Chapter - III

Materials and Methods
III. MATERIALS AND METHODS

3.1. Chemicals and softwares

Distilled ethanol was obtained from Shamsons Distilleries Pvt. Ltd., Duggavathi, Davanagere Dist, Karanataka, India; Hexane, petroleum ether (40 – 60 °C), chloroform (60 – 80 °C), ethyl acetate (60 – 80 °C), methanol (80 – 100 °C) (Merck, India); DMSO, silica gel for thin layer chromatography, silica gel (60-120 mesh) for column chromatography (HiMedia, India); Silymarin (Micro Labs, India). iTRAQ labels and reagents (Applied Biosystems Cat. No. 4352135); Trypsin (Promega); Protein assay reagents (Bio-Rad DC Reagent A – catalog No. 500-0113, Reagent B – catalog No. 500-0114); Cation exchange chromatography on PolySULFOETHYL A column (PolyLC, Columbia, MD); LC-MS/MS column Magic C18 AQ (Michrom Bioresources, Auburn, CA, USA); Peptide desalting C18 cartridge (Pierce, Rockford, USA); Trifluoroacetic acid, Acetonitrile, Formic acid, Sodium dodecyl sulphate, Ammonium persulphate, Acrylamide; Bis-acrylamide and TEMED (Sigma aldrich, India). LC-MS/MS of the peptide samples was carried out by using LTQ-OrbitrapVelos (Thermo Scientific, Bremen, Germany); Protein identification and quantitation was carried out by using Proteome Discoverer software (Thermo Fisher Scientific, Beta Version 1.3.0.208); MS/MS search was carried out using SEQUEST and MASCOT search algorithms; Signaling pathway was designed using ProteinLounge Pathway Builder online software, Molecular Docking was carried out using AutoDock 3.0 software.

3.2. Phytochemical evaluation

3.2.1. Plant resource

Flaveria trinervia herb was collected from the agricultural fields (dry lands) near by Chitradurga city of Karnataka, India (Fig. 1). Plant was authenticated by Dr. Manjunatha comparing with the voucher specimen
Figure 1. *Flaveria trinervia* (Sprengle) C. Mohr., herb.
deposited at Kuvempu University herbarium specimen FDD-No. 53 (Manjunatha et al., 2004).

This plant is locally referred as Bellary Halabu or Katte Kirubanagida. Local farmers of Chitradurga District imposed that this herb is seasonal and grows during late monsoon and winter from September to December. Hence, during November, 2008 the research plant was collected from Chitradurga region (Fig. 2).

3.2.2. Soxhlet extraction

The fresh whole plant was washed thoroughly, shade dried and porously powdered mechanically using blender. 1 kg of powdered plant material was packed in soxhlet timble and sequentially extracted with the solvents viz, petroleum ether, chloroform, methanol and water for about 48 hrs. The crude extracts were filtered and concentrated in vacuum under reduced pressure using rotary flash evaporator (Buchi, Flawil, Switzerland), complete evaporation of the solvent was done on water bath and finally vacuum dried. The yield of petroleum ether, chloroform, methanol and aqueous crude extracts for 1 kg of powdered whole plant material was 26 g, 16.5 g, 32.5 g and 90 g respectively. All the crude extracts were subjected to qualitative analysis for the detection of specific class phytoconstituents.

3.2.3. Isolation of phytoconstituents from petroleum ether extract

Crude petroleum ether extract was subjected for thin layer chromatography (TLC) studies, using the solvent system petroleum ether : ethyl acetate in the ratio of 9:1, which showed separation of three distinct spots, with \( R_f \) value: 0.98, 0.77 and 0.57. The constituents were eluted by column chromatography (180 g silica gel of 60-120 mesh, 60x4 cm) by gradient elution method using ethyl acetate/petroleum ether in combination and the fractions were collected at the intervals of 5 ml. Which yielded three different pure compounds, the purity of the isolated compounds was monitored by TLC.
Figure 2. *Flaveria trinervia* plant collection location in Chitradurga District.
examination, based on single spot separation. These isolated pure compounds were coded as P1, P2 and P3 (Fig. 3). Melting point of the isolated compounds was determined using scientific melting point apparatus and the observation was recorded. The characterization of the isolated compounds was carried out by subjecting to qualitative analysis followed by IR, $^1$H-NMR and mass spectral studies.

