6 SUMMARY
Liver plays a vital role in metabolism and excretion. In general, which perform an astonishing array of vital functions in the maintenance and performance of the body such as for metabolism, storage, biosynthesis and detoxification. Unfortunately, the liver is often abused by environmental toxins, alcohol and over-the-counter drug use (xenobiotics), which can damage the liver and eventually lead to hepatitis, cirrhosis and liver diseases. Liver ailments need to be treated with utmost care. In India, there are about 100 medicinal plants used in 33 herbal formulations. These hepatoprotective plants have the phytoconstituents such as phenyl compounds, coumarins, essential oils, monoterpenoids, diterpenoids, triterpenoids, steroids, alkaloids and other nitrogenous compounds. The conventional medicine is now pursuing the exploitation of natural products such as herbs to provide the support that liver needs on a daily basis. Numerous medicinal plants and their formulations are used for liver disorders in ethnomedical practices and in traditional system of medicine in India. Medicinal plants are believed to be an important source of new chemical substances with potential therapeutic effect. The research into plants with alleged folkloric use as pain relievers, anti-inflammatory and hepatoprotective agents, should therefore be viewed as a fruitful and logical research strategy in search for new drugs. Therefore, in the present study, the author conducted a thorough survey in Idukki Districts of Kerala, India in search of more effective, safe, reliable and scientifically unexplored ethnobotanically used hepatoprotective medicinal plants.

On the basis of survey, review of literature, medicinal uses and availability of the
plant, *Combretum albidum* G.Don were selected for this study. The plant is one of the widely used hepatoprotective medicinal plants of Muthuvans (considered as a superior tribal group of Chinnar Wildlife Sanctuary). In the survey, it is revealed that the commonly used hepatoprotective plants like *Phyllanthus amarus* and *P. airy-shawii* are available in this area and they are familiar with the use of these plants, though the tribe prefers bark juice of *C. albidum* administered orally against jaundice. For the further scientific validation and bioprospecting, we selected three parts (stem bark, heart wood and leaf) of the traditionally important hepatoprotective plant, *C. albidum*.

The study includes detailed evaluation of Taxonomical, Pharmacognostical, Phytochemical, Antioxidant, Antimicrobial, Cytotoxic and Pharmacological (Cell lines) screening of useful parts of selected plant. Special emphasis was given for pharmacognostical and chemical finger printing of genuine raw drug and its useful parts using modern analytical techniques like HPTLC, GC/MS and LC/MS.

The plant is a woody, deciduous shrub up to 30 m high, belongs to the family Combretaceae, flowering and fruiting season is December-March. Locally (in Malayalam), it is known as *Manjakody* and *Buffalo calf plant* in English. Its distribution restricted in semi-evergreen and deciduous forests, along river banks of Peninsular India and Sri Lanka. Habitat is semi-evergreen and deciduous forests, along river banks and generally associated with bamboo. In Kerala this plant is spot on Malappuram, Palakkad, Idukki and Thiruvanathapuram Districts. During expedition it is observed in Kiralur, Thrissur district of Kerala.
PHARMACOGNOSTIC STUDIES

The pharmacognostical study is a major and reliable criterion of identification of plant drugs. These parameters are necessary for the confirmation of identity and determination of quality and purity of crude drugs. To ensure reproducible quality of herbal products, proper control of genuine materials are utmost essential. Thus, in recent years there has been an emphasis on standardization of medicinal plants and evaluation of plant drugs by pharmacognostical studies is still more reliable, accurate and inexpensive means. According to World Health Organization (WHO) the macroscopic and microscopic description of a medicinal plant is the first step towards establishing its identity and purity and should be carried out before any tests are undertaken. The major diagnostic features of the raw drugs were identified.

**Stem bark (CaSB):** TS showed presence of storied cells and dead tissues of rhytidoma; the cells of the outer and inner cork filled with yellow brown contents; presence of bundles of stone cells associated with prismatic crystals of calcium oxalate in the cortex. Phloem fibre thick-walled, highly lignified and arranged in radial bands; crystal fibres containing prismatic crystals of calcium oxalate in each chamber. Medullary rays uni to bi-seriate, wavy, dialated towards outer side are the diagnostic features of T.S of CaSB. The major features of powder microscopy are groups of stone cells; fibres with reticulate thickening; stone cells; tannin masses; group of pitted parenchyma; fragments of fibres and groups of crystal fibres; cortical parenchyma containing tannin and oil globules; yellowish brown content, starch grains and prismatic crystals of calcium oxalate are scattered throughout the powder.

