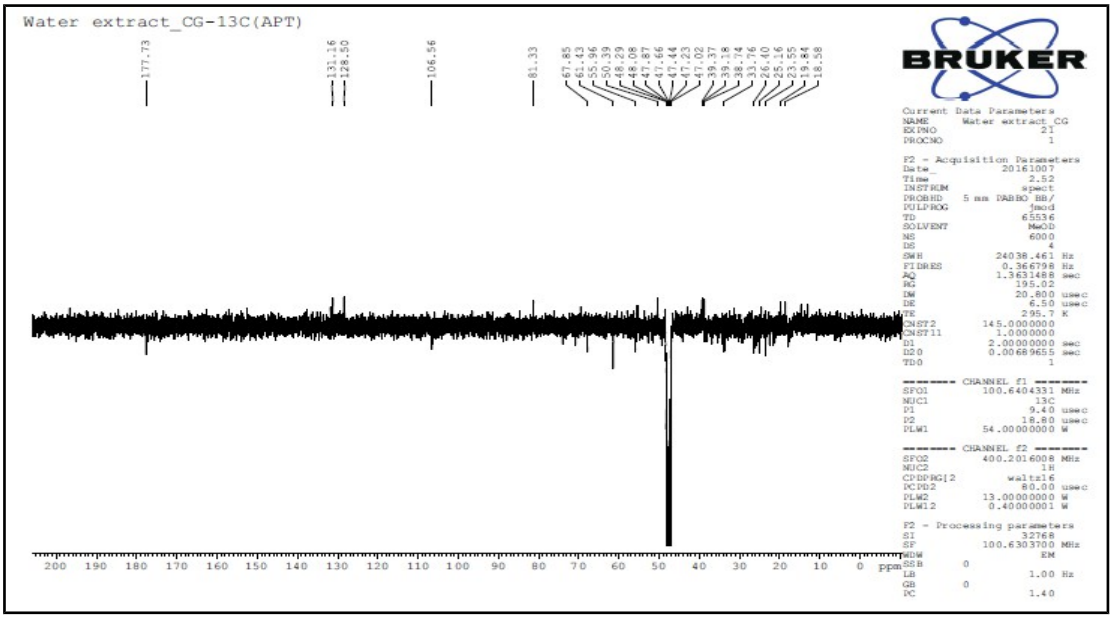


Figure 2.8. ^{13}C (jmod-APT) NMR analysis of WECGL. (Chemical shift is according to MeOD NMR solvent).

Table 2.6:Summary of NMR, LCMS and IR Spectroscopy

Compounds and peak numbering (Proton/Carbon); Present in EECGL/WECGL	*General chemical formula; Exact mass; Chemical nature*	*Important peak - 1H- δH (ppm) ; Number of H atoms ; (multiplicity)*	*Important peak (EECGL/WECGL) - 13C - δC(ppm)/Caron signal details*	*LCMS spectra (220 nm in channel 1 and 260 nm at channel 2 UV range (for Shimadzu)*	*IR υmax (KBr) cm-1:*	*Compound found in with Reference*
Calotropogenin ; H24,H19/C24, C21 WECGL	*C22H32O5; 388.22; Cardiac glycoside*	*~ 10.04 (1H24,s) ; 7.56 (1H19,s)*	*~ 177.73 (C21) (quaternary peak of carbonyl carbon).*	*WECGL : Fragment at 387.2 [M-H]*	*EECGL : ~ 3396.27 (-OH strong-Broad) ; 2923.28, 2853.65 (CH Stretch) ;*	*Latex part (Tiwari et al 1978; Chaudhary etal*

