CHAPETR 3

MATERIAL AND METHODS

This chapter describes the experimental procedures and protocols that were followed for these studies. On the bases of the wide use and reported efficacy as antidiabetic herbs, few plants mentioned below reported as antidiabetic and utilized alone or incorporated widely as one of the components in marketed multi component antidiabetic formulations. They were selected for studies to justify their presence in polyherbal formulations.

3.1 Preliminary Pharmacognostic Studies

3.1.1 Selection of the plants

A few plants mentioned below are reported as antidiabetic and utilized alone or incorporated widely as one of the components in marketed multi component antidiabetic formulations. On the bases of their wide use and reported efficacy as antidiabetic herbs they were selected for studies to justify their presence in polyherbal formulations.

- *Enicostemma hyssopifolium (Mamejavo)*
- *Tinospora Cordifolia (Guduchi)*
- *Gymnema Sylvestre (Madhunashini)*
- *Eclipta alba (Bhringraj)*

3.1.2 Collection of plant material

The fresh plants of *Enicostemma hyssopifolium* and *Tinospora cordifolia* were collected from the village nearby Junagadh district, Gujarat, India. *Eclipta alba* was collected from the river side places of village of Vadodara district, Gujarat, India. The fresh plants of *Gymnema sylvestre* was collected from the nursery of Vadodara city.

3.1.3 Authentication

The collected plants were authenticated as, *Enicostemma hyssopifolium* (willd.) Verdoon (Gentianaceae), *Tinospora cordifolia* (Willd.) Miers ex Hook.F. & Thoms (Menispermaceae), *Gymnema sylvestre* (Retz.) R.Br. ex Schult (Asclepediaceae), and *Eclipta alba* (L.) Hassk. (Compositae) at Botanical Survey of India, Pune. Voucher specimen has been deposited in herbarium of our laboratory for future reference and numbers are given as EH1/Samp1, TC1/samp3, GS1/Samp4, and EA1/Samp2 respectively. Aerial parts of *E. hyssopifolium, G. sylvestre, E. alba* and stemn of *T. cordifolia* were dried under shade and powdered.

3.1.4 Pharmacognostic studies

Selected plants were subjected to preliminary pharmacognostic studies as per the reported methods. Morphology of fresh plant material was studied and described. Different parts of selected plants were studied for their microscopic characteristics in their transverse sections which were obtained by microtome. Proximate analysis, phytochemical screening and other studies were performed as described below.

3.1.4.1 Proximate analysis

Physical and physico chemical parameters were determined using standard procedures.

3.1.4.2 Determination of foreign organic matter (WHO, 1998)

Foreign organic matter (FOM) is part of the organ or organs from which the drug is derived other than the parts named in the definition and description or for which the limit is prescribed in the monograph, or any organ other than those named in definition and description, or the matter not coming from the source plant or moulds, insects, or other animal contaminations). A weighed sample (500gm) spread out in a thin layer. The sample

was inspected with the eye and separated the FOM by hand. It was weighed and percentage was calculated.

3.1.4.3 Determination of loss on drying (WHO, 1998)

About 5 gm of air dried plant material was weighed into a previously dried and tarred flat weighing bottle and dried in an oven at 105 ^{0}C. Drying was continued until two consecutive weightings do not differ by more than 5 mg. The difference in the weight of the crude drug before and after drying was noted and loss on drying was calculated with reference to the air dried material.

3.1.4.4 Determination of extractive values (WHO, 1998)

3.1.4.4.1 Water soluble extractive value

Coarsely powdered air-dried material was weighed (4 gm) and macerated with 100 ml chloroform water in a glass stoppered conical flask for 24 hr shaking frequently during first 6 hr and then allowed to stand for 18 hr. It was then filtered rapidly taking care not to lose any solvent and evaporated 25 ml of the solvent in a tarred flat bottom dish on waterbath. The residue was dried at 105 ^{0}C for 6hr, colled and kept in a dessicator for 30 min and weighed. The content of extractable matter was calculated on the basis of air dried material.

3.1.4.4.2 Alcohol soluble extractive value

Coarsely powdered air-dried material was weighed (4 gm) and macerated with 100 ml of 95% ethanol in a glass stoppered conical flask for 24 hr shaking frequently during first 6 hr and then allowed to stand for 18 hr. It was then filtered rapidly taking care not to lose any solvent and evaporated 25 ml of the solvent in a tarred flat bottom dish on waterbath. The residue was dried at 105 ^{0}C for 6hr, colled and kept in a dessicator for 30 min and weighed. The content of extractable matter was calculated on the basis of air dried material.

3.1.4.5 Determination of ash values (WHO, 1998).

The weighed amount (2-3 gm) dried and powdered plant materials were incinerated in a silica crucible in muffle furnace at a temperature not exceeding 600 ^{0}C until the drug was free from carbon. Cooled and weighed. The total ash value was calculated with reference to the air

dried drugs. The total ash was then subjected to the determination of water-soluble ash and acid-insoluble ash using official procedure.

3.1.4.5.1 Ware-soluble ash

The total ash obtained was boiled with 25 ml of water for 5 min. It was filtered through ashless filter paper. The insoluble matter was collected in a Gooch crucible. It was then ignited for 15 min at a temperature not exceeding 450 ^{0}C. The weight of the insoluble matter was subtracted form the weight of total ash; the difference represents the water-soluble ash. The percentage of the water-soluble ash was calculated with reference to air-dried material.

3.1.4.5.2 Acid-insoluble ash

The total ash obtained was boiled with 25 ml of 2N hydrochloric acid for 5 min. It was filtered using ashless filter paper. The insoluble matter was washed with hot water, ignited for 15 min at a temperature not exceeding 450 ^{0}C. The weight of the insoluble matter was measured. The percentage of the acid-insoluble ash was calculated with reference to air-dried drug.

3.1.4.6 Estimation of elements and heavy metals (WHO, 1998)

About 5 gm of the powdered drug material was ignited in muffle furnace to obtain total ash. A 100 mg of the ash was dissolved in 10ml of 1 N HCl, the solution was filtered , filtrate was diluted to 50 ml with distilled water and used for quantitative determination of heavy metals by absorption spectroscopy.

3.1.5 Phytochemical studies

3.1.5.1 Successive solvent extraction

The presence of the different chemical constituents in the crude drugs can be detected by subjecting them to successive extraction using solvent in the order of increasing polarity and subjecting the extracts so obtained to qualitative tests for various chemical constituents. The selected drug sample in the present study were therefore, subjected to successive extraction followed by qualitative chemical tests in order to know the phyto profile on a preliminary basis.

The air dried powdered drugs weighing about 50 gm each were taken and extracted successively in soxhlet apparatus using solvents in the order of increasing polarity, as follows (Kokate, 1999)

Petroleum ether, benzene, chloroform, ethyl acetate, acetone and methanol. Each time before extracting with the next solvent, the material was dried in hot air oven at a temperature not exceeding 50 ^{0}C. All the extracts were concentrated by distilling off the solvents and evaporating to dryness on the water bath. Their percentage yield was recorded.

