Preface

Liver is one of the most exciting organs in entire body is so-named because it is essential for living. It is the largest internal organ and great alchemical factory with a very sophisticated shipping/receiving system. It detoxifies much of what comes into our body and ships off the many nutrients and tweaks for the requests of the rest of our body. Being the alchemist, the magician, its major function is detoxification turning raw bulk material into the golden nectars that run our body, still this sorcerer suffers from drug induced insult.

Hepatotoxicity implies chemical-driven liver damage. Certain medicinal agents, when taken in overdoses and sometimes even when introduced within therapeutic ranges, may injure the organ. Drugs often cause subclinical injury to liver which manifests only as abnormal liver enzyme tests. Drug induced liver injury is responsible for 5% of all hospital admissions and 50% of all acute liver failures.

Tuberculosis is a chronic infectious disease and a major global health problem because of hepatotoxicity of anti-tubercular drug. This is a serious adverse drug reaction and major health burden because it causes significant morbidity and mortality. Although better drugs are available for managing tuberculosis but treatment
failure is one of the common problems encountered. Drug induced hepatotoxicity is one amongst the various causes which can cause treatment interruption. Isoniazid, rifampicin and pyrazinamide each in itself are potentially hepatotoxic, when given in combination their toxic effects are enhanced.

Today’s Anti–TB drug regimen takes too long to be effective and requires too many medications. Treatment of drug-sensitive disease requires 6–9 months whereas treatment of drug-resistant TB is even lengthier, taking 18–24 months or longer. Second-line drugs are also much more toxic and considerably more expensive than the standard first-line anti TB regimen. A range of novel anti-tuberculosis drug are in preclinical development, several phase 2 and 3 trials are underway, and use of adjunct therapies is being explored for drug-sensitive and drug-resistant tuberculosis. Historical advances include approval of two new drugs, delamanid and bedaquiline. Combinations of new and existing drugs are being assessed to shorten the duration of therapy. But potential cardiologic side effects have been associated with their use, hence recently there has been an upsurge of interest in the therapeutic potential of medicinal plants - undoubtedly a valuable source for reducing tissue injury.

Approximately 80% of the world total population depends exclusively on plants for their health and healing as plants produce a diverse range of bioactive molecules, making them a rich source of different types of medicines. A rich heritage of knowledge to preventive and curative medicines was available in ancient scholastic works included in the Atharva veda, Charaka, Sushruta etc. Over 50% of all modern clinical drugs are of natural product origin and natural products play an important role in drug development programs in the pharmaceutical industry. Herbal drugs have gained importance in recent years because of their efficacy and cost effectiveness.

**Phyllanthus amarus** Linn. belongs to the family Euphorbiaceae, grows as small annual herb in India and other tropical regions of the world. It is commonly known as *bhumi amla*. It is a broad spectrum medicinal plant that traditionally used to treat flu, dropsy, diabetes, and jaundice arthritis, skin ulcer, diuresis, hepatic and urolitic diseases. Phytochemical analysis of *Phyllanthus amarus* extract showed that primarily it contains lignin- phyllanthin as active principle.
**Phyllanthin** is one of the pharmacologically active constituents which is known to possess anti-inflammatory and analgesic action.

Four drugs that form the core for first-line anti-TB treatment regimens are isoniazid (INH), rifampicin (RIF), ethambutol (ETH) and pyrazinamide (PZA). These drugs have been associated with hepatotoxicity and risk is enhanced when these drugs are used in combination.

**Adverse drug reactions commonly associated with anti-tuberculosis drug**

**Pyrazinamide**
- Hepatitis
- Increased transaminases
- Fibrosis and cirrhosis
- Focal cholestasis

**Isoniazid**
- Hepatic cellular damage
- Steatosis
- Alterations in hepatic gene expression
- Mitochondrial oxidative stress
- Hepatic dysfunction associated with metabolic abnormalities like lactic acidosis, hyperglycemia

**Rifampicin**
- Hepatocytic necrosis
- Jaundice
- Increased liver function tests
- Cholestasis

**Ethambutol**
- Liver damage
- Hyperuricemia
- Cholestasis
India is rich in having a vast medicinal flora that can be explored for treatment of many diseases and organ dysfunctions. The 21st century has seen a paradigm shift towards therapeutic evaluation of herbal products in liver disease models by carefully synergizing the strengths of the traditional systems of medicine with that of the modern concept of evidence based medicinal evaluation, standardization and randomized placebo controlled clinical trials to support clinical efficacy. Thus study was conducted taking following aim and objectives.

