Chapter VI

Summary
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Alcoholism is one of the major health problems and a primary cause of liver disorder all over the world. Prolonged consumption of alcohol leads to dreadful consequences associated with non-treatable liver conditions such as fibrosis or cirrhosis. Liver is the major site of metabolism and detoxification of alcohol and hence it is a system of choice for many investigators. Our study was also concerning ethanol toxicoproteomics and stress effects due to various pathobiological processes. Excessive consumption of alcohol at higher concentration causes a series of pathophysiological changes in the liver and led to a sequence of hepatic abnormalities that ranges from hepatic steatosis followed by steatohepatitis. With continued alcohol consumption for longer duration this stage may further proceed to chronic stages, that includes fibrosis, cirrhosis, hepatocellular carcinoma and finally to liver failure. Initial sign of alcoholic steatosis is the accumulation of triglycerides in the hepatocytes as micro or macrovesicles and this stage is generally considered as benevolent. Although, liver is endeavored with regeneration ability, the pathogenesis of alcoholic liver damage is reversible up to steatohepatitis stage. Beyond this stage the liver regenerating ability remains inefficient due to the complex pathogenesis that occurs during chronic stages of liver damage.

Data generated by proteomics analysis provides us with precious information to understand the molecular basis of alcohol induced hepatotoxicity and hepatoprotective effects of phytoconstituents isolated from Flaveria trinervia and also help us to follow the course of this disease. Eventually, it could lead to earlier diagnosis of liver pathology progressing into the irreversible fibrotic/cirrhotic stage, which is essential in determining the best course of treatment options and possible outcomes.
One of the most advanced and promising approach in quantitative proteomic analysis is the use of isobaric tags to label proteins from the two or more samples by iTRAQ followed by peptide mass spectrometry analysis. Isobaric Tags for Relative and Absolute Quantification (iTRAQ) is a set of amine-specific isobaric tags for multiplexed relative quantitation of proteins by peptide mass spectrometry. iTRAQ and peptide mass spectrometry are the advanced and superior technological applications in the field of proteomics to quantify the global changes in protein expression in relatively small amount of any complex biological system and yields efficient, reproducible and high-throughput information that can be used to identify potential biomarkers against any pathophysiological condition.

First part of this investigation focused on the isolation of potential bioactive phytochemicals from the research plant *Flaveria trinervia*, a member of Asteraceae. The choice of this plant was based on the fact that this plant is traditionally used to treat liver disorders, infected wounds and relieve pain (Shanthamma *et al.*, 1986; Yoganarasimhan and Chelladurai, 2000; Manjunath *et al.*, 2004; Umadevi *et al.*, 2004). However, reports on phytochemical studies of this plant are scarce in the literature. It is also reported that the leaf juice of the plant is used to cure jaundice, skin diseases, wound healing (Yoganarasimhan and Chelladurai, 2000; Umadevi *et al.*, 2004). Isolation of flavonoid patuletin-3- O-β-glucoside, oleanolic acid, β-sitosterol was reported from this plant. In our laboratory, Suresh Babu and Krishna (2008) conducted rigorous phytochemical investigations and isolated six phytoconstituents from this plant namely, Tridecan-1-ol, Flaveriopyranotriol from petroleum ether extract, Tricosan-1-ol from chloroform extract and Quercetin, β-sitosterol and Rutin from methanol extract. The comparative hepatoprotective activity of these constituents was screened against CCl₄ induced hepatotoxicity in rats.

Traditional herbal medicinal practitioners of Chitradurga and Davanagere Districts of Karnataka state, India are using *Flaveria trinervia* as a
promising drug for alcoholic liver disorders (Manjunath et al., 2004). The herbal practitioners noticed that this plant works as a best remedy for ethanol intoxicated liver cirrhosis than the other kinds of liver disorders. In the view of the above the present investigation was undertaken to authenticate the traditional medicinal claims of *F. trinervia* as a potent hepatoprotective drug against ethanol induced liver cirrhosis.