3.1.5.2 Qualitative evaluation of successive extract

The successive extracts were tested for presence of various phytoconstituents using reported methods (Kokate, 1999). Alkaloids were tested by Dragendroff, Wagner and Mayer test; phenolics and flavonoids were tested by ferric chloride, shinoda, alkali and lead acetate tests. Chlorosulphonic acid, Carr-price reagent and Salkowski tests were used for the detection of tri-terpenoial saponins. Terpenoids and steroids were detected by vanillin sulphuric acid and Liebermann-Burchard tests. Phytosterol in Unsaponifiable matter of petroleum ether extracts were detected by Liebermann-Burchard test. Carbohydrates were detected by Molisch's and Fehling's tests. Amino acid and proteins were detected by ninhydrin, and Millon's test, Biuret test respectively.

3.1.5.3 Thin layer chromatographic studies

The various extracts obtained in the successive extraction process were subjected to thin layer chromatographic studies using silica gel $60F_{254}$ precoated plates, to confirm the presence of various constituents described above.

Flavonoids were separated using ethylacetate: formic acid : acetic acid : water (100 : 11 : 11 : 32) as mobile phase and detected using Natural product-polyethylene glycol reagent (NP-PEG) as visualization agent (under UV 366 nm light). Terpenoids were chromatographed using toluene : ethylacetate (93 : 7) as mobile phase and vanillin-sulphuric acid as detecting agent. Alkaloids were detected by Wagner's and Dragendroff's reagent after development solvent system suitable for particular alkaloid. Sterols were detected after development in petroleum ether : ethyl acetate (4 : 1) mobile phase by Liebermann-Burchard reagent.

Steroids, amino acids, and carbohydrates were detected by TLC using reported solvent systems.

3.2 DNA Fingerprinting by RAPD

A pure genomic DNA of selected plants were isolated from fresh leaf and subjected to RAPD fingerprinting using ten random primers.

3.2.1 Chemicals and reagents

Plant DNA extraction kit for PCR amplification (GeNei, Bangalore) consisting of solution A (buffer for cell lysis) 3 M sodium acetate pH 5.2, and solution B (buffer for DNA solubilization).

Polyvinyl pyrrolidone (PVP)	:	A solution containing 1% w/v of PVP.
Ethanol	:	A solution of ethanol 70%, and 100% v/v.
TAE buffer (50X)	:	Tris base (24.2 gm), glacial acetic acid (5.71 ml) and EDTA (0.5M, pH 8, 10 ml) were mixed and diluted to 100 ml with distilled water.
Ethidium bromide	:	Ethidium bromide (2.5mg) was dissolved in distilled water to make 25 ml solution.
Gel loading dye	:	It contains bromophenol blue (50mg), xylene cyanol FF (50mg) and sucrose (8 gm) in 20 ml of solution made in distilled water.

3.2.2 Instruments

- Water bath (65-70 ^{0}C)
- Chilling centrifuge
- Micro pipette
- Gel electrophoresis Instrument
- Gel Doc Instrument
- Thermal cycler

3.2.3 DNA isolation protocol

Freshly harvested leaf sample (100 mg) was ground in liquid nitrogen using a mortar and pestle along with 500 µl of PVP. The pulverized leaves were quickly added with 500 µl of solution A from plant DNA extraction kit and transferred in a 1.5 ml sterile vial. It was kept into a water bath maintained at (65-70 ^{0}C) and incubated for 1 hr with intermittent crushing.

Vortex the vials for 10-15 sec and centrifuged at 10000 rpm for 7 minutes at room temperature. Supernatant was taken and added with 500 µl of cold ethanol and 40 µl of sodium acetate. Mixed gently by inverting the vials and kept at 4 ^{0}C for 10 minutes. Vials were then centrifuged at 14000 rpm for 20 minutes at 4 ^{0}C. Supernatant was discarded and a pellet was washed with 500 µl of 70% ethanol, centrifuged at 14000 rpm for 20 minutes at room temperature. Pellet was blot dried and solubilized in 40 µl of solution B from plant DNA extraction kit. Tubes were kept at 65-70 ^{0}C for 3-5 minutes for complete solubilization. Tubes were centrifuged at 12000 rpm for 15 minutes to remove any insoluble materials, supernatant was collected in a sterile vial and stored at -55 ^{0}C.

3.2.4 Amount and purity of DNA

The yield of DNA was measured using a Nano Drop ND-1000 spectrophotometer. DNA concentration and purity was also determined by running the samples on 1% agarose gel based on the intensities of band.

3.2.5 RAPD analysis

DNA extracted from plants collected from two different geographical location were subjected to RAPD analysis. Oligonucleotide primers with following series (Bangalore GeNei, Bangalore) were used for amplification.

5' – AG GG GT CT TG – 3'	**5' – GA GT CT CA GG – 3'**
5' – GT GA CG TA GG – 3'	**5' – GT GA CA TG CC – 3'**
5' – TG TC TG GG TG – 3'	**5' – TC TG GT GA GG – 3'**
5' – GA CG GA TC AG – 3	**5' – CA AA CG TC GG – 3'**
5' – GG TG CG GG AA – 3'	**5' – CA AT CG CC GT – 3'**

The reactions were carried out in a eppendorf realplex^4 (Master cycler, ep gradient S). Each 50 µl reaction volume contained about 50 ng of template DNA, 25 µl of PCR master mix (Bangalore GeNei, Bangalore), 0.5 µM of single primer (Bangalore GeNei, Bangalore). The thermocycler was programmed for an initial denaturation step of 3 min at 94°C, followed by 30 cycles of 45 s at 94 ^{0}C, annealing for 1 min at 37 ^{0}C, extension was carried out at 72 ^{0}C for 1 min and final extension at 72 ^{0}C for 7 min and a hold temperature of 4°C at the end. PCR

products were electrophoresed on 2% (w/v) agarose gels, in 1 X TAE Buffer at 75 V for 2 hrs. Gels with amplification fragments were visualized and photographed under UV light.

3.3 Extraction, fractionation and isolation of phytoconstituents

3.3.1 *Enicostemma hyssopifolium*

Coarse powder (# 30) of whole plant *E. hyssopifolium* was extracted exhaustively in soxhlet extractor (7 days) using methanol. The solvent was evaporated at 50 ^{0}C under reduced pressure till thick semisolid consistency achieved. Green thick paste was stored in tightly closed container at 2-8 ^{0}C.

3.3.1.1 Preparation of bitter fraction

Bitter fraction was prepared as per the method described in Ayurvedic pharmacopoeia. *E. hyssopifolium* powder (100 gm) was thoroughly mixed with calcium carbonate (2.5 gm) and boiled with 500, 300 and 200 ml of distilled water successively. Water was evaporated on waterbath at 70 ^{0}C till syrupy mass obtained. It was suspended in hot methanol and filtered while hot. Residues were washed with hot methanol. Combined methanol extract and washing were allowed to evaporate on waterbath. Residue left behind was dispersed in water shaken with ethyl acetate (100, 75, 50, 50 and 25 ml). Ethylacetate was recovered on rota evaporator and the residue were collected, dried and stored at 2-8 ^{0}C.