**Aim:**

*Scientific validation of Phyllanthus amarus and its active principle on anti-tuberculosis drug induced hepatic injury at biochemical and molecular level, to develop natural herb as a candidate therapy for TB patients.*

**Objectives:**

The present study was a meticulous endeavor with the following objectives:

- Evaluation of antioxidant potential of plant extract
- Restoration of membrane integrity of hepatocytes
- Detoxification strategy using drug metabolizing enzyme cytochrome P-450
- Measurement of modulating potential of effective therapy on cytokines production during experimental regimen
- Revitalization effect on DNA damage
- To understand mechanism of apoptosis
- Recovery pattern in cell viability of Hep-G2 lines
- To assess the degree of recovery pattern by observing histopathological and ultrastructural alterations

Thus, a thorough scientific study was conducted to arrive at vital inferences. The entire study was divided into 6 protocols that include 11 sets of experiments:
Experimental design

**Protocol-I: Determination of Antioxidant activity**
*Experiments 1-4:*
- Total phenolic contents
- Free radical scavenging activity - DPPH assay
- Hydrogen peroxide scavenging activity
- HPLC analysis

**Protocol-II: In vitro study**
*Experiment 5:*
- Determination of cell viability by SRB assay

**Protocol-III: Selection of optimum doses against ATD**
*Experiments 6-7:*
- Screening of PA
- Screening of PY

**Protocol-IV: Therapeutic effectiveness of PA and PY**
*Experiment 8:*
- Comparative evaluation of PA and PY

**Protocol-V: Evaluation of safety profile of selected drug**
*Experiments 9-10:*
- Choleretic activity
- Anti pyretic activity

**Protocol-VI: Chronic studies**
*Experiment 11:*
- Molecular mechanism of recovery

Materials and Methods

*Phyllanthus amarus* (PA) and Phyllanthin (PY) were evaluated for its protective efficacy against ATD induced liver injury.

Animals were administered combination of ATD { Isoniazid (70 mg/kg) + Rifampicin (52 mg/kg) + Pyrazinamide (175 mg/kg) + Ethambutol (140 mg/kg), p.o.} for 4 & 8 weeks (3 alternative days in a week). *Phyllanthus amarus* (100, 200, 300, 400 mg/kg, p.o.) and Phyllanthin (3, 6, 9 mg/kg, p.o.) were evaluated at different dose levels for its protective efficacy against ATD for 8 weeks (3 alternative days in a week).
Detailed investigations on effective doses were done. Optimum dose of *Phyllanthus amarus* (300 mg/kg, p.o.) and Phyllanthin (6 mg/kg, p.o.) were given for 8 weeks (3 alternative days in a week) along with the anti-TB drug.

Liver function tests (AST, ALT, LDH, SALP, albumin, bilirubin, ATPase, G-6-Pase), kidney function tests (urea, creatinine, uric acid, BUN) and oxidative stress markers (SOD, CAT, LPO and GSH) were estimated. Antioxidant status in liver tissues, GSH cycle as well as the levels of aniline hydroxylase, amidopyrine-N-demethylase and microsomal lipid peroxidation and reduced glutathione, cytochrome-P450-2EI, TNF-α and IL-6 were evaluated. Histological and ultrastructural studies were observed and DNA damage was assessed with comet assay.

PA was investigated for chronic study. Animals were administered with anti-tuberculosis drug for 12 weeks (3 alternative days in a week) followed by therapy with PA (300 mg/kg, p.o.). Detailed biochemical and molecular markers were evaluated. Histological and ultrastructural studies were also observed.

