**CHAPTER 6**

Chapter 6: Discussion of Result

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CHAPTER 6

Chapter 6: Discussion of Result

India is well known for its traditional medicinal value like Ayurveda, Unani, etc. From ancient days when allopathic medicine was not introduced, Ayurveda exist. Medicinal plants, herbs, spices and herbal remedies are known to Ayurveda in India since long times. In past the value of medicinal plants, herbs and spices as herbal remedies were getting lost due to lack of awareness, and deforestation. The result is many valuable medicinal herbs became rare and precious information got lost. Now slowly people are again moving towards natural remedies by knowing harmful side effects of allopathic drugs. The role of pharmacognocist is to preserve the knowledge of medicinal plants, herbs, spices and herbal remedies, which humankind has received from the past generations, for posterity. For this a thorough study is needed for every possible plant, plant part and in every possible extract\(^1\)\(^-\)\(^3\).

In the modern medicine no specific drug is available for treating hepatitis. Even allopathic practitioners prescribe herbal hepatoprotective agents. There are several herbs which are having proven hepatoprotective property. Whereas many herbs which are claimed to have hepatoprotective activity, remain without scientific and experimental evidences to justify the claims.

Thus this study includes isolation, characterization &
pharmacological screening of *Terminalia pallida* and *Boswellia ovalifoliolata*. Two solvents are used namely methanol and n hexane.

Preliminary qualitative phytochemical studies of METP revealed the presence of alkaloids, glycosides, carbohydrates, flavonoids, phytosterols/terpenes, proteins, tannins, saponins and lipids. HETP revealed that the presence of alkaloids, glycosides, carbohydrates, flavonoids, phytosterol and proteins. MEBO show improved response for of alkaloids, glycosides, carbohydrates, flavonoids, phytosterols/terpenes, proteins, tannins, saponins and lipids. HEBO revealed that the presence of alkaloids, glycosides, carbohydrates, flavonoids, phytosterols/terpenes, proteins and tannins.

### 6.1 Marker compounds

The spectral data of infrared spectra, mass spectra, H\(^1\)NMR-spectra revealed that *Terminalia pallida* roots contains ducosterol and *Boswellia ovalifoliolata* roots contains virtexin which can be considered as marker compounds in respective plant roots.

### 6.2 Anti-microbial Activity

Infectious diseases are the major cause of morbidity and mortality worldwide. The number of multidrug resistant microbial strains and the appearance of strains which reduced susceptibility to antibiotics are continuously increasing. Such increase has been attributed to
indiscriminate use of broad spectrum antibiotics, immunosuppressive agents, intravenous catheters organ transplantation and ongoing epidermis of human immunodeficiency virus (HIV) infections. This situation provided the impetus to the search for new antimicrobial substances from various source like medicinal plants.

The plants have traditionally provided a source of hope for novel drug compounds, as plant herbal mixtures have made large contributions to human health and well-being. The use of plant extracts with known antimicrobial properties can be of great significance for therapeutic treatment 37-39.

All the extracts at a concentration of 50 µg and 100 µg per each cup exhibited antibacterial and antifungal activities and against one or the other organisms in dose dependent manner. The plants extracts have exhibited considerable activity on the tested fungi. *Terminalia pallida* and *Boswellia ovalifoliolata* methanolic extract had produced good antibacterial activity against gram +ve and gram –ve bacteria and fungal strains when compared to n-Hexane extract. All the tested extracts have shown significant activity with that of the standard drugs.

The above results clearly demonstrated that the extracts had significant and considerable anti-microbial activity against variety of pathogens. All the pure compounds (TA-1 and BO-1) were tested for antimicrobial activity at a concentration of 25µg/cup. All the compounds
have shown significant activity with that of chloramphenicol (10µg/cup), where as these compounds had also shown moderate antifungal activity with that of nystatin (10µg/cup). The compounds were tested for minimum inhibitory concentration and all the tested compounds have shown significant activity with that of the standard drugs.

6.3 Anti-oxidant Activity

Reactive oxygen species (ROS) are an entire class of highly reactive molecules derived from the metabolism of oxygen. ROS, including superoxide radicals, hydroxyl radicals, and hydrogen peroxide, are often generated as byproducts of biological reactions or from exogenous factors. In vivo, some of these ROS play positive roles in cell physiology; however, they may also cause great damage to cell membranes and DNA, inducing oxidation that causes membrane lipid peroxidation, decreased membrane fluidity, and DNA mutations leading to cancer, degenerative, and other diseases\textsuperscript{57-61}.