3.3.1.2 Preparation of flavonoid fraction

The methanol extract (50 gm) was dispersed in distilled water (500 ml) and partitioned with hexane (3 X 200 ml) and then with n-butanol (3 X 200 ml). Butanol was allowed to evaporate at room temperature. Dried fraction was stored at 2-8 ^{0}C.

3.3.1.3 Isolation of compounds

TLC chromatogram of EH extract showed two distinct bands at R_f 0.71 (EH001) and 0.57 (EH002). Both these compounds were isolated from methanol extract. Methanol extract (10 gm) was redissolved in methanol (50 ml) and left as such at ambient temperature till green greasy precipitates start depositing on the surface of evaporating dish. Thick brown liquid devoid of greasy precipitates was separated by centrifugation (1000 X g) for 15 min.

Supernatant was subjected to column chromatography. Column was first eluted with petroleum ether (60 ^{0}C- 80 ^{0}C), then with ethyl acetate, followed by gradient of methanol (0-10%). Fractions (25 ml each) were monitored by thin layer chromatography. Fractions showing presence of compound 1 and 2 were pooled together and volume was reduced under vacuum to get 10 ml. Compounds were then isolated by repetitive preparative TLC using silicagel HF_{254} as stationary phase and ethyl aceate : methanol : water (77 : 15 : 8 v/v/v) as mobile phase. Bands at R_f value 0.71 (EH 001) and 0.57 (EH 002) were identified and eluted with methanol. Methanol was evaporated on waterbath (70 ^{0}C) and compounds were collected after repeated crystallization from methanol.

3.3.2 *Tinospora cordifolia*

3.3.2.1 Preparation of Alkaloid fraction

Defatted powdered drug was extracted with methanol in soxhlet apparatus (4 days). Methanol was recovered in rota-evaporator and semisolid extract was added with 10 % glacial acetic acid and digested for 30 min. It was then filtered and filtrate was made alkaline (pH 8) with ammonia. Filtrate was extracted with chloroform (5 X 100 ml). Chloroform was allowed to evaporate at room temperature and residue was stored at 2-8 ^{0}C.

3.3.2.2 Isolation of alkaloids

It was performed by preparative TLC using silica gel GF_{254} as stationary phase and ethyl acetate: formic acid: glcial acetic acid: water (100:11:1:26 v/v/v/v) as mobile phase.

Three major alkaloids at R_f 0.27, 0.35, and 0.42 were identified and eluted from silica gel with the help of methanol. Solvent was evaporated and solid powder was collected and labeled as TCA, TCB and TCY for the alkaloids at R_f 0.27, 0.35, and 0.42 respectively.

3.3.2.3 Identification of Tinsporaside

A clerodane furano-diterpene, tinosporaside was identified in methanol extract and methanol fraction of aqueous extract of TC by TLC using silica gel 60 F_{254} as stationary phase and chloroform : methanol (9:1 v/v) as mobile phase. Tinosporaside was identified at Rf 0.26 as fluorescent quenching band which turn brown after treatment with AS reagent and heated at 110 ^{0}C for 5 min.

3.3.3 *Gymnema sylvestre*

3.3.3.1 Isolation of crude gymnemic acid

Leaf powder (100 gm, # 30) was defatted with petroleum ether 60-80 ^{0}C and successively extracted with chloroform. Powdered material was dried by removal of chloroform and added with 50 % methanol in water (500 ml). The mixture was refluxed on waterbath for 30 min and filtered. Filtrate was subjected to evaporation of methanol. The aqueous portion remained was added with distilled water (200 ml) and pH was adjusted to 1.5. Black precipitates obtained were collected by filtration and dissolved in methanol, further boil for 10 min after addition of charcoal and filtered hot. Methanol was evaporated from filtrate, creamish powder obtained was considered as crude gymnemic acid.

3.3.3.2 Isolation of Gymnemagenin

Crude gymnemic acid (5 gm) was dissolved in 50 ml of 50% (v/v) methanol, 10 ml of potassium hydroxide solution (11 % in water) was added and the mixture refluxed on a water bath for 1 h. The mixture was cooled, 9 ml of concentrated hydrochloric acid added and the whole refluxed for 1 h. The resulting solution was cooled, adjusted to pH 7.5–8.5 with potassium hydroxide solution, made up to a volume of 250 ml with water. Partitioned with chloroform (5 X 100 ml), chloroform was evaporated in rota evaporator. Concentrated solution of genin was chromatographed over silica gel G by preparative TLC using mobile phase ethyl acetate : toluene : methanol (3.5 : 1 : 0.5). A band of gymnemagenin was identified by at Rf 0.45 by spraying with anisaldehyde sulphuric acid reagent on partially covered plate. Gymenagenin was eluted from silica gel G using chloroform : methanol (1 : 1) mixture.

3.3.4 *Eclipta alba*

3.3.4.1 Preparation of phenolic fraction

Powdered plant material (500 gm) was exhaustively extracted with methanol using soxhlet extractor (6 days). Methanol extract was reduced in volume under vacuum using rota evaporator. Thick green extract was suspended in distilled water (1:1) with heating for 15 min at 70 ^{0}C. Hydroalcoholic extract was partitioned successively

with hexane (3 X 150 ml) and ethylacetate (3 X 200 ml). Hexane fraction was used fro preparation of sterol fraction. Ethyl acetate fraction was used as phenolic fraction.

3.3.4.2 *Preparation of sterol fraction*

Hexane fraction was saponified by refluxing it with 10 % aqueous potassium hydroxide at 70 ^{0}C for 30 min. Dissolved in 50 % ethanol and partitioned with hexane. Hexane layer was separated, solvent was removed at room temperature and dried residue was used as sterol fraction.

3.3.4.3 *Isolation of wedelolactone*

Powdered plant material (500 gm) was exhaustively extracted with methanol with 2% acetic acid using soxhlet extractor. Methanol extract was filtered while hot. Filtrate was kept for 2 hrs during which wax, chlorophyll etc. precipitated out and stuck to the surface of porcelain dish. It was filtered; brown filtrate was allowed to evaporate in air to obtain a thick brown liquid. It was partitioned with diethyl ether till it is no longer fluorescent. Diethyl ether was evaporated to get concentrated solution. On cooling at 0-4 ^{0}C, a green yellow precipitates formed at the bottom of the vial were separated by centrifugation. Further purification was performed by preparative TLC using silicagel G as stationary phase and tolene : acetone : formic acid (11 : 6 : 1 v/v/v) as mobile phase. Wedelolactone was identified at R_f 0.56 as bright blue fluorescent band and eluted with methanol.

3.3.4.4 *Isolation of sesquiterpene (EA002)*

Coarse powder of *E. alba* (150 gm) was moistened with ammonia (10 %) and thoroughly mixed with basic aluminium oxide (100 gm), stirred with chloroform (500 ml) for 15 min and stand overnight for cold maceration. Chloroform extract was filtered out and evaporated under vacuum using rata evaporator. Green liquid obtained was chromatographed by preparative TLC using silicagel G as stationary phase and toluene : ethyl acetate (4:1 v/v) as mobile phase. A blue fluorescent band Rf 0.56 was scrapped off and eluted with chloroform : methanol (1:1). It was further subjected to preparative TLC using silicagel G as stationary phase and petroleum ether (60-80 ^{0}C) : ethyl acetate (4:1 v/v) as mobile phase. EA002 was identified at Rf 0.5 as blue fluorescent band and eluted with mixture containing chloroform and methanol in equal proportion.

