CHAPTER 4

SUMMARY AND CONCLUSION

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4.1 Summary

In India numbers of traditional systems of medicine are practiced since centuries. These include Ayurveda, Siddha, and Unani etc. apart from those having ethno botanical usage of herbs at regional level by the habitants or tribes as medicine. Although these systems possess rich heritage as health care, these were confined to Vaidyas, Hakeems, Traditional Dais, Bone Setters, and Massagers etc. and were not reached to industrial level of manufacture and distribution of different formulations practiced, due to lack of documentation of scientific facts regarding the properties of drugs and medicaments used.

Many of the important drugs of modern medicine are derived from plants as their constituents or those which are the modified form of the original constituents like digoxin, vincristine, quinine, taxol derivatives, artemisin and its derivatives etc. Investigations on plants for their biological utility therefore have become an important task throughout the world on a scientific basis. Generally the lead for these investigations in order to select the plant species comes from either the literature available in traditional systems or directly from among the folklore practitioners. This information provides a basis of screening these natural products or plants following a well accepted protocol in order to eliminate useless ones from useful one. Modern system of medicine although extremely equipped for combating many disorders by providing effective medicaments, still certain disease like hepatic disorders, viral infections, rheumatic disorders etc. could only be treated symptomatically. The available agents in alternative systems therefore need to be tapped for obtaining effective medicaments.

A significant number of populations in the country suffer from hepatic disorders of both known and unknown origin and the curative agents are still in developmental stage. The discovery of new drug entity from both natural and synthetic sources to combat hepatic disorders has become an identified area of thrust by many research groups.

The idea of present research study was evolved due to the extensive use of *Pergularia daemia*, commonly known as Dustapu teega in Telugu and Uttaravaruni in Hindi, by the folklore people of the Chittoor district of the Andhra Pradesh state in India, to treat jaundice. During the interaction it was known that powder of the aerial parts is directly dispensed in the form of small pills to affected hepatitis patients with water or honey as anupana for a period of two to three weeks showed successful results of the treatment. Similarly the Ayurvedic descriptions on the usage of roots of *Baliospermum montanum*, commonly known as Danti, in the treatment of jaundice also attracted.

Survey of literature on both these plants revealed that although these are used in many regions for hepatic disorders, a scientific data still unavailable as regards to their properties are concerned. Hence these two plants were taken up for a detailed study as regard to their hepatoprotective activity both in the extracted and isolates basis in order to verify claims made in the various systems of medicine regarding their effectiveness.

Liver, the largest and the most versatile by organ for metabolism and excretion, plays an important role in the maintenance of body's internal environment through its multiple and diverse functions. It is continually exposed to a variety of xenobiotics and therapeutic agents due to inadequately controlled environmental pollution and expanding therapeutic uses of potent drugs. Thus, the disorders associated with this organ are numerous and varied. Although a strict delineation of various hepatic disorders is not yet possible, from didactic point of view, these may be classified as acute or chronic hepatitis (inflammatory diseases), hepatosis (non-inflammatory disorders) and liver cirrhosis (degenerative disorder resulting in fibrosis).

Until recently it had been accepted almost as dogma that there was not and could not be any screening method for standardisation and evaluation of hepatoprotective drugs since most of the available methods do not simulate the clinical hepatic diseased conditions. Therefore evaluation of any compound with hepatoprotective claims in a single model does not suffice the purpose and needs to be based on multi models, which are in great demand

today. A review of literature reveals that several chemical substances and drugs having specific actions on liver are used as hepatotoxins in experimental animals to simulate ideal diseased conditions.

In all the test models, conditions for liver damage are implemented and an attempt is made to counteract this toxicosis with the substance/preparation under test. The magnitude of the protective effect can be measured by estimating the enzyme activities and the rate of survival and can be verified histologically. The available methods are *in vivo*, *ex vivo* & *in vitro* methods. All these methods are used to study the protective or curative effects of any compound under test. In order to test for hepatoprotective activity the test substance and the hepatotoxin are administered simultaneously whereas in case of antihepatotoxic or curative activity the test substance is generally administered after induction of hepatotoxicity.