The body posses’ defence mechanisms against free radical induced oxidative stress, which involve preventative mechanisms, repair mechanisms, physical defences and antioxidant defences. Enzymatic antioxidant defences include superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) etc., while non-enzymatic antioxidants are ascorbic acid (vitamin C), α-tocopherol (vitamin E), glutathione (GSH), carotenoids, flavonoids and etc. All these act by one or more of
the mechanisms like reducing activity, free radical scavenging, potential complexion of pro-oxidant metals and quenching of singlet oxygen. Reactive oxygen species (ROS) get special attention due to many factors such as drought, cold, heat, herbicides and heavy metals, because they harm the cell by raising the oxidative level through loss of cellular structure and function.\textsuperscript{58-61}

The METP, HETP, MEBO and HEBO shown significant and dose dependant reducing power and hydroxyl ion scavenging activities. Observed \textit{in vitro} antioxidant activity may be due to antioxidant principles present in METP, HETP, MEBO and HEBO.

However, METP, HETP, MEBO and HEBO was found to possess more potent in \textit{in-vitro} antioxidant activity. Therefore, METP, HETP, MEBO and HEBO were selected for screening \textit{in-vivo} antioxidant and hepatoprotective properties.

It is the normal practice to determine the LD\textsubscript{50} value, it is worthwhile to study an acute toxicity studies by employing several doses including reasonably high doses. Acute toxicity studies were conducted using a dose of 2000mg/kg, p.o. with METP, HETP, MEBO and HEBO in female albino rats according to the OECD guidelines. Even at this high dose METP, HETP, MEBO and HEBO did not exhibit any sign or symptoms of toxicity and mortality. Hence low dose (200mg/kg, p.o.)
medium dose (400 mg/kg, p.o.) and high dose of METP, HETP, MEBO and HEBO (600mg/kg, p.o.) were selected for further studies in animals.

### 6.4 Anti-inflammatory Activity

It is well known that inhibition of formalin-induced pedal oedema in rats is one of the most suitable test procedures to screen anti-arthritic and anti-inflammatory agents as it closely resembles human arthritis\(^{62-68}\). Injection of formalin subcutaneously into hind paw of rats produces localized inflammation and pain. The nociceptive effect of formalin is biphasic, an early neurogenic component followed by tissue mediated response. Thus formalin-induced arthritis is a model used for the evaluation of an agent with probable anti-proliferative activity. This experiment is associated with the proliferative phase of inflammation. Results with *Terminalia pallida* and *Boswellia ovalifoliolata* of METP, HETP, MEBO and HEBO (600mg/kg, p.o.) are showed quite compatible with those of the standard drug diclofenac sodium. Therefore, the drug appears to be effective against formalin-induced arthritis.

Formalin induced paw oedema is one of the most suitable test procedure to screen chronic anti-inflammatory agents. The mean response of standard 85.02% was inhibition of increase in paw thickness after 6 days respectively. In this model at 200, 400 and 600 mg/kg dose
level of METP, HETP, MEBO and HEBO extracts showed significantly inhibition of increase in paw thickness after 6 days.

### 6.5 Analgesic Activity

Acetic acid-induced writhing model represents pain sensation by triggering localized inflammatory response. Such pain stimulus leads to the release of free arachidonic acid from tissue phospholipids. The acetic acid induced writhing response is a sensitive procedure to evaluate peripherally acting analgesics. The response is thought to be mediated by peritoneal mast cells acid sensing ion channels and the prostaglandin pathway\(^{70-73}\).

The mean response of control and standard was 46.33 ± 3.712 and 18.50 ± 1.335 respectively. The respective test compounds METP and HETP in its 200, 400 and 600 mg/kg dose, showed mean writhing responses as 31.83 ± 2.455, 26.33 ± 1.085, 21.17 ± 1.108 and 34.17 ± 2.088, 29.67 ± 1.498, 24.50 ± 1.544. In terms of percentage inhibition of writhing by diclofenac sodium was 60.07% while with the test compound it was 31.30%, 43.17%, 54.31% and 26.25%, 35.96%, 47.12% respectively. The respective test compounds MEBO and HEBO in its 200, 400 and 600 mg/kg dose, showed mean writhing responses as 33.17 ± 1.621, 28.33 ± 1.783, 20.67 ± 1.116 and 36.83 ± 2.822, 31.33 ± 2.275, 25.33 ± 1.542. In terms of percentage inhibition of writhing by diclofenac sodium was 60.07% while with the test compound it was 28.40%, 38.85%, 55.39% and 20.51%, 32.38%, 45.33% respectively.
6.6 Hepatoprotective Activity

