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RESEARCH ARTICLE

PHYTOCHEMICAL AND PHARMACOLOGICAL EVALUATION OF GLORIOSA SUPERBA

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ABSTRACT

Gloriosa superba Linn. is an important medicinal plant (Liliaceae). It is a semi-woody herbaceous branched climber reaching approximately 5 meters height, with brilliant wavy-edged yellow and red flowers [1]. Being native form Indian especially Southern India it is known as glory lily and climbing lily- in English. In the world market glory lily considered as rich source of colchicines and gloriosine [4]. The flower has analgesic, anti-inflammatory potential, antimicrobial, larvicidal potential, antipoxviral potential, antithrombotic potential, antitumor potential, enzyme inhibition potential, and also used in treatment of snake bite, Skin disease, respiratory disorders [5, 6, 7, 8, 9]. Medicinal plants have been used as sources of medicine in virtually all cultures [10, 11]. In recent years, there has been a gradual revival of interest in the use of medicinal plants in developing countries because herbal medicines have been reported safe and without any adverse side effect especially when compared with synthetic drugs. Thus a search for new drugs with better and cheaper substitutes from plant origin is a natural choice. The medicinal values of these plants lie in some chemical substances that produce a definite physiological action on human body [12]. Different parts of *G. superba* have wide variety of uses especially in traditional system of medicine. The tuber is used for the treatment of bruises and sprains, colic, chronic ulcers, haemorrhoids, cancer, impotence, nocturnal seminal emission, and leprosy and also for including labour pains and abortions [13]. *Gloriosa superba* also used in wounds, skin related problems, Fever, Inflammation, piles, blood disorders, Uterine contractions, General body toner, Poisoning [14, 15]. Roots are acrid, anthelmintic, antipyretic, bitter, digestive, expectorant, highly poisonous and promoting expulsion of the placenta. Root paste is effective against paralysis, rheumatism, snake bite and insect bites [16]. This plant has gained the importance in medicine in recent years for the production of colchicine in large scale [17]. The aim of this study was to identify and determine the phytochemicals present in *G. superba* leaves, flowers, seeds and tubers. The main MeOH & dichloromethane extract were isolated and evaluated for anticancer activity.

Keywords: Gloriosa superba, MeOH extract, $CDCl_2$ Extract, anticancer activity

INTRODUCTION

Gloriosa superba is widely used as a medicinal plant & the alkaloid from plant are used in the treatment of gout .& rheumatism. Gloriosa superba rhizome which is perennial harb & important medicinal plant as a raw material for the collection of cochicine. The phytochemical constituent is useful in many ailments like cancer, malarial, gout, cholagouge, abortifacient in piles & constipation. The plant flowers are useful in religious functions & festivals. The tuber is widely used suicidal agent among men & womens in rural & interior areas. It is also used for homicide. The tuber of the plants is used as antidote from ancient time in snake bite. The tuber is commonly placed on window sills to determine snake presence. Rhizome of Gloriosa superba Linn (Colchicine) shows enzyme inhibitory activity

Extract of alcohol obtained from the Gloriosa superba rhizomes was investigated for enzyme inhibition activities. The crude & H_2O were checked for lipo0xygenase, acetylcholineesterase, butyrylcholineesterase & uricase activities. An extraordinary inhibition on lipo-oxygenase had been obtained. The maximum enzyme inhibition potency was expected by chloroform extract among the all tested extracts. The extract was obtained in 90% chloroform. The 60-70% inhibition was obtained for lipo-oxygenase. The inhibition of activity was obtained 49-69% for acetylcholinestrase & 10-33% for butyrylcholinesterase where uricase inhibitory activity is not found in extract.

Figure 1: Natural plant Gloriosa superba in Himalaya

Analgesic & anti-inflammatory activities of hydroalcohalic extract from gloriosa superba:-

Gloriosa superba has been widely utilized as phytochemical medicine & its alkaloids from plant (colchicines & Gloriosine) have been utilized in the treatment of rheumatism & gout. We investigated the anti-inflammatory & analgesic activities of extract had been checked by applying Eddy's hot plate method & acetic acid induced writhing procedure. The anti-inflammatory activity was checked by applying cotton wool granuloma model & carrageenan induced paw edema model. The % inhibition of writhes or % protection had been obtained to be 64.09%, 78.56% & 81.45% for extract at a dose 100, 200 & 400 mg/kg body weight with respect to in the CH_3COOH induced writhing method ($P<0.01$) when compared with control. The % increase in rxn time at 90 minuts were 21.02%, 79.96% & 158.05% for extract at a dose of 100, 200 & 400 mg/kg body weight with respect to in Eddy's hot plate procedure ($P<0.01$) when compared with control. The % inhibition of paw edema had been increased with time & gave max. effect at 2 hrs, then declined in case of st&ard extract 400 mg/kg body weights. Only the 200 & 400 mg/kg body weight. Only the 200 & 400 mg/kg body wt extracts exhibited important result ($P<0.05$) when compared with control.

The rats exhibited 9.59%, 28.72% & 45.8% inhibition of granuloma mass formation. After the 7 days treatment with 100, 200 & 400 mg/kg body weight of extract when compared with control ($P<0.05$) in cotton pellet granuloma.

MATERIAL AND METHODS

The rhizome of *Gloriosa superba* plant was collected from Himalaya region. The plant was identified & authenticated by botanist,, Department of Taxonomy, IIIM, Jammu. A voucher specimen is held in the institutional Herbarium. After authentification, rhizome portion was dried at room temperature until they were free from moisture & subjected to physical evaluation with different parameters. The factors which were used for evaluation are nature, odor, color, shape, size length & width, finally, rhizome portion was subjected to size reduction to get coarse powder & then passed through sieve no. 40 to get uniform powder. Then the uniform powder was subjected to st&ardization with different parameters.

Chemical required:

Growth medium (RPMI), Fetal calf serum (SIGMA), PBS (MERCK), Trypan blue, EtOH, Penicillin (SIGMA), Streptomycin (SIGMA), Trichloroacetic acid, Distilled water, Sodium Hydroxide, Tris-EDTA Buffer (Hi-Media), Sulphorhodamine (SIGMA), Tris Buffer (Hi-Media), Acetic acid (Rankem, Sodium Bicarbonat (Rankem), Mitomycin C (SIGMA), Paclitaxel (taxol) (SIGMA), 5-Flourouracil (SIGMA), Hydrochloric acid (Rankem, Isopropanol (SISCO), Tris Acetate-EDTA buffer.

Apparatus:

Tissue culture flask (NUNC), 96-well flat bottomed culture plates (NUNC) Micropipattes (Eppendorf), 1.5 ml & 0.5 ml eppendorf, centrifuse tubes (Tarsons), 15 ml centrifuse tubes (tarsons), 50 ml centrifuse tubes, sterile centrifuse tubes, Cryovials, Glass bottle to store media etc. (Schott Duran), Glass pipattes (Tarsons), Syringes (Dispo van).

Instruments:

Hemocytometer, Cryocontainers (Thermo Electron), Water bath (Genei), Filtration assembly (Millipore), Deep Freezer (Scien Temp), Mechanical shaker, Centrifuge, (Beckman), Autoclave, ELISA Reader (Tecan), Liquid Nitrogen Cylender, CO_2 gas cylinder, CO_2 gas cylinder, CO_2 gas Incubator (Hera Cell)

Reagents

1) Incomplete growth medium: Prepare RPMI-1640 with 2 mML glutamine medium or MEM in double distilled water. pH is adjusted to 7.2. add Pencillin (100 unites/ml dissolved in PBS) & sterilize by filtering through 0.2 µm filters in sterile laminar flow. Store the media in refrigerator (2-8°C).

2) Complete growth medium: contains 10% FCS & 1% Penicillin. The amount of FCS may vary depending upon the requirements of cell lines used. Freezing medium for cryopreservation contains 20% FCS & 10% DMSO in growth medium (RPMI or MEM).