3.2.4. Isolation of phytoconstituents from chloroform extract

Crude chloroform extract was also subjected for thin layer chromatography studies, which showed a spot with the $R_f$ value 0.56 using the solvent system hexane : ethyl acetate at the ratio of 9:1. The constituent was eluted by column chromatography (180 g silica gel of 60-120 mesh, 60x4 cm) and the fractions were collected at the intervals of 5 ml. Which yielded one pure compound, the purity of the isolated compound was monitored by TLC examination, based on single spot separation. The isolated pure compound was vacuum dried, melting point recorded and coded as C1 (Fig. 3). The characterization of the isolated compound was carried out by subjecting to IR, $^1$H-NMR and mass spectral studies.

3.2.5. Isolation of phytoconstituents from methanol extract

Initially the methanolic extract was subjected for thin layer chromatography studies using chloroform : Hexane at the ratio of 9:1, which showed 6 separated spots. Among them 3 spots were UV active, as observed on UV transilluminator. Using same solvent proportion column chromatography was carried out. But no pure compound was harvested from the elutants. Methanol extract was eluted completely using ethyl acetate, concentrated and made acidic of pH 5 with 1% HCl. 10 g of this concentrated ethyl acetate fraction was subjected for thin layer chromatography using ethyl acetate : petroleum ether 8:2, which showed 2 spot separation with $R_f$ value: 0.67 and 0.52. The constituents were eluted by column chromatography (180 g silica gel of 60-120 mesh, 60x4 cm) by gradient elution method using ethyl
Figure 3. Flowchart representing the isolation of phytoconstituents.
acetate/petroleum ether in combination and the fractions were collected at the intervals of 5 ml of each. Which yielded two different pure compounds, the purity of the isolated compounds was monitored by TLC examination, based on single spot separation. These isolated pure compounds were coded as M1 and M2 (Fig. 3). Melting points of the isolated compounds were determined using scientific melting point apparatus and were characterized based on IR, $^1$H-NMR and mass spectral studies.

3.2.6. Qualitative chemical analysis of the crude extracts and the isolated compounds

The crude petroleum ether, chloroform, methanolic and aqueous extracts and their isolated phytoconstituents were subjected to the qualitative tests to detect the major chemical groups following the methods of Harborne, 1984; Trease & Evan, 1989 and Kokate, *et al.*, 1996; Kamaraswamy and Krishna, 2008.

i) Tests for Alkaloids

*Dragendorff's test:* To 2 mg of the test extract/compound, 5 ml of distilled water was added, 2M hydrochloric acid was added until an acid reaction occurs. To this 1 ml of dragendorff's reagent was added. Formation of orange or orange red precipitate indicates the presence of alkaloids.

*Hager's test:* To 2 mg of the test extract/compound taken in a test tube, a few drops of Hager's reagent were added. Formation of yellow precipitate indicates the presence of alkaloids.

*Wagner's test:* 2 mg of test extract/compound was acidified with 1.5 % v/v of hydrochloric acid and a few drops of Wagner's reagent were added. A yellow or brown precipitate indicates the presence of alkaloids.

*Mayer's test:* To a few drops of the Mayer's reagent, 2 mg of test extract/compound was added. Formation of white or pale yellow precipitated indicates the presence of alkaloids.
ii) Tests for Flavonoids

*Shinoda's test:* In a test tube containing 0.5 ml of the test extract/compound, 10 drops of dilute hydrochloric acid followed by a small piece of magnesium was added. Formation of pink, reddish or brown colour indicated the presence of flavonoids.

*Ferric chloride test:* Test solution with few drops of ferric chloride solution shows intense green colour, indicating the presence of flavonoids.

*Zinc-Hydrochloric acid reduction test:* Test solution with zinc dust and few drops of hydrochloric acid shows magenta red colour, indicating the presence of flavonoids.

*Alkaline reagent test:* Test extract/compound solution when treated with sodium hydroxide solution, shows increase in the intensity of yellow colour which becomes colourless on addition of few drops of dilute acid, indicating the presence of flavonoids.

*Lead acetate solution test:* Test extract/compound solution with few drops of lead acetate (10%) solution gives yellow precipitate, indicated the presence of flavonoids.

iii) Test for Triterpenoids

*Liebermann - Burchard's test:* 2 mg of test extract/compound was dissolved in acetic anhydride, heated to boiling, cooled and then 1 ml of concentrated sulphuric acid was added along the sides of the test tube. Formation of a violet coloured ring indicated the presence of triterpenoids.

