CHAPTER 6

SUMMARY & CONCLUSION
Current scenario of cancer, a global threat, proposed the challenge in its early diagnosis and successful prevention through target specific drugs. Nitrosamines are one of the important causes of cancer. NPYR (N-Nitrosopyrrolidine), a nitrosamine, is a potent hepatocarcinogen that is found in the side-stream of cigarette smoke, fried bacon and other cured meats and also in drinking water. NPYR cause hepatic cancer via the formation of reactive oxygen species through liver microsomes. It exerts its carcinogenicity through CYP450 mediated metabolic activation in liver that leads to the formation of various intermediate products (alkylating agents) i.e. 4-oxobutanediazohydroxide, 2-hydroxytetrahydrofuran (2-OH-THF), crotonaldehyde. All have capability to form various DNA adducts that further cause mutations followed by hepatic cancer. Increasing side effects of allopathic medicines further burdened the health problems. Ayurvedic scenario of medicinal plants would be a better option to invent the target specific herbal drugs that would be full of no side effects in patient’s body due to herbal origin. Chemoprevention with the help of phytoconstituents rich *Indigofera tinctoria* crude extracts was already reported gave an idea for the preparation of novel extract (hydroethanol) from this plant which was followed by isolation of active constituents and then *in vivo* protective role against hepatic cancer. Results significantly reported the protective potential of hydroethanol and its isolated compound (ITC-1) against hepatic cancer and suggested *I. tinctoria* as the promising source in treatment of hepatotoxicity/cancer due to high antioxidant potential. Overall, the summarized form of complete research work is as follows-

- Shade dried *Indigofera tinctoria* aerial parts were purchased from Tindivanam, Tamilnadu, India and then authenticated and submitted at herbarium section of Department of Bioscience and Biotechnology, Banasthali University (BURI-13515).
- Plant powder was used as sample for the analysis of various physico-chemical parameters whereas various sequential extracts i.e. petroleum ether, chloroform, ethyl acetate and ethanol and non sequential extract i.e. hydroethanol were used as sample for phytochemical analysis.
- Physico-chemical study of plant powder revealed moisture content, foreign organic matter, ash values including total ash, acid insoluble and water soluble ash values, pH and extractive values including alcohol and water. Different extractive yields were also calculated that denotes that hydroethanol extract has much higher yield
(25.825±0.256 %) in comparison to other extracts. Behavior of plant powder was also evaluated in both day light and U.V. light after treated with different chemicals and reagents. Microscopic study of *I. tinctoria* powder also revealed the presence of several determinants like oil globules, xylem vessels, lignified fibers, trichomes, starch grains and calcium oxalate crystals. Pharmacognostical study of aerial parts powder of *Indigofera tinctoria* is helpful in identification and preparation of its monograph. These diagnostic indices have major role in study of crude drug (plant powder).

- Phytochemical or qualitative analysis of various sequential and non sequential extract revealed the presence of various phytoconstituents. Comparative study pointed that ethanol and hydroethanol extract (polar) possess varieties of important phytoconstituents (primary and secondary metabolites) i.e. alkaloids, steroids and terpenoids, tannins, phenols, flavonoids, saponins, anthroquinones, cardiac glycosides, carbohydrates, proteins, amino acids and was further selected for *in vitro* quantitative assays.

- Radical scavenging activity of *I. tinctoria* was established via study of DPPH, hydroxyl, superoxide, nitric oxide and metal chelating activities. Antioxidant prospective was also evaluated through the study of various parameters i.e. FRAP, total antioxidant capacity and reducing power assay. Quantitative assays for various phytoconstituents i.e. phenols, flavonoids, proanthocyanidine, saponins and tannins were also performed. Good radical scavenging and antioxidant activity of both ethanol and hydroethanol extracts were observed but comparatively hydroethanol extract showed better potency in comparison to ethanol extract. High scavenging and antioxidant potential of *I. tinctoria* was observed due to presence of good amount of polyphenols as confirmed by Karl Pearson correlation analysis that gave positive linear relationship between antioxidant and radical scavenging activity and phytoconstituents present in *I. tinctoria* extracts. This study indicated that hydroethanol extract of *I. tinctoria* is the good source of natural antioxidants that had shown potent therapeutic efficacy and was further selected for the isolation of one of the important bioactive compound i.e. Isothiocyanate.