3) Phosphate Buffer Saline (PBS): dissolve 9.6 gm/Lt. in distilled water. PBS is used to prepare solutions of Penicillin & Trypsin EDTA.

4) Penicillin Solution: Dissolve 100 units/ml or 625 µg/ml in PBS. Penicllin is mixed in RPMI Medium to avoid contamination.

5) Gentamycin Solution: Dissolve 50 µg/ml in PBS. Gentamycin is added in the medium used in the preparation of dilutions of the test sample to avoid contamination. Because of plant extracts.

6) Trypsin ETDA: 0.005 % trypsin & EDTA (Disodium Salt) are dissolved in PBS. Trypsin EDTA is used to detach the cell while sub-culturing & splitting the cell lines.

7) TCA: 50% (w/v) TCA solution is prepared in double distilled water. TCA is used for fixing the culture cells before washing.

8) Acetic acid: Prepare 1% in distilled water. Acetic acid is utilized to prepare solution of SBR dye crystals & remove unbound dye from cells after staining.

9) SRB Dye: Dissolve 0.4% in 1% acetic acid. SRB Dye is mainly used to stain the basic proteins of cancer cells fixed by 50% TCA.

10) Tris buffer: 10 mM (pH 10.5). tris buffer is used to dissolve protein bound dye.

11) Special Requirements: cell lines, test materials & a well equipped cell culture laboratory.

Extraction of plant material (Gloriosa Superba rhizomes)

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The Gloriosa superba rhizomes were prepared coarse powder in grinder. 1 kilogram of powdered plant substance had been divided into major 3 parts & extract of every part was made. The plant material was extracted with MeOH, Dichloromethane & Et acetate separately & labelled as GS-ME, GS-D, GS-E respectively.

a) Preparation of Methanol Extract

Dried plant material (50 mg) was placed in a conical flask submerged with MeOH for 24 hrs. after st&ing for 24 hrs, the percolate was collected. It was filtered & filterate was concentrated on rotatory evaporator & dried & labeled as (GS-ME). The dried extract was transferred to a tared wide mouth container.

b) Preparation of dichloromethane Extract

Dried plant material (50 g) was placed in a conical flask submereged with dichloromethane for 24 hrs. after st&ing for long time, the percolate was collected. It was filtered. Then filterate was concentrated on rotary evaporator & dried & labeled as (GS-D). the dried extract was transferred to a tarred wide mouth container.

Column Chromatography of Dichloromethane extract

Slurry formation

Dried dichloromethane extract 3.5 gram was taken. Slilica gel 60-120 mesh (3.5 gram) was added & drying at room temperature free flowing slurry.

Packig of the column

Weight of silica gel used (column): 35 gm

Diameter of column used: 5.5 cm

Length of the column used: 90 cm

Elution of the column

The column was first eluted with petroleum ether. After that the column was eluted with Et acetate, dichloromethane & MeOH fractions of 300 ml of each were collected & concentrated on Rota-vapor. Thin layer chromatography of fractions were done simultaneously by applying various developing solvents accoradingly in different proportions & the proportions were pooled on the basis of thin layer chromatographic pattern shown by them.

Column Chromatography of first fraction:

Amount of silica taken (to form slurry): 200 mg

Wight of extract: 150 mg

Amount of silica charged in column: 500 mg

Dimensions of column used:

Length of column: 60 cm

Breadth of column: 2.0 cm

Figure 2: Solvent evaporation apparatus

50 fractions were collected from this column eluted first by using pet. Ether alone & further by Petrolium – ethyl acetate mixture (starting from 5% Et acetate to 50%) fractions 25-30 eluted using 30% Et acetate in petroleum ether gave a pure compound & was named GS-6. The structure of the compound was elucidated with the help of PCR. ^{13}CNMR, MS & FTIR.

COMPD (GS-6)

Physical state: compound	It is pale yellow powdered
Yield:	50 mg
Melting point:	146°C
Mass MS/MS:	422 (M^+ + Na)
IR/KBr cm^{-1}:	3285.34, 2925.99, 99.1662.60, 1588.68, 1556.04, 1254.12, 12, 1048.83, 754.62
$[\alpha]_D$	-143 $CHCl_3$, c 0.1)
R_f	0.51

Figure 3: TLC PLATE OF GS-6

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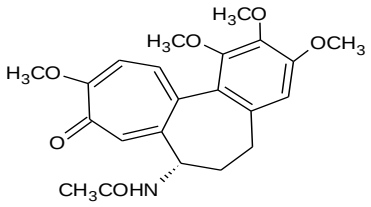
Table 1: ^{1}H NMR spectral data of GS-6 ($CDCl_3$)

S. No.	Chemical shift (δ)	Multiplicities of the Signals	No of protons	Coupling constant J(Hz)	assignments
1.	7.47	S	1 H	-	H_8
2.	7.33	D	1 H	10.66	H_{11}
3.	6.85	D	1 H	10.66	H_{12}
4.	6.76	D	1 H	6.38	NH
5.	6.54	S	1 H	-	H_4
6	4.65	M	1 H	-	H_7
7.	4.01	S	3 H	-	C-3 OCH_3
8.	3.95	S	3 H	-	C-10 OCH_3
9.	3.91	S	3 H	-	C-1 OCH_3
10.	3.65	s	3 H	-	C-2 OCH_3
11.	2.53	M	1 H	-	H_5
12.	2.41	M	1 H	-	H_5
13.	2.29	M	1 H	-	H_6
14.	1.99	S	1 H	-	H_{14}
15	1.85	M	1 H	-	H_6

Table 2: ^{13}C NMR spectral data of GS-6 ($CDCl_3$)

S. no	Chemical shift	DEPT	Assignment
1	151.18	C	C_1
2	141.63	C	C_2
3	153.56	C	C_3
4	107.34	CH	C_4
5	134.12	C	C_{4a}
6	29.89	CH_2	C_5
7	36.54	CH_2	C_6
8	52.56	CH	C_7
9	152.37	C	C_{7a}
10	130.53	CH	C_8
11	179.58	C	C_9
12	164.03	C	C_{10}
13	112.97	CH	C_{11}
14	135.66	CH	C_{12}
15	136.93	C	C_{12a}
16	125.57	C	C_{12b}
17	170.17	C	C_{13}
18	22.83	CH_3	C_{14}
19	61.59	CH_3	C-1 OCH_3
20	61.42	CH_3	C-2 OCH_3
21	56.13	CH_3	C-10 OCH_3
22	56.44	CH_3	C-3 OCH_3

The compound was characterized as colchicines. The structure of cholchine is shown below



Colchicines

g) Column chromatography of second fraction

Amount of silica taken (to form slurry): 250 mg

Wight of extract: 150 mg

Amount of silica charged in column: 400 mg

Dimensions of column used:

Length of column: 60 cm

Breadth of column: 2.0 cm

25 fraction were collected from this column eluted first by using petroleum ether alone & further by petroleum – Et acetate mixture (starting from 5% ethyl acetate to 50%) fractions 20-27 eluted using 12% Et acetate in petroleum ether gave a pure compound & was named GS-3. The structure of compound was elucidated with the help of PMR, ^{13}C NMR, MS & IR.

COMPD (GS-3)

Physical state: colorless needle crystals.