					1640.04(C=O stretch);1458.40 (Ring C=C stretch stretch) ; 1381.03 (CH3 C-H bend) ; 1258.94 (Ester-aromatic) ; 1111.05 (RR'CH-OH (2o) or C=C-CRR'-OH); 1044.42 (Ethers-Ar-O-R); 874.74,752.42,	*1966)*
Calotoxin ; H35,H30/C35,C31 EECGL	*C29H40O10 ; 548.26; Cardiac glycoside*	*~ 10.04 (1H39,s) ; 5.91(1H30,s)*	*~ 208.05 (C35, CHO peak of aldehyde),~ 176.85, 175.86 (C31) (quaternary peak of carbonyl carbon)*	*EECGL : Fragment at m/z 547.2 [M-H] (260nm) ; [M-H] 547.2 (220 nm)*		*Latex part (Tiwari et al 1978; Chaudhary et al 1966)*
Calotropin; H25,H20/C25,C21 EECGL and WECGL (May be present)	*C29H40O10; 532.27; Cardiac glycoside*	*~ 10.04 (1H25,s) ; 5.91(1H20,s)*	*EECGL ~ 208.05 (C25, CHO peak of aldehyde) , ~ 176.85, 175.86 (C21) (quaternary peak of carbonyl carbon)*	*EECGL : Fragment at m/z 519,535.2 [M+H]+ (260nm)*		*Latex part (Tiwari et al 1978; Chaudhary etal 1966)*

			(EECGL); WECGL ~ 177.73 (C21) (quaternary peak of carbonyl carbon).		*801.45 (stretching and Meta/Para distributed benzene ring).*	
Uscharin ; H30,H25,H32/C30, C26,C32 EECGL and WECGL	*C31H41NO8S; 587.26; Cardiac glycoside*	*EECGL: ~ 10.04 (1H30, s); 5.91(1H 25, s) WECGL : 10.04 (1H30, s); 5.91(1H 25, s) ;~ 7.56 (H32) (1H, t)*	*EECGL ~ 208.05 (C30, CHO peak of aldehyde),~ 176.85, 175.86 (C26), (quaternary peak of carbonyl carbon); ~ 161.69 (C32, CH of heterocyclic imine ring)*	*EECGL : Fragment at m/z 586.2 [M-H],632.4 [M+HCOO-] (260nm) WECGL : m/z 646.4 [M+CHCOO-]*	*WECGL : 3155.50 (-OH strong-Broad;Secondary amine) 1729.16 (C=O stretch); 1639.44 (C=O stretch); 1400.50*	*Latex part (Tiwari et al 1978; Chaudhary et al 1966), Parhira et al 2014*

Calactin H5,H32/C5,C35 EECGL and WECGL (May be present)	*C29H40O10; 532.27; Cardiac glycoside*	*~ 10.04 (1H5 ,s); 5.91(1H 32 ,s)*	*EECGL ~ 208.05 (C5, CHO peak of aldehyde),~ 176.85, 175.86 (C35) (quaternary peak of carbonyl carbon), ~ 161.69 (C32) (Primary - CH of phenyl ring); WECGL ~ 177.73 (C26) (quaternary peak of*	*EECGL : Fragment at m/z 519,535. 2 [M+H] + (260nm)*	*(ring C=C stretch); 1246.26 (Ester-aromatic).*	*Latex part (Tiwari et al 1978; Chaudhary et al 1966)*

			carbonyl carbon).			
Coroglaucigenin (H23); EECGL and WECGL (May be present)	*C23H34O5 ; 390.24 ; Cardenolide glycoside*	*~ 5.91(1H 23,s)*	*Only information in aliphatic region which is very clumsy and difficult to interpret.*	*EECGL : Fragment at m/z 395.4 [M+H]+ (260nm)*		*Root part (Kiuchi et al1998)*
Frugoside (H23); EECGL	*C29H44O9 ;536.3 ; Cardenolide glycoside*	*~ 5.91(1H 23,s)*	*~ 118 (C23) and information in aliphatic region which is very clumsy and difficult to interpret.*	*EECGL : Fragment at m/z 535.2 [M-H] (260nm)*		*Root part (Kiuchi et al 1998)*
4-0 beta-D-glucopyranosylfrugoside (H23); EECGL	*C35H54O14 ; 698.35 ; Cardenolide glycoside*	*~ 5.91(1H 23,s)*	*~ 118 (C23) and information in aliphatic region which is*	*EECGL : Fragment at m/z 700.2 [M+H] (220 nm)*		*Root barks (Kiuchi et al 1998)*