3.4 Fingerprinting of extracts, fractions, formulations and quantitation of marker compounds

3.4.1 High Performance Thin Layer Chromatography (HPTLC) studies

3.4.1.1 Materials and chemicals

Study samples (extracts and fractions).

Marker compounds.

Aluminium backed precoated silicagel $60F_{254}$ HPTLC plates (Merk KGaA, Germany).

Whatman filter paper (No. 41).

Ethyl acetate, methanol, chloroform, toluene, acetone used were of analytical grade.

Distilled water.

3.4.1.2 Instruments

Sample applicator: LINOMATE 5, Hamilton's glass syringe (100 µl).

Development chamber: Twin trough chamber (10 X 10 or 20 X 10 cm).

Scanner: CAMAG SCANNER 3.

U V Cabinet with short wave (254 nm) and long wave (366 nm) tubes.

Dipping chamber, plate dryer.

3.4.1.3 Fingerprinting

Fingerprint of the ethanol extracts of plants and studied formulations were obtained in three different solvent systems; one of which was non polar (hexane : ethyl acetate , 4 : 1) second was of medium polarity (chloroform : methanol, 4.5 : 0.5) and third solvent system was polar in nature (ethyl acetate : acetic acid : formic acid : water, 25 : 2.75 : 2.75 : 6.5). Developed tracks were scanned densitometrically under 254 nm (absorption mode), 366 nm (fluorescence mode) and 540 nm (after derivatization with anisaldehyde sulphuric acid reagent). HPTLC quantitation of marker compounds was also performed.

3.4.1.4 Estimation of markers in Enicostemma hyssopifolium

HPTLC quantitation of markers, swertiamarin and swertisin was performed to standardize the extract. Plate was developed in a twin trough chamber saturated with the mobile phase, ethyl

acetate : methanol : water (77 : 15 : 8). Detection was carried out by scanning the developed and air dried plates at 238 nm (for swertiamarin, in absorption mode) and at 342 nm (for swertisin, in absorption mode).

Method for their simultaneous estimation in plant extracts has also been developed and validated.

3.4.1.5 Estimation of markers in Tinospora cordifolia

Fingerprinting of alkaloid fraction was performed using HPTLC. Plates were developed in the mobile phase ethyl acetate : formic acid : acetic acid : water (100 : 11 : 11 : 26) and scanned at 254 nm in absorption mode and at 366 nm at fluorescence mode.

Quantitation of marker compound, tinosporaside in plant extracts was performed using the mobile phase chloroform : methanol (4 : 1). Scanning was done at 255 nm in absorption mode.

3.4.1.6 Estimation of markers in Gymnema sylvestre

Fingerprinting of aqueous and methanol extract was performed by keeping all the HPTLC parameters same as mentioned above, except the mobile phase. Chloroform : methanol : acetic acid (5 : 1 : 1) was used to develop the TLC plates. Also isopropylalcohol : chloroform : methanol : acetic acid (5 : 3 : 1 : 0.5) was used to get fingerprint alongwith gymnemic acid and gymnemagenin. Plate was sprayed with AS reagent and heated for 5 min. at 110 ^{0}C.

Plant leaves, methanol extract and aqueous extract were quantified for their gymnemagenin content. A sample (100 mg) of *Gymnema sylvestre* leaves/methanol extract/aqueous extract was first subjected to hydrolysis by dissolving it in 5 ml of 50% methanol and added with 1 ml of 11% potassium hydroxide solution. The mixture was refluxed on a water bath for 1 hr. The resulting solution was cooled, 0.9 ml of concentrated hydrochloric acid was added and the mixture refluxed again for 1 hr. After cooling, the pH of the solution was adjusted to 7.5 – 8.5 with potassium hydroxide solution, the volume made up to 25 ml with 50% methanol and filtered. The filtrate was used for HPTLC analysis. Chloroform : methanol : acetic acid (5 : 1 : 1) was used as mobile phase and plates were scanned at 450 nm after derivatization with AS reagent under absorption mode.

3.4.1.7 *Estimation of markers in Eclipta alba*

Fingerprinting of methanol extract and phenol fraction was performed using toluene : acetone : formic acid (11 : 6 : 1) as mobile phase. Plates were scanned at 366 nm under fluorescence mode. Fingerprinting of sterol fraction was performed using petroleum ether : ethyl acetate (4 : 1) mobile phase. Scanning was done at 366 nm under fluorescence mode.

Quantitation of marker compound, wedelolactone was performed by using the same mobile phase and scanning of HPTLC plate was performed at 351 nm under fluorescence mode.

3.4.2 High Performance Liquid Chromatography (HPLC) studies

3.4.2.1 *Materials and chemicals*

Study samples (extracts and fractions).
Marker compounds.
Excil ODS C-18 column (5 µm, 4.6 X 250 mm) (SGE, India).
Nylon membrane filter paper (0.2 µm).
Vacuum filtration assembly.
Sonicator.
Methanol and acetonitrile were of HPLC grade (Qualigens, India)
Double distilled water.

3.4.2.2 *Instruments*

Isocratic HPLC system with LC-20AT pump and SPD-20A UV/VIS detector (Prominence, Shimadzu, Japan).

3.4.2.3 *HPLC studies of Enicostemma hyssopifolium*

HPLC finger printing of bitter fraction and flavonoid fraction were performed by preparing their stock solution in methanol (1 mg/ml) and dilutions were made with the mobile phase i.e, acetonitrile : water (25 : 75). Flow rate was kept at 0.8 ml/min and detection was carried out by using UV detector at 238 and 342 nm separately.

Quantitation of markers, swertiamarin and swertisin in both the fractions were carried out at 238 and 342 nm wavelength respectively keeping the flowrate of mobile phase at 1 ml/min. Method for the estimation of marker compound in plant fraction was also been validated.

3.4.2.4 HPLC studies of Tinospora cordifolia

HPLC fingerprint of alkaloidal fraction was obtained by injecting 20 µl of solution (0.5 mg/ml) prepared in mobile phase, acetonitrile : Britton Robinson buffer (245 : 5, pH 3). Flow rate was adjusted to 1 ml/min, UV detection was performed at 345 nm. Isolated alkaloid, TCA was detected in aqueous extract by using methanol : acetic acid : triethanolamine : water (30 : 1 : 0.5 : 68.5) as mobile phase. Detection was performed at 270 nm.

3.4.2.5 HPLC studies of Gymnema sylvestre

Methanol extract was subjected to HPLC fingerprinting. Mobile phase was optimized as acetonitrile : 0.1% KH_2PO_4 (40 : 60) at the flow rate of 0.8 ml/min. Detection was performed at 210 nm.