Yield: 25 mg

Melting point: 136°C

Mass MS/MS: 437 (M^+ + Na)

IR/KBr cm^{-1}: 3414, 2936, 1637, 1617

$[\alpha]_D$ -30 $CHCl_3$, c 0.1)

R_f 0.61

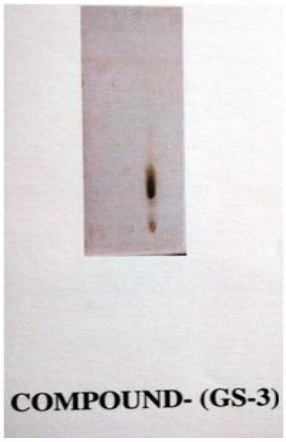


Figure 4: TLC PLATE OF GS-3

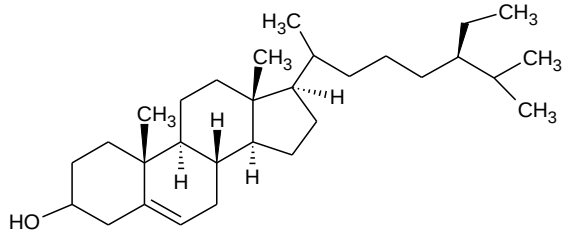
Table 3: ^{1}H NMR spectral data of GS-3 ($CDCl_3$)

S. No.	Chemical shift (δ)	Multiplicities of the Signals	No of protons	Coupling constant J(Hz)	Assignments
1.	0.68	S	3 H	-	H_{18}
2.	0.82	D	3 H	6.7	H_{27}
3.	0.85	D	3 H	6.7	H_{26}
4.	0.92	T	3 H	7.4	H_{29}
5.	1.01	D	3 H	6.5	H_{21}
6	3.58	S	3 H	-	H_{19}
7.	5.36	M	1 H	-	H_3
8.	3.95	M	1 H	-	H_6

Table 4: ^{13}C NMR spectral data of GS-3 ($CDCl_3$)

S. no	Chemical shift	DEPT	Assignment
	12.48	CH_3	C_{29}
	12.60	CH_3	C_{18}
	19.67	CH_3	C_{21}
	20.02	CH_3	C_{26}
	20.02	CH_3	C_{27}
	20.45	CH_3	C_{19}
	21.70	CH_2	C_{11}
	23.67	CH_2	C_{28}
	24.92	CH_2	C_{15}
	26.67	CH_2	C_{23}
	28.52	CH_2	C_{16}
	29.55	CH	C_{25}
	32.20	CH_2	C_2
	32.51	CH_2	C_7
	32.52	CH	C_8
	34.55	CH_2	C_{22}
	36.77	CH	C_{20}
	37.11	C	C_{10}
	37.88	CH_2	C_1
	40.30	CH_2	C_{12}
	42.93	CH_2	C_4
	42.95	C	C_{13}
	46.42	CH	C_{24}
	50.74	CH	C_9
	56.67	CH	C_{17}
	57.37	CH	C_{14}
	72.34	CH	C_3
	117.06	CH	C_5
	141.37	C	C_6

The compound was characterized as colchicines. The structure of cholchine is shown below



β - Sitosterol

h) Column chromatography of third fraction

Amount of silica taken (to form slurry): 400 mg

Wight of extract: 300 mg

Amount of silica charged in column: 700 mg

Dimensions of column used:

Length of column: 60 cm

Breadth of column: 2.0 cm

50 fractions were collected from this column eluted first by using petroleum ether alone & further by petroleum – Et acetate mixture (starting from 5% Et acetate to 50%) fractions 24-34 eluted using 30 % Et acetate in petroleum ether gave a pure compound & was named GS-7. The structure of compound was elucidated with the help of PMR, ^{13}C NMR, MS & IR & the compound was characterized as colchicines.

i) Flash Chromatography of MeOH: H2O Extract

Slurry formation

Dried MeOH : H2O extract 80 mg was taken. Silica gel 200-400 mesh (80 gm) wa added in this & drying at room temperature. Free flowing slurry was obtained.

Packing of the flash column

Weight of the silica column : 800 gm

Diameter of column used : 13 cm

Length of column used : 32 cm

Elution of column

The column was run in increasing order of polarity & small fractions (75 ml) each were collected & monitored on thin layer chromatography by proper solvent system first of all the column was run with pet. Ether (100%). 10 fractions of each 0f 75 ml were collected. No spot was visualized on TLC plate. Then column was run with increasing conc. (2.5% Et acetate in hexane). 30 fractions were taken & TLC was monitored. Then column was run with 5% Et acetat in hexane. 25 fractions had been taken. Again polarity had been increased upto 10% Et acetate in hexane. Now column had been run with DCM (dichloromethane). 45 fractions had been taken. But there was no spot seen on thin layer chromatographic sheet. Then the column had been run with 2.5 % methanol in Dichloromethane.

35 fractions had been collected. Fraction no 22 – 28 had given one spot on then layer chromatographic plate. The fractions were poured & concentrated on rotary evaporator. The compound isolated from these fractions had been submitted for PMR. The compound was labaled as GS-21 characterized as 3-demethylcolchicine by PMR, ^{13}C NMR, FT-IR & Mass spectroscopy. Now column had been eluted with 5% methanol in Di Chloro Methane & Thin layer chromatographic plat was monitored. Now polarity had been increased upto 7.5 % MeOH in dichloromethane. 49 fractions were collected. Fraction no. 40-45 gave a pure compound. These fractions had been pooled & concentrated on rotatory evaporator. The compound isolated from these fractions was labelled as GS-23 characterized as β-sitisterol-3-O-β-D glucoside. The column had been eluted with 10% MeOH.in Di chloro methane. A mixture was obtained which was need to be purified. Finally column was eluted with MeOH alone.

COMPD (GS-21)

Physical state: compound It is pale colored powdered

Yield: 35 mg

Melting point: 142°C

Mass MS/MS: 407 (M^+ + Na)

IR/KBr cm^{-1}: 3285.34, 2925.99, 1662.60, 1588.68, 1556.04, 1254.12, 12, 1048.83, 754.62

$[\alpha]_D$ -143 $CHCl_3$, c 0.1)

R_f 0.47

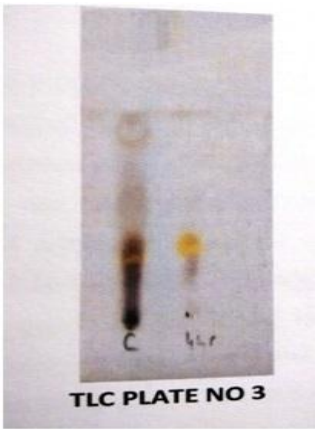


Figure 5: TLC Plate of GS-21

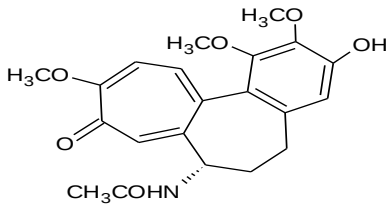
Table 5: ^1H NMR spectral data of GS-21 ($CDCl_3$)

S. No.	Chemical shift (δ)	Multiplicities of the Signals	No of protons	Coupling constant J(Hz)	assignments
1.	7.49	S	1 H	-	H_8
2.	7.31	D	1 H	10.66	H_{11}
3.	6.85	D	1 H	10.66	H_{12}
4.	7.09	D	1 H	6.38	NH
5.	6.59	S	1 H	-	H_4
6	4.65	M	1 H	-	H_7
7.	4.01	S	3 H	-	C-3 OCH_3
8.	3.95	S	3 H	-	C-10 OCH_3
9.	3.91	S	3 H	-	C-1 OCH_3
10.	3.65	s	3 H	-	C-2 OCH_3
11.	2.53	M	1 H	-	H_5
12.	2.41	M	1 H	-	H_5
13.	2.29	M	1 H	-	H_6
14.	1.99	S	1 H	-	H_{14}
15	1.85	M	1 H	-	H_6

Table 6: ^{13}C NMR spectral data of GS-21 ($CDCl_3$)

S. no	Chemical shift	DEPT	Assignment
	150.27	C	C_1
	139.24	C	C_2
	149.99	C	C_3
	110.41	CH	C_4
	134.75	C	C_{4a}
	29.47	CH_2	C_5
	36.21	CH_2	C_6
	52.84	CH_3	C_7
	152.96	C	C_{7a}
	179.57	CH	C_8
	179.58	C	C_9
	164.02	C	C_{10}
	113.13	CH	C_{11}
	135.66	CH	C_{12}
	137.23	C	C_{12a}
	124.77	C	C_{12b}
	170.44	C	C_{13}
	22.68	CH_3	C_{14}
	61.41	CH_3	C-1 OCH_3
	61.33	CH_3	C-2 OCH_3
	56.13	CH_3	C-10 OCH_3

The compound was characterized as colchicines. The structure of cholchine is shown below



3 – Demethyl Colchicines

COMPD (GS-23)

Physical state: amourphus white powder..