*Salkowski test:* When few drops of conc. sulphuric acid was added to the test extract/compound solution, shaken and allowed to stand, lower layer turns yellow indicating the presence of triterpenoids.

iv) Test for Acids

1 mg of test extract/compound was dissolved in 1 ml of distilled water and sodium-bicarbonate was added to this solution. Effervescence indicated the presence of resins.
v) Tests for Saponins

*Foam test:* In a test tube containing about 5 ml of test extract/compound, a drop of sodium bicarbonate solution was added. The test tube was shaken vigorously and left for 3 minutes. Formation of honeycomb-like froth indicates the presence of saponins.

*Haemolysis test:* 2 ml of 18% sodium chloride solution in two test tubes were taken. To one test tube distilled water was added and to the other 2 ml of extract/compound. Few drops of blood were added to both the test tubes. Mixed and observed for haemolysis under microscope.

vi) Tests for Steroids

*Liebermann-Burchard's test:* 2 mg of dry test extract/compound was dissolved in acetic anhydride, heated to boiling, cooled and then 1 ml of concentrated sulphuric acid was added along the sides of the test tube. Formation of green colour indicated the presence of steroids.

*Salkowski reaction:* 2 mg of dry test extract/compound was shaken with chloroform, to the chloroform layer sulphuric acid was added slowly by the sides of test tube. Formation of red colour indicated the presence of steroids.

*Sulphur test:* Sulphur when added to the test solution, it sinks to the bottom.

vii) Test for Tannins

*Ferric chloride test:* To 1-2 ml of the test extract/compound, few drops of 5% w/v FeCl₃ solution were added. Appearance of green colour indicated the presence of gallotannins, while brown colour indicated the presence of pseudotannins.

*Gelatin test:* Test extract/compound solution when treated with gelatin solution gives white precipitate, indicated the presence of pseudotannins.

viii) Tests for Quinones

*Test with potassium iodide:* Potassium iodide gives hydrogen iodide on reaction with dilute sulphuric acid. The liberated hydrogen iodide reacts with
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quinones producing iodine, which can be tested with starch paper, which turns into blue colour.

Quenching in UV at 254 nm shows the presence of a naphthoquinone.

* After spraying with 10% methanolic potassium hydroxide, the test extract/compound sample shows red fluorescence in UV-365 nm and red to red-brown colour (vis). This confirms the presence of a naphthoquinone.

ix) Tests for Glycosides

Baljet test: The test extract/compound solution when treated with sodium picrate gives yellow to orange colour, indicating the presence of glycosides.

Keller-Killiani test: The test extract/compound solution was treated with few drops of ferric chloride solution and mixed. When conc. sulphuric acid containing ferric chloride solution was added, it forms two layers, lower layer reddish brown and upper acetic acid layer turns bluish green, indicating the presence of glycosides.

Raymond’s test: The test solution when treated with dinitrobenzene in hot methanolic alkali gives violet colour, indicating the presence of glycosides.

Bromine water test: Test solution when dissolved in bromine water gives yellow precipitate, indicating the presence of glycosides

Legal’s test: Test solution when treated with pyridine (made alkaline by adding sodium nitroprusside solution) gives pink to red colour, confirming the presence of glycosides.

3.3. Pharmacological evaluation

3.3.1. Animals

Wistar albino rats of both sex weighing 100-150 g were procured from central animal House, Soniya Education Trust’s College of Pharmacy, Dharwad and were maintained at standard environmental conditions (25 ± 2 ºC and 45 to 55% of relative humidity). Animals were fed with commercial diet (Durga feeds and foods, Bangalore) and watered with ad libitum during the experiment. The animals were acclimatized for one week before the initiation
of each experiment. For the use of animals in pharmacological experiments ethical clearance was obtained from the institutional animal ethical committee (Reg.No.SETCP/IAEC/2010-11/165).

3.3.2. Acute toxicity studies

The staircase method (Ghosh, 1984) was adopted for the determination of acute toxicity by oral administration. This method involved the determination of LD<sub>50</sub> value in biphasic manner. The animals were starved of feed but allowed access to water 24 hrs prior to the study. In the initial investigatory step (phase I), a range of doses of the extract and the constituent producing the toxic effects was established. This was done by oral administration of widely differing doses of the extract (10, 100, 1000, 1500 mg/kg b.w.) to the rats based on the pharmacological study. Based on the results obtained, a phase II investigatory step was followed by giving more specific doses (100, 200, 300, 500 and 700 mg/kg b.w.). Animals were observed for 24 hrs for behavioural signs such as nervousness, excitement, dullness, ataxia or death. The maximum non-lethal and the minimum lethal doses were recorded. One tenth of LD<sub>50</sub> dose was considered as the safer dose for oral administration for the evaluation of pharmacological activity of the extracts and the constituents (Ghosh, 1984; Jalalpure, et al., 2004).