3.4.2.6 HPLC studies of Eclipta alba

HPLC fingerprinting of phenolic fraction (0.1 mg/ml) was done using mobile phase acetonitrile : methanol : acetic acid (0.5%), 10 : 45 : 45. Flow rate was kept at 1 ml/min and detection was performed at 351 nm using UV detector. Fingerprint of phenolic fraction was also obtained using pure acetonitrile as mobile phase at flow rate of 0.8 ml/min and detection was performed at 360nm. Fingerprint of sterol fraction (0.5 mg/ml) was obtained by using pure acetonitrile as mobile phase at flow rate of 0.8 ml/min. Detection was performed at 360 nm using UV detector.

Quantitation of markers, wedelolactone and isolated EA2 was performed in aerial parts as well as in phenolic and sterol fraction using the parameters mentioned above.

3.5 Biological Studies

3.5.1 Glucose uptake study on rat everted gut sac

A preparation of isolated small intestine was used for convenient measurement of active transference of D-Glucose across the intestinal wall. The difficulty of adequate oxygenation

is overcome by everting a piece of intestine, tying it at both ends and filling it with sufficient fluid to distend the wall. The eversion exposes the highly active mucosa to the well-oxygenated suspending medium, while the distension increases the surface area of the sac and reduces the thickness of the sac wall. The relatively small volume of fluid contained in the sac (serosal side) allows a rapid rise in concentration of transferred substance (D-Glucose). A number of adjacent segments of intestine from the same animal were studied simultaneously.

3.5.1.1 Materials and chemicals

Krebs-Henseleit bicarbonate buffer (KHBB): $NaHCO_3$ 25 mM/L; NaCl 118 mM/L; KCl 4.7 mM/L; $MgSO_4$ 1.2 mM/L; NaH_2PO_4 1.2 mM/L; $CaCl_2$ 1.2 mM/L and Na_4EDTA 9.7 mg/L.

Carbon dioxide incubator.

3.5.1.2 Experimental design

Adult male Swiss albino rats weighing 100-150 gm were used. Rats were sacrificed by severe blow on the head against a hard surface. The abdomen was opened by a midline incision. The entire small intestine was removed. The small intestine was washed out with normal saline solution (0.9% w/v NaCl) using a syringe equipped with blunt end. Intestinal segments (5 ± 1 cm) were everted. A 1 gm glass weight was fixed and tied to the end of the everted gut segment to make an empty gut sac. After weighing, the empty sac was filled with 0.5 mL of KHBB. Glucose (2 gm/L) was added to the medium just before the start of the appropriate experiment. The pH was maintained at 7.4. The compartment containing the buffer in the sac was named serosal fluid compartment. The distended sac was placed inside a 40 mL KHBB bath (mucosal fluid compartment) and mounted. For studying the effect of the plant extracts on the uptake of glucose (substrates), glucose at varying concentrations was added into mucosal compartment fluid. The plant extracts/fractions were also added in the same compartment after digestion in 2 mL of simulated gastric fluid (5 mg/mL). At the end of the incubation period (60 min), the sacs were removed from the gut sac bath. Glucose concentrations in both the compartment were measured using a commercially available glucose oxidase kit (Span Diagnostics Ltd. Sachin, India). The amount D(+)-glucose transported from the mucosal compartment was characterized as 'uptake'. Glucose uptake was expressed as mM/gm tissue wet weight/hr.

3.5.1.3 *Data analysis*

In terms of enzyme kinetics, glucose transported per hour was analogue to the velocity of transfer, in other words, to the concentration difference of glucose between compartments at the beginning and end of an experiment. The Michaelis – Menten constant (K_m), which is the affinity of the transferring enzyme for the substrate, and maximal velocity (V_{max}), which is the rate of transfer reaction, in the presence as well as in the absence of studied plant extracts and fractions were determined from the differences of uptake and release values using the Michaelis-Menten and Lineweaver-Burk Plots in Microsoft Excel. Comparison of D(+)-glucose uptake difference between the controls and experimental groups were examined using paired t - test for mean ± SEM. Any difference with p values less than 0.05 were considered as statistically significant.

3.5.2 Alpha amylase Inhibitory Activity

3.5.2.1 *Materials and Reagents*

Pancreatic α-amylase (Hi Media, India). Sodium dihydrogen phosphate, disodium hydrogen phosphate, and sodium chloride were of LR grade (S D fine chemicals, India). Soluble starch (Qualigens, India). Glucose estimation kit (Span diagnostics, India).

α-amylase solution: A solution containing 10 mg/ml of pancreatic α-amylase was prepared in 0.4% bovine serum albumin.

Sörensen buffer (20 mM, pH 6.9): Solution A- 0.55 gm sodium dihydrogen phosphate in 20 ml solution, Solution B – 0.57 gm of disodium hydrogen phosphate in 20 ml solution. Solution A (11.25 ml) and solution B (13.75 ml) was mixed and diluted with distilled water to make 250 ml of the final volume.

Sodium chloride (10 mM): Sodium chloride (5.83 gm) dissolved in distilled water and diluted up to 100 ml.

3.5.2.2 *Experimental Design*

Various concentrations of the samples were dissolved in 10 ml of 20 mM Sörensen buffer, pH 6.9 and 10 mM NaCl. Pancreatic α-amylase solution (0.1 ml) was incubated with various

concentrations of samples at 25 ^{0}C for 10 min. The enzymatic reaction was started by addition of 1.0 ml, 2 % soluble starch solution and allowed to continue for 30 min. After 30 min, 0.1 ml of the reaction mixture was mixed with 1 ml of glucose oxidase reagent. The absorbance was measured after 15 min at 505 nm against the reagent blank. The absorbance of sample without enzyme was treated as 100% inhibition.

3.5.3 Aldose Reductase Inhibitory Activity

3.5.3.1 Materials and chemicals

Sheep eyes were obtained from a local abattoir. A saturated solution of ammonium sulfate was prepared by dissolving excess of ammonium sulfate in distilled water.

Sodium phosphate buffer (0.067 M, pH 6.2): Potassium dihydrogen phosphate (0.264 gm) and disodium hydrogen phosphate (1.085 gm) were dissolved in sufficient distilled water to make the volume 100 ml and pH was adjusted 6.2.

Nicotinamide dinucleotide phosphate reduced (NADPH) solution: Accurately weighed 31.3 mg of NADPH was dissolve in sodium phosphate buffer to make 10 ml of the solution.

Lithium Sulfate: Lithium sulfate (26.4 gm) was dissolved in sufficient quantity of distilled water to make the volume 100 ml.

D-xylose solution (10 mM): Accurately weighed quantity of D-xylose (450 mg) was dissolved in minimum amount of distilled water and volume was made up to 10 ml with distilled water.

Positive control: Quercetin (0.33, 3.33 and 33.3 mM)

A. Quercetin (1.51 gm) dissolved in sodium phosphate buffer to produce 5 ml solution (0.33 mM).
B. Ten time diluted solution A in sodium phosphate buffer (3.33 mM).
C. Ten time diluted solution B in sodium phosphate buffer (3.33 mM).

Galactose (30 mM): Galactose (540 mg) was dissolved in Dulbecco's modified Eagle's medium (to produce 100 ml).