Yield: 10 mg

Melting point: 270 - 271°C

IR/KBr cm^{-1}: 3400, 3044, 1646

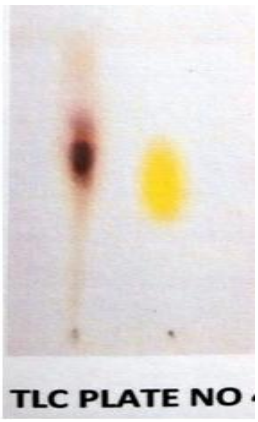
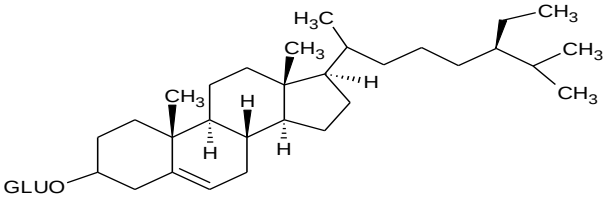


Figure 6: TLC Plate of GS-23

^{1}H NMR 500 MHz of GS-23 ($CDCl_3$)

5.26 (1H, m, H-6), 5.08 (1 H, J¼ 7.7 Hz H-10) 4.59 (1 H, dd J¼ 11.6 Hz, 2.1 Hz, H-60b), 4.47 (1 H, dd J¼ 11.7 Hz 5.2 Hz H-60a), 4.32 (2 H, m J¼ H-30, H-40), 4.10 (1 H, t J¼ 7.9 Hz H-20), 4.02 (1 H, m H-50), 3.08 (1 H, m H-3), 0.91 (3 H, J¼ 6.4 Hz Me-21), 0.90 (3 H, s Me-10), 0.89 (3 H, t J¼ 7.3 Hz Me-29), 0.85 (3 H, d, J¼ 6.8 Hz Me-10), 0.86 (3 H, d J¼ 6.9 Hz Me-27), 0.08 (3 H, s Me-10). The compound isolated was characterized as beta-sitisterol-3-O-β-D-glucoside.

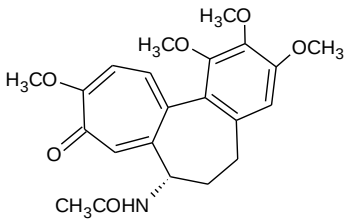


β-sitisterol-3-O-β-D-glucoside

RESULTS AND DISCUSSION

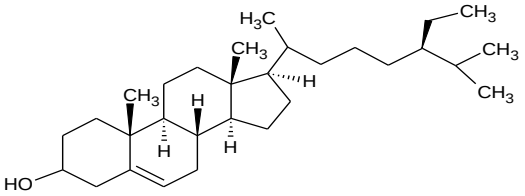
On column chromatography of dichloromethane extract of Gloriosa superba overall two compounds were isolated labelled as GS-6 & GS-3 respectively. The yield of GS-6 & GS-3 was 260 mg & 30 mg. the compounds had been characterized by ^{1}H NMR, ^{13}NMR, DEPT, Mass & IR.

(1) Colchicine: structure of colchicine is given below



Total no. of CH3, CH2, CH, C & Mass of the compound were found in agreement with the literature values.

(2) β- sitosterol structure is given below



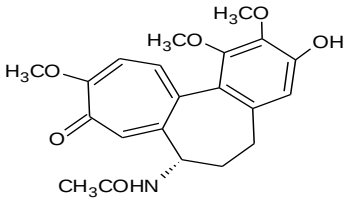
22, 23-Dihydrostigmosterol, stigmast-5-en-3-ol

Total no. of CH3, CH2, CH, C & Mass of the compound were found in agreement with the literature values.

In case of β-sitosterol acetylation was done & a monoacetate was formed which confirm the presence of hydroxyl group.

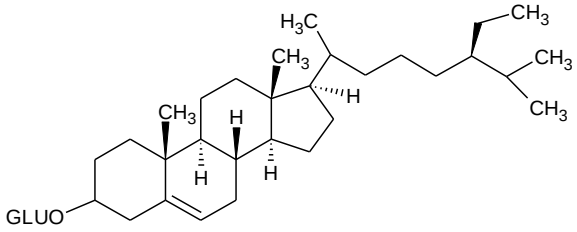
On flash chromatography of aqueous MeOH extract two compounds had been isolated labelled as GS-21 & GS-23 & Characterized by ^{1}H NMR, ^{13}NMR, DEPT, Mass & IR. The yield of compound was 35 mg & 10 mg respectively. Compounds had been characterized as 3-demethylcolchicine & beta-sitosterol-3-O-beta-D-glucoside.

(3) 3-Demethyl Colchicine: structure of colchicine is given below



Total no. of CH3, CH2, CH, C & Mass of the compound were found in agreement with the literature values.

(4) β- sitosterol-3-O-β-D-glucoside structure is given below



22, 23-Dihydrostigmosterol, stigmast-5-en-3-ol glycoside

Total no. of CH3, CH2, CH, C & Mass of the compound were found in agreement with the literature values.

In case of β-sitosterol-3-O-β-D-glucoside acetylation was done & a monoacetate was formed which confirm the presence of hydroxyl group.

Pharmacological activities

The solvent extracts coded as GS-PE, GS-D, GS-MW, GS-6, GS-2 along with compound coded asGS-6 had been tested for antibacterial & antifungal activity. The samples had been tested against pathogens i. e. bacteria i.e. S. aureous, MRSA & P. aeruginosa & fungus i.e. C. albicans. A. fumigates, amphotericin-B had been used as a st&ard antifungal agent in this study.

Table 7: showing the antibacterial activity of the given samples.

Plant code	MIC (µg/ml)		
	S. aureus ATCC	MRSA 15187	P. aeruginosa
GS-PE	>256	>256	>256
GS-D	>256	>256	>256
GS-MW	>256	>256	>256
GS-6	>256	>256	>256
GS-2	>256	>256	>256
Ciprofloxacin	**0.25**	**16**	0.03

Table 8: showing the antifungal activity of the given samples

Plant code	MIC (µg/ml)	
	C. albicans ATCC 90028	A. fumigatus LSI-II
GS-PE	>256	>256
GS-D	>256	>256
GS-MW	>256	>256
GS-6	>256	>256
GS-2	>256	>256
Amphotericin-B	**1**	**1**

Ciprofloxacin was used as standard antibacterial agent & amphotericin-B was used as standard antifungal agent.

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Table 9: showing the Anti-inflamatory activity of GS-D (Dichloromethane extract) sample

S. No.	Group/Dose	Animal Labelled	Intial paw Vol.	Uninjected paw vol.	Injected paw vol.	Oedema	% I
	GS-D 250 mg/kg p.o.	H	1.0	1.0	1.9	0.9	
		B	1.0	1.0	2.0	1.0	
		T	1.0	1.0	2.0	1.0	
		NC	0.9	0.9	2.1	1.1	**45.35**
		1.00±0.0250					
	STD (Ibu) 100mg/kg p.o.	H	1.1	1.1	1.9	0.8	
		B	1.1	1.1	2.1	1.0	
		T	1.2	1.2	2.2	1.0	
		NC	1.0	1.0	1.9	0.9	**49.18**
	0.93±0.0666						
	control	H	1.1	1.1	3.0	1.9	
		B	1.1	1.1	2.9	1.8	
		T	0.9	0.9	2.7	1.8	
		NC	1.0	1.0	2.8	1.8	-
		1.83±0.0333					

Table 10: showing the Comparative analysis of anti-inflammatory activity

S. No.	Group	Dose (mg/kg p.o.)	%I@4th hr
1.	GS-D 250 mg/kg p.o.	250	45.35
2.	STD (Ibu) 100 mg/kg p.o.	100	49.18
3.	Control (Vehicle)	-	-

Route of administration of test compound:

Oral route was used for the study.