In the present study, the sequential solvent extraction of *Flaveria trinervia* whole plant was carried out and the extracts were collected and vacuum dried separately. 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 four extracts of *F. trinervia* showed negative test for the presence of alkaloids but showed positive tests for the presence of triterpenoids and sterols. The chloroform, methanolic and aqueous extracts showed positive for flavonoids and tannins. The presence of glycosides was noticed in methanol and aqueous extract. Each extract was profiled for its quantitative phytochemical constitution on the basis of their adsorbance using thin layer chromatography (TLC) and this complex of compounds in crude extracts were separated by column chromatography using silica gel by chromatographic techniques. The isolated compounds were characterized by IR, 1H NMR and Mass spectral studies.

The rigorous phytochemical study on this plant resulted in the isolation and purification of six compounds, among them five phytoconstituents are not yet reported by our previous investigators, Suresh Babu and Krishna (2008). However, the phytochemical quercetin was yielded in higher concentration as compared to other phytochemicals and this compound was also isolated by Suresh Babu and Krishna (2008).

Among the six phytochemicals isolated from the sequential extracts of *F. trinervia*, the three phytochemical isolated from the petroleum ether extract
using solvent proportion petroleum ether : ethyl acetate (8:2). The yield of phytoconstituents from crude petroleum ether extracts were P1 (700 mg/10g), P2 (900 mg/10g), P3 (1.2 g/10g). The phytochemicals isolated from petroleum ether extract are found to be novel and are identified following the rules of IUPAC and were identified as compound-P1: 4-(3-Hydroxy-but-1-enyl)-3-(2-hydroxy-1-methyl-propyl)-5,5-dimethyl-cyclohex-2-enone; compound-P2: 15 Hydroxy-5,10,14-trimethyl-pentadecanoic acid and compound-P3: 1-(Decahydro-naphthalen-2-yl)-ethanol.

The phytochemical Oleanolic acid was isolated from the chloroform extract using solvent proportion Hexane : ethyl acetate (8:2) with the yield of 850 mg/10g of chloroform extract. The earlier investigator Umadevi et al., (2005) also isolated this compound from F. trinervia. Whereas, in our laboratory the previous investigators isolated Tricosan-1-ol from the chloroform extract (Suresh Babu and Krishna, 2008). The methanol extract of this plant yielded two flavonoid compounds namely Syringetin and Quercetin using solvent proportion petroleum ether : ethyl acetate (2:8). Of these, quercetin was isolated in higher concentration (900 mg/10g of methanol extract) and it was also isolated by our previous investigators, Suresh Babu and Krishna (2008).

Plants are the store houses of a vast of array of phytoconstituents. Popularity of herbal remedies is increasing globally especially to treat the ailments of liver. Except the liver transplantation no other chemical drug can act as a potent remedy for liver cirrhosis, necrosis and cancer. Hence, more efforts need to be directed towards the methodological scientific evaluation of herbal medicine for their safety and efficacy by subjecting to vigorous preclinical studies followed by clinical trials to unravel the mysteries hidden in the plants. This approach will help in exploring the real therapeutic value of these natural pharmacotherapeutic agents and standardized the dosage regimen on evidence-based findings to become more than a fashionable trend (Stickel
and Schuppan, 2007). Many herbals drug are available in the market to support health, relieve symptoms and cure liver diseases. However, most of these products lack scientific validation and due to lack of scientific-based pharmacological data, most of the herbal formulations can not be recommended for the treatment of liver diseases.

In the preliminary investigations, the other preliminary pharmacological models were also carried out to justify the traditional medicinal claims of *F. trinervia*. We have screened the therapeutic property of this plant for antibacterial and anthelmintic property, CNS depressant activity and antinociceptive activity. The results obtained from the above said pharmacological models were published in the peer reviewed international journals *viz.*, Antinociceptive activity of crude extracts of *F. trinervia* (Joy *et al.*, 2011, *Natural Product Research*); Anhelminthic and bactericidal activity of crude extracts of *F. trinervia* (Joy *et al.*, 2011, *European Journal of Medicinal Plants*); CNS depressant activity of *F. trinervia* extracts (Joy *et al.*, 2011, *Phytopharmacology*).