**Observations**

**Protocol I**

The first protocol dealt with the evaluation of antioxidant and free radical scavenging properties of plant extract by determining DPPH free radicals, H$_2$O$_2$ scavenging activity and total phenolic contents. The HPLC analysis was also conducted.

The first experiment dealt with free radical scavenging activity of plants extract by DPPH assay, which is an easy, rapid and sensitive way to estimate antioxidant activity of specific compound or plant extracts. DPPH is a relatively stable free radical, which when encounters proton donors such as antioxidants, the radicals get quenched and absorbance gets reduced. Result indicated definite scavenging activity of the extract. *Phyllanthus amarus* at different doses *i.e.*, 10 to 50 µg/ml showed free radical scavenging activity in dose dependent manner. Up to 75.9% of DPPH inhibition was observed at the highest dose of 50 µg/ml. The result was comparable to standard vitamin C.
Second experiment dealt with H$_2$O$_2$ scavenging activity in which *Phyllanthus amarus* showed concentration dependent efficacy, maximum inhibition 75.9% was observed at 50 µg/ml. The result was comparable to standard ascorbic acid.

Third experiment dealt with the estimation of total phenolic contents in plant extract by Folin-Ciocalteu reagent. Phenolic compounds are known as powerful chain breaking antioxidants because of their scavenging ability due to their hydroxyl groups. These phenolic compounds may contribute directly to anti-oxidative action mainly due to the redox properties which showed an important activity in adsorbing and neutralizing free radicals, entrapments of singlet and triplet oxygen or oxidising peroxides. The amount of phenolic contents was calculated as tannic acid equivalents. It was found to be 291µg/mg in the PA sample, which indicated considerable free radical scavenging activity.

Fourth experiment dealt with the high performance liquid chromatography analysis. HPLC based phytochemical analysis showed presence of phyllanthin in PA. Thus, plant could be a good source of natural antioxidants.

**Protocol II**

Experiment-5 dealt with in vitro evaluation of therapeutic agents by determining cell viability. Percentage of cell viability of Hep2 cell lines were carried out by using SRB assay. The cell viability rate was significantly decreased (14.2%) in HepG2 cell lines by ATD (60 µM) administration. PA and PY (30, 50 and 100 µg/ml) treated cells enhanced the proliferation of HepG2 cell lines in culture media in a concentration-dependent manner. PA was more effective as compared to PY. Higher doses of PA and PY at 100 µg/ml showed 89%; 87% cell viability, whereas 50 µg/ml and 30 µg/ml showed 84%; 82% and 79%; 77% cell depicting significant regeneration. No adverse effects were found in cells after per se treatment of PA, PY and silymarin concentrations of 30, 50 and 100 µg/ml.

**Protocol III**

Experiments-6 and 7 was planned to evaluate the optimum doses of *Phyllanthus amarus* (PA) and phyllanthin (PY) against anti-tuberculosis drug
(INH+RIF +PZA+ETH) intoxication. In 6th experiment, ATD (70, 52, 175, 140 mg/kg, p.o.) was administered for 8 weeks (3 alternative days in a week) followed by the treatment with PA (100, 200, 300 and 400 mg/kg, p.o.) and in experiment 7th treatment with PY (3, 6 and 9 mg/kg, p.o.) for 8 weeks (3 alternative days in a week considering every next day of anti-TB drug treatment). Results revealed that administration of toxicant caused significant increase in levels of serum transaminases (AST & ALT) and SALP, in cases of liver damage with hepatocellular lesions and parenchymal cell necrosis, these marker enzymes are released from the damaged tissue into the blood stream.

ATD treated animals showed significant elevation in albumin, bilirubin, urea, uric acid, creatinine and BUN level and decrease in the level of ATPase and G-6-Pase. Subchronic exposure with ATD caused drastic elevation in the LPO levels and a further decrease in glutathione (GSH) levels which suggests excessive formation of free radicals, resulting in failure of antioxidant defense system. Significant decline was also found in SOD and CAT activities. Therapy with PA and PY both showed recovery in blood and tissue biochemistry. PA at all four doses (100, 200, 300 and 400 mg/kg) and PY at all three doses showed almost same pattern of recovery in all the parameters against toxicant. Maximum protection was seen with PA 300 mg/kg. PY at 6 mg/kg dose showed maximum recoupment. This study revealed significant improvement in the functional status of the liver cells and thus, showed marked hepatoprotective effect.