Inference

The result of the coded material GS-D revealed it to possess 45.35% anti-inflammatory activity against carrageenan induced oedema in rats at a dose of 250 mg/kg p.o. the values are highly important ($P<0.05$; student-Newman- Keuls test) in comparision to the vehicle control. The anti-inflammatory activity of the compound was comparable to the standard drug Ibuprofen.

Anti-Cancer Activity

Human cancer cell lines:

The cancer cell lines of different tissue origin available at IIM, Jammu for in vitro cytotoxicity testing are as follows:-

BREAST : MCF-7

CERVIX : HeLa, SiHa

CNS : IMR-32, SK-N-SH, SNB-78, SF-295

COLON : CoLo-205, HCT-15, HT-29, SW-620, 502713

LUEKEMIA : K-562, MOLT-4

LIVER : Hep-2

LUNG : A-549, MOLT-4

PROSTATE : DU-145, PC-3

ORAL : KB

Sources of cell lines:

- National center for Cell Science, Ganeshkhind, Pune-4111007 (India).
- National Cancer Institute, Biological Testing Branch DTP/DCTD/NCI, Federick Cancer Research & Development Center, Fairview, Suite 205, 1003 West 7th Street, Frederick, MD 21701-8527 (USA)

In-vitro anti-cancer studies

Selection of cell lines:

For primary screening, cell lines representing different types of human cancer & tissues are utilized. The cell lines are selected in such a way that all the cell lines grow in growth medium (RPMI & MEM) in tissue culture plates & mass doubling time in such that enough cells are obtained for screening. It is best to use these cell lines although others can be used. Cells to be utilized must be free from bacteria, yeast, moulds, mycoplasma & in certain cases, from viruses, at all the stages. If at any stages contamination appears, the stock in which it occurs must discarded immediately.

Table 11: In Vitro Anti-tumor activity

S. No.	Name ofcell lines	Medium growth	Positive control	Source
	502713	RPMI	5-flourouracil	Colon
	Colo 205	RPMI	5-flourouracil	Colon
	A-549	RPMI	DDP, taxol	Lung
	HeLa	RPMI	DDP, 5-flourouracil	Cervix
	Hep-2	MEM	DDP, Mitomycin-C	Liver
	HCT-15	RPMI	5-flourouracil	Colon
	SiHa	RPMI	DDP, 5-Fluorouracil	Cervix
	MOLT-4	RPMI	Adiriamycin	Leukemia
	HT-29	RPMI	5-flourouracil	Colon
	IMR-32	RPMI	Adriamycin, Vincristine	CNS
	K-562	MEM	Adriamycin, Vincristine	Leukemia
	MCF-7	RPMI	Adriamycin	Breast

Healing of cell lines on arrival:

- Immediately on receipt of the original stock of cell lines from authentic source, the cells are observed under microscope, for apparent contamination & proper growth. If there is any contamination, the cell lines are discarded & fresh one is obtained
- If it is observed under microscope that cells have been detached themselves from the flask & monolayer is not present the cells are removed, by taking out growth medium aseptically, centrifuged & then the cells are suspended in enough growth medium & incubated in carbon dioxide incubator to grow.
- If there is a proper growth & monolayer is present, excess growth medium is colledctd aseptically in sterilized bottles which can be utilized again & the cells are incubated at 37°Cin atmoshphere of 5% CO_2 & 90% Relative humidity, in CO_2 incubator. The flask is checked daily for proper growth & complete growth medium (prewarmed at 37°C) is changed whenever necessary. When growth is 70-90% (sub-confluent) the passage is given

Revival & seeding of cells:

- Remove the desired cryovial from liquid nitrogen & thaw it to 37°C as soon as possible.
- Wipe the cryovial with 70% alcohol before taking to laminar flow to avoid contamination.
- Pour nearly 10 ml of complete growth medium to tissue culture flask.
- Mix the cells in the cryovial properly & aspirate them with the help of an autoclaved syringes tip.
- Add the cells to tissue culture flask & rinse the cryovial with media & add tissue culture flask.
- Mix properly to ensure uniform distribution of cells in the growth medium.
- Mark the flask properly & incubate it at 37°C, 5% carbon di oxide atmosphere & 90% humidity.

Stages of cell growth during culturing:

- **Attachment Stage:** within 24 hrs of incubation after seeding or trypsinization, the cells get attached to the base of tissue culture flask.
- **Sub-confluent Stage:** It is a stage of rapid growth of cells. In this stage, some space remains in between the growing cells. The cells are in log phase of their growth & are used for experimental purposes.
- **Confluent Stage:** The medium turns turbid as nutrients are utilized & cell derbis & metabolic wastes accumulate in the medium. When the cells are observed under microscope, they are found to form a complete monolayer.

Maintenance of cell lines:

The cell lines should be observed daily. If medium has become turbid, it should be changed. It helps to avoid contamination. Procedure for changing the medium is:

Sub-culturing of cell lines:

Sub-culturing of cell lines has been done by trypsinisation to maintain the cells in log phase of growth. The cells are detached when they reach confluent stage & to make single cell suspension for experimental purposes. The trypsinisation of a cell line should not be done more than 5 times as the cells lose their potential to grow.

Crypreservation of the cells:

- Take the flasks, which have the cells in log phase of growth.
- The cells are trypsinized as usual.
- Prepare the single cell suspension by passing the cells through pipatte.
- Pour the cell suspension in sterilized centrifuge tubes & centrifuge at 2000 rpm for 10 minutes at 0°C.
- Remove the supernantant.
- Add the freezing medium to the pellet & make single cell suspension by pipetting.
- Pour the single cell suspension in cryovials upto 2/3 of their volume.
- Cryovials are stored in cryocontainer (having isopropanol to avoid sudden drop in temperature which may damage the cells)
- Store the dryocontainers at -85°C in deep freezer.

Preparation of test material:

- **Stock solution:** prepare a stock solution of 20 mg/ml. DMSO is used for dissolving 95% MeOH/EtOH extract, 50 % aqueous MeOH/EtOH extracts & distilled water for hot water extracts.

Stock solutions are prepared one day in advance. The working solutions of test samples are prepared in the complete growth medium. The samples, which do not dissolve properly, are kept in hot water hath at 50°C. the solution of test sample is prepared twice concentrated 1 of media. Hence the final than the final concentration as it is added to 100 concentration is reduced to half of the intial concentration. Gentamycin (50 g/ml) is added to check the microbial contamination in growth medium.

- **Positive control:** it is essential for comparision of the activity of the test material. Some of the compounds, which are generally used as positive control, are
- **5-fluorouracil:** it is a prodrug undergoing a series of biotransformation reactions to ribosyl & desoxyribosyl nucleotide metabolites. One of these metabolites is 5-fluor-2-deoxyribosyl nucleotide metabolites. (FdUMP) which gets incoroporated in to RNA where it interferes with t-RNA & m-RNA translation.
- **Mitomycine-C:** It is an antibiotic isolated from Streptomyces caespitosus. It is an alkylating agent that undergoes metabolic activation through an enzyme-mediated reduction, to generate an alkylating agent that cross-links DNA.
- **Blank control:** Blank control is essential to reduce the OD of medium & test samples. In each plate 5-8 wells of each row are blank control. In blank control, gentamycin media & test sample solution are added but cell suspension is not added.

Determination of cytotoxicity:

In a single 96 well plate, the cytotoxicity of sample can be determined on 2 cell lines at a time. The number of plates depends on the number of test samples.