Several chemical reagents and drugs which induce liposis, necrosis, cirrhosis, carcinogenesis and hepatobiliary dysfunctions in experimental animals are classified as hepatotoxins. The most important ones used are carbon tetrachloride, D-galactosamine, paracetamol, chloroform, ethyl alcohol and thioacetamide.

The present studies were planned to assess the hepatoprotective activity scientifically in rats against carbon tetrachloride, paracetamol and thioacetamide as hepatotoxins to prove its claims in traditional medicine against liver disorders and to isolate bio active molecules from the bio active guided extracts. Therefore, studies on the standardisation of the selected plants were carried out taking WHO guidelines for quality control methods for medicinal plant materials, into consideration.

Aerial parts of *P. daemia* were collected from foot hills of Tirumala, Andhra Pradesh state and their identity was confirmed at The Botanical Survey of India, Southern circle, Coimbatore, India. The voucher specimen (BSI/SC/5/21/05-06/Tech 1512) was also deposited at The Madras herbarium, The Botanical Survey of India, Coimbatore.The roots of the *B. montanum* were procured from Centre for Medicinal Plants Research, Arya Vaidya Sala, Kottakkal, Kerala state, India and authentified at the same centre. The

voucher specimen (HDT/CMPR/04/SVSK/2005) has been deposited in the Herbarium of the Institute. Pharmacognostic evaluations were also carried out to identify the diagnostic features, by studying morphological, microscopical characters of the organs under test. The presence of uni to multi cellular covering trichomes, collapsed covering trichomes, biseriate covering trichomes, rosettes and acicular raphides types of calcium oxalate crystals, stratified cork and anomocytic stomata forms the important diagnostic features of the *P. daemia*. Similarly presence of rosettes of calcium oxalate crystals, stratified cork, stone cells forms the important diagnostic features of the roots of *B. montanum* which helps in the identification and evaluation.

The plant drugs were then subjected to proximate analysis in order to develop standards, like determination foreign organic matter, loss on drying, extractive values (alcohol and water soluble), ash values (water soluble and acid insoluble), heavy metals, foaming index, total phenolic content and total flavonoid content etc.

The presence of different chemical constituents in crude drugs were detected by subjecting them to successive extraction using solvents in the order of increasing polarity and subjecting the successive extracts so obtained to qualitative tests for various chemical constituents. The successive extracts were then further subjected to TLC studies to confirm the presence of phytoconstituents. In aerial parts of *P. daemia* cardenolides, phenolic compounds and flavonoids, phytosterols, saponins, carbohydrates, amino acids and fixed oils; while in roots of *B. montanum* phenolic compounds and flavonoids, terpenoids and steroids, sterols, saponins, carbohydrates, amino acids and fixed oils were detected.

Ethanol and aqueous extracts of aerial parts of *P. daemia*; methanol and aqueous extracts of roots of *B. montanum* were prepared separately to determine the hepatoprotective activity on preliminary basis against CCl_4 induced toxicity at the dose levels of 200 mg/kg p.o. after subjecting them to acute toxicity determinations as per OECD guidelines. Silymarin was used as positive control. The ethanol extract of *P. daemia* and methanol extract of

B. montanum exhibited significant ($p<0.05$) hepatoprotective activity similar to silymarin.

The biologically active ethanol extract of aerial parts of *P. daemia* and methanol extract of roots of *B. montanum* were further subjected for fractionations in order to determine activity guided fractions of the plants. Bio active ethanol extract of *P. daemia* was fractionated using chloroform and 95% ethyl alcohol. The fractions obtained were concentrated under vacuum and the ethanol fraction was again fractionated using solvents of increasing polarity i.e. benzene, chloroform, acetone and 95% ethyl alcohol. The bio active methanol extract of roots of *B. montanum* was subjected to fractionation using chloroform and methanol. The fractions obtained were concentrated under vacuum. Methanol fraction of methanol extract was again fractionated with solvents, ethyl methyl ketone and methanol. All the fractions and sub-fractions were subjected to TLC studies to identify the constituents separated into various solvents. Phenolics, flavonoids, cardenolides and terpenoids were found to contain in fractions and sub-fractions of P. *daemia*; while phenolics, flavonoids, terpenoids and steroids in *B. montanum*.