3.3.3. Drug formulations

The crude sequential solvent extracts and their isolated compounds were used for drug formulation based on their water solubility/insolubility factors. The one tenth of the lethal dose of extracts and their isolated compounds were mixed with 1% gum tragacanth (w/v) or 5% DMSO (v/v) for oral administration. Based on traditional medicinal claim and reviews the crude extracts and the isolated constituents of Flaveria trinervia were used for the evaluation of following pharmacological models.
3.3.4. Prophylactic effect of *Flaveria trinervia* against ethanol induced liver damage

Female rats were randomly divided into twelve groups of six animals in each group. All the group animals except group 1 were fed with the ethanol containing liquid diet by incrementing the ethanol content from 0 - 30% for one week, this dose was progressively increased to 35% ethanol for one week followed by 40% of the caloric content for 5 more weeks by using oral gavage (Billy *et al.*, 2009). Group 1 animals were adapted to ethanol free liquid diet over the same period. Thereafter, animals were maintained on 0% or 40% ethanol-containing diets throughout the study. Rats were monitored daily to ensure adequate nutritional intake and maintenance of body weight (Rajakrishnan *et al.*, 1997; Joharapurkar *et al.*, 2003; Pornpen *et al.*, 2007a; Vipul *et al.*, 2007; Pornpen *et al.*, 2007b; Billy *et al.*, 2009).

Group I rats were maintained as control by administering 5% DMSO orally. Group II rats were fed with only ethanol containing diet daily. Group III, IV and V rats were treated with suspension of PE (75 mg/kg of petroleum ether extract), CE (75 mg/kg of chloroform extract) and ME (75 mg/kg of methanol extract) respectively, prepared in 5% DMSO. Group VI, VII, VIII and IX rats were treated with P1 (100 mg/kg of 4-(3-Hydroxy-but-1-enyl)-3-(2-Hydroxy-1-Methyl-propyl)-5,5-Dimethyl-Cyclohex-2-Enone); P2 (100 mg/kg of 15 Hydroxy-5,10,14 –Trimethyl-Pentadecanoic acid); P3 (100 mg/kg of 1-(Decahydro-naphthalen-2-yl)-Ethanol) and C1 (100 mg/kg of oleanolic acid) respectively were prepared in 5% DMSO. Group X and XI animals were treated with M1 (50 mg/kg of Syringetin) and M2 (50 mg/kg of Quercetin) respectively, prepared in 5% DMSO. Group XII animals were administered with the standard drug silymarin at the dose of 250 mg/kg, b. w. orally once daily. All the extracts and the isolated phytoconstituents and the standard drug were administered to the animals after an interval of 3 h after the administration of ethanol during the last week of 7 week study. This treatment was slightly modified as reported by Billy *et al.*, 2009. Animals were sacrificed
24 h after the last treatment. Blood was collected into a sterilized centrifuge tube, allowed to clot and serum was separated at 2500 rpm for 15 min.

i) *In vivo* antioxidant activity by the estimation of superoxide dismutase (SOD), catalase (CAT), peroxidase (PER) and TBARS.

Liver samples dissected out of each animal were washed immediately with ice cold phosphate buffer saline to remove as much blood as possible. Liver homogenates (5% w/v) were prepared in cold 50 mM potassium phosphate buffer (pH 7.4) using a glass homogenizer. The unbroken cells and cell debris were removed by refrigerated centrifugation at 3000 rpm for 10 min. The supernatant was used for the study. 0.5 ml rat liver homogenate was taken to carryout superoxide dismutase (SOD) assay and 1 ml of 50 mM sodium carbonate, 0.2 ml of 0.1mM EDTA and 0.4 ml of 24μm NBT were added. The reaction was initiated by adding 0.4 ml of 1 mM hydroxylamine hydrochloride. Absorbance was taken at 560 nm. SOD activity was expressed based on the reduction of NBT by half of its total amount (Beauchamp *et al.*, 1971).