These findings were also supported by light microscopy of liver. In our study, ATD administration produced central venous congestion with heavy lymphocytic infiltration, hypertrophied nuclei, dialation in sinusoidal spaces, reduced number of kupffer cells loss of lobular architecture with damaged cellular outlines, pycnotic nuclei and fatty changes. With administration of PA at 300 mg/kg there was reduction in necrosis and normal histoarchitecture was observed. Distinct sinusoids, hexagonal hepatocytes with well-preserved cytoplasm and no lymphocytic infiltration were clearly observed. PA at 100, 200 mg/kg and 400 mg/kg also showed retrieval in the hepatocytes with mild degree of degeneration, thus clearly showing less improvement when compared to 300 mg/kg. Treatment with PY presented recoupment at 6 mg/kg
in the hepatic sections. Treatment of PY at 3 mg/kg dose showed presence of normal hepatic cords minor vacuolization, lymphocytic infiltration, and absence of necrosis. Treatment of PY at 9 mg/kg showed comparatively maintained chord arrangement, cuboidal hepatocytes, well-preserved cytoplasm, clear sinusoidal spaces, absence of necrosis and moderated hepatocytes swelling. However, regeneration was more pronounced in animals treated with PY at 6 mg/kg as it restored most of its normal architecture. Hepatocytes were radially arranged with well-preserved cytoplasm and minimum perinuclear vacuolization in hepatocytes. PY at 6 mg/kg showed almost same recovery as in silymarin positive control. Therapy with PA and PY stabilized the anti-tubercular drug induced histopathological alterations. This signifies the improved functional status, accelerated regeneration of parenchyma cells, defended against membrane fragility and decreased leakage of the markers enzymes into the circulation. Thus, 300 mg/kg dose of PA and 6 mg/kg dose of PY were investigated further and evaluated against ATD exposure.

Protocol IV

Experiment 8 dealt with the Comparison of effectiveness of PA and PY against Anti TB drug. In this experiment ATD were administered to the animals for 8 weeks (3 alternative days in a week) followed by therapy PA (300 mg/kg) and PY (6 mg/kg) for 8 weeks (3 alternative days in a week considering every next day of anti-TB drug treatment). The toxicity was manifested by decrease in the activities of GSH cycle enzymes glutathione reductase (GR), glutathione-S-transferase (GST), glutathione peroxidase (GPx) and glucose-6-phosphate dehydrogenase. GSH and its linked enzymes especially GR, plays a vital role in scavenging ROS, maintaining GSH pool and responsible for recoveries. It is reported that, the toxic metabolites of isoniazid and pyrazinamide bind to and damage cellular macromolecules in the liver which contains GSH and the antioxidant enzymes SOD, CAT and GPx. In the present study, the glutathione levels were restored to normal with therapy PA and PY. This may be due to an initial reduction in hepatic peroxidative activities followed by inhibition of the activities of the GSH-dependent enzymes, thereby leading to restoration of the GSH content. PA was found to be more effective in recouping the
levels of GR, GPx GST and Glucose-6-phosphate dehydrogenase when compared to PY.

The activity of drug metabolizing enzyme aniline hydroxylase (AH) is specific for CYP2E1 and aminopyrine N-demethylase (AND) is specific for CYP1A2, which were estimated in microsomal fraction. The damage inflicted by ATD caused a loss of drug metabolizing capacity of the liver. The microsomes of liver originats from the ER are the most susceptible site of LPO. In the present investigation, results demonstrated that ATD caused decrease in AH and AND activity and augmented LPO in microsomal fraction which might be due to damage to the smooth endoplasmic reticulum. This fact was also well supported by ultrastructural observations, which showed degeneration in smooth endoplasmic reticulum (the main sites for MDMEs) as a result of ATD intoxication. Our results indicated that PA and PY counteracted inhibition in AH and AND activity and inhibited the event of microsomal LPO significantly thus, showed their hepatoprotective effects by regulating the levels of drug metabolizing enzymes. Maximum recovery was observed by PA.

