CHAPTER - 3
EXPERIMENTAL

3.1 Development of quality control parameters

All solvents, chemicals and reagents used were of analytical grade.

3.1.1 Collection and authentication of plant materials

Leaves of *Clerodendrum phlomidis* were collected from out-skirts of Trichy city, Tamilnadu, India, and leaves of *Nymphaea stellata* were collected from suburb ponds of Vadodara city, Gujarat, India. Both the plant materials were authenticated by Prof. Daniel Mammen, Botany Department, The M.S. University of Baroda, Vadodara. Voucher specimen (Pharmacy/HDT/CP/08-09/MKM/15 and Pharmacy/HDT/NS/08-09/MKM/16) of both the plant were deposited in the herbarium of medicinal plants, Pharmacy Department, The M.S. University of Baroda, Vadodara, Gujarat, India.

3.1.2 Determination of foreign matter

Accurately weighed 250 g of the leaf material was spread in to thin layer and the foreign matter was sorted into groups by visual inspection, using a magnifying lens. The remaining sample was sifted through a number 250 sieve; dust was regarded as mineral admixture. The content of each group was calculated in grams per 100 g of air-dried sample (WHO, 2005).

3.1.3 Morphology, anatomy, histology and powder microscopy

The leaf specimens were cut and fixed in FAA [Formalin (5 ml) + Acetic acid (5 ml) + 70 % Ethyl alcohol (90 ml)]. After 24 h of fixing, the specimens were dehydrated with graded series of tertiary butyl alcohol. Infiltration of the specimens was carried by gradual addition of paraffin wax (melting point 58-60 °C) until TBA solution attained supersaturation. Then the specimens were cast into paraffin blocks.

3.1.3.1 Sectioning

The paraffin embedded specimens were sectioned with the help of Rotary Microtome with thickness of 10 – 12 μm. Sections were stained with toluidine

blue after dewaxing. Wherever necessary sections were stained with safranin, fast-green and iodine (for starch). For studying the stomatal morphology, venation pattern and trichome distribution, paradermal sections (sections taken parallel to the surface of leaf) were taken after clearing with 5 % sodium hydroxide or epidermal peeling by partial maceration employing Jeffrey's maceration fluid. Glycerin mounted temporary preparations were prepared for macerated/cleared materials (Solerder, 1899; Johanson, 1940; Metcalfe and Chalk, 1979).

3.1.3.2 Photomicrographs

Photographs of different magnification were taken with Nikon Labphot 2 microscopic unit. For normal observations bright field was used. For the study of crystals, starch grains, and lignified cells, polarized light was employed. Magnifications of the figures were indicated by the scale-bars (Sass, 1940; O'Brien et al., 1964).

3.1.4 Determination of ash values

3.1.4.1 Determination of total ash

About 3 g of the coarse leaf powder was accurately weighed in a silica crucible which was previously ignited and weighed. The powdered drug was spread as a fine even layer at the bottom of the crucible. The crucible was incinerated at a temperature not exceeding 450 °C until free from carbon. The crucible was cooled and weighed and the procedure was repeated until a constant weight was obtained. The content of total ash was calculated in mg per g of air-dried material (WHO, 2005; Anonymous, 1996).

3.1.4.2 Determination of acid-insoluble ash

The ash obtained as described in total ash was boiled with 25 ml of 2 M hydrochloric acid for five minutes. The insoluble ash was collected on an ashless filter paper and washed with hot water. The insoluble ash was transferred into pre-weighed silica crucible, ignited, cooled, weighed and the procedure was

repeated until a constant weight was obtained. The content of acid-insoluble ash was calculated in mg per g of air-dried material (WHO, 2005; Anonymous, 1996).

3.1.4.3 Determination of water-soluble ash

The ash obtained as described in total ash was boiled in 25 ml of water for five minutes. The insoluble matter was collected in a crucible and washed with hot water. The insoluble ash was transferred into pre-weighed silica crucible, ignited for 15 minutes at a temperature not exceeding 450 °C, cooled, weighed and the procedure was repeated until a constant weight was obtained. The weight of the insoluble matter was subtracted from the weight of the total ash. The difference in weight was considered as water soluble ash. The water-soluble ash was calculated in mg per g of air-dried material (WHO, 2005; Anonymous, 1996).

3.1.4.4 Determination of sulphated ash

Silica crucible was heated to redness for about 10 minutes, allowed to cool in desiccator and weighed. About 1 g of coarse leaf powder was accurately weighed and taken in the crucible. The crucible was ignited gently first until the drug was thoroughly charred. The crucible was cooled and the residue was moistened with 1 ml of sulphuric acid, heated gently until white fumes no longer evolved and then ignited at 800 °C until all black particles disappeared. The crucible was allowed to cool, few drops of sulphuric acid were added and again heated. The ignition was carried out as before, allowed to cool and weighed. The operation was repeated until two successive weights did not differ by more than 0.5 mg. The percentage of sulphated ash was calculated in mg per g of air-dried material (WHO, 2005; Anonymous, 1996).

3.1.5 Determination of extractive values

3.1.5.1 Determination of water-soluble extractive

5 g of air-dried coarse leaf powder was mixed with 100 ml of chloroform water and kept in a closed flask for 24 h, shaking frequently during the first 6 h and then

allowed to stand for 18 h. Thereafter, it was filtered rapidly, taking precautions against loss of the solvent. About 25 ml of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish, dried at 105 °C and weighed. The percentage of water-soluble extractive was calculated in mg per g of air-dried material (WHO, 2005; Anonymous, 1996).

3.1.5.2 Determination of ethanol-soluble extractive

5 g of air-dried coarse leaf powder was mixed with 100 ml of ethanol and kept in a closed flask for 24 h, shaking frequently during the first 6 h and then allowed to stand for 18 h. Thereafter, it was filtered rapidly, taking precautions against loss of the solvent. About 25 ml of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish, dried at 105 °C and weighed. The percentage of ethanol-soluble extractive was calculated in mg per g of air-dried material (WHO, 2005; Anonymous, 1996).

3.1.5.2 Determination of ether-soluble extractive

5 g of air-dried coarse leaf powder was mixed with 100 ml of ether and kept in a closed flask for 24 h, shaking frequently during the first 6 h and then allowed to stand for 18 h. Thereafter, it was filtered rapidly, taking precautions against loss of the solvent. About 25 ml of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish, dried at 105 °C and weighed. The percentage of ether-soluble extractive was calculated in mg per g of air-dried material (WHO, 2005; Anonymous, 1996).

3.1.6 Determination of loss on drying (gravimetric determination)

Accurately weighed 3 g of the air-dried leaf powder was placed in a previously dried and tared flat weighing bottle. The bottle was dried in an oven at 100-105 °C until two consecutive constant weighings. Loss on drying was calculated in mg per g of air-dried material (WHO, 2005).

3.1.7 Determination of bitterness value

3.1.7.1 Stock and diluted quinine hydrochloride solutions

Accurately weighed 100 mg of quinine hydrochloride was dissolved in sufficient safe drinking-water to produce 100 ml. Further 5 ml of this solution was diluted to 500 ml with safe drinking water to produce a stock solution of 0.01 mg/ml of quinine hydrochloride. Nine serial dilutions of 10 ml each were made with safe drinking water to produce the concentration 0.042, 0.044, 0.046, 0.048, 0.052, 0.050, 0.054, 0.056 and 0.058 mg.