- Presence of organosulfur compound in hydroethanol extract was confirmed qualitatively through PbS formation and then subjected to column chromatography to obtain large active subfraction (327-450) with yield 192 mg/5 g of extract. From this large fraction, a small bioactive subfraction 437-448 was collected that gave
positive test after sprayed with NaOH-Lead acetate solution in solvent system chloroform : methanol (6:4). This subfraction was then subjected to HPLC to see the extent of mixing and for further purification also.

- Reverse phase HPLC of subfraction 437–448 was done through gradient elution of mobile phase Water : Acetonitrile in RP-C18 column. HPLC gave 10 prominent peaks in chromatogram that suggested the presence of 10 different compounds in this fraction. Peak of interest at retention time 5.3 was successfully purified and again chromatographed with HPLC to see its purity and also by TLC. The prominent single peak of desired compound gave purity of 97% with yield 22 mg/5 g of crude hydroethanol extract. The compound was then lyophilized to get it in purest form. This purified compound was completely soluble in water and gave resin like appearance with light pale yellow color and polar in nature.

- For the possible structural elucidation and characterization of purified compound, various spectroscopic techniques were used i.e. FT-IR (Fourier Transform-InfraRed), 1H-NMR (Proton Nuclear Magnetic Resonance) and LC-MS (Liquid Chromatography-Mass Spectrometry) and possible structure has been established. The isolated compound was designated as ITC-1 having IUPAC name 1-[1, 2-Diisothiocyanato-2-(3-isothiocyanato-2,2-dimethyl-propylsulfanyl)-ethoxy]-3-isothiocyanato-2,2-dimethyl-propane; C_{16}H_{22}N_{4}OS_{5} (m/e 446.70). This compound is a isothiocyanate derivative because of the presence of N=C=S group and considered as natural derivative.

- The purified compound “ITC-1” was then further tested for its biological and therapeutic activity in vivo. Further in vivo study of both crude hydroethanol and ITC-1, has enlightened the therapeutic role of Indigofera tinctoria and its isolated isothiocyanate derivative ITC-1 against NPYR-CCl_{4} induced hepatic toxicity/cancer.

- For in vivo study, total 36 Swiss albino male mice has been divided into 6 group (n = 6). Group 1 was considered as control that received only vehicle (olive oil). Group 2 was carcinogen treated that received single dose of NPYR (120 mg/kg b.w.; intraperitoneal) on first day of treatment. On 14th day, first dose of CCl_{4} (3 ml/kg b.w.) was administered subcutaneously followed by another 5 doses (total 6 doses once in a week). Group 3 and 4 have been received hydroethanol extract of I. tinctoria (HEIT) as low dose (100 mg/kg b.w.) and high dose (300 mg/kg b.w.) orally respectively for 21 days regularly after carcinogen treatment. Similarly group
5 and 6 have been received standard herbal commercial hepatoprotective drug “Silymarin” (25 mg/kg b.w.; oral) and purified compound “ITC-1” (15 mg/kg b.w.; intraperitoneal) for 21 days regularly. The whole duration of treatment was 70 days.

- Blood was collected by heart puncture to isolate serum. Liver tissues of mice from each group were excised out. Various biochemical parameters were studied by using serum, liver homogenates and isolated liver microsomes to see the therapeutic role of crude HEIT and ITC-1 against carcinogen induced hepatic toxicity/cancer. Multiple comparisons of obtained results in different groups were done with control, NPYR and Silymarin that provided the effect of treatment on animals.

- Acute oral toxicity studies of HEIT reported that HEIT at dose 1000 and 500 mg/kg b.w. is safe up to some extent because some changes at tissue level were seen but not externally whereas 100 and 300 mg/kg b.w. were considered as completely safe and did not show any toxic symptoms externally as well as at tissue level thus have been selected for the in vivo study.

- Increment in % change in body weight and relative organ weight showed the toxic conditions whereas fewer changes were observed in HEIT, ITC-1 and Silymarin treated groups. Behavioral changes in mice along with morphological changes in mice and excised liver tissues were also observed that denotes the toxic symptoms of NPYR-CCl₄.

- Various oxidative stress markers i.e. LPO, SOD, CAT, GSH, GST, GPx and GR were evaluated because it is well reported that obliterations in these markers lead to the abnormal production of reactive oxygen species (ROS) that are responsible to induce toxicity, cell damage and cancer. It is also reported previously that oxidative stress play a major role in development of hepatocellular carcinoma and favours the proliferation of cancerous cells. NPYR has capability to induce the ROS generation via liver microsomes may have capability to alter the activity of these enzymes because these markers have key role in detoxification pathway. NPYR induced DNA adducts during its metabolism that hepatocarcinogenic potential by exerting DNA damage and mutations. CCl₄ works as an inducer of hepatotoxicity through metabolic activation and ROS generation thus cause a state of abnormal metabolic function.