Perchloric acid (0.6N): Perchloric acid (7.9 ml) was diluted up to 100 ml with distilled water.

Potassium hydroxide (2 N): Potassium hydroxide (11.2 gm) was dissolved in distilled water and volume was made up to 100 ml.

Periodic acid (0.03 M): Periodic acid (684 mg) was dissolved in distilled water and volume was made up to 100 ml with distilled water.

Stannous chloride (0.125 M): Accurately weighed quantity (2.37 gm) of stannous chloride was dissolved and volume was made up to 100 ml with distilled water.

Chromotropic acid (0.2%): Accurately weighed 200 mg chromotropic acid was dissolved in water to make 100 ml of the solution.

Sample prepration: Samples were prepared by dissolving 2 mg of the sample in 50 µl DMSO. After 30 min or more, a 1 ml volume was completed by adding phosphate buffer pH 6.2.

3.5.3.2 Enzyme Preparation

Sheep eyes were obtained from a local abattoir soon after slaughtering. Lenses were enucleated by lateral incision. Lenses (10-12 gm) were homogenized in 3 volumes of cold distilled water. Saturated was added to the supernatant fluid to 40% saturation, kept for 15 min and was centrifuged (2500 RPM, 10 min), and the precipitate was discarded. Additional inert protein was removed by increasing the ammonium sulfate concentration to 50% saturation and centrifuging the mixture. Aldose reductase was then precipitated from the 50% supernatant solution by the addition of saturated ammonium sulfate solution up to 75'% saturation and was recovered by centrifugation. The pellet obtained by centrifugation was dissolved in the phosphate buffer (0.067 M, pH 6.2). Further purification of the fraction was done with the use of Sephadex LH 20 and used as enzyme preparation. Enzyme preparation was analyzed for its protein content using Lowry's method (Lowry et al, 1951).

3.5.3.3 Determination of AR activity

The reaction mixture was prepared at 25° C, with a total volume of 3.0 ml, by adding 0.1 ml $LiSO_4$, 0.3 ml of enzyme preparation, 0.1 ml NADPH, 0.1 ml D-xylose as a substrate and sodium phosphate buffer (50 mM pH 6.2), with or without sample (0.1 ml). In case of positive control 0.1 ml of the standard solution A, B or C was added in both reference and sample cuvette.

The reaction was initiated by addition of NADPH and continued for 5 min. Absorbance readings were conducted in a double beam spectrophotometer at 340 nm, at every 30 s intervals for 5 min.

A negative control was prepared using 5 % DMSO in phosphate buffer (pH 6.2). The bioassays were run in triplicate and the average inhibitory activities were measured. ΔOD/min/mg protein was calculated for each sample. Percent inhibition of AR activity was calculated using the following formula. IC50 value was obtained from a dose–response curve (DRC) calculated by plotting dose concentration versus percent inhibition.

$$\% \text{ AR Inhibition} = \frac{\Delta \text{ Abs. (Negative Control)} - \Delta \text{ Abs. ((Extract)}}{\Delta \text{ Abs. (Negative Control)}}$$

Enzyme kinetic parameters like maximal velocity (V_{max}) and Michaelis-Menten constant (K_m) were also measured for bio active compounds.

Bio active fractions and compounds were studied further for their effect on polyol accumulation in sheep lenses under hyperosmotic condition.

3.5.3.4 Effect on polyol level under osmotic stress

Sheep eyes were enucleated and lenses were dissected from the eyes. Each isolated lens was incubated in 5 ml of (DMEM) at 37 ^{0}C in an incubator at 95% air and 5% CO_2. The medium was supplemented with 10% fetal calf serum and 0.9 g/l sodium bicarbonate. Streptomycin, 100 μg/ml and penicillin, 100 IU/ml were also added to prevent bacterial contamination. After 2 h of incubation, transparent lenses were taken for the subsequent experimental studies. Osmotic stress was generated by supplementing galactose (30 mM) in the DMEM culture medium. The transparent rat lenses were divided into normal, negative control, positive control and treated groups. Normal, negative control, positive control and treated group's lenses were incubated for 3 h in DMEM, DMEM supplemented with 30 mM galactose, DMEM supplemented with 30 mM galactose and quercetin (33.3 mM), and DMEM supplemented with 30 mM galactose as well as the concentration of the fraction/compound with maximum AR inhibitory activity. After incubation for 3 h, the lenses

from different groups were washed, the fresh weights recorded and processed for polyol estimation by the reported method (West and Rapoport, 1949).

The lenses were homogenized in 0.6N perchloric acid and centrifuged at 5000×g for 20 min. The supernatant so obtained was neutralized with 2N potassium hydroxide and again centrifuged. The supernatant was reacted with 0.25 ml of periodic acid (0.03 M) for 10 min, followed by the addition of 0.25 ml of freshly prepared stannous chloride (0.125 M) and 2.5 ml of chromotropic acid (0.2%). The reaction mixture was heated in a boiling water bath for 30 min. The absorbance of the purple-colored complex was measured at 570 nm using a spectrophotometer. A parallel standard was also prepared using dulcitol.

3.5.3.5 Kinetic parameters

K_m and V_{max} of lens AR were determined for the most effective bio markers with varying concentrations of substrate (D-Xylose) in the absence and presence of test samples. K_m and V_{max} were estimated by Lineweaver-Burk double reciprocal plots.

3.5.3.6 Statistical Analysis

Experimental results of polyol accumulation study were presented as mean ± SEM of three parallel measurements. Analysis of variance was performed by one way ANOVA. Significant differences between means were determined by Bonferroni's multiple comparison test (GraphPad prism 3.02). $p<0.05$ was considered as significant.

3.5.4 Alpha Glucosidase Inhibitory Activity

3.5.4.1 Material and chemicals

Phosphate buffer (80 mM pH 7.0): Potasium dihydrogen phosphate (1.05 gm) and disodium hydrogen phosphate (4.34 gm) was dissolved in distilled water separately, mexed and pH of the clear solution was adjusted to 7.0. Final volume was made up to 250 ml with distilled water.

α –glucosidases solution: Accurately weighed α –glucosidases (3.8 mg, Sigma USA) from total 19.38 mg (750 IU) was dissolved in phosphate buffer to produce 25 ml solution.

Disaccharide solutions:

Sucrose solution (37 mM): Sucrose (126.7 mg) was dissolved in distilled water and volume was made up to 10 ml with distilled water.

Maltose solution (37 mM): Maltose monohydrate (133.3 mg) was dissolved in distilled water and volume was made up to 10 ml with distilled water.

Sample solutions: All studied samples were dissolved in phosphate buffer (minimum quantity of DMSO was used to solubilize extract wherever required). Samples were prepared in the range of 10-100 µg/ml). Bioactive fractions and compounds were used in form of solution with necessary dilution.