To carryout catalase assay, 1 ml of rat liver homogenate was taken and 1.9 ml of phosphate buffer 50 mM (pH 7.4) was added. Similarly, blank was prepared by using phosphate buffer instead of liver homogenate with 1.9 ml of phosphate buffer. The reaction was initiated by the adding 1 ml of H$_2$O$_2$ (30 mM) to both the reaction mixtures. The decrease in optical density was measured at 240 nm. Unit of catalase activity was expressed based on the amount of 1 μM H$_2$O$_2$ reduced per min (Aebi, 1984).

To carryout peroxidase assay, 0.5 ml of rat liver homogenate and 1 ml of 10 mM KI solution with 1 ml of 40 mM sodium acetate was added. The absorbance was taken at 353 nm then to this 20 μl of H$_2$O$_2$ (15 mM) was added, and the change in the absorbance was observed after 5 min. Unit of peroxidase activity was expressed based on the amount of 1 μM H$_2$O$_2$ reduced per min (Nicholos, 1962).
The antioxidant status was also assessed from the levels of malondialdehyde (MDA) an end product of lipid peroxidation (Esterbauer et al., 1990). As 99% of the thiobarbituric acid reactive substance is malondialdehyde (MDA), thiobarbituric acid reactive substance concentrations in the samples were expressed as nmol of MDA/g of wet tissue using molar extinction coefficient of the chromophore \(1.56 \times 10^{-5} \text{ M}^{-1} \text{ cm}^{-1}\) (Shenoy et al., 2001).

**ii) In vivo hepatoprotective activity**

The activity levels of hepatospecific marker enzymes *viz.*, serum aspartate transaminase (AST), serum alanine transaminase (ALT) were estimated by the method reported by Reitman and Frankel, 1957. The activity level of alkaline phosphatase (ALP) in serum was estimated by the method of King, 1965 and bilirubin was estimated by the method reported by Malloy et al., 1937.

**iii) Histopathological studies**

The liver tissue was dissected out from the animals of each group after draining out the blood and was washed with the normal saline and fixed in 10% formalin, dehydrated in gradual ethanol (50–100%), cleared in xylene and embedded in paraffin. Tissue sections of 5 μm thickness were prepared, processed in alcohol-xylene series and were stained with alum-haematoxylin and eosin (H–E) dye for photomicroscopic observation for the evaluation of histological changes including fibrotic tissue, lymphocyte infiltration, cell necrosis, macro and micro vesicular fatty change, tissue degeneration etc (Shanmugasundaram and Venkataraman, 2006).

**vi) Statistical analysis**

The data of all pharmacological activities were expressed as mean ± S.E.M of six animals in each group. The statistical analysis was carried out using one
way ANOVA followed by Tukey’s t-test. The difference in values at *P* ≤ 0.05, **P** ≤ 0.01 was considered as statistically significant.

3.4. Quantitative proteomic analysis

3.4.1. Sample preparation and normalization for iTRAQ experiment

The livers were dissected out from the sacrificed animals; livers were thoroughly washed in phosphate buffer saline. Immediately stored in Thermo ultra deep freezer (-80 °C) until further study. This stored tissue sample was thawed before processing. Liver tissues from each of the five lobes of all the six animals from each group were pooled together. One gram of liver tissue were freeze-dried and crushed to powder in liquid nitrogen, homogenized and lysed in 0.5% sodium dodecyl sulphate (SDS) using Dounce homogenizer followed by sonication (3 pulse, 10 seconds each) using Microson Ultrasound Cell Disruptor. Lysed tissue samples were centrifuged at 3000 rpm for 15 minutes using Eppendorf Centrifuge 5810R, supernatant was collected and further centrifuged at 13,000 rpm for 10 minutes using Eppendorf Centrifuge 5417R. Lipid content present in the tissue sample appeared at the top of supernatant, was meticulously skimmed and removed, such that the supernatant was free from lipid contamination. Protein quantification was carried out by Lowry’s method using Bio-Rad DC protein assay reagents (Reagent A – catalog No. 500-0113, Reagent B – catalog No. 500-0114) and analyzed using Eppendorf Biophotometer plus. For sample normalization by PAGE profiling, tissue protein samples were vacuum dried using eppendorf concentrator plus, such that 25 µl volume of sample contained 50 µg of protein. 25 µl of protein sample was gel profiled by SDS-PAGE. Samples were normalized based on protein concentration and PAGE profile and subjected to iTRAQ labelling.