3.1.7.2 Stock and diluted solutions of the plant material

Accurately weighed 10 mg of the leaf powder was dissolved in sufficient safe drinking water to produce 100 ml. Serial dilutions of 10 ml each were made with safe drinking water by pipetting 1 to 10 ml of the stock solution. After rinsing the mouth with safe drinking water, 10 ml of the most dilute solution was tasted by swirling it in the mouth mainly near the base of the tongue for 30 seconds. If the bitter sensation was no longer felt in the mouth after 30 seconds, the next highest concentration was tested. The mouth was rinsed thoroughly with safe drinking water after every test and at least 10 minutes was kept as interval between two testing. The lowest concentration at which the standard/sample provoked a bitter sensation after 30 seconds was considered as the threshold bitter concentration. All solutions and the safe drinking water for mouth washing were between 20-25 °C. Bitterness value was calculated in units per g using the following formula;

$$\frac{2000 \times c}{a \times b}$$

where,

a = the concentration of the sample stock solution (mg/ml),

b = the volume of sample (in ml) in the tube with the threshold bitter concentration,

c = the quantity of quinine hydrochloride (in mg) in the tube with the threshold bitter concentration (WHO, 2005).

3.1.8 Determination of arsenic and heavy metals

The coarsely powdered leaf samples were subjected to determination of arsenic, mercury, lead, cadmium, manganese, zinc, calcium, magnesium, nickel, copper, sodium, iron and potassium as per WHO guidelines (WHO, 2005).

3.1.9 Determination of microbial content

The coarsely powdered leaf samples were subjected to determination of microorganisms including total viable, bacterial and fungal count as per WHO guidelines (WHO, 2005).

3.2 Successive extraction

The air-dried leaf materials were grounded to coarse powder and extracted successively in a soxhlet apparatus until exhaustion with solvents of increasing polarity viz., petroleum ether (60-80 °C), benzene, diethyl ether, chloroform, ethyl acetate, acetone, methanol and water. The extracts were concentrated in a rotary evaporator and dried in a dessicator. The unsaponified matter of petroleum ether extract was prepared. The petroleum ether extract (2.5 g) was added with 1 g of KOH in methanol (20 ml) and refluxed for an hour. The extract was transferred to a separating funnel containing 50 ml of water and shaked with ether 3X50 ml. The ether layer was washed with 2X20 ml followed by 3 %w/v of KOH wash. Finally the ether layer was washed with water till it was no longer alkaline to phenolphthalein. The ethereal layer was evaporated to dryness to obtain the unsaponified matter (Khandelwal, 2002). The percentage yield, appearance and odour were noted for all the extracts.

3.2.1 Preliminary qualitative phytochemical screening

All the extracts were studied for the presence and absence of secondary metabolites like, alkaloids, glycosides, saponins, phytosterols, phenolics,

terpenoids, flavonoids, coumarins and tannins by qualitative chemical tests (Rosenthaler, 1930; Peach and Tracey, 1955; Middelton, 1956; Shah and Quadry, 1980; Wagner et al., 1984; Kokate, 1991; Wallis, 1960).

3.2.1.1 Detection of alkaloids

About 50 mg of solvent free extract was stirred with little quantity of dilute hydrochloric acid and filtered. The filtrate was tested carefully with various alkaloid reagents as follows.

Mayer's test - To a few ml of filtrate, two drops of Mayer's reagent were added along the sides of the test tube. Formation of white or creamy precipitate was considered as positive.

Wagner's test - To a few ml of the filtrate, few drops of Wagner's reagent were added along the sides of the test tube. Formation of reddish brown precipitate was considered as positive.

Hager's test - To a few ml of filtrate 1 or 2 ml of Hager's reagent were added. A prominent yellow precipitate indicated positive.

Dragendroff's test - To a few ml of filtrate, 1 or 2 ml of Dragendroff's reagent were added. A prominent reddish brown precipitate indicated positive.

3.2.1.2 Detection of carbohydrates

About 100 mg of the extract was dissolved in 5 ml of distilled water and filtered. The filtrate was subjected to the following tests.

Molish's test - To 2 ml of filtrate, two drops of alcoholic solution of α-naphthol was added. The mixture was shaken well and 1 ml of concentrated sulphuric acid was added slowly along the sides of the test tube, the test tubes were cooled and allowed to stand. A violet ring at the junction was considered positive for the presence of carbohydrates.

Fehling's test - 1 ml of filtrate was boiled on a water bath with 1 ml of each Fehling's solution A and B. Formation of red precipitate indicated the presence of sugar.

Barfoed's test - To 1 ml of the filtrate, 1 ml of Barfoed's reagent was added and heated on a boiling water bath for 2 minutes. Red precipitate indicated the presence of sugar.

Benedict's test - To 0.5 ml of filtrate 0.5 ml of Benedict's reagent was added. The mixture was heated on a boiling water bath for 2 minutes. A characteristic colored precipitate indicated the presence of sugar.

3.2.1.3 Detection of glycosides

For detection of glycosides, about 50 mg of the extract was hydrolyzed with concentrated hydrochloric acid for 2 h on a water bath, filtered and the hydrolysate was subjected to the following tests.

Borntrager's test - To 2 ml of filtrate hydrolysate, 3 ml of chloroform was added and shaken, chloroform layer was separated and 10% ammonia solution was added to it. Formation of pink color indicated the presence of glycosides.

Legal's test - About 50 mg of the extract was dissolved in pyridine. Sodium nitroprusside solution was added and the solution was made alkaline using 10% sodium hydroxide solution. Presence of glycoside is indicated by a characteristic pink color.

3.2.1.4 Detection of saponins

Foam or froth test - A small quantity of extract was diluted with distilled water to 20 ml. The suspension was shaken in a graduated cylinder for 15 minutes. A two centimeter layer of stable foam or froth for 10 minutes indicated the presence of saponins.

3.2.1.5 Detection of proteins and amino acids

About 100 mg of extract was dissolved in 10 ml of distilled water and filtered through Whatmann no. 1 filter paper and the filtrate was subjected to tests for proteins and amino acids.

Millon's test - To 2 ml of filtrate, few drops of Millon's reagent were added. A white precipitate indicated the presence of proteins.

Biuret test - An aliquot of 2 ml of filtrate was treated with one drop of 2 % copper sulphate solution. Then 1 ml of 95 % ethanol was added, followed by excess of potassium hydroxide solution. Pink color in the ethanolic layer indicated the presence of proteins.

Ninhydrin test - About 2 drops of ninhydrin solution were added to two ml of aqueous filtrate. A characteristic purple color indicated the presence of amino acids.

3.2.1.6 Detection of steroids

Libermann - Burchard's test - The extract was dissolved in acetic anhydride, boiled, cooled and then 1 ml of concentrated sulphuric acid was added along the side of test tube. Red, pink or violet color at the junction of the liquids indicated the presence of steroids / triterpenoids and their glycosides.

3.2.1.7 Detection of phenolic compounds and tannins

Ferric chloride test - About 50 mg of the extract was dissolved in distilled water and few drops of neutral 5 % ferric chloride solution was added. Formation of blue, green and violet color indicated the presence of phenolic compounds.