3.5.4.2 Experimental design

The inhibitory activity is determined by incubating a solution of an enzyme (100 µl) with 80 mM sodium phosphate buffer, pH 7.0 (700 µl) containing various concentrations of the samples at 37 ^{0}C for 10 min. Sucrose or maltose (100 µl) was added and the reaction mixture was allowed to incubate at 37 ^{0}C for 30 min. After incubation the amount of liberated glucose was measured by the glucose oxidase method by mixing 0.5 ml of the mixture with 1.0 ml of glucose oxidase peroxidase reagent. After 15 min the absorbance was read at 505 nm in double beam spectrophotometer.

3.5.4.3 Data analysis

After screening of effective compound, enzyme kinetic parameters like, Michaelis – Menten constant (K_m), and maximal velocity (V_{max}), in the presence as well as in the absence of sample were determined. The statistical significance was checked by comparing the control and experimental groups by one way ANOVA followed by Bonferroni's multiple comparision test for mean ± SEM. Any difference with p values less than 0.05 were considered as statistically significant. IC_{50} value was determined by plotting dose vs % inhibition curve.

3.5.5 Cytoprotective Activity (MTT Assay)

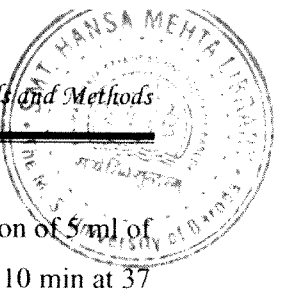
3.5.5.1 Experimental Design

An EC_{50} study and a 24 hr time course exposure study were carried out in sterile flat bottom 96 well plates. A hemocytometer was used for cell counts. Concentrations of $1x10^5$ cells/well were plated (3 wells per treatment group) in each well, in 200 µl of media without test sample. The cells were allowed to reach a 70% - 80% confluent state. Once the desired confluent state was reached, media was removed and cells were dosed with 100 µl of their respective media with and without test samples. In the first series of experiment, screening of cytoprotective fractions has performed. Then the compounds isolated from bio active fractions were subjected to MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide) assay and EC_{50} values were determined thereof.

A series of concentrations (10-100 µg/well) of different fractions were studied by dissolving the sample in phosphate buffer saline (PBS, pH 7.2). The first set of experiment was followed by a series of time course studies to evaluate the effects of isolated compounds over a 24 hr period of time. The plates were submitted to the MTT viability assay at their designated time of 24 hr. All experiments were carried out in triplicates. The cellular viability of the control group was considered to be 100%. Cytoviability of treated cells (with streptozotocin (STZ) alone and with STZ + samples) was expressed as a percentage of control cells.

3.5.5.2 Cell Cultivation

Rat insulinoma RINm5F cell line was obtained from National Centre for Cell Sciences (NCCS, Pune, India). RINm5F cells were selected based on their established use to study cytoprotective effects (Kang et al, 2007). Cells were cultured in open vented $75cm^3$ culture flasks (Tarsons, India) in a vertical laminar flow hood and incubated in a cell incubator at 37 ^0C in an atmosphere of 5% CO_2 and 95% Air. RINm5F cells were provided with a growth media liquid growth media of RPMI-1640 (Hi Media, India) supplemented with 2 mM L-glutamine adjusted to contain 1.5 gm/L sodium bicarbonate, 4.5 gm/L glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate, 90%; fetal bovine serum 10% (Hi Media, India). Media was added with streptomycin (100 µg/ml) and penicillin (100 units/ml). Media was refreshed two times a week (15 ml) and aseptic techniques were employed rigorously to avoid cell culture contamination. RINm5F cell were sub-cultured when 80% confluency was



reached. Subculturing consisted of pouring off the media in one stoke, and addition of 5 ml of 0.25% Trypsin-0.03% EDTA solution to remaining cell layer and incubation for 10 min at 37 ^{0}C. Cells were then washed with a 10 ml aliquot of RPMI-1640 media which causes an inhibitory effect on trypsin. Cells were then transferred to a 14 ml sterile centrifuge tube (Tarsons, India) and centrifuged for 10 min at 1500 rpm. RPMI-1640 and trypsin mixture was decanted and cells were aspirated with fresh media, transferred to new 75cm^3 culture flasks, supplemented with fresh media (10 ml) and incubated in 5% CO_2 atmosphere at 37 ^{0}C.

3.5.5.3 Sample Preparation

All fractions and isolated compound were weighed accurately (100 mg) and dissolved in phosphate buffer saline (PBS, pH 7.2) and the volume was made up to 10 ml with PBS. Samples were dissolved in minimum amount of dimethyl sulfoxide (DMSO, Qualigens, India) wherever required and the final volume was made up with PBS. Incase of formulations, dried water extracts were used.

3.5.5.4 Cell Viability Assay

To assess cell viability, 10 µl of a 10 mg/ml solution of MTT (Hi Media, India) was added directly to RINm5F cells in the 96 well plates. The yellow tetrazolium is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes to generate reducing equivalents such as NADPH and NADH, a process that was allowed to occur over a period of 4 hr. After 4 hr, media containing MTT was removed and 200 µl of DMSO was added, the resulting intracellular purple formazan was solubilized and quantified by spectrophotometric means. MTT absorbance was measured at a wavelength of 540 nm using ELISA reader.

3.5.5.4 Statistical Analysis

Statistical analysis was performed using GraphPad Prism 3.02 software. One way analysis of variance (ANOVA) was applied to the cell viability studies to assess the statistical significance of mean values of varying percent concentration of the samples. The overall p level selected for the statistical analysis was 0.05. When an overall significant difference was observed, Bonferroni comparison was used to perform the multiple comparison procedure.

3.5.6 Glucose Production Assay in Rat Hepatocytes

3.5.6.1 Materials and chemicals

HEPES buffer I (pH 7.4): HEPES (0.01M), NaCl (0.142M) and KCl (0.0067M).

HEPES buffer II (pH 7.6): HEPES (0.01M), NaCl (0.142M) and KCl (0.0067M).

Collagenase type IV (Himedia, India): A solution (0.5% w/v) in HEPES buffer II.

Trypan blue dye solution: A solution (0.2% w/v) in distilled water.

Culture medium : RPMI-1640 (Himedia, India), 10% Fetal bovine serum (Himedia), streptomycin and penicillin 100 µg/ml each.

Trypsin EDTA (ethylene diamine tetra acetic acid) solution: A solution containing 0.25% Trypsin and 0.1% of EDTA.

Sodium pyruvate, sodium lactate, dexamethason (Injection), and insulin (Injection)

Sterilized 24 well plates (Tarsons, India).

3.5.6.2 Instruments

- Orbital shaker
- Vertical laminar air flow chamber
- Chilling centrifuge
- Carbon dioxide incubator.
- Inverted microscope
- Hemocytometer

3.5.6.3 Isolation and primary culture of rat hepatocytes

Hepatocytes were isolated by the collagenase perfusion technique (Sarkar et al, 2006). Rat liver was isolated under aseptic conditions and placed in chilled HEPES buffer I. Liver was minced in small pieces and digested with collagenase IV for about 45 min in orbital shaker at 100 rpm and 37 ^{0}C temperature. Hepatocytes were separated by filtration through sterile muslin cloth and cold centrifugation (4°C, 500 rpm for 5 min three times) and resuspended in 5 ml HEPES buffer I. The viability of the hepatocytes was assessed by trypan blue exclusion method (Kiso et al, 1983). Cells with an intact plasma membrane exclude this dye whereas damaged cells become stained particularly at the nucleus. The stained hepatocytes were observed under neaubaur's chamber under inverted microscope and counted in four different fields to calculate the viability of cells.