Preparation of cell suspension for the assay:

The desired human cancer cell lines are grown in tissue culture flask at 37°C, in an atmosphere of 5% carbon di oxide & 90% relative humidity in complete growth medium to obtain enough number of cells.

The wells of 1 to 4 & 9 to 12 of first row in each plate is control growth (CG) i.e. having the cell suspension & medium. The growth of the cells in these wells is the maximum as no test sample is added.

The second row of one plate have 100 miroliter positive control (PC) i.e. having 5-FU of Mitimycin-C etc. the growth of these wells is less, as it is less, as it is the known inhibitor of cells.

Addition of test material:

- Incubate the plates for 48 hrs at 37 degree centigrade in an atmosphere of 5% CO_2 & 90% relative humidity.
- Determine the cell growth after 48 hrs of adding sample.

Cytotoxicity assay by Sulphorhodamine B Dye:

SRB assay is a rapid, sensitive & expenxive method for measuring the cytotoxity potential of test substances, based on the cellular protein content of adhered suspension cultures in 96 well plates. This method is suitable for ordinary laboratory purposes & for large-scale applications like high throughput screening in anti-tumor drug development.

Procedure of assay:

- The plates are taken out from the incubator after 48 hrs of adding test samples.
- To stop the reaction, gently add 50 µl of chilled 50% TCA (trichloroacetic acid) to each well of the plate, making final concentration of 10%.
- Incubate the plates at 4°C for one hour to fix the cells attached to bottom of the wells.
- Wash the plates 5-6 times with distilled water.
- Plates are air-dried.
- Add 100 µl of SRB dye (0.4% in 1% acetate acid) to each well of the plate & leave the isolates at room temperature for 30 minutes.
- With the plates with 1% acetic acid after 30 minutes.
- Plates the again air-dried.
- Add 100 µl of tris buffer (10.5 M) to each well.
- Shake the plates gently for 10-15 minutes on a mechanical shaker.
- Record the optical density with ELISA reader at 540 nm wavelength & maintain the data.

CALCULATIONS:

The viability & growth in the presence of test material is calculated as:

OD Change = Mean of OD of Test sample – mean of OD of Blank

% Growth in presence of the Control = 100 / OD change in presence of control

% Growth in the presence of Test sample =

(% growth of in presence of control) × (OD change in presence of test sample)

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Table 12: In vitro cytotoxicity report

Tissue			Lung	Ovary	Leukemia	Prostate	Breast	Liver
Cell line type			A-549	IGR-OV-1	THP-1	PC-3	MCF-7	HEP-2
CCL codes	Inst. Codes	Conc.	% Growth Inhibition					
M-3824	GS-6	10µM						
		30µM	**48**	**67**	**90**	**67**	**80**	**90**
		100µM	**58**	**70**	**91**	**69**	**96**	**93**
	Paclitaxel	10µM	**70**	**76**	**91**	**74**	**99**	**95**
	Mitomycin	10µM	**69**	**59**				
	5-FU	20µM				**61**		
	Adriamycin	1µM			**75**		**76**	**72**

Quantification of colchicines by HPTLC

In order to quantify colchicines content in the extract in the extracts prepared by using different solvents, it was envisaged to use High Performance Thin Layer Chromatography (HPTLC) procedure to achieve the objective. In this direction, 10 mg of each extract (five extracts in all) were taken & dissolve in 1 ml of MeOH .or MeOH/H2O samples were coded as I-0770-(01). I-0770 (02), I-0770 (03), I-0770 (04), I-0770 (05) 20 mg of colchicines was taken as the st&ard sample & dissolved in 1 ml MeOH. St&ard curve was achieved with r squar = 0.99080125, an aliquot of 2 µl of each extract was charged on the TLC plate & the plate was analyzed for colchicines content. The % of colchicines in each extract was calculated using the following procedure.

1. from HPTLC it was detected that the content of colchicnes in petroleum either extract I-0770-(01) was below detection limit (BDL).

2. Calculation of content of colchicines in DCM extract-I-0770-(02)

2 µl of DCM extract showed presence of = 321.16 mg of colchine

1000 µl contained colchicines = 321.16 × 500 = 1600580 ng of colchicines.

1 ml of DCM extract contained colchicines = 160580 ng of colchine.

10 mg extract contains = 0.160580 mg of colchicines

Therefore 35000 mg contains = 0.160580 × 3500 = 562.03 mg

3. calculation of content of colchicines in MeOH: water extact I-0770-(03)

Each 2 microliter injection of sample name I-0770 (03) contains <90 mg of colchicines

So 1000 µl contains colchicines = 90 × 500 = 45000 ng of colchicines.

I ml of MeOH water extract contains colchicines = 45000 ng

10 mg of extract contains colchicines = 0.045 mg

Therefore 80 mg contains colchicines.

10 mg of this extract gives colchine = 0.045 mg

80000 mg gives = 0.45 × 8000 = 360 mg

4. Calculation of content of colchicines in sample name I-0770- (04) Et acetate extract each 2 µl injection contains colchicines = 231.77 mg of colchicines

1 ml contains colchicines = 115885 ng

10 mg of extract contains colchicines = 0.115885 mg

Therefore 520 mg contains colchicines = 520 × 0.115885 = 6.0262 mg

Total quantity of colchicines in all extracts = 562.03 + 360 + 6.0262 = 598.632mg

1050 mg powdered plant material gives colchicines = 0.59863262 mg

1 gm powdered plant material gives colchicines = 5.9863262/1050 = 0.000570126 gm

100 gm gives = 0.000570126 × 100 = 0.0570126

So the content of colchicines in whole plant = 0.0570126%

Table 13: HPTLC Result

Sample name	Colchicines
I-0770-(01) (GS-PE)	BDL
I-0770 (02) (GS-D)	321.16 ng
I-0770 (03) (GS-E)	< 90
I-0770 (04)(GS-ME)	231.77 ng
I-0770 (05) (GS-MW)	174.57 ng

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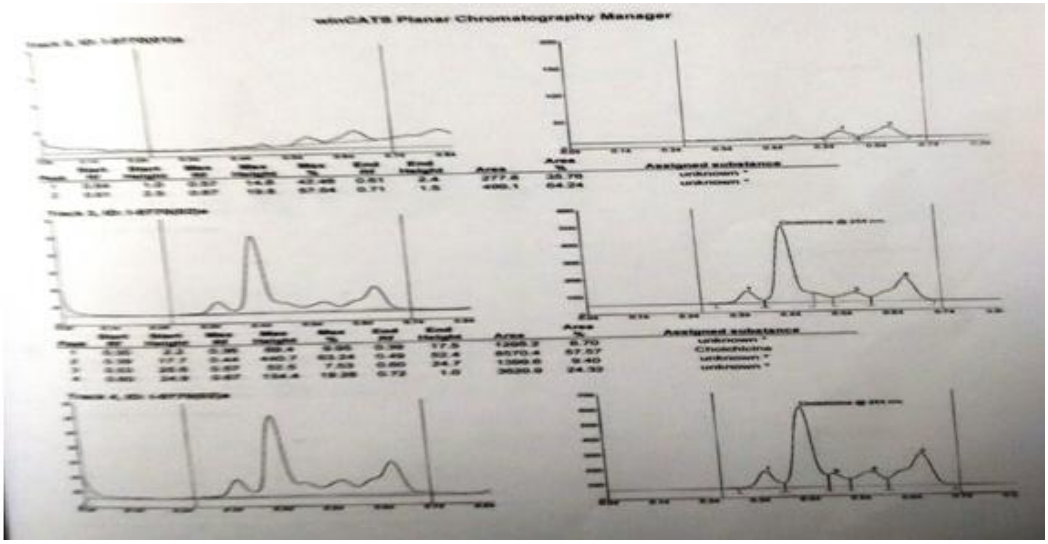

Figure 7: HPTLC graph of Trace 1-4

Light yellowish needle shaped crystalline compound (30 mg) isolated from chloroform fraction using silica gel (100-200 mesh) column chromatography & 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.