In case of toxic liver, Wet liver weight and wet liver volumes are increased. Toxicants induced hepatotoxicity produce fatty changes and also it is observed that there is a fall in serum lipids in another series of experiments. In this case water is retained in the cytoplasm of hepatocytes leading to enlargement of liver cells, resulting in increased total liver mass and volume. It is reported that liver mass and volume are important parameters in ascertaining the hepatoprotective effect of the drugs. Treatment with METP, HETP, MEBO and HEBO significantly reduced the wet liver weight and wet liver volumes of animals and hence it possesses statistically significant hepatoprotective activity.

The hepatoprotective activity was assessed by measuring the biochemical markers like SGPT, SGOT, total protein, bilirubin (total and direct triglycerides (TG), total cholesterol (TC), HDL-Cholesterol (HDL-C), LDL-Cholesterol (LDL-C), VLDL-Cholesterol (VLDL-C) and ALP in all the four hepatotoxic models (D-GalN/LPS and ethanol induced hepatotoxicity).

The D-GalN/LPS represents a model very much similar to that of viral hepatitis, the efficacy of METP, HETP, MEBO and HEBO in preventing virus mediated liver hepatotoxicity needs to be investigated.
D-GalN/LPS induced hepatoceullar damage, a well-established model of hepatitis takes advantage of the ability of D-GalN to potentiate the toxic effects of LPS producing fulminant hepatitis within a few hours of administration. A high dose of D-GalN causes necrosis of the liver by UTP depletion and inhibition of protein synthesis, although D-GalN is often used in combination with lipopolysaccharide or tumour necrosis factor. Accumulation of UDP-sugar nucleotides may contribute to the changes in the rough endoplasmic reticulum and to the disturbance in the protein metabolism\textsuperscript{100,101}. Further, intense galactosamination of membrane structure is thought to be responsible for loss in the activity of ionic pumps. The impairment in the calcium pump, with consequent increase in the intracellular calcium is considered to be responsible for cell death\textsuperscript{196}. In recent years, apart from the well documented inhibition of protein synthesis, it has been suggested that reactive oxygen species produced by activated macrophages might be the primary cause in D-GalN-induced liver damage. Treatment with METP, HETP, MEBO and HEBO (200, 400 and 600mg/kg.p.o.) significantly reduced dose dependently all the biochemical markers enzymes and increase tissue SOD and CAT.

The Liver damage induced by D-GalN/LPS generally reflects disturbances of liver cell metabolism which lead to characteristic changes in the activities of serum enzymes. The disturbance in the transport function of the hepatocytes as a result of hepatic injury causes
the leakage of enzymes from cells due to altered permeability of membranes. In this context, we have also observed a significant increase in the serum activities of SGPT, SGOT, total protein, bilirubin (total and direct triglycerides (TG), total cholesterol (TC), HDL-Cholesterol (HDL-C), LDL-Cholesterol (LDL-C), VLDL Cholesterol (VLDL-C) and ALP levels, which is in accordance with the earlier findings. Because the levels of these marker enzymes are proportional to the extent of damage, the activity of these enzymes can be used for diagnosis as indicators of prognosis of the disease.

Pretreatment with METP, HETP, MEBO and HEBO brought back the enzyme level to near normal indicating clearly the therapeutic value of METP, HETP, MEBO and HEBO.

The histological evidence authenticated the injury caused by D-GalN/LPS and the protection offered by METP, HETP, MEBO and HEBO to hepatocytes. Microscopical examination revealed loss of architecture and cell necrosis with inflammatory collections in the central zone in DGalN/LPS - induced rats. Prior oral administration with METP, HETP, MEBO and HEBO extracts prevented completely the histopathological changes in liver induced by D-GalN/LPS. Thus the histopathological studies serve as a direct evidence of efficacy of drug as protectant. The results of histopathological study also support the result of biochemical
parameters and explain the hepatoprotective activity of METP, HETP, MEBO and HEBO.

In this toxicity there is increased formation of lipoperoxides, conjugated dienes and malondialdehyde (MDA) and reduced levels of antioxidants like vitamin E and glutathione in the tissues have been demonstrated in experimental animals administered with ethanol as well as alcoholic human subjects. The increased level of AST, ALT, ALP, and bilirubin are conventional indicator of liver injury. Ethanol produces a constellation of dose-related deleterious effects in the liver. In chronic alcoholics, hepatomegaly occurs due to accumulation of lipids and proteins in hepatocytes with an impaired protein secretion by hepatocytes. Oxidative stress is one major factor in etiology of ethanol injury, mainly by Kupffer cells through the action of a substance called endotoxin, which is released by certain gram-negative bacteria present in the intestine, activates Kupffer cell to generates ROS and pro inflammatory cytokines (TNF alpha, IL-1), both of them can lead to liver damage.