The fractions and sub-fractions were then subjected to acute toxicities studies followed by hepatoprotective activity determination at various dose levels. Acute toxicity studies were performed according to the acute toxic classic method as per guideline no. 423 prescribed by OECD. Female albino rats were used for acute toxicity study. The groups of rats were administered orally with ethanol extract, ethanol faction of ethanol extract and acetone and ethanol sub-fractions of ethanol extract of aerial parts of *P. daemia* and methanol extract, methanol fraction of methanol extract and ethyl methyl ketone and methanol sub-fractions of methanol extract of roots of *B. montanum* up to a dose of 2000 mg/kg. None of these showed mortality even at the dose level of 2000 mg/kg and therefore considered safe.

The extracts, fractions and sub-fractions were then screened for hepatoprotective activity at various dose levels. The assessment of hepatoprotective activity was carried out by estimation of various biochemical parameters i.e. Glutamic oxaloacetic transaminase (GOT), glutamic pyruvic

transaminase (GPT), alkaline phosphatase (ALKP), total bilirubin (TBL), total cholesterol (CHL), total protein (TPTN), and albumin (ALB) in serum. The biochemical observations were supported by histological examination of liver sections of rats.

The active ethanol extract and its ethanol fraction of *P. daemia*; active methanol extract and its methanol fraction of *B. montanum* were subjected to evaluation of hepatoprotective activity in vivo using different toxicants, CCl_4, paracetamol and thioacetamide at various dose levels. Ethanol extract of *P. daemia* and methanol extract of *B. montanum* were tested at dose levels of 100, 200 and 300 mg/kg, while the fractions at dose levels of 50, 150 and 250 mg/kg. The ethanol extract (200 and 300 mg/kg) and its fraction (150 and 250 mg/kg) from *P. daemia* and methanol extract (200 and 300 mg/kg) and its methanol fraction (150 and 250 mg/kg) from *B. montanum* showed significant ($p<0.05$) hepatoprotective activity against all the selected hepatotoxins as observed with silymarin (100 mg/kg) a standard hepatoprotective agent. As a next step the acetone and ethanol sub-fractions from *P. daemia*; ethyl methyl ketone and methanol sub-fractions from *B. montanum* were subjected to hepatoprotective activity against carbon tetrachloride, paracetamol and thioacetamide induced toxicities at dose levels of 50, 100 and 150 mg/kg. The acetone sub-fraction (150 mg/kg) from *P. daemia* and methanol sub-fraction (150 mg/kg) from *B. montanum* exhibited significant ($p<0.05$) hepatoprotective activity in all the selected models. The activities exhibited by them were similar to silymarin.

In vitro hepatoprotective activity of acetone and ethanol sub-fractions from *P. daemia*; ethyl methyl ketone and methanol sub-fractions from *B. montanum* were carried out using different toxicants, carbon tetrachloride, paracetamol and thioacetamide on primary cultures rat hepatocytes. The hepatocytes were isolated from albino rats. The isolated hepatocytes are subjected to primary culturing using culture medium RPMI-1640 supplemented with calf serum (10%), HEPES and gentamicin (1 µg/ml). These cells were 97-98% viable as confirmed by trypan blue exclusion test. The acetone and ethanol sub-fractions of ethanol extract of *P. daemia*; ethyl

methyl ketone and methanol sub-fractions of methanol extract of *B. montanum* were subjected to hepatic cytotoxicity testing up to the dose level of 1500 mg/kg. From the results obtained it was found that these sub-fractions have no significant ($p<0.05$) toxic effects on primary cultured hepatocytes.