3.4.2. iTRAQ labeling and SCX fractionation

Processed liver samples from all the four group animals were used for iTRAQ experiment. Trypsin digestion and iTRAQ (Applied Biosystems Cat.
No. 4352135) labeling was carried out according to manufacturer’s protocol using the reagents provided unless otherwise mentioned. Briefly, 80 μg of each liver lysate was treated with 2 μL of reducing agent (tris(2-carboxyethyl) phosphine (TCEP)) at 60 °C for 1 h and alkylated with 1 μL of cysteine blocking reagent, methyl methanethiosulfonate (MMTS)) for 10 min at room temperature. Protein sample was digested using sequencing grade trypsin (Promega) (1:10), digestion reaction mixture of liver lysate and trypsin was incubated for 16 h at 37 °C (Chen et al., 2007). Trypsin digested liver samples of four groups were labelled with one of the four iTRAQ reagents at room temperature (114 reporter iTRAQ was labelled to normal liver proteome and 115, 116 and 117 were labelled to ethanol intoxicated, quercetin treated and oleanolic acid treated liver proteomes respectively). After labeling tryptic digests, all the four group labeled samples were pooled, vaccum-dried and subjected to fractionation through a strong cation exchange chromatography on PolySULFOETHYL A column (PolyLC, Columbia, MD) (100 × 2.1 mm, 5 μm particles with 300 Å pores) using an LC Packing HPLC system connected to a Agilent fraction collector. Twenty eight SCX fractions (0.5 mL) were collected from a 0-350 mM KCl gradient in the presence of 10 mM potassium phosphate buffer (pH 2.85), containing 25% acetonitrile for 70 min at a flow rate of 0.2 mL/min. Solvent A contained 10 mM potassium phosphate buffer, pH 2.85, 25% acetonitrile and solvent B contained 10 mM potassium phosphate buffer, 350 mM KCl, pH 2.85, 25% acetonitrile. The fractions were vacuum-dried and desalted using C18 cartridge (Pierce, Rockford, USA) as per the manufacturer’s instructions. After desalting, consecutive fractions with comparable peptide quantities as approximated from SCX chromatograms were pooled to obtain a total of nineteen fractions and were taken for LC-MS analysis. The fractions were dried and stored at until mass spectrometry analysis.
3.4.3. Mass spectrometry

LC-MS/MS of the peptide samples was carried out by nanoflow electrospray ionization tandem mass spectrometric analysis using LTQ-OrbitrapVelos (Thermo Scientific, Bremen, Germany) interfaced with Agilent’s 1200 Series nanoflow LC system. The chromatographic capillary columns used were packed with Magic C18 AQ (particle size 5 μm, pore size 100Å; Michrom Bioresources, Auburn, CA, USA) reversed phase material in 100% ACN. The peptides were eluted using a linear gradient of 5-40% acetonitrile in 0.1% formic acid over 120 min and peptide samples from each fraction were enriched using a trap column (75 μm × 2 cm) at a flow rate of 3 μl/min and separated on an analytical column (75 μm × 10 cm) at a flow rate of 350 nl/min. The MS spectra were acquired in a data dependent manner at a mass resolution from m/z 350 to 1200 Da. with full scans acquired using the Orbitrap mass analyzer targeting twenty most abundant ions in the survey scan and those ions selected were excluded from MS/MS data. The ions selected for fragmentation were excluded for 30 sec. The automatic gain control for full FT MS was set to 1 million ions and for FT MS/MS was set to 0.1 million ions with a maximum time of accumulation of 500 ms, respectively. For accurate mass measurements, the lock mass option was enabled.

3.5. Protein identification and quantification

The raw data has been processed through Proteome Discoverer software (Thermo Fisher Scientific, Beta Version 1.3.0.208). The SEQUEST and MASCOT algorithms were used that consisted of a spectrum selector and a reporter ion quantifier for peptide identification, MS/MS was searched against the NCBI RefSeq database (release 45) containing 31,811 proteins (Carr et al., 2004). Search parameters included trypsin as the enzyme with 1 missed cleavage; oxidation of methionine was set as a dynamic modification while carbamidomethyl at cysteine as static modification and iTRAQ modification at N-terminus of the peptide. Precursor and fragment mass tolerance were set to 20 ppm and 0.1 Da. respectively. The peptide and protein data were extracted
using high peptide confidence. Unique peptide(s) for each protein identified was used to determine relative protein content in the two samples. The FDR was calculated by enabling the peptide sequence analysis using a decoy database. High confidence peptide identifications were obtained by setting a target FDR threshold of 1% at the peptide level. Relative quantitation of proteins was carried out based on the relative intensities of reporter ions released during MS/MS fragmentation of peptides they were derived from. Whenever a protein was identified with multiple peptides, relative intensities of the four reporter ions for each of the peptide identifiers for a protein were used for averaging and assessing percentage variability to determine relative quantity of a protein.