The main objectives of this investigation are to investigate the prophylactic effect of the phytoconstituents of *Flaveria trinervia* against liver cirrhosis followed by proteomic analysis of ethanol induced hepatotoxicity and the effect of phytochemicals in suppression of differentially expressed protein in the ethanol intoxicated liver in rats. Since the main objectives were revolving on the proteomics analysis of the hepatotoxicity and hepatoprotectivity, the present investigation was focused on the prophylactic effect of the isolated constituents and proteomic studies.

Acute toxicity study revealed that the animals treated orally with the sequential extracts and the isolated phytoconstituents of *Flaveria trinervia* possessed bioactivity, based on the observable changes in the behavioural pattern and mortality of experimental. The LD₅₀ value of petroleum ether and
chloroform extracts was found to be 700 mg/kg b.w. Where as, the LD<sub>50</sub> value of methanol and aqueous extracts were 500 and 900 mg/kg b.w. respectively. Among the isolated constituents, 15-Hydroxy-5,10,14-trimethyl-pentadecanoic acid and Oleanolic acid showed highest LD<sub>50</sub> value at 700 mg/kg b.w. The compounds isolated from the petroleum ether extracts namely, 4-(3-Hydroxy-but-1-enyl)-3-(2-hydroxy-1-methyl-propyl)-5,5-dimethyl-cyclohex-2-enone and 1-(Decahydro-naphthalen-2-yl)-ethanol showed LD<sub>50</sub> value at 500 mg/kg b.w. The two flavonoids isolated from the methanol extract namely, Syringetin and Quercetin showed LD<sub>50</sub> values 300 and 200 mg/kg b.w. respectively. One-tenth of these doses were considered as safer dose for drug administration.

All the six phytochemicals isolated from the <i>F. trinervia</i> were screened for their <i>in vivo</i> antioxidant property by estimation of the activity levels of superoxide dismutase (SOD), catalase (CAT), peroxidase (PER) and TBARS in rat liver intoxicated with ethanol for 7 consecutive weeks. All the isolated phytoconstituents were administered orally at the therapeutic doses. The <i>in vivo</i> antioxidant activity of <i>Flaveria trinervia</i> revealed that among the animal groups administered with the isolated phytochemicals, oleanolic acid isolated chloroform extract and quercetin isolated from methanol extract showed significant antioxidant effect by maintaining the levels of oxidative stress enzymes upto normalcy. The levels of these oxidative stress enzyme markers were almost similar with that of the standard drug silymarin treated group. The restoration of levels of oxidative stress enzymes was moderate in the animal groups treated with the constituents of petroleum ether extract. But the compound-P1 [4-(3-Hydroxy-but-1-enyl)-3-(2-hydroxy-1-methyl-propyl)-5,5-dimethyl-cyclohex-2-enone] isolated from the petroleum ether extract effectively brought the oxidative stress enzyme levels nearer to normalcy.