**Heart wood (CaSHW):** Detailed TS of heart wood showed xylem region consists of vessels and parenchyma filled with starch grains and tannin contents; crystal fibres
embedded with prismatic crystals of calcium oxalate. Medullary rays are uni to biseriate loaded with starch grains. Radially and longitudinally cut section showed broad lumened bordered pitted vessels, thick walled crystal fibres associated with rows of parenchymatous cells embedded with starch grains and patches of rows of medullary rays crossing these elements at places. Powder microscopy showed fragments of crystal fibers, vessels with bordered pitted thickening, fragments of xylem parenchyma embedded with starch grains, stone cell; starch grains measures upto 18 µm and prismatic crystals of calcium oxalate as such scattered throughout the powder.

**Leaf (CaL):** Detailed anatomy passing through the midrib region showed upper epidermis covered with thin cuticle and glandular trichomes and peltate hairs at the lower side. Two to three rows of collenchymatous hypodermis with few cluster crystals of calcium oxalate. The vascular strand was large and omega shaped. The prismatic type of calcium oxalate crystals occur in the outer phloem zone. The lignified xylem elements and calcium oxalate crystals appear bright under polarised light. The mesophyll contains calcium oxalate crystals of druses mostly in palisade tissue. Powder shows calcium oxalate cluster and druse crystals. The stomata were anisocytic type. The stoma was surrounded by three dissimilar subsidiary cells.

**PHYSICO-CHEMICAL ANALYSIS**

The physical constant evaluation of the powder is an important parameter in detecting adulteration or improper handling of drugs. The loss on drying of plant material is high in C1SB (11.30%) and low in C1SHW (7.27%). The total ash is particularly important in the evaluation of purity of drugs, *i.e.* the presence or absence of foreign inorganic matter such as metallic salts and/or silica. The total ash
and acid insoluble ash were found to be more in C2L (13.91%, 0.54% respectively) and less in C2SHW (3.48%, 0.05% respectively). The extractive values are useful to evaluate the chemical constituents present in the crude drug and also help in estimation of specific constituents soluble in a particular solvent. Extractive values of ethanol and water were more in C2L (14.72%, 21.25% respectively) and less ethanol soluble extractive in C1SB (8.21%) and water soluble extractive in C3SHW (7.51%). Sequential extractive values of sample in petroleum ether, ethyl acetate and methanol were studied. Leaf samples possessed more value in all the solvents. Direct methanol value also reported more in leaf samples followed by heart wood and stem bark.

**PHYTOCHEMICAL STUDIES**

The phytoconstituents are known to play an important role in bioactivity of medicinal plants. Flavonoids, triterpenoids and tannins are well known for their hepatoprotective activities. In qualitative phytochemical analysis, tannins and triterpenoids were present in high amount as compared to other phytoconstituents analyzed. In quantitative phytochemical analysis, phenolic content was much more than flavonoid content. Methanol extract of CaSB, CaSHW and CaL showed the presence of carbohydrates, glycosides, saponin, tannin, coumarins, steroids, flavonoid & phenolics compounds and triterpenoids in all the three extracts. Quinone and antraquinone glycoside are present only in the stem extract, while alkaloids are totally absent in all the extracts. Total phenolics, flavanoid and tannin content are maximum in leaf compared to stem bark and heart wood.

**TLC comparison studies**

TLC finger printing technique evolved a method for checking the purity of herbal drugs. The present drug sequentially extracted with Petroleum ether, Ethyl acetate
and Methanol to get low, medium and highly polar extractives of the drug. The HPTLC comparison revealed that the finger printing profiles of leaf consists of more prominent bands corresponding to chemical constituents than that of stem bark and heart wood.

**GC/MS analysis of essential oil of CaL**

About 18 compounds were identified in leaf oil accounting for 89.21% of the total in which, caryophyllene predominates in the oil with 21.58%. linalool, β-phellandrene and phytol were the other major compounds present in the oil analysed. All these compounds have specific reported biological activities.

**Isolation and Characterisation of Secondary metabolites**

In the present study, we report our work on isolation of three bioactive compounds i.e., β-sitosterol, ursolic acid and gallic acid from the stem bark of *C. albidum*. Preliminary TLC experiments indicated the presence of β-sitosterol, ursolic acid and gallic acid from the selected parts of the plant. They were fractionated and isolated by column chromatography. Further the compounds were quantified by thin layer chromatography and densitometry methods using high performance thin layer chromatography. Accuracy of the method checked by conducting recovery studies at three different levels for the three compounds and the average percentage of recoveries obtained were 99.09, 99.58 and 99.89. *C. albidum* samples were found to contain 0.017 - 0.041% w/w of β-sitosterol, 0.019 - 0.079% w/w of ursolic acid and 0.016 - 0.062% w/w of gallic acid. Three compounds viz., β-sitosterol, ursolic acid and gallic acid have been reported for the first time from the plant. These compounds are reported to be antioxidant and hepatoprotective. The present findings provide
certain evidence to the ethno medicinal property of *C. albidum* in treating against acute jaundice.