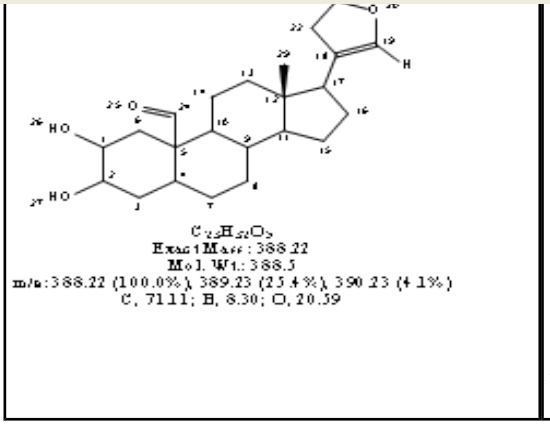
			very clumsy and difficult to interpret.			
Isorhamnetin-3-O-rutinoside ; (H7, H 8 and H14, H15, H22); EECGL (May be present)	*C28H32O 16 ; 624.17 ;Flavanol*	*~ 6.9 (1H7, s); ~ 5.93 (2H15,2 2,s), ~ 6.7 (2H8,14, d)*	*EECGL: Some peak at aromatic region is the supporting data from APT NMR (~ 118.80, 118.67, 116.51, 114.70, 109.58, 110.26, 101.03, 95.38, and 91.67). Other information is from aliphatic region*	*EECGL : Fragment at m/z 632.4 [M+H]+ (260 nm),*		*Arial parts Sen S et al., 1992.*

			-which is very clumsy and difficult to interpret.			
Isorhamnetin-3-O-Glucopyranoside; (H7, H 8 and H14, H15, H22; EECGL	*C22H22O12 ; 478.11 ;Flavonol*	*~ 6.9 (1H7, s); ~ 5.93 (2H15,22,s), ~ 6.7 (2H8,14, d)*	*EECGL: Some peak at aromatic region is the supporting data from APT NMR (~ 118.80, 118.67, 116.51, 114.70, 109.58, 110.26, 101.03, 95.38, and 91.67). Other information is from aliphatic*	*EECGL : Fragment at m/z 477.0 [M-H] (260 nm),*		*Arial parts ; Sen S et al., 1992.*

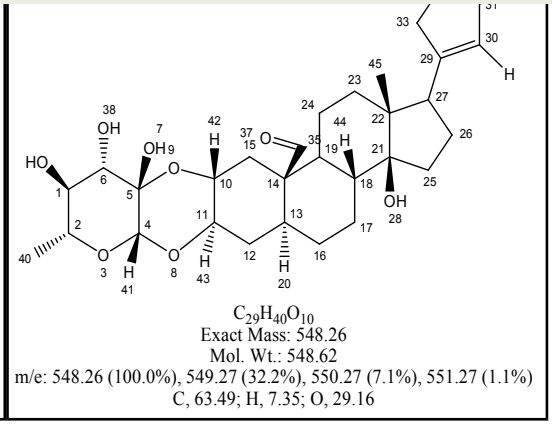
			region -which is very clumsy and difficult to interpret.			
Giganticine; H1, H5, H2, H4 / C1, C5, C2, C4 WCGGL	*C13H16N2O5; exact mass:280.11; Sterols*	*~ 7.73, (2H 1, 5, d) and ~ 7.70 (2H 2, 4 d).*	*WCGGL ~128.50 (C1, C5), 131.16 (C2, C4) (Primary - CH of phenyl ring).*	*WECGL : Fragment at m/z 279.2 [M-H] and 280.3)*		*burk part, Pari K et al., 1998.*
Taraxasteryl acetate ; C30 ; EECGL (May be present)	*C32H52O2 ; 468.4 ;Flavanol*		*~ 98.28 (C30) ; (Olefinic CH2).*			*Arial parts; Sen S et al., 1992.*
Lupeol; C31; WECGL	*426.39; C30H50O ; Triterpenoid*	*Olefinic CH2 peak may merge with MeOD*	*~ 106.56 (C31); Olefinic CH2)*	*WECGL : Fragment at m/z 427.2 [M+H] and*		*Latex, Sarath a et al2011*