Gelatin test - A little quantity of the extract was dissolved in distilled water and 2 ml of 1 % solution of gelatin containing 10 % sodium chloride was added to it. Development of white precipitate indicated the presence of phenolic compounds.

Lead acetate test - A small quantity of the extract was dissolved in distilled water and 3 ml of 10 % lead acetate solution was added. A bulky white precipitate indicated the presence of phenolic compounds.

Alkaline reagents - An aqueous solution of the extract was treated with 10 % ammonium hydroxide solution. Yellow fluorescence indicated the presence of flavonoids.

Shinoda test or Magnesium hydrochloric acid reduction – A little quantity of the extract was dissolved in alcohol and few fragments of magnesium turnings and concentrated hydrochloric acid (drop wise) were added. Appearance of pink or crimson red color indicated the presence of flavonoids.

To 50 mg of the extract, ferric chloride solution was added. Dark green solution indicated the presence of phenolic compounds.

3.2.2 Thin layer chromatographic study

Thin layer chromatography (TLC) is an important analytical tool in separation, identification and estimation of different classes of natural products. Comparative TLC (co-TLC) with marker compounds can be used for identification of chemical constituents and to standardiże the herbal raw materials.

3.2.2.1 High performance thin layer chromatography

A Camag TLC system equipped with Camag Linomat V, an automatic TLC sample spotter, Camag glass twin trough chamber (20 X 10 cm) were used for the analysis. Chromatography was performed using pre-activated (60 °C for 5 min) silica gel $60F_{254}$ TLC plates (20 X 10 cm; layer thickness 250 μm) (Merck, Darmstadt, Germany). Samples and standards were applied on the plate as 8 mm wide bands with an automatic TLC sampler under a flow of N_2 gas, 10 mm from the bottom and 10 mm from the side and the space between two spots were 15 mm of the plate. The linear ascending development was carried out in a Camag twin trough chamber saturated with 20 ml mobile phase for 20 min at room temperature (25 ± 2 °C and 40 % relative humidity). The plates were developed up to 8 cm under chamber saturation conditions. Subsequent to the development, TLC plates were dried in current air with the help of a hair dryer. The post chromatographic derivatization was carried out with specific detecting agents. Evaluations of the plates were performed with Camag scanner 3 (win CATS 4.0 integration software). Densitometric scanning was performed in the absorption-reflection mode, using a

slit width of 6 X 0.45 mm, data resolution 100 μm step and scanning speed 20 mm/s with a computerized Camag TLC scanner.

3.2.2.2 Identification and quantification of chemical constituents

Based on the preliminary qualitative phytochemical screening, co-TLC studies of extracts were performed with known standards. Accurately weighed extracts were dissolved in respective solvents to produce a known concentration. The extracts were separated in suitable mobile phase along with standards. The identified chemical constituents were quantified from the calibration curve of peak area versus concentration of the standards. All quantification was performed by external standard method.

3.2.2.3 Method development and validation

Specificity of the method was determined by analyzing standard and the unknown sample. The spot sample spot was confirmed by comparing the R_f multi-wavelength scanning and spectral overlay of the standard spot. The peak purity was assessed by comparing the spectra at three different levels, i.e., peak start, peak apex and peak end positions of the spot. The method was validated for precision, accuracy and repeatability (ICH, 1996/2005).

Instrumental precision was checked by repeated scanning of the same standard spot at different concentrations and expressed as coefficient of variance (% RSD). Method precision was studied by analyzing standard at lower and higher concentration under the same analytical procedure and laboratory condition on the same day (intra-day precision) and on different day (inter-day precision), the results were expressed as % RSD.

Accuracy of the method was tested by performing the recovery studies of pre-analyzed sample with standard at three levels 80, 100 and 120 % and % recovery was calculated.

3.2.2.4 Identification and quantification of adrenaline in *C.phlomidis*

Preparation of extract: Accurately weighed 2.5 g of the coarse powder of *C. phlomidis* leaves were extracted separately with 1 % glacial acetic acid in water (4 X 50 ml) under reflux (30 min each time). The combined extracts were filtered, concentrated and transferred to a 25 ml volumetric flask and the volume was made up with same solvent.

Preparation of standard solution: A stock solution (100 µg/ml) was prepared by dissolving 5 mg of accurately weighed adrenaline (Himedia Laboratories Pvt. Ltd., Mumbai, India) in 1 % glacial acetic acid in water and making up the volume of the solution to 50 ml. Further dilutions were prepared by transferring 1.0–5.0 ml aliquots of the stock solution (100 µg/ml) to 10 ml volumetric flasks. The obtained standard solutions contained 10, 20, 30, 40 and 50 µg ml^{-1} adrenaline, respectively. All solutions were protected from light and stored in refrigerator at 2 to 4 °C.

Calibration curve for adrenaline: Ten microlitre of the standard solutions (100, 200, 300, 400 and 500 ng $spot^{-1}$) were applied on a TLC plate for preparing calibration curve of peak area versus concentration.

Quantification of adrenaline in test sample: Ten microlitre of sample solution was applied in triplicate on a TLC plate, developed and scanned. Peak areas were recorded and the amount of adrenaline was calculated using the calibration curve.

Specificity: Specificity of the method was determined by analyzing standard and the unknown sample. The spot for adrenaline in the sample was confirmed by comparing the R_f and spectra of the spot with that of the standard. The peak purity of adrenaline was assessed by comparing the spectra at three different levels, i.e., peak start, peak apex and peak end positions of the spot.

Method validation: The method was validated for precision, accuracy and repeatability (ICH, 1996/2005). Instrumental precision was checked by repeated

scanning of the same standard spot 100 and 500 ng three times and was expressed as coefficient of variance (% RSD). Method precision was studied by analyzing the standards 100 and 500 ng $spot^{-1}$ under the same analytical procedure and laboratory condition on the same day and on different day, the results were expressed as % RSD. Accuracy of the method was tested by performing the recovery studies of pre-analyzed sample with standard at three levels (80, 100 and 120 %), and % recovery was calculated.

3.2.2.5 Identification and quantification of l-dopa in *C. phlomidis*

Preparation of sample and standard solution: Accurately weighed 5 g of the coarse powder of *C. phlomidis* leaves were extracted with water:methanol (7:3) (4 X 50 ml) under reflux (30 min each time) on a water bath. The combined extracts were filtered, concentrated and transferred to a 50 ml volumetric flask and the volume was made up with same mixture of solvents. A stock solution of l-dopa (Himedia Laboratories Pvt. Ltd., Mumbai, India) (1 mg/ml) was prepared in water:methanol (7:3). Working solutions were prepared by appropriate dilution of the stock solution with the same mixture of solvents. All solutions were protected from light and stored in refrigerator at 2 to 4 °C.

Calibration curve for l-dopa: Standard l-dopa solution in the range of 100 to 500 ng $spot^{-1}$ was applied on TLC plate for preparation of calibration curve of peak area versus concentration.

Quantification of l-dopa in test sample: Quantification was performed by external standard method, using pure l-dopa as standard (Mennickent et al., 2007). 5 µl of the sample solution was applied in triplicate on the TLC plate and developed with the mobile phase acetone: chloroform: n-butanol: glacial acetic acid: water (60:40:40:40:35, v/v/v/v/v). The post chromatographic derivatization was carried out with ninhydrin 0.5 % in ethanol placed in a dipping chamber (CAMAG) followed by heating for 5-10 min under observation (Wagner and Bladt, 1996).