After confirming the safety of the sub-fractions on hepatocytes the sub-fractions were subjected to evaluation of hepatoprotective activity using carbon tetrachloride, paracetamol and thioacetamide as hepatotoxins at concentrations of 100, 500 and 1000 µg/ml. The results were compared with standard hepatoprotective agent silymarin 100 µg/ml. The assessment of hepatoprotective activity was carried out by measuring an increase in the percentage of viable cells in that group of cells incubated with sub-fractions, compared with the control and toxicant groups. Reversal of toxin-induced alterations in the levels of enzymes and proteins were also considered as an important criterion of protective activity. The acetone (at concentrations of 100, 500 and 1000 µg/ml) and ethanol (at concentrations of 500 and 1000 µg/ml) sub-fractions of ethanol extract of *P. daemia* exhibited significant ($p<0.05$) protective effect as observed with silymarin treated hepatocytes. Maximum protection was observed with acetone sub-fraction at a concentration of 1000 µg/ml and it also showed similar spectrum of activity to that of silymarin at this dose level. Similarly methanol (at concentrations of 100, 500 and 1000 µg/ml) and ethyl methyl ketone (at a concentration of 1000 µg/ml) sub-fractions of methanol extract exhibited significant ($p<0.05$) protective effect as with silymarin. Maximum protection was observed with MFMFME 1000 µg/ml and the activity was statistically similar to silymarin. Though EFEFEE was able to show significant ($p<0.05$) hepatoprotective activity in vitro at high dose levels it fails to show the same in vivo.

Active acetone and methanol sub-fractions from *P. daemia* and methanol sub-fraction from *B. montanum* were then subjected to isolation of the active principles by column and preparative thin layer chromatography techniques. PD1 and PD2 were isolated form the acetone sub-fraction of ethanol extract of *P. daemia*. The compound PD2 was found to be unstable and only PD1 was selected for further studies. Only BM1 was isolated form

with sufficient yield from methanol sub-fraction of methanol extract of *B.* montanum. Both the isolated compounds PD1 and BM1 which gave positive response to NP-PEG reagent were purified and subjected for hepatic cytotoxicity testing up to the concentration of 1000 µg/ml and in vitro hepatoprotective activity screening at concentrations of 10, 100 and 500 µg/ml using CCl_4, paracetamol and thioacetamide as toxicants. Both the compounds did not show significant ($p<0.05$) toxic effects on isolated primary cultured hepatocytes and were considered safe. Though the compounds PD1 and BM1 exhibited significant ($p<0.05$) protection against the selected toxicants at concentrations of 100 and 500 µg/ml the activity shown at concentration of 500 µg/ml was statistically similar to silymarin (100 µg/ml).

Then isolated compounds were subjected to the characterisation with the help of physical characterisation and spectral studies (IR, Mass, NMR studies). The compound PD1 isolated form *P. daemia* was identified as chlorogenic acid, a good hepatoprotective agent through its anti-oxidant properties. The compound BM1 isolated from *B. montanum* on the basis of data was identified as quercetin-3-O-galactosyl-7-O-rhamnoside, a flavonoid glycoside.

Finally the bio active extracts, fractions and sub-fractions from *P. daemia* and *B. montanum* were subjected to HPTLC studies to generate qualitative finger prints for flavonoids, in an attempt to develop standardised bio active extracts and fractions. The isolated compound PD1 and reported compound quercetin-3-glucoside (a good anti oxidant) from *P. daemia* were quantified using HPTLC and were found to be 0.174 and 0.086% w/w respectively. The estimation of these compounds with known hepatoprotective activity will resulted the quantification of ethanol extract and there by of plant material as part of their standardisation protocol. Similarly BM1 from *B. montanum* was quantified in methanol extract and was found to be 1.13% w/w.

4.2 Conclusion:

In present investigations ethanol extract, ethanol fraction of ethanol extract and acetone sub-fraction of ethanol extract of *P. daemia* and methanol

extract, methanol fraction of methanol extract and methanol sub-fraction of methanol extract of *B. montanum* exhibited good hepatoprotective activity in vivo against carbon tetrachloride, paracetamol and thioacetamide induced toxicities. Acetone sub-fraction of ethanol extract of *P. daemia* and methanol sub-fraction of methanol extract of *B. montanum* also showed protective effect against artificially induced toxicities in vitro. The activities were comparable to silymarin and were even similar to silymarin at higher doses tested. The isolated compounds PD1 (chlorogenic acid) and BM1 (quercetin-3-O-galactosyl-7-O-rhamnoside) also showed similar spectrum activity to that of silymarin when tested in vitro at a dose level of 500 µg/ml. Hence it may be hypothesized that flavonoids and phenolics, are responsible for the exhibited hepatoprotective activity of these plants. On the whole, these findings offer justification for their usage in alternative system of medicine for treatment of liver disorders.

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