The method of Tinstorm and Obrink (1989) with slight modification was adopted for preparation of primary culture of rat hepatocytes. The freshly isolated viable hepatocytes (appx. 2 X 10^6 cells) were suspended in the culture medium (7 ml) and incubated at 37°C in atmosphere of 5% CO_2. A monolayer of hepatocytes was formed upon incubation for 24 hrs. The cells were then trypsinized by incubation with Trypsin-EDTA solution at 37 ^{0}C in 5% CO_2 atmosphere for 10 min. Cell suspension was centrifuged at 1500 rpm for 10 min and supernatant was discarded. Cell pellet (hepatocytes) was suspended in culture medium and assessed for cell viability. These cells were 96-97% viable as confirmed by trypan blue.

3.5.6.4 Experimental design

The glucose production was measured by incubating the culture in glucose free RPMI-1640 medium. The glucose release in to the medium was determined enzymatically with glucose oxidase.

Rat hepatocytes were treated with 500 nM of dexamethasone (DEX) and 0.1mM of 8-(4-chlorophenylthio) adenosine 3', 5'-cyclic monophosphate sodium salt (pCPT-cAMP) in the presence or absence of insulin (10 nM) or test samples for 5 h at 37 ^{0}C. Cells were incubated for an additional 3 h in glucose production buffer (glucose-free Dulbecco's modified essential medium, pH 7.4, containing 20 mM sodium lactate and 2 mM sodium pyruvate without phenol red) with DEX/pCPT-cAMP in the presence or absence of insulin or test samples. At the end of this incubation, 0.5 ml of medium was taken to measure the glucose concentration in the culture medium using a glucose assay kit (Beackon Diagnostics, India). Cells were collected and digested in 0.2N NaOH and an aliquot was taken for protein determination (Lowry et al, 1951). The total protein concentration was measured to correct for cell count.

3.5.6.5 Statistical Analysis

Data are expressed as mean + SEM. Statistical significance was evaluated by One way ANOVA followed by Bonferroni multiple comparisons test; $p < 0.05$ was considered statistically significant.

3.5.7 Insulin Secreting Activity

3.5.7.1 Experimental Design for In vitro study

An insulin secretion effect of fractions and isolated compounds was studied on RINm5F cell line. After trypsinization, RINm5F cells were seeded at a concentration of 2 X 10^5 cells per well, in 24-well plates. The cells were grown overnight and allowed to reach a 7 -80% confluent state. Culture medium was then replaced with 0.5 ml of phosphate saline buffer (PBS, pH 7.2) followed by 40 min incubation in fresh Krebs-Ringer Balanced Buffer containing NaCl (115 mM/L), KCl (4.7 mM/L), $CaCl_2$ (1.28 mM/L), $MgSO_4.7H_2O$ (1.2 mM/L), KH_2PO_4 (1.2 mM/L), $NaHCO_3$ (10 mM/L), and Hepes (25 mM/L), supplemented with glucose (1.1 mM) and bovine serum albumin (0.5%), pH 7.4. The buffer was replaced after 40 min and the cells were incubated (37 ^{0}C, 5% CO_2) with different concentrations of test and the standard compounds for 30 min in the presence of both 16.7 mM and 1.1 mM glucose load. The supernatant was collected and the insulin amount was measured by enzyme-linked immunosorbent assay using commercial rat insulin ELISA Kit (Linco Research, USA). This assay is a sandwich ELISA based, sequentially, 1) capture of insulin molecules from samples to the wells of a microtiter plate coated by pre-titered amount of a monoclonal mouse anti-rat insulin antibodies and the binding of biotinylated polyclonal antibodies to the captured insulin, 2) wash away of unbound materials from samples, 3) binding of horseradish peroxidase to the immobilized biotinylated antibodies, 4) wash away of free enzyme conjugates, and 5) quantification of immobilized antibody-enzyme conjugates by monitoring horseradish peroxidase activities in the presence of the substrate 3,3',5,5'-tetramethylbenzidine. The enzyme activity is measured spectrophotometrically by the increased absorbency at 450 nm, corrected from the absorbency at 590nm, after acidification of formed products. The total insulin content was calculated in ng/10^5 cells per/30 min.

3.5.7.2 Cell Cultivation

Rat insulinoma RINm5F cell line was obtained from National Centre for cell Sciences (NCCS, Pune, India) and cultivated as described in section *3.5.5.2.*

3.5.7.3 Sample Preparation

Stock solution of 1 mg/ml of each sample was prepared in in phosphate buffer saline (pH 7.2). The treatment groups for fraction study were consisted 10 µg/ml. Isolated compounds were studied at the concentration levels of 1, 5 and 10 µg/ml.

3.5.7.4 Animal studies

Wistar rats of either sex (180 ± 35 gm) obtained from Zydus Research Centre, Ahmedabad, Gujarat, were used at 21–24 weeks of age. The rats were housed in an air conditioned room (25 ± 5 ^{0}C, 60-65 % relative humidity) with a lighting schedule of 12 hr light and 12 hr darkness. Animals had free access to a standard pellet diet tap water. The *in vivo* insulin secreting and glucose lowering properties of swertisin, TCA and gymnemagenin alone and in their combination were evaluated in rats. Two days prior to the study, the animals were randomized and divided into 10 groups (n=5). On the day of experiment, food was withdrawn from all the cages, water was given *ad libitum* and animals were kept for overnight fasting. An intraperitoneal glucose tolerance test was used to study the *in vivo* effect. In rats fasted overnight, vehicle (normal saline)/test/standard compounds were administered intraperitoneally (i.p.) on a body weight basis 90 min before the i.p. injection of glucose (3 gm/kg body weight). The control group was given only saline i.p. soon after the administration of glucose (0 min), blood was collected via retro-orbital route, under light ether anesthesia and the samples were marked, the subsequent blood collection was done at 60 min. Blood samples were centrifuged and the separated serum was subjected to glucose estimation. Serum for insulin estimation was stored at -70 ^{0}C until used for the insulin estimation. The glucose estimation was conducted with GOD/POD method (Span Diagnostics Ltd., Sachin, India). Furthermore, the insulin estimation was conducted using rat insulin ELISA kit. Changes in the blood insulin and glucose levels with compounds alone and in combination thereof were studied.

3.5.7.5 Statistical Analysis

Statistical analysis was performed using GraphPad Prism 3.02 software. One way analysis of variance (ANOVA) was applied to assess the statistical significance of mean values of varying concentration of the samples. Groups were considered to be significantly different if

$p<0.05$. When a significant difference was observed for ANOVA the differences between all pairs were tested using the Bonferroni multiple comparisons test.

3.5.8 Characterization of Bioactive Molecules

Isolated compounds which were found to have promising activity on either of the studied models were subjected to various spectroscopic studies for their identification.