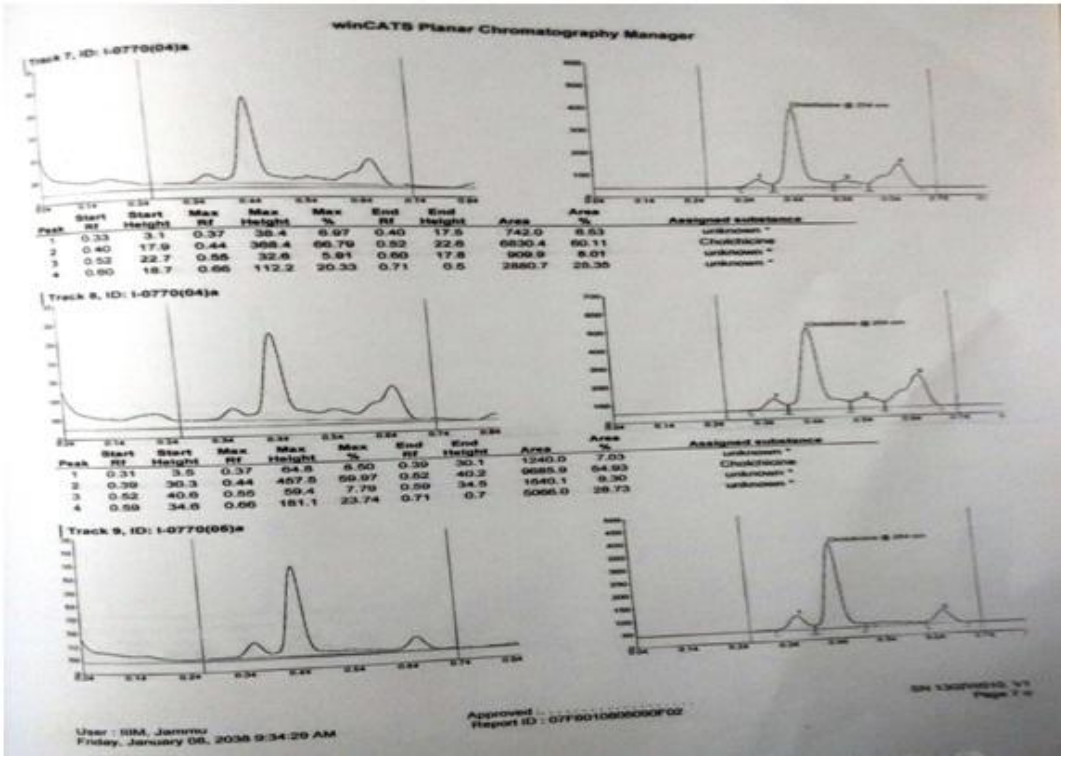

Figure 8: HPTLC graph of Trace 5-8

White amorphous compound (100 mg) isolated from hexane fraction using silica gel (100-200 mesh) column chromatography & eluted in 2-4% of ethyl acetate in hexane.

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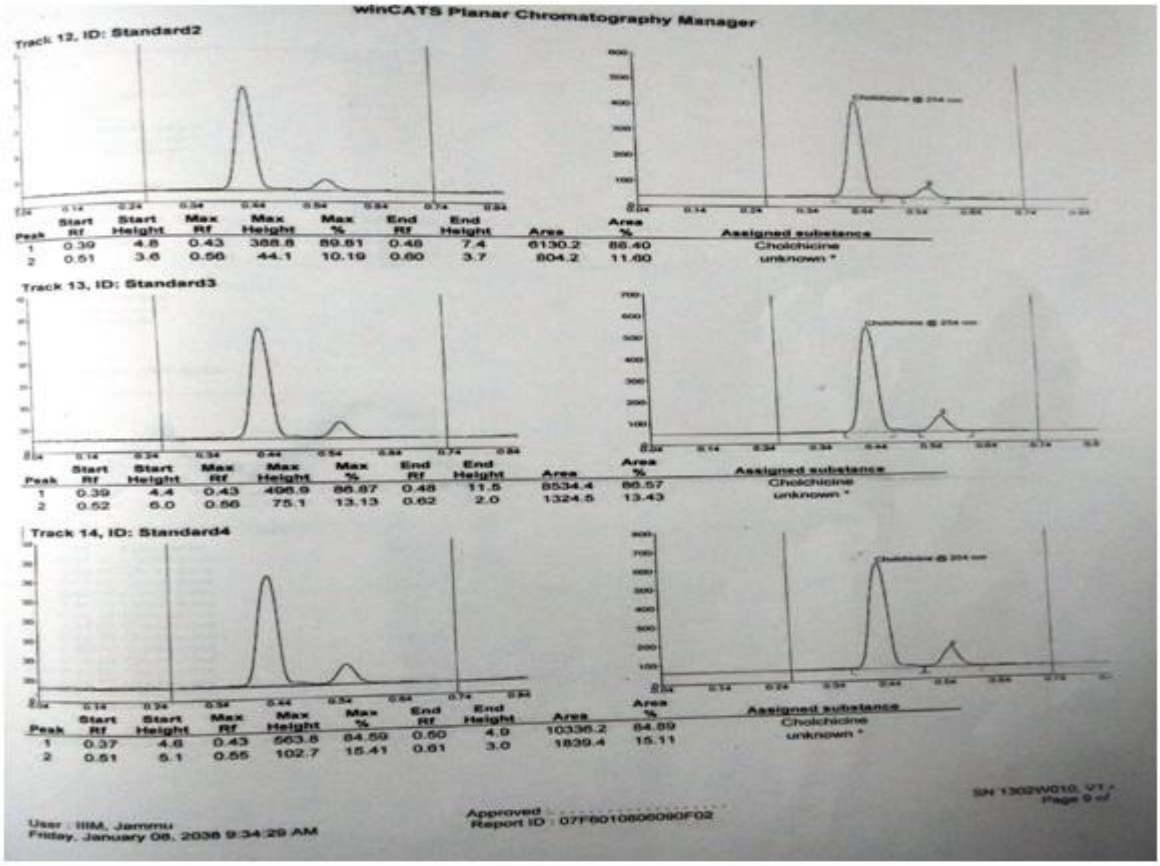

Figure 9: HPTLC graph of Trace 8-12

White needle shaped crystalline compound (50 mg) isolated from chloroform fraction using silica gel (100-200 mesh) column chromatography & 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-03 was characterized as 4-O-Metylglabridin by comparison of its spectral data with reported data.