Densitometric scanning was performed in absorption-reflection mode at 497 nm. Peak areas were recorded and the amount of l-dopa was calculated using the calibration curve.

3.2.2.6 Identification and quantification of lupeol in *C. phlomidis* and *N. stellata*

Preparation of sample and standard solutions: Accurately weighed (5 g for *C. phlomidis* and 7.5 g for *N. stellata*) coarse leaf powder were extracted with methanol (4 X 50 ml) under reflux (30 min each time) on a water bath. The combined extracts were filtered, concentrated and transferred to a 50 ml volumetric flask and the volume was made up with methanol. A stock solution of lupeol (Sigma Chemicals, Bangalore, India) (100 µg/ml) was prepared in methanol. Working solutions were prepared by appropriate dilution of the stock solution with the same solvent.

Calibration curve for lupeol: Standard lupeol solution in the range of 50 to 250 and 100 to 500 ng $spot^{-1}$ for *C. phlomidis* and *N. stellata*, respectively were applied on TLC plate for preparation of calibration curve of peak area versus concentration.

Quantification of lupeol in test sample: Quantification was performed by external standard method, using pure lupeol as standard (Padashetty and Mishra, 2007). 5 µl of the sample solution was applied in triplicate on the TLC plate and developed with the mobile phase toluene: chloroform: ethyl acetate: glacial acetic acid (10:2:1:0.03, v/v/v/v).

The post chromatographic derivatization was carried out with freshly prepared antimony trichloride reagent (20 % solution of antimony III chloride in chloroform) placed in a dipping chamber (CAMAG) followed by heating in an oven at 110 °C for 5-6 min (Wagner and Bladt, 1996). Densitometric scanning was performed in fluorescence mode at 366 nm. Peak areas were recorded and the amount of lupeol was calculated using the calibration curve.

3.2.2.7 Identification and quantification of β-sitosterol in *C. phlomidis* and *N. stellata*

Preparation of sample and standard solutions: Accurately weighed 5 g of the coarse powder of leaves were extracted with methanol (4 X 50 ml) under reflux (30 min each time) on a water bath. The combined extracts were filtered, concentrated and transferred to a 50 ml volumetric flask and the volume was made up with methanol. A stock solution of β-sitosterol (Himedia Laboratories Pvt. Ltd., Mumbai, India) (100 µg/ml) was prepared in methanol. Working solutions were prepared by appropriate dilution of the stock solution with the same solvent.

Calibration curve for β-sitosterol: Standard β-sitosterol solution in the range of 100 to 600 ng $spot^{-1}$ and 100 to 500 ng $spot^{-1}$ for *C. phlomidis* and *N. stellata* respectively were applied on TLC plate for preparation of calibration curve of peak area versus concentration.

Quantification of β-sitosterol in test sample: Quantification was performed by external standard method, using pure β-sitosterol as standard (Murthy and Mishra, 2009). 5 µl of the sample solution was applied in triplicate on the TLC plate and developed with mobile phase toluene: chloroform: methanol (4:4:1, v/v/v). The post chromatographic derivatization was carried out with anisaldehyde-sulphuric acid placed in a dipping chamber (CAMAG) followed by heating in an oven at 100 °C for 5-10 min (Wagner and Bladt, 1996). Densitometric scanning was performed in absorption-reflection mode at 527 nm. Peak areas were recorded and the amount of β-sitosterol was calculated using the calibration curve.

3.2.2.8 Identification and quantification of β-carotene in *C. phlomidis* and *N. stellata*

Preparation of sample and standard solutions: Accurately weighed 5 g of the coarse powder of leaves were extracted with methanol (4 X 50 ml) under reflux (30 min each time) on a water bath. The combined extracts were filtered,

concentrated and transferred to a 50 ml volumetric flask and the volume was made up with methanol. A stock solution of β-carotene (Himedia Laboratories Pvt. Ltd., Mumbai, India) (100 μg/ml) was prepared in methanol. Working solutions were prepared by appropriate dilution of the stock solution with the same solvent.

Calibration curve for β-carotene: Standard β-carotene solution in the range of 100 to 500 ng $spot^{-1}$ was applied on TLC plate for preparation of calibration curve of peak area versus concentration.

Quantification of β-carotene in test sample: Quantification was performed by external standard method, using pure β-carotene as standard. Sample solution was applied in triplicate on the TLC plate and developed with mobile phase n-hexane: benzene (9:1, v/v). Densitometric scanning was performed in absorption-reflection mode at 445 nm. Peak areas were recorded and the amount of β-carotene was calculated using the calibration curve.

3.2.2.9 Identification and quantification of oleanolic acid in *N. stellata*

Preparation of sample and standard solutions: Accurately weighed 10 g of the coarse powder of leaves were extracted with methanol (4 X 50 ml) under reflux (30 min each time) on a water bath. The combined extracts were filtered, concentrated and transferred to a 50 ml volumetric flask and the volume was made up with methanol. A stock solution of oleanolic acid (Himedia Laboratories Pvt. Ltd., Mumbai, India) (100 μg/ml) was prepared in methanol. Working solutions were prepared by appropriate dilution of the stock solution with the same solvent.

Calibration curve for oleanolic acid: Standard solution of oleanolic acid in the range of 100 to 500 ng $spot^{-1}$ was applied on TLC plate for preparation of calibration curve of peak area versus concentration.

Quantification of oleanolic acid in test sample: Quantification was performed by external standard method, using pure oleanolic acid as standard. Sample solution

was applied in triplicate on the TLC plate and developed with mobile phase toluene: ethyl acetate: glacial acetic acid (7:3:0.1, v/v/v). The post chromatographic derivatization was carried out with anisaldehyde-sulphuric acid placed in a dipping chamber (CAMAG) followed by heating in an oven at 100 °C for 5-10 min (Wagner and Bladt, 1996). Densitometric scanning was performed in absorption-reflection mode at 540 nm. Peak areas were recorded and the amount of oleanolic acid was calculated using the calibration curve.

3.2.2.10 Identification and quantification of betunilic acid in *N. stellata*

Preparation of sample and standard solutions: Accurately weighed 2.5 g of the coarse powder of leaves were extracted with methanol (4 X 50 ml) under reflux (30 min each time) on a water bath. The combined extracts were filtered, concentrated and transferred to a 50 ml volumetric flask and the volume was made up with methanol. A stock solution of betunilic acid (Himedia Laboratories Pvt. Ltd., Mumbai, India) (100 µg/ml) was prepared in methanol. Working solutions were prepared by appropriate dilution of the stock solution with the same solvent.

Calibration curve for betunilic acid: Standard solution of betunilic acid in the range of 100 to 500 ng $spot^{-1}$ was applied on TLC plate for preparation of calibration curve of peak area versus concentration.

Quantification of betulinic acid in test sample: Quantification was performed by external standard method, using pure betunilic acid as standard. Sample solution was applied in triplicate on the TLC plate and developed with mobile phase toluene: ethyl acetate: glacial acetic acid (7:3:0.03, v/v/v). The post chromatographic derivatization was carried out with anisaldehyde-sulphuric acid placed in a dipping chamber (CAMAG) followed by heating in an oven at 100 °C for 5-10 min (Wagner and Bladt, 1996). Densitometric scanning was performed in absorption-reflection mode at 527 nm. Peak areas were recorded and the amount of betulinic acid was calculated using the calibration curve.