		water peak.		*428.2*		

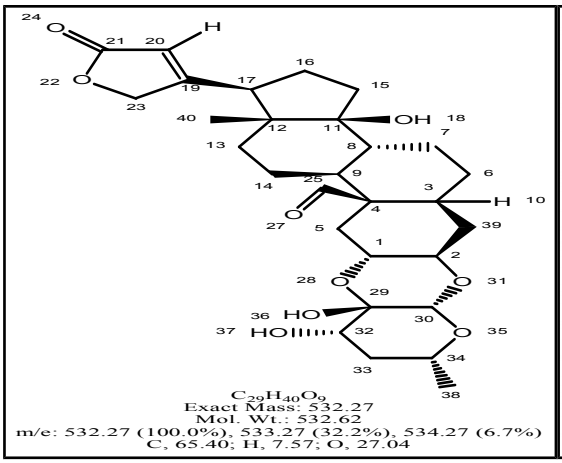
Above study indicates the presence of Calotoxin, Uscharin, Frugoside**,** and Isorhamnetin-3-*O*-Glucopyranoside, 4-0 beta-D-glucopyranosylfrugoside in EECGL and Calotropogenin, Uscharin, Giganticine, Lupeol in WECGL. Many other unknown peaks are present in LCMS spectra, some of which are the daughter fragments and m/z of some peaks are very close to some already isolated compounds from latex of C. *gigantea* in EECGL and WECGL which may have been identified or yet to be discovered.