- Lipid peroxidation is significantly associated with the destruction and impairment of membrane function, inactivation of membrane bound receptors and enzymes that leads to membrane damage. Increased LPO level due to NPYR intoxication
replenished by terminating efficiency of lipid peroxidation reaction and MDA formation by HEIT low and high dose, Silymarin and ITC-1 thus protected the membrane integrity of liver tissues from the detrimental effects of NPYR.

- Remarkable depletion in ROS scavenging enzymes i.e. SOD and CAT were observed in NPYR intoxicated mice suggested the accumulation of hydrogen peroxide in cells that may lead to oxidative damage but oxidative stress protecting efficacy of HEIT, Silymarin and ITC-1 restored the enzymatic activity of SOD and CAT efficiently (ITC-1 has efficiency to hold CAT level as in NPYR treatment) and protect the cell from cytotoxic effect of hydrogen peroxide.

- Increment in GSH, GST and GR whereas reduction in activity of GPx after NPYR intoxication unshielded the protective layer of hepatotoxic condition due to obliteration in detoxification processes, reduction in defense against lipid peroxidation etc. HEIT, ITC-1 and Silymarin significantly restored their level thus showed therapeutic effect against oxidative stress. According to results, the purified compound possesses biological significance in contrast to oxidative stress.

- As results stated ROS mediated imbalanced level of various oxidative stress markers may lead to hepatotoxicity, was further measured by levels of various hepatotoxic markers i.e. AST, ALT, ACP, ALP and Bilirubin. Significant increment in aminotransferases activities (AST and ALT) whereas declined level of ACP and ALP have been observed in NPYR intoxicated mice suggested the hepatotoxic condition possibly due to obliterations in metabolites transportation across cell membrane and secretary enzyme synthesis etc. It has been reported that elevation in total and direct bilirubin is also associated with the hepatic injury as indicated by NPYR intoxication in our results. Briefly, HEIT, ITC-1 and Silymarin treatments have significant ability to restore the level of various hepatotoxic markers that enlightened the anti hepatotoxic role of crude hydroethanolic extract of *Indigofera tinctoria* and its isolated ITC-1. In comparison to Silymarin, a commercial hepatoprotective drug, the purified compound ITC-1 showed better response against hepatotoxicity.

- Various liver lipid indices i.e. total lipid content, cholesterol level and triglyceride level were evaluated. Total lipid content and triglyceride (TG) level were found to be decreased after NPYR intoxication showed the condition of “dislipidemia” and might be due to inhibition of TG synthesis via involvement of various cytokines like IL-6, TNF-α, IL-1. Obliterations in various lipid indices in cancerous tissues
associated with their direct effect on membrane integrity, fluidity and regulation of cellular processes is related to growth and cell survival. Cholesterol level was found to be increased in hepatic tissues of NPYR intoxicated mice. Cholesterol, a major component of animal cell membrane, is required for the membrane integrity and fluidity. During cancer development, blockage of evacuation of cholesterol cause changes in its level that obliterates the membrane fluidity and integrity. Results showed that HEIT and its isolated ITC-1 have potent capability to normalize various lipid indices and suggested that I. tinctoria and its isolated ITC-1, isothiocyanate derivative exerts their therapeutic activities against hepatic toxicity/cancer potentially. Therapeutic activity of HEIT may be due to the presence of various phytoconstituents that have potent efficacy to regulate the action of cytokines and Protein Kinase C (PKC) expression in which ITC-1 is might be one of them. PKC is a signaling molecule that might be involve in the overproduction of cholesterol.

- To validate the condition of hepatic cancer and its curative aspect, various hepatic cancer markers were elucidated i.e. Cytochrome P4502E1, Cytochrome b5, 5’-Nucleotidase (NT), Gamma glutamyl transpeptidase (GGT) and α-Feto protein (AFP). Cytochrome P4502E1 and Cytochrome b5 were estimated in isolated liver microsomes whereas AFP was estimated in serum samples of treated mice.

- **GGT**, a membrane bound enzyme which is involved in transportation of some amino acids and peptides through the cell membrane to the extracellular fluids, was found to be overexpressed in NPYR intoxicated mice which is associated with the hepatobiliary damage, hepatocellular damage, liver tumors and hepatocellular carcinoma as reported previously. Increased GGT level causes resorption of GSH by preneoplastic cells that induce cell proliferation. HEIT, ITC-1 and Silymarin showed marked improvement in its level by decreasing its level upto normal. I. tinctoria and its isolated ITC-1 treatment may express their potential to modulate GSH transport and metabolism that further helpful in prevention of hepatic cancer.