3.2.2.11 Identification and quantification gallic acid in *N. stellata*

Preparation of sample and standard solutions: Accurately weighed 7.5 g of the coarse powder of leaves were extracted with methanol (4 X 50 ml) under reflux (30 min each time) on a water bath. The combined extracts were filtered, concentrated and transferred to a 50 ml volumetric flask and the volume was made up with methanol. A stock solution of gallic acid (Himedia Laboratories Pvt. Ltd., Mumbai, India) (100µg/ml) was prepared in methanol. Working solutions were prepared by appropriate dilution of the stock solution with the same solvent.

Calibration curve for gallic acid: Standard solution of gallic acid in the range of 500 to 900 ng $spot^{-1}$ was applied on TLC plate for preparation of calibration curve of peak area versus concentration.

Quantification of gallic acid in test sample: Quantification was performed by external standard method, using pure gallic acid as standard. Sample solution was applied in triplicate on the TLC plate and developed with mobile phase toluene: ethyl acetate: methanol: formic acid (6:3:1:0.5, v/v/v/v). Densitometric scanning was performed in absorption-reflection mode at 282 nm. Peak areas were recorded and the amount of gallic acid was calculated using the calibration curve.

3.2.2.12 Amino acid identification in *C. phlomidis*

Accurately weighed 2.5 g of coarse leaf powder was extracted separately with 1% glacial acetic acid in water (4 X 50 ml) under reflux (30 min each time). The combined extracts were filtered and concentrated. TLC for the extract was performed with the mobile phase n-butanol: glacial acetic acid: water (4:1:1, v/v/v) along with generally recognized 20 'protein' amino acids (Glycine, alanine, serine, cysteine, threonine, valine, leucine, isoleucine, methinonie, aspartic acid, asparagine, glutamic acid, glutamine, arginine, lysine, proline, phaenylalanine, tyrosine, trytophan and histidine). The post chromatographic derivatization was

carried out with ninhydrin 0.5 % in ethanol followed by heating for 5-10 min under observation (Wagner and Bladt, 1996).

3.2.2.13 Amino compounds in *C. phlomidis*

3.2.2.13.1 Preparation of crude polyamine extract (CPECP)

Accurately weighed 100 g of coarse leaf powder was stirred in 400 ml of 5 % trichloroacetic acid for 1 h at room temperature. After centrifugation to separate the extract, the residue was re-extracted with 200 ml of 5 % trichloroacetic acid. The trichloroacetic acid in the combined extracts was removed by shaking the solutions five times with several volumes of ether. After removing residual ether under reduced pressure, the solution was adjusted with sodium hydroxide to pH 13 to free the polyamines. The alkaline solution was then extracted five times with 50 ml of n-butanol. The n-butanol solution was concentrated under reduced pressure and dried. The resulting solids were dissolved in water (50 ml). The solution was neutralized with hydrochloric acid and then centrifuged to remove insoluble materials. The clear supernatant was collected, dried and designated as crude polyamine extract (CPECP) (Wang, 1972).

3.2.2.13.2 TLC of crude polyamine extract

The extract was applied in varying concentration on the TLC plate and developed with the mobile phase n-butanol: methyl ethyl ketone: ammonium hydroxide: water (5:3:1:1, v/v/v/v) along with standard spermidine (Himedia Laboratories Pvt. Ltd., Mumbai, India). The post chromatographic derivatization was carried out with ninhydrin 0.5 % in ethanol followed by heating for 5-10 min under observation (Harborne, 1998).

3.2.2.13.3 Preparation of crude alkaloidal fraction (CAFCP)

Dried and ground leaves of *C. phlomidis* (750 g) were extracted with methanol for 48 h in soxhlet apparatus. The extract was evaporated under reduced pressure and the residue was dissolved in 0.2 N Hcl; the filtered solution was basified with

aqueous NaOH, extracted with $CHCl_3$ and evaporated; the residue was distributed between $CHCl_3$ and aqueous citric acid pH 2.2. Basification of the aqueous solution and extraction with $CHCl_3$ yielded the crude alkaloidal fraction (CAFCP) (Lumbu and Hootele, 1993).

3.3 DNA sequence for internal transcribed spacer (ITS) region of *C. phlomidis*

Total DNA was extracted from silica gel dried leaf tissue (Chase and Hills, 1991) using the protocol of Doyle and Doyle (1990). The 5.8S nr DNA and flanking ITS regions were amplified using the polymerase chain reaction (PCR) with primers ITS 5 and ITS 4 (White et al., 1990). The amplification conditions were those described by Baldwin (1992), 97 °C for 1 min, 48 °C for 1 min and 72 °C for 45 seconds increasing by 4 seconds/cycle over 40 cycles. Single-stranded DNA was produced by including 10 µl of double-stranded DNA in a second 100 µL reaction mixture containing only one of the two primers (Kaltenboeck et al., 1992). Twenty-five cycles of PCR were required for the single-stranded amplifications (Steane et al., 1999). Single-stranded PCR products were sequenced with TAQuence (Amersham, Arlington Heights, Illinois), using ^{32}P dATP, in accordance with the recommendations of the manufacturer. The sequencing reactions were primed using the ITS 5 and ITS 4 primers externally, and ITS 3 and ITS 2 internally. Both strands of DNA were sequenced. To overcome band compressions in the gels, reactions containing 7-deazadGTP were run in addition to reactions containing dGTP. Sequences were aligned using the DNA sequence alignment program Clustal V (Higgins, 1991) followed by visual inspection (GenBank database). The new sequence were also aligned by eye to the aligned ITS sequences from Steane et al., (1999) and Steane et al. (1997). Alignment gaps were scored as separate binary characters following the 'simple gap coding' method of Simmons and Ochoterena (2000).

Parsimony analyses were carried out using PAUP* ver. 4.0b10 (Swofford, 2002) using heuristic searches with 100 replicates, each with ten random order entry

starting trees, TBR branch swapping, and saving multiple trees at each step (MULTREES on). All data sets were bootstrapped 250 times following DeBry and Olmstead (2000) using 10 random order entry starting trees per replicate, TBR branch swapping and MULTREES off. The consensus analysis was carried out by combining the sets of trees that had been obtained in previous analyses and calculating a semistrict consensus.

3.4 Isolation and characterization of chemical constituents

3.4.1 Preparation of unsaponified petroleum ether fraction of methanol extract (UPFMCP and UPFMNS)

Coarsely powdered leaves of *C. phlomidis* and *N. stellata* were extracted with methanol in soxhlet apparatus until exhaustion; the extract was concentrated *in vacuo* by rotary evaporator and dried in desiccator. The methanol extract was fractioned with petroleum ether. The dried petroleum ether fraction of methanol extract was saponified to obtain the unsaponifiable matter (Khandelwal, 2002). The unsaponified petroleum ether fraction of methanol extract of *C. phlomidis* and *N. stellata* were designated as UPFMCP and UPFMNS respectively.