The in vivo comet assay (single-cell gel electrophoresis assay) was done for evaluation of DNA damage and assessing the potential of therapeutic agent in DNA repair by measuring tail movement, tail length and % DNA damage in liver of rats. In toxicant groups, the tail lengths and tail movement were significantly increased in liver. DNA damage was raised. Our data suggests that ATD administration was associated with oxidative stress induced DNA damage in liver. Therapies controlled DNA damage significantly indicating that test drugs rich in antioxidants might contribute to delay or prevent DNA damage. PA presented better collaborative effect in scavenging ROS as compared to PY.

These findings were also supported by transmission electron microscopic observations of liver. Eight weeks exposure to ATD showed unclear liver cell ultrastructure along with blurring of the mitochondria, ruptured membranes and unclear cristae, cholestasis, heavy vacuolation and scanty glycogen rosettes. Treatment of PA at 300mg/kg protected to a large extent of hepatic lesions produced
by ATD. The nucleus was normal in appearance and mitochondria were normal. Extensive ER with diminished centrilobular hepatocytes was observed. PA 300 mg/kg treated animals revealed improvement in nucleus, mitochondria and endoplasmic reticulum. Treatment with PY restored ultra-structure of hepatocytes but to a lesser extent when compared to PA.

On the basis of above all observations, it was statistically comprehended that PA exhibited better protective effect in comparison to PY. Thus, PA was analysed further to study its effect on specific hepatocellular markers and mechanism of resurgence of therapy by chronic study.

**Protocol V**

Studies on specific hepatocellular markers were conducted in protocol 5. The ninth experiment was conducted for determination of bile flow and bile solids. Bile is partially an excretory product of liver, which plays an important role in digestion. It removes drugs, toxins, pigments and various inorganic substances either derived from diet or synthesized by the body as cholesterol or cholic acid. Results represent choleretic activity which was estimated by the rate of bile flow and amount of bile solid contents. Treatment of Phyllanthus amarus (300 mg/kg) slightly stimulated liver activity, this was compared to DHC (standard drug), thus indicating no adverse effect of PA on liver cells.

The tenth experiment was conducted to determine antipyretic activity of plant extract. An increase in the body temperature is associated with elevated lipid peroxidation level, suggesting that yeast induced pyrexia in rat was associated with increased oxidative stress. antipyretic effect of PA was observed. This was well compared to silymarin group.

**Protocol VI**

This protocol dealt with chronic exposure to ATD to evaluate the mechanism of recovery by PA. Animals were administered ATD for 12 weeks (3 alternative days in a week) followed by therapy with PA 300mg/kg (3 alternative days in a week considering every next day of anti-TB drug treatment for 12 weeks). Administration
of ATD for 12 weeks resulted in significant elevation in AST, ALT, LDH, SALP, albumin, bilirubin, urea, uric acid, creatinine and BUN level into blood circulation when compared with control group. Tissue biochemical parameters such as ATPase, G6Pase, LPO, GSH and antioxidant enzymes i.e., CAT, SOD were also altered after ATD intoxication. GSH cycle, which includes GR, GPx and GST were altered after exposure to ATD. The ATD exposure significantly increased the activity of hepatic CYP-450 2E1 and decreased AH and AND as compared with control group. PA showed protective influence on Microsomal LPO and GSH by fighting against free radicals. ATD induced inflammatory response was expressed by enhanced the level of TNF-α and IL-6 in serum.

Treatment of PA significantly attenuated the blood and tissue biochemical alterations, this might be due to destruction of free radicals that were already formed or by supplying a competitive substrate for unsaturated lipids in the membrane and/or by accelerating the repair mechanism of damaged cell membrane.