Among the three phytochemicals of petroleum ether extract namely, Compound-P1 [4-(3-Hydroxy-but-1-enyl)-3-(2-hydroxy-1-methyl-propyl)-5,5-dimethyl-cyclohex-2-enone]; compound-P2 [15 Hydroxy-5,10,14-trimethyl-
pentadeconoic acid] and Compound-P3 [1-(Decahydro-naphthalen-2-yl)-ethanol], the compound-P1 exhibited significant hepatoprotective activity viz., AST (347.83 ± 4.29 IU/L), ALT (161.17 ± 3.3 IU/L), ALP (310.17 ± 6.32 IU/L), total bilirubin (6.38 ± 0.22 mg/dL), and direct bilirubin (1.52 ± 0.04 mg/dL) and the compound-P2 showed least toxic amelioration effect by bring the levels of AST (473.67 ± 4.08 IU/L), ALT (181.33 ± 3.92 IU/L), ALP (377.67 ± 1.45 IU/L), total bilirubin (10.11 ± 0.3 mg/dL), and direct bilirubin (1.77 ± 0.06 mg/dL) as compared with the standard drug silymarin viz., AST (201.37 ± 2.71 IU/L), ALT (133.5 ± 4.27 IU/L), ALP (231.08 ± 2.36 IU/L), Total bilirubin (2.4 ± 0.25 mg/dL), and Direct bilirubin (1.17 ± 0.13 mg/dL) (Table 4). The compound-C1: oleuropeolic acid isolated from the chloroform extract at 100 mg/kg b.w. showed more significant hepatoprotective effect viz., AST (195.45 ± 2.61 IU/L), ALT (113.83 ± 2.14 IU/L), ALP (222.5 ± 5.22 IU/L), Total bilirubin (2.4 ± 0.25 mg/dL), and Direct bilirubin (1.02 ± 0.08 mg/dL) and its value is nearer to the standard drug silymarin. The flavonoid compounds namely, compound-M1 (Syringetin) and compord-M2 (Quercetin) isolated from the methanol extract also exhibited significant hepatoprotective effect but the amelioration effect was more in quercetin treated animals viz., AST (192.58 ± 1.61 IU/L), ALT (102.02 ± 2.55 IU/L), ALP (212.88 ± 5.06 IU/L), Total bilirubin (1.99 ± 0.04 mg/dL), and Direct bilirubin (0.96 ± 0.06 mg/dL). The prophylactic effect the extracts and the constituents against ethanol induced toxicity was also supported with the histopathological studies of liver and in vivo antioxidant activity.

Histology of the liver sections of the control animals showed the normal hepatic architecture, absence of lymphocyte infiltration, well preserved cytoplasm, lack of necrosis, visible central vein and without fatty lobulation. The sections of 40% ethanol-intoxicated rats (for 7 weeks) showed total loss of hepatic architecture, stress fiber formation (Fig. 17b), lymphocyte infiltration, microvesicular and macrovesicular fatty changes (Fig. 17d and e). Further, it also exhibited areas of hemorrhage and intuition of fibrosis in the damaged...
region with excessive fat accumulation (Fig. 17c and d). In the case of rats treated with the standard drug silymarin (250 mg/kg b.w.) showed the regeneration of hepatocytes with compact cellular integration and prominent nucleus (Fig. 17f).

Among the six isolated constituents of *F. trinervia* significant hepatoprotective activity against ethanol induced liver damage were noticed in the liver samples of the animals treated with oleanolic acid and quercetin. They exhibited significant liver protection against ethanol induced liver damage as evident by the presence of normal hepatic cords, absence of necrosis, normal lobular pattern and well preserved cytoplasm (Fig. 20a and 20c). Among these phytochemicals hepatoprotective activity was well emphasized in the animals treated with the constituent quercetin, which is almost comparable to the control and silymarin treated groups (Fig. 20c).

The mechanism of alcohol induced liver fibrosis was investigated by studying the abnormal protein expression in alcohol intoxicated rat liver by iTRAQ and peptide mass spectrometry based quantitative proteomic analysis.

Quantitative proteomic analysis by iTRAQ and peptide mass spectrometry revealed that the proteins upregulated in ethanol induced liver damage in rats and this abnormal regulation was controlled by the effect of quercetin and oleanolic acid. This proteomics strategy helped us to identify and quantify the differentially expressed proteins which are previously described in several hepatic damage biomarker analysis studies and as well as non reported proteins. The results of iTRAQ studies revealed that 83 proteins were found to be differentially expressed. The proteins that were found to be expressed above 1.5 folds were considered as upregulated and proteins expressed below 0.7 folds were considered as downregulated. Among 69 proteins that were found to be upregulated in this study, 31 proteins have no previous description in context with hepatotoxicity and 21 of them were previously shown to be
associated with hepatic damage or hepatotoxicity and others were are reported either during liver cancer. Among the 14 proteins that were found to be downregulated, 11 proteins were not reported previously to be associated with hepatotoxicity.