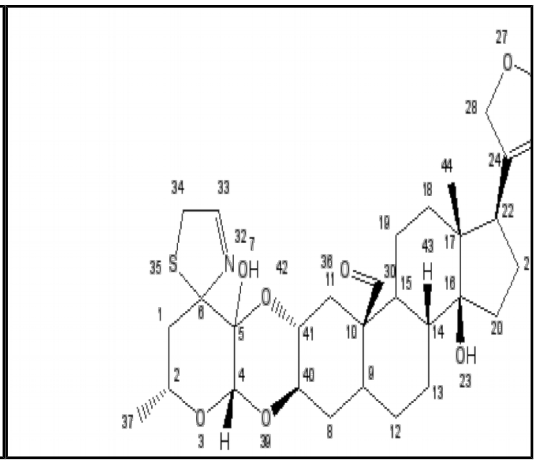


Calotropogenin*

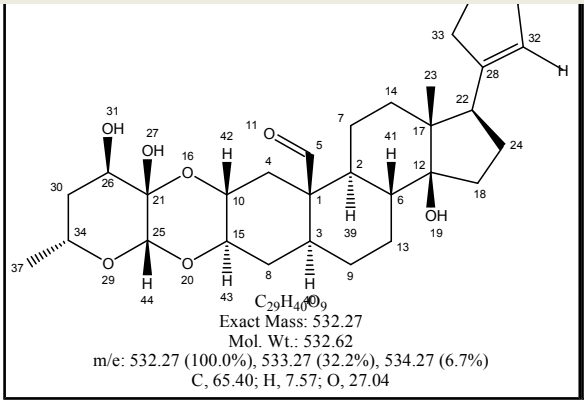


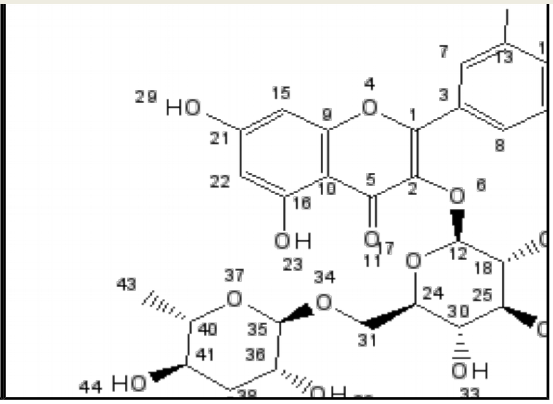
Calotoxin**

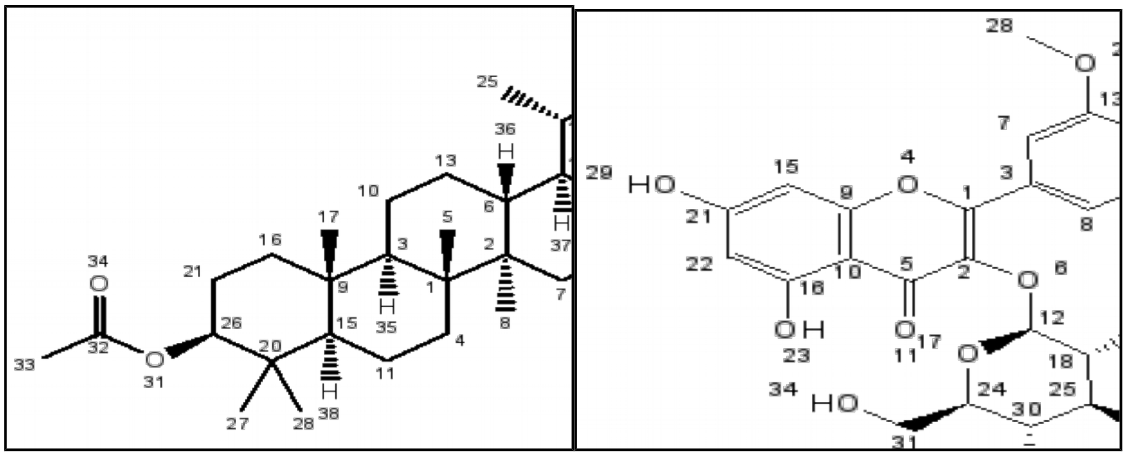




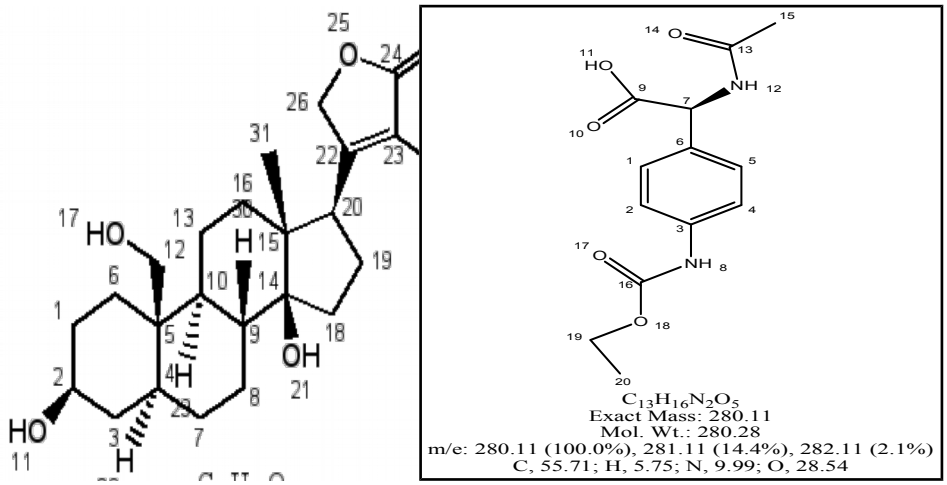
Calotropin **Uscharin***

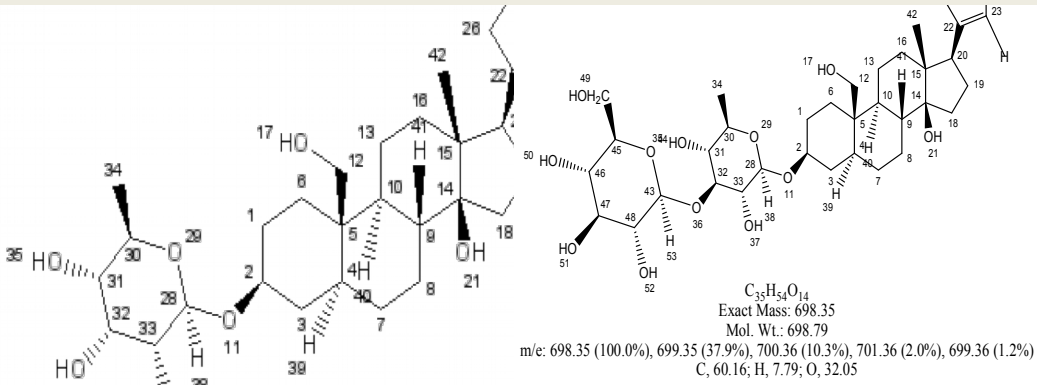




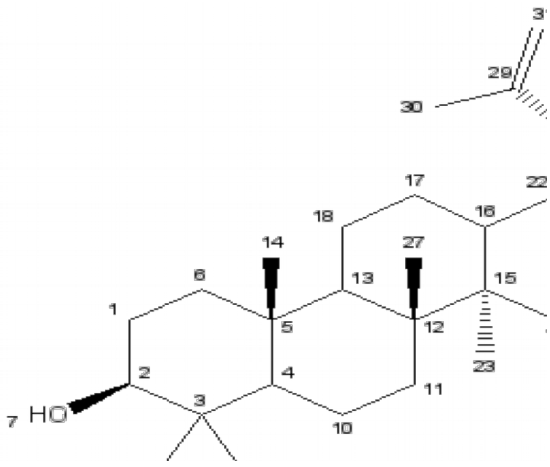


. **Isorhamnetin-3-*O*-Glucopyranoside**** **Taraxasteryl acetate**





Frugoside** **4-0 beta-D-glucopyranosylfrugoside4****



Lupeol*

Figure 2.9 Compounds present in EECGL and WECGLwithmolecular weight and general chemical formula. (*), (**) and (***) indicate the presence of themolecules in WECGL, EECGL and both in WWECL and EECGL respectively according to IR, LCMS and NMR Spectroscopy data.Others molecule may be present in latex extract of these extracts.

2.4 Discussion

The preliminary phytochemical screening tests are useful for the detection of the bioactive components and subsequently may be helpful in the drug discovery and development. The phytochemical screening in the present study has revealed the presence of triterpenoids, glycosides, flavonoids, tannins in the latex extract. Flavonoids have been found to have antimutagenic and antimalignant effects (Brown et al; 1980). Moreover its protective effect against cancer may be by their effect on signal transduction in cell proliferation and angiogenesis. The presence of different phytocompounds in two different extracts may be responsible for the therapeutic properties of *C. gigantea*latex. Flavonoids and tannins are phenolic compounds and plant phenolics are a major group of phytocompounds that may act as primary antioxidants or free radical scavengers. The extracts possess these compounds which may be responsible for the effective antioxidant capacity of *C. gigantea*latex. Phytochemicals as secondary metabolites and other chemical components of medicinal plants account for their medicinal value. For example, saponins have hypotensive and cardio-depressant (Olaleye et al., 2007) properties. Glycosides are naturally cardioactive drugs used in the treatment of congestive heart failure and cardiac arrhythmia. The yield of bioactive metabolites in a plant extract varies significantly with the solvent of extraction (Clark et al.,1997; Marston et al., 1993). Approximately, 60% of the anticancer drugs currently used have been isolated from plant natural products. At this time, more than 3000 plants worldwide have been reported to possess anticancer properties. On a whole, our goal was to determine whether *C. gigantean* latex extracts could exert an inhibitory effect on cancer cell proliferation and death.