**PHARMACOLOGICAL ACTION OF ISOLATED COMPOUND**

**β-sitosterol**

Anti-hepatotoxic, antitumor, anti-inflammatory, antiarthritic, nutraceutical, chemopreventive agent, antimicrobial, antiedemic, antihyperglycemic, antioxalate, hypotensive and antiperoxidant activities. Hence one of the reasons behind the observed hepatoprotective activity of CaSB may be attributed to the presence of β-sitosterol.

**Ursolic acid**

Both oleanolic acid and ursolic acid have been identified as active components in producing hepatoprotective effects. Ursolic acid (UA), one of pentacyclic triterpene acids, is ubiquitous in the plant kingdom and is found in fruits, vegetables and medicinal plants. It is well known for its hepatoprotective effects for both acute chemically induced liver injury and chronic liver fibrosis and cirrhosis. The observed hepatoprotective activity of CaSB may be due to the presence of ursolic acid.

**Gallic acid**

Gallic acid (GA), a trihydroxybenzoic acid, possesses promising hepatoprotective effects. Hepatoprotective activity of CaSB may be because of the presence of Gallic acid.

The three isolated compounds *i.e.*, β-sitosterol, Ursolic acid and Gallic acid were also observed in leaf and heart wood samples, which provides a lead to explore the possibilities of using these parts as hepatoprotective and other relevant activities. Further pharmacological investigation is required to prove the activity scientifically.
Thus the three isolated compounds can serve as biological markers for the plant and also the presence of these hepatoprotective agents proving its usage as hepatoprotective remedy by the tribals.

ANTIOXIDANT STUDIES

A large number of medicinal plants as well as *Combretum* species and their purified constituents have shown beneficial therapeutic potentials. The majority of the antioxidant activity is due to the flavones, isoflavones, flavonoids, anthocyanin, coumarin lignans, catechins and isocatechins. The free radicals can be scavenged by the *in vivo* produced antioxidant compounds, the endogenous antioxidants are insufficient to completely remove them and maintain a balance. As a result, dietary antioxidants are required to counteract excess free radicals. Antioxidants help to neutralise free radicals. Tannins are known as free radicals scavenger and could inhibit the lipidi peroxidation of biomembranes caused by reactive free radicals and they could also be used as hepatoprotective substances against the CCl$_4$-intoxication. Flavonoides and anthocyanes are scavengers of free radicals. In fact they react with them and prevent damages related to their reaction with membrane phospholipids.

The antioxidant activity of the various parts of different samples of *C. albidum* was determined using the DPPH scavenging assay. The radical scavenging activity of the extracts is expressed as percentage inhibition and IC$_{50}$ values. Most of the tested extracts showed scavenging activity above 80% with IC$_{50}$ values ranging between 28.4 and 48.3µg/ml. All the three tested extracts of heart wood showed very low as indicated by their high IC$_{50}$ values. Leaf extract showed to have maximum DPPH scavenging activity followed by stem bark and heart wood. Among the extracts studied C3L, C1L and C2L showed better activity respectively IC$_{50}$ of 28.4, 29.2 and
30.5 µg/ml. IC$_{50}$ value obtained for standard quercetin was 8.1 and for gallic acid were 6.25. The results showed that the methanol extracts of *C. albidum* have a considerable free radical scavenging activity.

The histochemical and preliminary Phytochemical screening revealed the presence of high percentage of phenolics, flavanoid, saponin, tannin contents and the isolated compounds ursolic acid, gallic acid are reported to be natural antioxidants. They can scavenge off free radicals. So the increased level of free radicals scavenging activity is due to the presence of these antioxidants. The percentage of gallic acid in leaf is directly proportional to the antioxidant activity. However, the chemical constituents present in the extract, which are responsible for this activity, need to be further investigated, and it is obvious that the constituents like tannins, flavonoids and proteins present in the extract may be responsible for such activity. The phytochemical tests indicated the presence of glycosides, tannins, and flavonoids in the crude methanolic extract. Such compounds are known to possess potent antioxidant activity. Hence, the observed antioxidant activity may be due to the presence of any of these constituents. The plant exhibited strong hepato-protective, antibacterial and antifungal activities. These properties may be due to its antioxidant activity.