3.4.2 Column chromatography

The unsaponified petroleum ether fraction of methanol extract (UPFMCP and UPFMNS; 2.5 g each) were subjected to column chromatography on silica gel 60-120 mesh and eluted with petroleum ether (60-80 °C): ethyl acetate in varying proportions. 100 fractions of 25 ml each were collected. Similarly another 2.5 g of UPFMCP and UPFMNS each were also subjected to column chromatography on silica gel 60-120 mesh and eluted with chloroform: ethyl acetate in varying proportions. 100 fractions of 25 ml each were collected. The collected fractions were either kept separate or pooled based on their TLC profile. The fractions with single spot in TLC were further purified using the same procedure. The purified compounds were dried and stored in dessicator.

3.4.3 Characterization of isolated compounds

The isolated compounds were characterized by IR, ^{1}H NMR, ^{13}C NMR, Mass, and CHNOS data. The IR, NMR, Mass, and CHNOS analysis were done in IR Department, Choksi Laboratories, Vadodara; SAIF, Chandigarh University, Chandigarh; Oxygen Research Healthcare Private Limited, Ahmedabad and SAIF-IIT, Mumbai respectively. The chemical structures of isolated compounds were unambiguously elucidated by the available data and comparing with literature values. To check the purity of the isolated compounds, a TLC study was performed.

3.5 Acute toxicity study

Toxicity study was conducted as per internationally accepted protocol drawn under OECD guidelines 423 in Albino rats Wistar strain (200-250 g). Rats were fasted overnight and maintained with water *ad libitum*. The rats were separated into groups of 3 each. Fractions were administered at a dose level of 5, 50, 300 and 2000 mg/kg, per rat orally as a fine suspension in 2 % gum acacia solution, while the isolated compounds were administered at a dose of 5, 50 and 300 mg/kg. After administration of fractions/isolated compounds, rats were observed individually and continuously for 30 min, 2 h and 24 h to detect changes in the autonomic or behavioural responses and also for tremors, convulsion, salivation, diarrhoea, lethargy, sleep and coma and then monitored for any mortality for the following 14 days. A group of rats treated with vehicle alone served as control (Ghosh, 1984).

3.6 Antidiabetic study

3.6.1 Preparation of samples

C. phlomidis: Unsaponified petroleum ether fraction of methanol extract (UPFMCP), residual methanol extract left after petroleum ether fractionation (RMECP), crude polyamine extract (CPECP) were prepared and studied along with the isolated compounds CP I, CP II and CP III.

N. stellata: Unsaponified petroleum ether fraction of methanol extract (UPFMNS), chloroform fraction of methanol extract (CFMNS) and residual fraction of methanol extract (RFMNS) were prepared and studied.

3.6.2 Selection and maintenance of animals

Healthy adult albino rats of Wistar strain weighing 200-250 g were procured from animal house of Zydus-Cadila Pharmaceuticals, Ahmedabad. The animal house was well ventilated and rats had 12±1 h day and night cycle with temperature between 25±3 °C; 35–55 % humidity. Rats were housed in large spacious hygienic polypropylene cages during the course of the experimental period. Rats were fed with rat pellet feed supplied by Nav Maharashtra Oil Mills, Maharashtra, India and water *ad libitum*.

3.6.3 Induction of experimental diabetes

Type 2 diabetes mellitus (NIDDM) was induced in overnight fasted rats by a single intraperitoneal injection of 65 mg/kg streptozotocin (STZ) (Himedia Laboratories Ltd, Mumbai) in citrate buffer 0.1 M (pH 4.5), 15 min after the i.p. administration of 110 mg/kg nicotinamide (Himedia Laboratories Ltd, Mumbai) in normal physiological saline (Masiello et al., 1998). Hyperglycemia was confirmed by the elevated blood glucose levels determined at 72 h. Those rats with fasting blood glucose concentration of more than 180 mg/dl were divided into fourteen groups and six groups of 6 rats each for antidiabetic study on *C. phlomidis* and *N. stellata* respectively.

Groupings for antidiabetic study on *C. phlomidis* were;

Group 1 : Diabetic control (2% gum acacia solution)

Group 2 : Standard control (Metformin, 11.3 mg/kg) (Dhanabal et al., 2007)

Group 3 : UPFMCP 100 mg/kg

Group 4 : UPFMCP 200 mg/kg

Group 5 : RMECP 100mg/kg

Group 6 : RMECP 200mg/kg

Group 7 : CPECP 100mg/kg

Group 8 : CPECP 200mg/kg

Group 9 : CP I 15 mg/kg

Group 10 : CP I 30 mg/kg

Group 11 : CP II 15 mg/kg

Group 12 : CP II 30 mg/kg

Group 13 : CP III 15 mg/kg

Group 14 : CP III 30 mg/kg

Groupings for antidiabetic study on *N. stellata* were;

Group 1 : UPFMNS 100 mg/kg

Group 2 : UPFMNS 200 mg/kg

Group 3 : CFMNS 100 mg/kg

Group 4 : CFMNS 200 mg/kg

Group 5 : RFMNS 100 mg/kg

Group 6 : RFMNS 200 mg/kg

Fractions/isolated compounds were administered orally as a fine suspension in 2 % gum acacia solution for 30 days. Rats were allowed to access food and water *ad libitum*. Changes in body weight were also noted in control, standard and treated groups. Blood samples were collected by orbital sinus puncture under light ether anesthesia (Waynforth, 1980) in Eppendroff's tubes containing EDTA-sodium (1 mg/ml). The tubes were centrifuged at 3000 rpm for 10 minutes in a Remi centrifuge and the resultant plasma was used for the estimation of different parameters..

3.6.4 Estimation of glucose

Glucose content was measured by GOD-POD enzymatic kit (Span Diagnostics Ltd, Surat, India) (Trinder, 1969).

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} \text{Gluconic acid} + H_2O_2$$

$$2\,H_2O_2 + \text{4-Aminoantipyrine} + \text{Phenol} \xrightarrow{\text{POD}} \text{Quinoneimine} + 4\,H_2O$$

The concentration of glucose was measured at 505 nm in a UV-Vis spectrophotometer 1800 (Shimadazu, Japan) and expressed as mg/dl.

$$\text{Concentration of glucose} = \frac{\text{Absorbance of sample X Concentration of standard}}{\text{Absorbance of standard}}$$

3.6.5 Estimation of insulin

Plasma insulin was determined by using radioimmunoassay kit based on the method of Yalow et al., (1974). Insulin estimation of all the samples were carried out in Endocrine laboratory and *in vitro* allergy testing, Ahmedabad.

3.6.6 Collection of tissues/organs

After the treatment period, rats were euthanized by cervical dislocation. Liver was isolated for the estimation of hexokinase, glucose-6-phosphatase, glycogen and pancreas was isolated for histopathological studies.

3.6.7 Estimation of hexokinase

Glucose-6-phosphate dehydrogenase (Sigma Aldrich, Germany), ATP and NAD (Himedia Laboratories Ltd, Mumbai, India) were procured for the study. The hexokinase assay is based on the reduction of NAD^+ through a coupled reaction with glucose-6-phosphate dehydrogenase. The excised liver tissue homogenate was prepared in saline. To 0.1 ml of homogenate were added 2.28 ml of Tris (200 mmol l^{-1})–MgCl2 buffer (20 mol l^{-1}), pH 8, 0.5 ml of 0.67 M glucose, 0.1 ml of 16 mM ATP, 0.1 ml of 6.8 mM NAD and 0.01 ml of 300 U ml^{-1} glucose-6-phospate dehydrogenase. The solution was mixed thoroughly, and the absorbance was

measured at 340 nm. The enzyme activity was expressed as unit per gram per minute in tissue (Brandstrup et al., 1957).