It is possible that the ethanolic extracts were generally more potent than the water extracts probably for the bioactive principles of the plant latex dissolved in more readily and were better extracted by a less polar solvent like ethanol than water. This is in covenant with many literatures reporting the differences in the activities of extracts found from the same morphological part of a plant using different solvents.

Medicinal plants establish a common alternative for cancer prevention and treatment in many countries worldwide (Mehta et al., 2010; Soobrattee et al., 2006).

The data presented in this study demonstrate that *C. gigantea* latex extract has special kind of phytochemicals present in the latex.Particularly in oriental medicine, plant flavonoids have been used for centuries in conjunction with their *anti-oxidant*, *anti-inflammatory*properties. Flavonoids like Isorhamnetin-3-*O*-rutinoside, Isorhamnetin-3-*O*-Glucopyranoside, Taraxasteryl acetate (Sen et al.,1992)have been already reported in aerial part of *C. gigantea.* Alkaloids have medical value as well in the form of salts and been used as low toxic pesticides. Saponin are amphipathic glycosides which involve their complication with cholesterol to form pores in cell membrane bilayers and give them activity as surfactants that can be used to enhance penetration of macromolecules such as proteins through cell membranes and used as use in combination with immunotoxin or other targeted toxins for patients with leukaemia, lymphoma and other cancers. Cardinoids like 16alpha-hydroxycalactinic acid methyl ester; 15beta-hydroxycardenolides have already been isolated from leaves of *C. gigantea* which have very good medicinal value. Cardiac glycosides are drugs used in the treatment of congestive heart failure and cardiac arrhythmia and calotropogenin, calotropin, uscharin, calotoxin, calactin have been isolated from root and latex of *C. gigantea*. Presence of flavonoids, alkaloids saponin, glycosides, triterpenoids and cardiac glycosides in latex extract indicates very possibility of these

important components and other special kind of chemicals in latex part too. The knowledge of the chemical constituents of plants is important because such information will be valuable for synthesis of complex chemical components and to screen for biological activities as well (Francis et al., 2002; Mojab et al., 2003; Kumari et al., 2013; Roy et al., 2013).