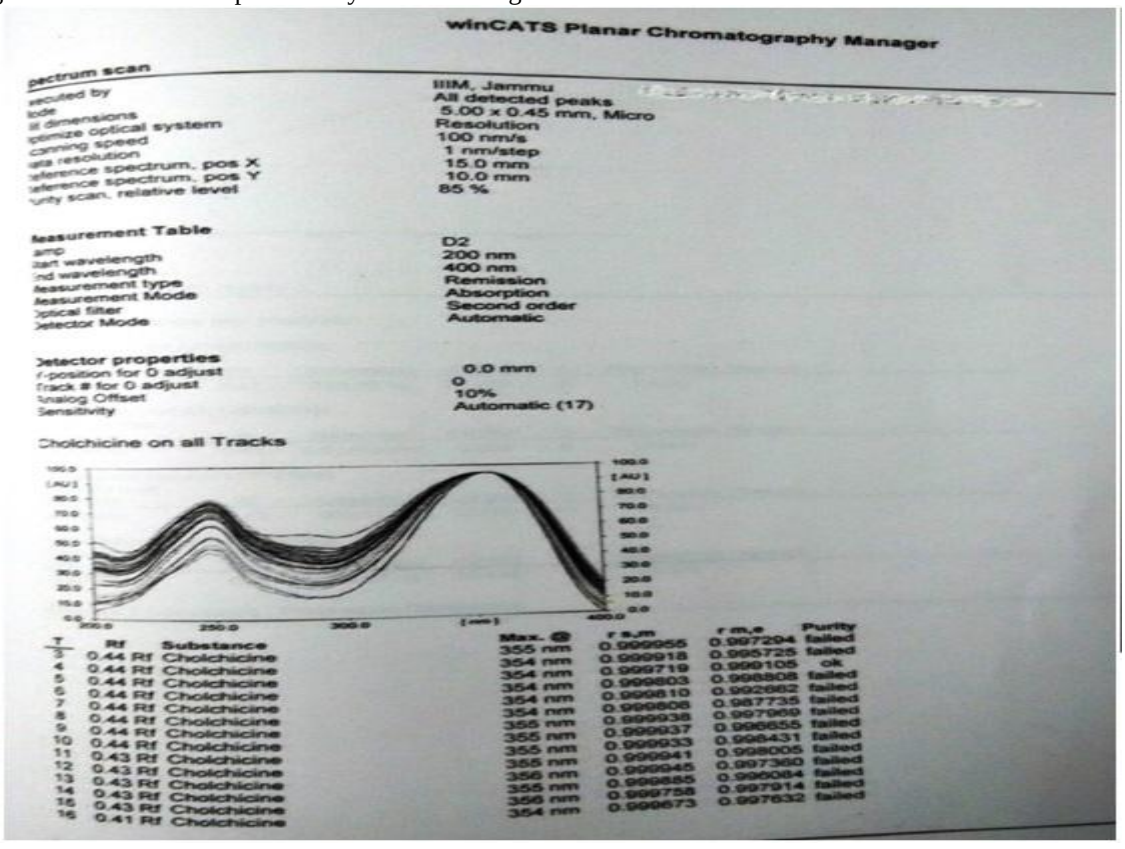

Figure 10: Spectrum scan results

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The spctra of HPTLC is shown in the graph, the density of compound was measured by absorbance of each compo0unds then calculated individually as given in the calculation.

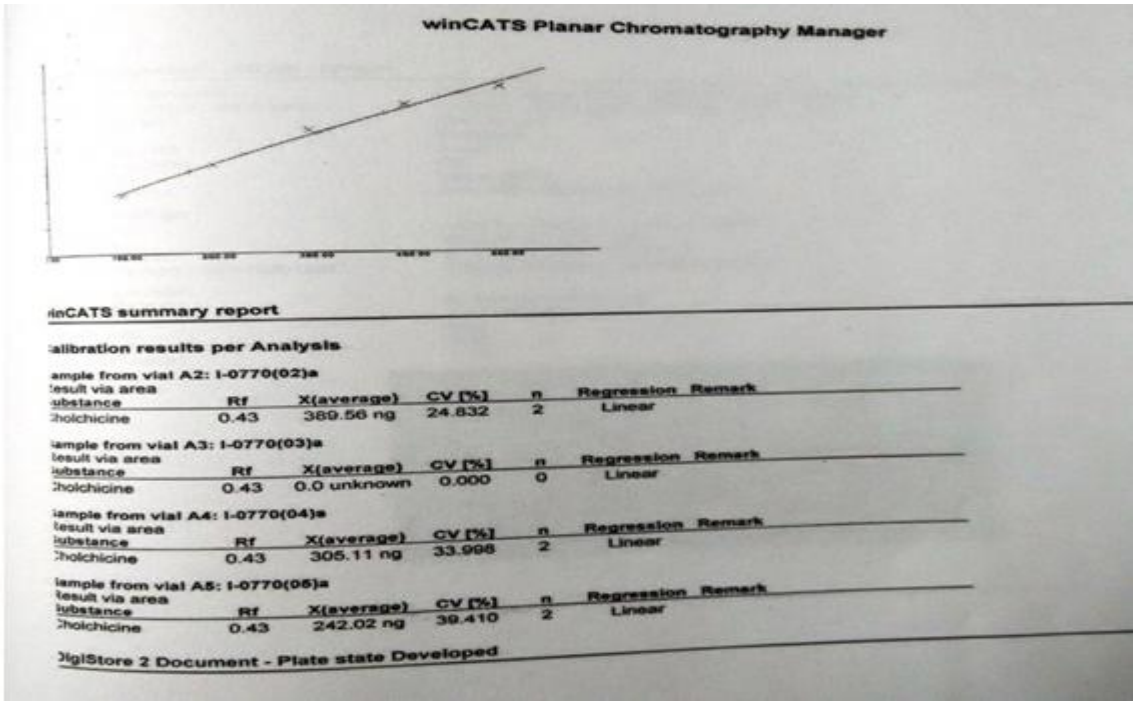

Figure 11: Summary Report of HPTLC graph

In the given graph summary of all compounds high pressure thin layer chromatographic revaluation is given. The compound which showed best result may be used in daily routine life.

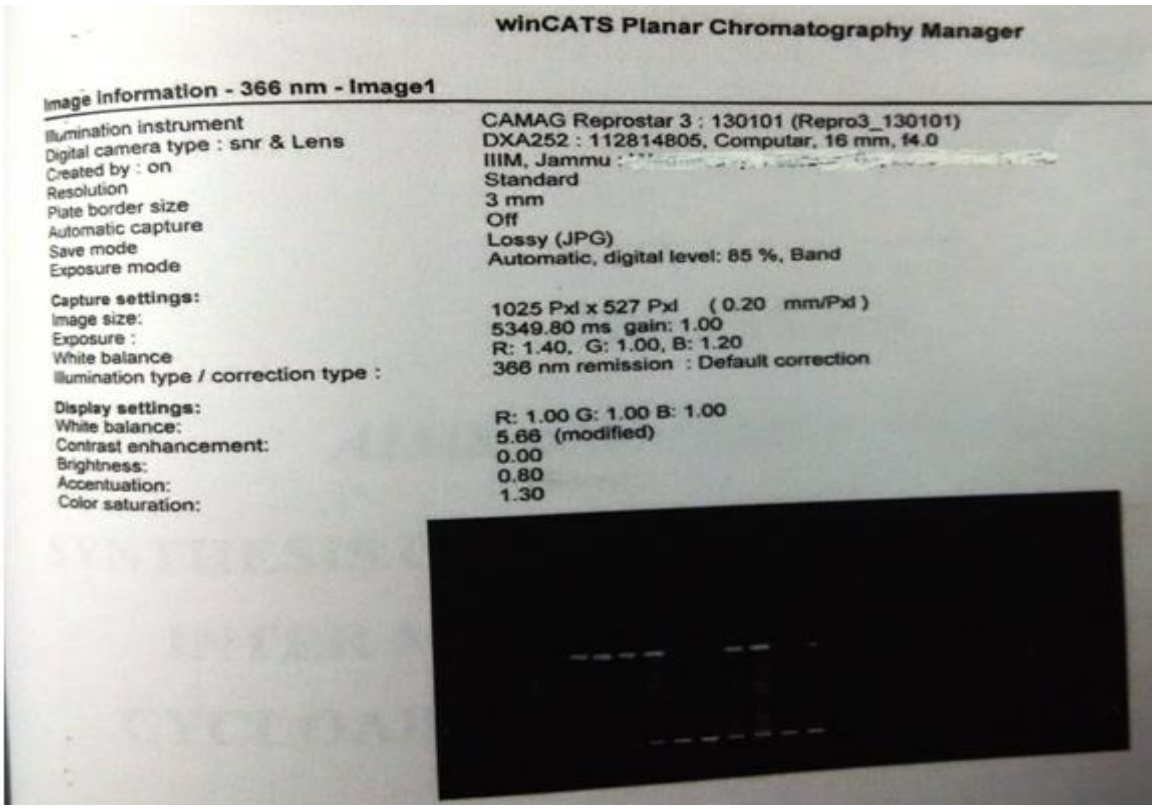

Figure 12: HPTLC Images

In the given diagram the high pressure thin layered chromatographic images of each compound is given to compare the each isolated compounds.

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