3.6.8 Estimation of glucose-6-phosphatase

Glucose-6-phosphate was procured from Sigma Aldrich, Germany. Glucose-6-phosphatase catalyzes the conversion of glucose-6-phosphate to glucose. The excised liver was homogenized in ice-cold sucrose (250 mM) solution. To 0.1 ml of sucrose/EDTA buffer were added 0.1 ml of glucose-6-phosphate (100 mM), 0.1 ml of imidazole buffer (100 mM, pH 6.5) and 0.1 ml of homogenate, with thorough mixing. The tubes were incubated at 37 °C for 15 min. The enzymatic activity was terminated by the addition of 2 ml of TCA/ascorbate (10 %/2 %, w/v), and the solution was centrifuged at 3000 rpm for 10 min. To 1 ml of clear supernatant were added 0.5 ml of ammonium molybdate (1 %, w/v) and 1 ml of sodium citrate (2 %, w/v). The absorbance was measured at 700 nm. The enzyme activity was expressed as unit per gram per minute in tissue (Baginsky et al., 1974).

3.6.9 Glycogen estimation

Liver glycogen was estimated by anthrone method (Stafford et al., 1955).

Anthrone reagent – 1 liter of 72% v/v sulphuric acid was prepared by placing 280 ml of distilled water in a suitable flask and then cautiously adding 720 ml of concentrated sulphuric acid to it. Then, 500 mg of anthrone (Sigma Aldrich, Germany) and 10 g of thiourea was added. The mixture was then warmed at 80-90 °C with occasional shaking till anthrone and thiourea were dissolved. Then the mixture was cooled and stored in a refrigerator.

Standard glucose solution – A stock solution (1 mg/ml) of glucose solution was prepared by dissolving 100 mg of glucose in 100 ml of saturated benzoic acid solution.

Each liver tissue was homogenized with 5 ml of 5 % trichloroacetic acid for 3 minutes. The homogenate was centrifuged at 3000 rpm for 5 minutes. The supernatant was decanted on an acid washed filter paper to a graduated cylinder. The residue was homogenized with 5 ml of 5 % trichloroacetic acid again for 1 minute. The homogenate was centrifuged and the supernatant was filtered and homogenized similarly. The process was repeated two more times. The total volume of filtrate collected in the cylinder was noted. 10 µl of this filtrate was pippetted into 15 ml pyrex centrifuge tubes.

To each tube, 5 ml of 95 % ethanol was added and the tubes were allowed to stand at room temperature. After completion of precipitation of glycogen, the tubes were centrifuged at 3000 rpm for 15 minutes. The clear liquid was gently decanted out from the packed glycogen free from alcohol and then dissolved in 2 ml of distilled water.

A reagent blank was prepared by pipetting 2 ml of distilled water into a clean boiling tube. A standard was prepared by pipetting out 2 ml of standard glucose solution (containing 0.05 mg glucose). Then to each tube, 10 ml of anthrone reagent was added. All the tubes were capped tightly and placed in cold water bath. After 20 minutes all tubes were immersed in boiling water for 15 minutes. The tubes were again immersed in cold water bath and cooled to room temperature. This procedure was repeated 10 times. Optical density of all tubes was measured at 620 nm with reagent blank.

$$\mu g \text{ of glucose /mg of liver} = \frac{DU}{DS} \times 0.05 \times \frac{\text{Total volume of extract (ml)}}{\text{Weight of tissue (mg)}} \times \frac{0.9}{0.01}$$

where,

DU = optical density of sample

DS = optical density of standard

0.05 = mg of glucose in 2 ml of standard solution

3.6.10 Statistical analysis

The quantitative measurements in all the experiments were made on 6 rats in each group and the values are expressed as Mean ± SD. GraphPad Instat Version 4 software was used. Data were subjected to the analysis of variance (one way ANOVA) to determine the significance of changes followed by Dunnett's multiple comparisons.

3.6.11 Histopathological study

A small portion of pancreatic tissue was fixed in a 10% solution of formalin (formaldehyde) in 0.9 % saline. These tissues were processed for paraffin embedding and sections were stained with Haematoxylin-Eosin (H and E) reagent. The histological results were recorded on microphotographs and examined for intracellular changes.

3.7 GC-MS analysis of unsaponified matter of petroleum ether fraction of methanol extract of *C. phlomidis* (UPFMCP)

The unsaponified matter of petroleum ether fraction of methanol extract (UPFMCP) was subjected to GC-MS analysis

GC-MS Programme

Column	: Elite-1 (100% Dimethyl poly siloxane), 30m X 0.25mm ID X 1µm df
Equipment	: GC Clarus 500 Perkin Elmer
Carrier gas	: Helium 1 ml/min
Detector	: Mass detector-Turbo mass gold-Perkin Elmer
Software	: Turbo mass 5.1
Sample injected	: 2 µl
Split	: 10:1

Oven Temperature Programme

110 deg C-2 min hold

Up to 280 °C at the rate of 5 deg/min-9min hold

Injector temp: 250 °C

Total GC time: 45 min

MS Programme

Library used : NIST Ver.2.0-Year 2005

Inlet line temperature: 200 °C

Source temperature : 200 °C

Electron energy : 70 eV

Mass scan : (m/z) 45-450

Total MS Time : 45 min

3.8 PTP1B inhibition study

3.8.1 Preparation of samples

C. phlomidis: Powdered leaves (each 10 g) were individually extracted with methanol / 50 % methanol / water (200 ml) and then filtered. Filtrates were concentrated individually, dried under vacuum and used for PTP1B inhibition study. Crude polyamine extract (CPECP), unsaponified petroleum ether fraction of methanol extract (UPFMCP), Residual methanol extract (RMECP), crude alkaloidal fraction (CAFCP), CP I, CP II, CP III, β-sitosterol, lupeol, l-dopa, adrenaline and β-carotene were also studied.

N. stellata: Powdered leaves (each 10 g) were individually extracted with methanol / 50 % methanol / water (200 ml) and then filtered. Filtrates were concentrated individually, dried under vacuum and used for PTP1B inhibition study. Unsaponified petroleum ether fraction of methanol extract (UPFMNS), chloroform fraction of methanol extract (CFMNS), residual fraction of methanol extract (RFMNS) were also prepared and studied along with oleanolic acid, betulinic acid and gallic acid.

The antidiabetic activity of the samples were tested against PTP1B enzyme by using Calbiochem® PTP1B colorimetric assay kit (Calbiochem® PTP1B Colorimetric Assay Kit. User Protocol. 2008, Catalogue No: 539736, USA). The kit components were; PTP1B enzyme (5 µg, 100 ng/µl in 50 mM HEPES, 1 mM EDTA, 1 mM DTT, 10 % (v/v) glycerol, 0.05 % NP-40, pH 7.2), PTP1B substrate (1 mg, lyophilized solid, amino acids 1142-1153, pY^{1146}, M.W. 1703 kDa), 2X assay buffer (20 ml, 300 mM NaCl, 100 mM MES, 2 mM DTT, 2 mM EDTA, 0.1 % NP-40, pH 7.2), red reagent (5 ml, concentrated phosphate detection reagent), phosphate standard (500 µl, 100 µM solution in 1X Assay) and suramin (10 mg).