Chronic ATD exposure caused liver damage as seen in kupffer cell activation with the release of TNF-α and interleukins indicating considerable inflammation. Treatment with PA along with ATD markedly suppressed the level of cytokines IL-6 and TNF-α may be due to anti-inflammatory role of test drug. Anti-inflammatory effects of PA can be related to inhibition of the transcription factor nuclear factor-κB (NF-κB), which regulates and coordinates the expression of various genes involved in inflammation. PA also protected DNA damage induced by ATD.

Ultrastructure of ATD intoxicated liver revealed deformed nucleus, disarrangement in rough endoplasmic reticulum and heavy vacuolation in cytoplasm as a result of degeneration of cellular organelles. Therapy with PA extract showed normal nucleus with intact nuclear membrane and well organized mitochondria. Thus, it may be PA possesses significant hepatoprotective effects.

Antitubercular drug induce hepatitis by a multiple step mechanism involving combination of four drugs. ATD are extensively metabolized by the liver and induces multiple hepatic enzymes including the CYTP_{450} enzymes. Rifampicin known to
increase isoniazid’s toxicity when used together with it. Isoniazid is acetylated and then hydrolyzed, resulting in isonicotinic acid and monoacetyl hydrazine; the later compound is activated to a toxic species by cytochrome P450. The conversion of acetylhydrazine to a toxic metabolite via cytochrome P450 leads to hepatotoxicity. Reactive metabolites of acetyl hydrazine are probably toxic to tissues through free radical generation. In rats, the free radical scavenger glutathione-related thiols, and the antioxidant GPx, SOD and CAT activities are diminished by isoniazid. The combination of INH and RIF was reported to result in higher rate of inhibition of biliary secretion and an increase in liver cell lipid peroxidation. Pyrazinamide in combination with these drugs is also associated with an increased incidence of hepatotoxicity. It alters nicotinamide acetyl dehydrogenase levels in rat liver, which might result in generation of free radical species. This acts as stimulator of LPO and source for destruction and damage to the cell membrane. Weakened cellular membranes allow sufficient leakage of calcium into the cytosol to disrupt intracellular calcium homeostasis. High calcium levels in the cytosol activate calcium-dependent proteases and phospholipases that further increase the breakdown of the membranes. Similarly, it may cause increase in intracellular calcium that activates endonucleases that may cause chromosomal damage and also contribute to cell death as seen by DNA damage. Thus, ATD administration caused significant hepatic damage playing a crucial role in the postulated mode of action.

*Phyllanthus amarus* and phyllanthin showed strong hepatoprotective potential, however, *Phyllanthus amarus* was found to be more effective in ameliorating liver abnormalities. This data provides a scientific validation for the folkloric uses in the treatments of hepatic disorders. We hypothesize that therapy showed ameliorative effects, which might be due to following facts.

- Increased cell viability in Hep 2 cultured cell lines.
- Stabilized activity of IL-6 and TNF-α.
- Recovery at molecular level by DNA damage assessment.
- Ability to scavenge free radicals.
- No side effect on the secretary activity of the liver as seen by enhanced release of bile.
• Inhibition in lipid peroxidation.
• Increase of activities of enzymes of antioxidant defense system (GR, GPx, G-6-PDH, SOD and CAT) and nonenzymatic antioxidant status in terms of GSH contents.
• Improved MDME status in liver and restoration in CYP450 enzymatic activities.
• Retrieval in liver function tests.
• Regenerative pattern in histopathological and ultrastructural studies.
• Presence of antipyretic activity.
• Their ability to reduce toxic metabolitics and recycling the status of antioxidant defense system.

CONCLUSION

In conclusion, this study revealed that plant *Phyllanthus amarus* and phyllanthin significantly decreased ATD induced oxidative damage in liver. However, plant extract was more significantly effective in comparison to its active principle. These findings warrant future studies for the prevention and treatment of liver disease. The results of the proposed study will definitely add new information against liver abnormalities that will ultimately be helpful in developing a suitable and cost effective hepatoprotective drug for mankind.

This will help to understand the wisdom of nature cure for encouraging new innovations in treatment of liver ailments.