The phenolic and flavanoids are most common secondary metabolites in plants having antioxidant activity and have wide range of biological activities asanti-inflammation, anti-atherosclerosis, anti-apoptosis, anti-aging, anti-carcinogen, cardiovascular protection and improvement of endothelial function, as well as inhibition of angiogenesis and cell proliferation activities (Catherine et al., 1996). Some recent studies reported that many dietary polyphenolic constituents from plants are more effective anti-oxidants *in-vitro* than vitamins E or C, and thus might contribute significantly to the protective effects *in vivo*(Catherine et al., 1997).

2.5 Conclusion

From the above study it was shown that EECGL and WECGL both have important phytochemicals present in latex extract which is responsible for antioxidant, anti-inflammatory and anti-cancer property. Due to low extraction yield experiments like 2D-NMR were not possible from crude extracts or from the Prep-TLC separated compounds of these extracts for further structure elucidation. However^{1}H NMR, ^{13C} IR and LCMS have shown that EECGL and WECGL have known and unknown important phytochemicals hidden into the extract and need further study to extract these precious components for future drug discovery.

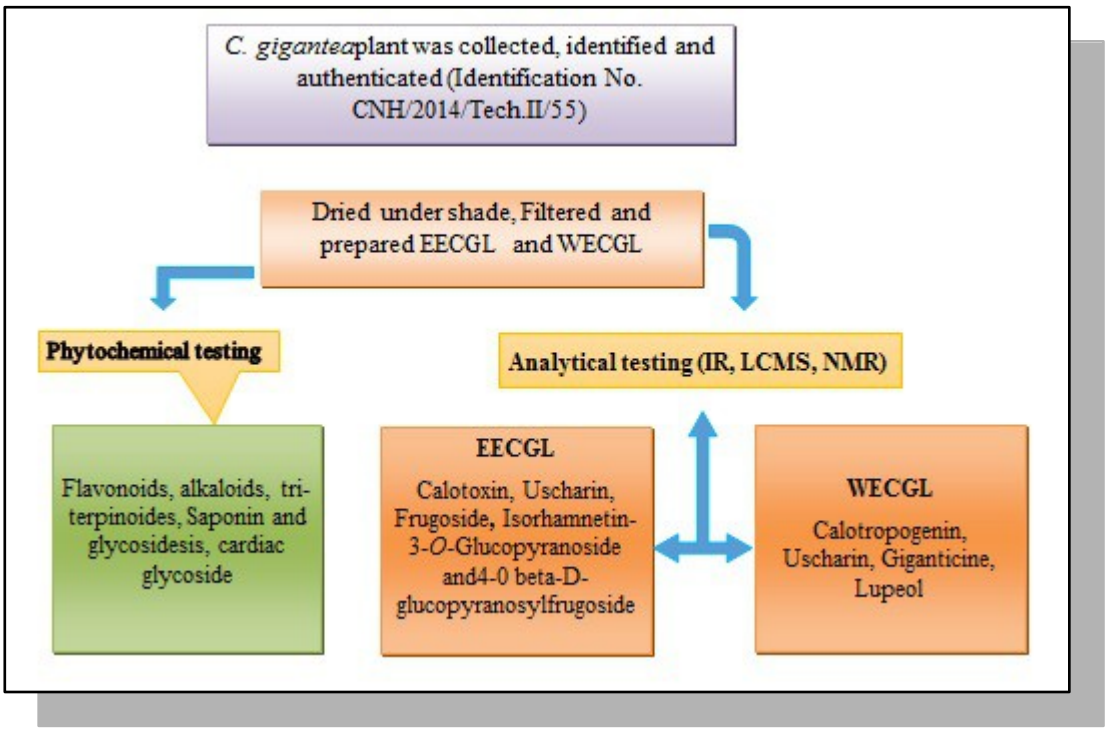


Figure 2.10. Schematic representation of phytochemical analysis and analytical testing of *C. gyganteal*atex extracts.

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