Preparation of phosphate standard curve: All kit components were thawed and the PTP1B enzyme, PTP1B substrate (IR5), and assay buffer were placed on ice bath; red reagent was stored at room temperature. Reconstitution of PTP1B substrate (1.5 mM) was done by adding 88 µl 2X assay buffer and 88µl of distilled water to 500 µg net peptide. After vortexing the aliquot was freezed (-70 °C). 1.2ml of 1X assay buffer was prepared by diluting 600 µl 2X assay buffer with 600µl of distilled water. 100, 97.5, 95, 90, 80 and 70 µl 1X assay buffer was pipetted into duplicate sets of six wells. 0, 2.5, 5, 10, 20 and 30 µl of the 100 µM phosphate standard was pipetted into those same wells, in the same order so that these wells contained respectively, 0, 0.25, 0.5, 1.0, 2.0 and 3.0 nmol of inorganic phosphate. After incubating at 30 °C for 30 min the reaction was terminated by adding of 25 µl red reagent. The plates were agitated plate and allowed to stand for 20-30 min. The absorbance was measured at 620 nm (A_{620}) on microplate reader (BioRad 680XR, France). Standard curve of A_{620nm} versus nmol PO_4^{2-} released was plotted.

Test sample/inhibitor Assay: 1 ml 1X Assay Buffer was prepared by diluting 500 µl of 2X assay buffer with 500 µl of distilled water and placed on ice. Dilution of PTP1B enzyme in cold 1X assay buffer was prepared, such that each 5 µl contained 150 µM of enzyme per well. Test sample solutions was prepared in 1X assay buffer and warmed to assay temperature (30 °C). 10 mM stock of suramin was prepared

by dissolving 10mg of solid in 0.7 ml of 1X assay buffer. For a final concentration of 10 μM, a 10X stock (100μM) was prepared by mixing 10 μl of the 10 mM stock with 990 μl assay buffer. 35 μl of 1X assay buffer was added to each well and warmed to 30 °C. 10 μl of test sample/inhibitor was added to appropriate wells. 10 μl of 1X assay buffer was added to control wells. 5 μl of the PTP1B enzyme dilution (2.5 nmole/well) was added to each well. After incubating at 30 °C for 30 min the reaction was terminated by adding of 25 μl red reagent. The plates were agitated plate and allowed to stand for 20-30 min. The absorbance was measured at 620 nm on micro plate reader. The results of the test compound were expressed as amount of phosphate released in nmoles. The percentage inhibition of test compounds was calculated as percentage of control. ("Time zero" measurement was obtained by adding 25 μl of red reagent, 50 μl of 2X enzyme and 50 μl of 2X substrate. These values were subtracted from the values, expressed as nmol of phosphate released from both the control and test sample/inhibitor values).

3.9 Brine shrimp lethality bioassay

Brine shrimp lethality bioassay was performed as per the method of Meyer et al., (1982). Brine shrimps (*Artemia salina*) were hatched using brine shrimp eggs (Ocean Star International Inc., Snowville, UT, USA) in a conical shaped vessel (1L), filled with sterile artificial seawater (prepared using sea salt 38 g per liter and adjusted to pH 8.5 using 1N NaOH) under constant aeration for 36 h. After hatching, active nauplii free from egg shells were collected from brighter portion of the hatching chamber and used for the assay. Ten nauplii were drawn through a glass capillary and placed in each vial containing 4.5 ml of brine solution (24 % of sodium chloride in water). In each experiment, 0.5 ml of the extracts/fractions/isolated/identified compounds was added to 4.5 ml of brine solution and maintained at room temperature for 24 h under the light and surviving larvae were counted. Experiments were conducted at different concentrations (up to 4000 μg/ml for extracts/fractions and 2000 μg/ml for isolated/identified compounds) of the test substances in a set of six tubes per dose.

Extracts/fractions or Isolated/identified compounds were dissolved in minimum volume of DMSO and made up with water. The concentration of DMSO used was also studied as vehicle control.

The percentage lethality was determined by comparing the mean surviving larvae of the test and control tubes. LC_{50} values were obtained from the best-fit line plotted concentration verses percentage lethality.

$$\% \text{ lethality} = \frac{\text{Number of surviving nauplii in control - Number of surviving nauplii in test}}{\text{Number of surviving nauplii in control}} \times 100$$

3.10 Anti-platelet aggregation activity

The extracts/fractions/compounds of *C. phlomidis and N. stellata* were studied for their *in vitro* ADP (Chrono-Log, USA) induced platelet aggregation inhibitory activity on whole human blood in comparison with aspirin as standard drug. Platelet aggregation inhibition in whole blood was measured with Chrono-Log 540 aggregometer (Chrono-Log Corp., Haverton, PA, U.S.A.). The readings were taken in triplicate with control and aspirin each time for comparative study.

Blood was obtained from non-smoking healthy human volunteers who had not taken any medications or alcohol for at least two weeks. Blood was drawn into vacuette tubes containing 0.1 ml/4.5 ml of 3.38 % of trisodium citrate solution. 0.5 ml citrated whole blood and 0.5 ml saline was added to glass cuvettes (both reference and sample cell) preheated to 37 °C and incubated for 5 minutes with siliconized stirring bars in each cuvette. Aspirin (10 µM) was added in reference cell and the sample (10 µl) in the sample cell and further incubated for 5 min. The electrodes were placed in the cuvettes and 10 µM of ADP was added in each cuvette. Baseline correction was done and aggregation initiated within 1-2 min after ADP addition. Aggregation was measured in terms of change in impedance, either until the maximal amplitude was reached or for eight minutes. The extent of aggregation was recorded in ohms. Experiments were conducted at different

concentrations, 4000 µg/ml for extracts/fractions and 400 µg/ml for isolated/identified compounds. The percent inhibition was calculated by comparing the change in impedance after the addition of ADP, taking the impedance of the control curves as 100% aggregation.

$$\% \text{ inhibition of platelet aggregation} = \frac{\text{Control- sample/standard}}{\text{Control}} \times 100$$

3.11 Anti-acetylcholineesterase study

Acetyl cholinesterase inhibition was assayed by the method of Ellman et al. (1961). In the 96-well plates, 25µl substrate, 15mM ATCI (Sigma Aldrich, Germany) in Millipore water, 125µl 3mM DTNB (Himedia Laboratories Pvt Ltd, Mumbai) in buffer C (50mM Tris–HCl, pH 8, 0.1M NaCl, 0.02M $MgCl_2 \cdot 6H_2O$), 72.5µl buffer B (50mM Tris–HCl, pH 8, 0.1 % bovine serum albumin) and 2.5µl test compound solution dissolved in DMSO were added. Then 25µl 0.22 U/ml AChE (Sigma Aldrich, Germany) in buffer B were added to the wells and the absorbances were read in a microplate reader (BioRad 680XR, France) at 405 nm after three minutes. The percentage inhibition was calculated by comparing the rates for the samples to the control (Lopez et al., 2002; Adsersen et al., 2007). Galanthamine (1 µM) was used as standard. The experiment was done in triplicate.