**ANTIMICROBIAL STUDIES**

**Antibacterial**

The family Combretaceae contains a diversity of antimicrobial compounds. The increasing occurrence of bacterial resistance against available antibiotics, it has now become essential to look for newer antibiotics. Most of the antibiotics available today come from natural origin, especially from various microbial or plant sources. Higher
plants also produce compounds to protect themselves from microbial attacks. The study thus reveals the effectiveness of antibacterial activity of *C. albidum* against *Escherichia coli*, *Klebsiella pneumonia*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Salmonella typhimurium*.

**Antifungal**

The results of the antifungal study revealed the significant dose dependent antifungal activity of methanolic extract of CaSB and CaSHW. The extract CaSHW found to be more efficient in inhibiting the fungal growth than CaSB extract. CaL possesses negligible antifungal activity on studied fungal groups. *Aspergillus flavus* was found to be the most sensitive in both extracts and *Rhizopus species* and *Candida albicans* are the least sensitive organism to CaSB and CaSHW respectively. The higher concentration of methanol extract of CaSHW and CaSB (100µl) inhibited the growth of *Aspergillus flavus* by 26 and 24 respectively. The same concentration of the extracts showed a growth inhibitory zone measuring 18 and 16 for CaSHW and CaSB respectively towards *C. albicans*. The extract also showed an efficient inhibition of the growth of *Aspergillus niger* and *Pencillium chrysogenum*. The results signify that the extracts possess almost same growth inhibitory activity with the standard antibiotics against all the tested organisms

**TOXICITY STUDY**

Toxicology is a science to study adverse-effects of chemicals or physical agents on biological system and preclinical toxicology is a science to evaluate safety of one drug (mostly) in animals to decide if the drug is safe for human use or not. In the present study, cytotoxicity of the CA-H2O, CA-EtOH and CA-Hex were done in three different concentrations (200, 100 and 50µg/ml) on HEK293 cell lines (100
µg/ml) at 48 hrs duration. Compare to the Normal Control, CA-H₂O extract showed slight cytotoxic and other two extracts, CA-EtOH and CA-Hex, showed no cytotoxicity and possess cell proliferation activity on HEK293. From the result it is concluded that the extracts are not making any harm to the Human Embryonic Kidney cells up to a concentration of 100µg/ml.

ANTICANCEROUS ACTIVITY ON K562 CELL LINES

Methanol extract of CaSB was checked for Anticancerous activity on cell line K562 (Chronic Mylogenous leukemic cell lines) in six different concentration (10, 25, 50, 75, 100, 150µg/ml) at 48 hrs duration. The three extracts in different concentrations showed anticancerous activity as compare to the normal sample and IC₅₀ value observed in the concentration in between 75 to 100 µg/ml.

HEPATOPROTECTIVE ACTIVITY ON HEP3B CELL LINES

Natural products and their active principles as sources for new drug discovery and treatment of diseases have attracted attention in recent years. Hepatic fibrosis is usually initiated by hepatocyte damage. Biologic factors such as hepatitis virus, bile duct obstruction, cholesterol overload, etc. or chemical factors such as CCl₄ administration, alcohol intake are known to contribute to liver fibrosis. The incidence of chronic fibrosis is high, but there are no satisfactory agents with ascertained effectiveness and with fewer side effects on liver. So, finding effective ways to inhibit liver fibrosis and prevent the development of cirrhosis are of great significance.

Sylimarin, the available chemoprotectant drug, on Hep3b cells reduced the level of activity and the methanolic extract of CaSB also showed significant arrest in the activity of Hep3B cells, when compared to Sylimarin treated cells. From the result it
is for the first time identified the anti-proliferating result and associated molecular mechanism of CaSB extract in human hepatocellular carcinoma cells. At the same time from our cytotoxicity study on Human Embryonic Kidney cells (HEK cell lines) were showed no significant cytotoxic effect. This is an important preliminary finding that the cell death is occurred only in the canrcinogenic cells without affecting the normal Human Kidney embryonic cells. These results strongly suggest that importance of further investigation especially using in vivo model to promote the use of the active fraction of *C. albidum* as a chemopreventive agent in Hepatocellular carcinoma.

It is earnestly anticipated that this will give scientific validation of the plants for its botanical and chemical identity and pharmacological efficacy of the raw drugs used by the tribals of Idukki districts of Kerala, India. We hope that the result from this study will lead to develop a scientific data of the selected plant. It is also expected that through bio prospecting of the selected plant, the details of the plant species have been scientifically proven for the future benefit of the researchers and herbal industry.