- **5’-Nucleotidase (5’-NT)**, an intrinsic membrane glycoprotein hydrolyzes AMP to produce adenosine. It is the remarkable enzyme of liver damage resulting from interference with bile secretion. This enzyme has been found to be overexpressed in NPYR intoxicated mice that was normalized by high dose of HEIT (300 mg/kg b.w.) and ITC-1. Down regulation of this enzyme through HEIT and ITC-1 results the lower concentration of adenosine that slows the process of proliferation of
hepatic cancerous cells and showed the antihepatic cancerous activity of *Indigofera tinctoria* and its isolated compound. Silymarin also showed regulatory effect on this enzyme approximately equal to ITC-1.

- **AFP (α-Fetoprotein)**, a tumor-assisted oncofetal protein was found to be increased in serum of NPYR intoxicated mice that suggested the cancerous state. Both doses of HEIT i.e. 100 and 300 mg/kg b.w. and its isolated compound ITC-1 have potential to lowered the expression of AFP that might be unable to inhibit PTEN activity and cause particular changes in PI3K/AKT pathway followed the lower expression of CXCR4 that prevent the carcinogenic hepatocytes to further proliferate and migrate.

- **Cytochrome P4502E1 and Cytochrome b5** both have been found to be decreased when estimated in liver microsomes of NPYR intoxicated mice. This might be due to obliterations in allosteric interaction between Cytochrome b5 and CYP4502E1. Their levels were further recovered by the treatment of HEIT (100 and 300 mg/kg b.w.) and ITC-1. From this, it can be concluded that that HEIT and ITC-1 acts as inducer of CYP450 thus working as cancer blocking agent. The isolated and purified compound “ITC-1” showed better anticarcinogenic activity in comparison to Silymarin.

- Biochemical analysis was also validated by the histopathological evaluation of excised hepatic tissues of various treated groups that interpret the obliterations at cellular level. Microscopic results depicts several morphological changes in NPYR treated liver section of mice like distorted architecture, thickening of various lobule parts, distorted portal vein, dilated bile ductules and hepatic arteries, accumulation of infiltrated RBCs, granular and dispersed cytoplasm in swallow hepatocytes, loss of reticulin fibers, distorted hepatic chords and nucleus etc. that clearly showed the cancerous state of liver. Trabecular growth pattern, a characteristic feature of hepatocellular carcinoma was also observed. This damage was overcome by the treatment with *I. tinctoria* hydroethanolic extract and its isolated isothiocyanate derivative ITC-1. Plant and ITC-1 treatment showed remarkable improvement of cellular architecture i.e. properly arranged flattened epithelial cells with normal central vein, less infiltrated RBCs, well shaped portal vein and bile ductules, regenerated cytoplasm and reticulin fibers, normal sinusoids and proper shaped nuclei (ovoid or round).
The observed histopathological results clearly represent the curative aspect of hydroethanolic extract of *Indigofera tinctoria* and its isolated, purified and characterized isothiocyanate derivative “ITC-1” against hepatic cancer/toxicity. Overall, the possible mechanisms/reasons behind to exert their anticarcinogenic and anti toxic activities might be-

- Presence of the range of important phytoconstituents in hydroethanolic extract of *Indigofera tinctoria* in a good amount
- Good scavenging and antioxidant potential that might be helpful in ROS depletion
- Potential to protect the membrane integrity of cells
- Capability to halt the accumulation of various toxic products like hydrogen peroxide
- Improved detoxification process
- Capability to regulate the expression of various cytokines and kinases esp. Protein kinase C
- Ability to modulate GSH transportation, lowered expression of adenosine, increase expression PTEN followed the lower expression of CXCR4 via PI3K/AKT pathway that slower the process of development, proliferation and migration of hepatic cancerous cells