The proteins identified with high confidence were subjected to Bioinformatics analysis which included annotations of the proteins based on their subcellular localizations and molecular functions using Human Protein Reference Database (HPRD, http://www.hprd.org) and gene ontology (GO) standards. Annotations were in the context of hepatotoxicity or liver damage in rats and human based on previously published reports available in PubMed (Raghothama et al., 2008).

3.6. Signaling pathway study

An extensive curation of scientific literature was carried out to catalog signalling pathway stimulated reactions. The reactions in signalling pathway were collectively represent the individual biochemical events such as, protein–protein interactions (PPIs), enzyme–substrate reactions or protein translocation events and the reactions which proved to be induced or enhanced by the protein-receptor interaction. The curation of signalling pathway reactions was based on the following criteria: (i) reactions should be stimulated by protein/receptor system, (ii) reactions must be either induced or enhanced in vivo, (iii) proteins involved in reactions should be from human system, however other mammalian signalling pathway data which are made available
using different community standards allows interoperability with other data visualization and analysis software such as PathVisio or Protein Lounge Pathway Builder (Rajesh et al., 2011). Moreover, various software tools are currently under development which converts user-defined formats into these standard pathway formats. A signalling network of signaling pathway was created using pathvisio software and designed using Protein Lounge Pathway Builder online tool. The number of steps which leads to any reaction from the stimulated receptor currently depends on the interconnectivity of molecules and their reactions were reported in a designed pathway.

3.7. *In-silico* molecular docking of the drug isolates with the target protein

Structural database survey revealed that the crystal structure of Rock1 protein of *Rattus norvegicus* (rat) is not available. Since the over all investigation is carried out using *Rattus norvegicus*. Hence further *in silico* drug designing studies by molecular docking needs Rock1 protein from rat source. Hence, homology modeling of rat Rock1 protein was carried out by using amino acid sequence of rat Rock1 protein and matched with human ROCK1 which showed 100% identity. Further, human ROCK1 protein structure was used as template (PDB ID: 2ETRA) for modeling rat Rock1 protein structure using swiss modeler and SPDBV tool, which was validated by Ramachandran Plot analysis using RAMPAGE server (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php). This modeled rat Rock1 protein structure was used for *in silico* drug designing studies.

Automated computational docking was used to determine the orientation of inhibitors bound in the active site of Rock1 protein. A Lamarckian genetic algorithm method implemented in the program AutoDock 3.0 was employed in molecular docking studies. The 3D structure coordinate file of ligand molecules viz., quercetin and oleanolic acid were generated using PRODRG server (Ghose and Crippen, 1987) and their structures were analyzed by using Chem Draw Ultra 6.0. The rat Rock1 protein structure file obtain by homology
modeling was collected and edited by removing the heteroatoms, adding C-terminal oxygen using SPDBV tool (Binkowski et al., 2003). For docking calculations, Gasteigere Marsili Partial charges were assigned to the ligands, nonpolar hydrogen atoms were merged, kollman charges were added and total charge was spread on all the residues (Gasteiger and Marsili, 1980). All torsions were allowed to rotate during docking. The grid map was set by adjusting the grid box centered at the following active pocket residues of the protein Rock1 [(Arg84(B), Gly85(B), Glu88(B), Val90(B), Ala103(B), His136(B), Val137(B), Met153(B), Met156(B), Asp202(B), Leu205(B), Ala215(B) and Asp216(B)]. The active pocket was predicted by studying the reports on protein interaction with the inhibitor and crystal structure of Rock1 protein with the inhibitors using PDBSum and CASTp server. These active pocket residues were used to generate with the AutoGrid file. The Lamarckian genetic algorithm was applied for minimization, using default parameters. The number of docking runs was 10. Number of energy evaluations was 100,000 and the maximum number of iterations 10,000.