Hepatotoxin gets converted into radicals in liver by action of enzymes & these attacks the unsaturated fatty acids of membranes in presence of oxygen to give lipidperoxides consequently. The functional integrity of hepatic mitochondria is altered, leading to liver damage. During hepatic damage, cellular enzymes like AST, ALT and ALP present
in the liver cells leak into the serum, resulting in increased concentrations. Ethanol administration for 25 days significantly increased all these serum enzymes.

Serum levels of SGPT can increase due to damage of the tissues producing acute hepatic necrosis, such as viral hepatitis and acute cholestasis. Alcoholic liver damage and cirrhosis also can associate with mild to moderate elevation of transaminases. SGPT, SGOT, total protein, bilirubin (total and direct triglycerides (TG), total cholesterol (TC), HDL-Cholesterol (HDL-C), LDL-Cholesterol (LDL-C), VLDL Cholesterol (VLDL-C) and ALP. Treatment with METP, HETP, MEBO and HEBO (200, 400 and 600mg/kg.p.o.) significantly reduced dose dependently all the biochemical markers enzymes and increase tissue SOD and CAT. The METP, HETP, MEBO and HEBO have improved the liver architecture as similar to other models of hepatotoxicity. Hence, it can be informed that the test extract possess hepatoprotective activity.

In case of toxic liver, alkaline phosphatase levels are very high, which may be due to defective hepatic excretion or by increased production of ALP by hepatic parenchymal or duct cells. In case of toxic liver, bilirubin levels are elevated. Hyperbilirubinemia can result from impaired hepatic uptake of unconjugated bilirubin. Such a situation can occur in generalized liver cell injury. Certain drugs (e.g., rifampin and probenecid) interfere with the net uptake of bilirubin by the liver cell and
may produce a mild unconjugated hyperbilirubinemia. Bilirubin level rises in diseases of hepatocytes, obstruction to conjugation of bilirubin pigment such as in Gilbert’s disease. Toxicant induces hypercholesteremia and hypertriglyceridemia, perhaps be due to the activation of enzyme HMG CoA reductase, the rate-limiting step in cholesterol biosynthesis. The increased serum triglyceride level in ethanol-treated rats may be due to the decreased activity of lipoprotein lipase, which is involved in the uptake of triglyceride-rich lipoprotein by the additional hepatic tissues.

Our study further revealed that chronic exposure to ethanol decreased the activities of the ROS scavenging enzymes, viz. SOD and CAT. This is in line with assumption suggested earlier that decrease in the activity of antioxidant enzymes SOD and CAT following ethanol exposure may be due to the damaging effects of free radicals, or alternatively could be due to a direct effect of acetaldehyde, formed from oxidation of ethanol, on these enzymes. In our studies, it reveals that METP, HETP, MEBO and HEBO could restore the activity of both these antioxidant enzymes and possibly could reduce generation of free radicals and hepatocellular damage.

Formation of ROS, oxidative stress and hepatocellular injury has been implicated to alcoholic liver disease. It has been documented that Kupffer cells are the major sources of ROS during chronic ethanol
consumption, and these are primed and activated for enhanced formation of pro-inflammatory factors.

Even if various enzymatic and non-enzymatic systems have been developed by cell to cope up with the ROS and other free radicals, when a condition of oxidative stress establishes, the defense capacities against ROS becomes inadequate. ROS also affects the antioxidant defence mechanisms, reduces the intracellular concentration of GSH, and decreases the activity of SOD and CAT. It has also known to decrease the detoxification system produced by GST. Increasing evidence indicates that oxidative stress causes liver injury, cirrhosis growth and carcinogens. In our studies, it reveals that METP, HETP, MEBO and HEBO could restore the activity of both these antioxidant enzymes.

It seems the protective activities of the plant may be by strengthening the inbuilt antioxidant systems by of the antioxidant principles that are present in the plant. However further studies are needed to completely establish the mechanism of hepatoprotective effect of the plants in this model.

The observed *in-vitro, in-vivo* and hepatoprotective activity may be due to presence of phenols, alkoloids, tannins, flavanoids, triterpenoid sand sterols present in METP, HETP, MEBO and HEBO.