The present research work has been carried out to elucidate the protective role of *I. tinctoria* and its isolated compound against hepatic toxicity/cancer and on the basis of results, it can be concluded that *I. tinctoria* is the phytoconstituents rich medicinal and historical plant that possess potential protective aspect to treat hepatic cancer/toxicity. It’s isolated isothiocyanate derivative ITC-1 (1-[1, 2-Diisothiocyanato-2-(3-isothiocyanato-2,2-dimethyl-propylsulfanyl)-ethoxy]-3-isothiocyanato-2,2-dimethyl-propane) also possess potential curative aspect in this way. In the best of my knowledge, ITC-1 is the novel herbal isothiocyanate compound that is isolated first time from this plant. Plants are god gifted valuable source of drugs but it should be our willingness to save these plants and invent new and potent drugs due to their specificity and potentiality to treat various health problems. Increasing cases of cancer every year develop the great fear in heart of peoples thus to get the success in cancer prevention, it is very important to formulate novel cancer blocking agents and obviously, the medicinal plants are the richest source of
novel compounds that may have therapeutic properties. ITC-1 is the one anticancer agent of *Indigofera tinctoria* plant but still many are unknown that have great prospects in future medicines.
SIGNIFICANT FINDINGS
Pharmacognostical analysis serve as initial preliminary but important step in identification of crude drugs before carry out next one. In this way, pharmacognostical analysis of *I. tinctoria* powder reveals its various properties like pH, extractive values, ash content, moisture content etc. whereas microscopic studies introduced the presence of various characteristic moieties.

Phytochemical screening reveals the presence of various primary and secondary metabolites like carbohydrates, proteins i.e. sulphur containing, flavonoids, cardiac glycosides, steroids, triterpenoids, tannins and phenols etc. in polar extracts i.e. ethanol and hydroethanol of *Indigofera tinctoria*. Overall, polar extracts are rich in varieties of phytoconstituents in comparison to non polar and mid polar extracts and was further selected for *in vitro* study.

*In vitro* quantitative, radical scavenging and antioxidant activity revealed that hydroethanol extract of *I. tinctoria* possess more potential aspect for further studies in comparison to ethanol extract because of the presence of good amount of phenols and flavonoids along with free radical scavenging and antioxidant activities. Good positive correlation was also observed between antioxidant and phytoconstituents suggested the possible involvement of phytoconstituents to exerting the potent activities.

Polyphenols esp. flavonoids and phenols, tannins, saponins, steroids, terpenoids etc. provide the antioxidant potential along with various therapeutic properties to the plant thus makes the plant valuable in medicinal system.

Hydroethanol extract of *I. tinctoria* was subjected to isolation and characterization of Isothiocyanate compound via various techniques i.e. chromatographic separation, HPLC, FT-IR, 1H NMR and LC-MS. The isolated isothiocyanate derivative was characterized as 1-[1,2-Diisothiocyanato-2-(3-isothiocyanato-2,2-dimethyl-propylsulfanyl)-ethoxy]-3isothiocyanato-2,2-dimethyl-propane with molecular formula C_{16}H_{22}N_{4}OS_{5} with m/z 446.70; ITC-1. Presence of –N=C=S group, reveled that this compound is the natural derivative of isothiocyanate.

Overall, crude *I. tinctoria* hydroethanolic extract and ITC-1 both showed hepatoprotective activity by normalizing several hepatic toxicity and cancer marker enzymes. Both also have capability to restore the NPYR induced oxidative stress through normalize the enzymatic activity of various oxidative stress marker enzymes.

Histopathological evaluation of liver tissues of various treated groups also showed remarkable changes. Crude *I. tinctoria* hydroethanolic extract and ITC-1 showed improvement in morphological nature of hepatocytes, hepatic chords, sinusoidal
spaces, cell architecture, nuclei morphology; hepatic portal triads etc. in comparison to NPYR treated groups.

- Silymarin also showed improvement at both i.e. histological basics (tissue level) and biochemical level but showed less activity in comparison to \textit{I. tinctoria} hydroethanolic extract. At some cases, Silymarin showed less positive response in comparison to ITC-1. At tissue level, this statement also validates the protective role of ITC-1.

- The new potent herbal derivative i.e. ITC-1 from \textit{I. tinctoria} that belongs to the family Fabaceae showed no side effect that serves as the potent chemotherapeutic agent and also helpful in growth of hairs as shown by investigated findings in experimental mice.

- Hydroethanolic extract of \textit{I. tinctoria} and isolated compound ITC-1 both are completely novel because no data regarding these are present. ITC-1 is the first novel herbal isothiocyanate derivative that is extracted from \textit{I. tinctoria} medicinal plant.

Therefore, the findings significantly enlightened the medicinal values of \textit{I. tinctoria}. The study also suggested that \textit{I. tinctoria} is a phytoconstituents rich medicinal plant that has great aspect in treatment of several health problems including cancer, a global threatening health problem in future. Hydroethanolic extract of \textit{I. tinctoria} may act as the reservoir of various therapeutic agents including anticancerous agents that further lighted the importance of god gifted valuable medicinal plants.