The protein **Rho-kinase 1 (Rock1)** is reported as a liver fibrotic factor in several animal models. Rho-GTPase is an important regulator of cytoskeletal organization, migration and activation of hepatic stellate cells and myofibroblasts, which is actively mediated by Rho-kinase. In the present proteomic study elevated levels of Rock1 was observed with 2.3-folds which is an indicative of fibrotic condition of the liver. On the contrary, the liver samples of the animals administered with the constituents oleanolic acid and quercetin brought down the upregulated levels of Rock1 protein to the considerable extent i.e., 1.4-folds and 0.7-folds respectively. In the present research work, we have observed that Rock1, an importance protein involved in Rho-kinase signalling was found to be upregulated, revealing that alcohol treatment for 7 weeks had produced a fibrotic condition in rat liver (evidenced by histological studies). Hence, Rho kinase signaling pathway was studied based on the information obtained from proteomics analysis, literature survey and other knowledgebases. Rho-kinase signaling pathway pertaining to Rock1 was designed and built by using PathVisio and Protein Lounge pathway builder software tools.

The crystal structure of Rock1 of *Rattus norvegicus* was not available in the PDB structure database. Hence, this protein structure was modelled by homology modelling process using crystal structure of human ROCK1 protein with PDB. ID.: 2ETRA as the template. Further, the isolated small-molecule phytocompounds were fit into the target Rock1 protein structure using a docking program Autodock 3.0. The energies of the resulting complexes were evaluated and the one that show the most promising binding was considered as potent inhibitor.
Generally, small molecules isolated from plant source work as drugs by binding very specifically to certain locations on important target protein that play important role in liver disease, by targeting only catalytic sites that are uniquely present in the Rock1 protein, the mode of suppression of stress fiber formation has been evaluated. The same principle is also employed in the present study to hypothesize the mode of action of the phytoconstituent ligands oleanolic acid and quercetin on the candidate protein Rock1 by in silico molecular docking studies.

In the present investigation, the ligand molecules oleanolic acid and quercetin were prepared with their 3D co-ordinate files using PRODRG server. The homology modelled Rock1 protein structure was prepared for docking by removing the heteroatom, adding C-terminal oxygen, adding kollman charges, spreading total charges over all residues and retained the polar hydrogens. Further, the ligand molecules oleanolic acid and quercetin were separately docked in the catalytic pocket of Rock1, which was predicted by using PDBSum, CASTp and literature survey. Docking results revealed that quercetin exhibited significant inhibition constant as compared to oleanolic acid. It was also documented that quercetin could form two hydrogen bonds with MET156 and ASP160 amino acid residues within the catalytic pocket (Fig. 31b and 31e), but oleanolic acid could form only one hydrogen bond with only MET156 (Fig. 30b and 30e). Results of docking studies also revealed that oleanolic acid with Rock1 showed binding energy: -9.71, Docking energy: -9.71, Inhibition constant: 7.65e-008 and 1 hydrogen bonding with MET160 (bond distance: 2.098, bond energy - 4.304) (Table 9). Where as, docking of quercetin with Rock1 showed binding energy: -6.95, Docking energy: -7.47, Inhibition constant: 8.08e-006 and 2 hydrogen bonding with MET156 (bond distance: 1.829, bond energy: -0.203) and ASP160 (bond distance: 2.076, bond energy: -7.519) (Table 9). The in silico molecular docking study revealed that both the phytoconstituents quercetin and oleanolic acid proved to
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be the potent inhibitors of Rock1 protein and this investigation also supported
the results of pharmacological and proteomic analysis studies.

To conclude with, the outcome of this investigation revealed that the
phytoconstituents, quercetin and oleanolic acid isolated from the
hepatoprotective herb *Flaveria trinervia* potentially inhibited the function of
Rock1 protein, which is an important mediator involved Rho-kinase signalling
pathway. Therefore, inhibition of Rock1 directly corroborates with the
arresting of Rho-kinase signalling cascade and thus the pathogenesis of
hepatofibrosis was controlled by suppressing the production of stress fibers
during ethanol induced liver fibrosis in rats.