5

DISCUSSION
5. DISCUSSION

GENERAL DISCUSSION

India has over 84.3 million tribals, belonging to diverse ethnic groups. In India, there are 550 communities of 227 ethnic groups as per the classification made by the anthropologists on linguistic basis (Pushpangadhan, 2008). In Kerala the major tribal communities are nearly forty in number. Some of them are still nomadic and living in the forests. The major tribes of Kerala are Kani, Kurichiar, Kurumar, Eravallan, Kattunaikkan, Muthuvan etc. (Joseph & Antony, 2012). Forest has been the home for many of these tribes and they have deep rooted association with the forest and nature around. Their relationship with the forest has always been harmonious and their whole life revolves forest and forest resources. They have acquired unique knowledge about the use of many wild flora and fauna through generations most of them are lesser known or hitherto unknown to the outside world. This treasure of traditional knowledge, if subjected to scientific scrutiny could be highly beneficial for the tribals, the country and the humankind in many ways. The inroads of modernization are presently posing a threat to this traditional knowledge and these are in the imminent danger to losing out, this age old wisdom and expertise can be lost for alltimes to come. There are several medicinal plants, it enables traditional healers to develop effective therapies against various ailments. These plants are used only at the tribal level are found to have very good hepatoprotective, antidiabetic, anticancerous, antiobesity and antivenomous activity. But scientific validation and authentication of these species is needed to establish with evidence based therapeutic activity. It
was in this background the present exercise of survey, documentation and scientific validation of a hepatoprotective plant was carried out, which is commonly used by the tribes of Chinnar, Idukki district of Kerala.

Liver is one of the largest organs in human body and the major site for metabolism and excretion. It is involved with almost all the biochemical pathways of growth, fight against disease, nutrient supply, energy provision and reproduction (Ward & Daly, 1999). Jaundice and hepatitis are two major liver disorders that account for a high death rate. Modern medicines have little to offer for alleviation of hepatic diseases and it is chiefly the plant based preparations are employed for the treatment of many liver disorders (Pang et al., 1992). Therefore, many folk remedies from plant origin are to be tested for their potential hepatoprotective and antioxidant properties in liver damage in experimental animal models.

The major criteria that can be used for the standardization of raw drugs are Pharmacognosy and Phytochemistry. These are species specific with characters and can be exploited by developing anatomical and chemical fingerprints of each raw drugs.

5.1. SURVEY AND TAXONOMIC STUDIES

The objective of the present study was to find out a scientifically unexplored, effective, reliable and non toxic tribal level hepatoprotective medicinal plant of Idukki district of Kerala, India. As per the survey conducted in this area revealed that, the tribes are using several plants for jaundice and other liver ailments but many of these plants like Phyllanthus sp, Andrographis paniculata were already scientifically evaluated. Other plants like Arenga wightii and Ceasalpinia...
culcullata are not very commonly used among the tribes. Hence *Combretum albidum* G. Don, one of the widely used hepatoprotective medicinal plants of Muthuvans (considered as a superior tribal group of Chinnar Wildlife Sanctuary) are selected for the present study. The plant is very important because the tribes prefer using bark juice of *C. albidum* (commonly known as Manjakody) administered orally against jaundice though *Phyllanthus airy-shawii* and *P. amarus* are known for their use against jaundice. As per the survey report and review of literature is concerned, it is noted that the plant *C. albidum* is not scientifically evaluated. In the present study, the author attempted to develop standards based on anatomical, histochemical and phytochemical characterization of three parts of *C. albidum* i.e., Stem bark (CaSB), Heart wood (CaSHW) and Leaf (CaL) and their therapeutic effects.

In Peninsular India, especially in Kerala, two species of *Combretum* is occurred commonly, *i.e.*, *C. albidum* G. Don and *C. latifolium* Blume. Both species are large climbing shrubs, the distinguishing key characters are:

Calyx tube campanulate; leaves pale, yellow when dry................. *C. albidum*

Calyx tube funnel-shaped; leaves shining, reddish when dry........ *C. latifolium*

A new species *C. malabaricum* (Bedd.) Sujana, Ratheesh, & Anil (Syn. *Quisqualis malabarica* Bedd.), is also reported, which is so far spotted only from Aralam Wildlife Sanctuary, Kannur district of Kerala, India (Sujana *et al*., 2012).

**Ethnobotanical survey**

Some *Combretum* species are extensively used in traditional medicine against inflammation, infections, diabetes, malaria, bleeding, diarrhea, digestive disorders and others as a diuretic in Africa and Asia (De Morais *et al*., 2012).
Chinnar Wildlife Sanctuary, Idukki district, Kerala, South India is rich in tribal population, comprises the Muthuvans and Hill Pulayas. Muthuvans is using many plants for their health care and day to day ailments, but many of them are not yet been scientifically validated. This tribe is using *C. albidum* as an effective remedy for jaundice (Sajeev *et al.*, 1997).

Ganesan *et al.*, (2009) listed 60 plants in sacred groves of Pallapatty village (Reserved forest), Tamil Nadu, India. Among these, the bark of *C. albidum*, locally know as *Odaikodi*, is used against certain skin diseases. A similar study conducted by Swamynathan *et al.*, (2011) in sacred groves of Chidambaram Taluk, Cuddalore district, Tamil Nadu ensures the same practice. Kadavul *et al.*, (2009) recorded Ethnomedicinal studies of the woody species of Kalrayan and Shervarayan Hills of Eastern Ghats, Tamil Nadu. The people of this area is using wiry stem, seed oil, root bark and stem of *C. albidum* against eye problems, eczema and it is also having antimalarial activity. Madhava (2008) listed 44 plants, which possess anti-ulcer activity, in his book *flowering plants of Chittor District*, Andhra Pradesh, among these the leaves of *C. albidum* also included (Ramasubramaniaraja *et al.*, 2011). In the present study, the stem bark and heart wood and leaf of this plant showed positive results towards the antibacterial and antifungal activities.

5.2. PHARMACOGNOSTIC STUDIES

Pharmacognosy according to Youngken, (1950), Trease, (1966) and Wallis, (2004) which is an applied science aims at a complete and systematic knowledge of crude drugs of vegetable, animal or mineral origin. It implies not only drugs but also includes a knowledge of sources from which the drugs are prepared, their
history, properties and use, distribution, cultivation, collection and selection. It is an important link between pharmacology and medicinal chemistry. The knowledge of pharmacology is essential for understanding action of drugs on animals and the human system. Pharmacognosy is the infrastructure on which depends evolution of novel medicines, as it seen that several crude drugs are utilized for preparation of galenicals or as a sources of therapeutically significant substances that cannot be synthesized economically (Kokate et al., 1999).

No previous pharmacognostic study was reported in the selected plant, the author made an attempt to report the pharmacognostic analysis of stem bark (Sreedhar et al., 2012), heart wood (Sreedhar et al., 2013) and leaf (Sreedhar et al., 2013 in press) of C. albidum. In this study various standardization parameters such as macroscopy, microscopy, histochemical and powder microscopy were published.

**STEM BARK (CaSB):** The characteristic features of stem bark are 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 multiseriate, wavy, dialated towards outer side are the diagnostic features of T.S of stem bark of C. albidum (Sreedhar et al., 2012). 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 (Sreedhar et al., 2012).

**HEART WOOD (CaSHW)** Diagnostic features 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 18.50 µm and prismatic crystals of calcium oxalate as such scattered throughout the powder (Sreedhar et al., 2013).

Metcalfe and Chalk (1972) reported the anatomy of Combretaceae and identified the diagnostic features like islands of sclerenchyma in the secondary phloem, chambered fibres with crystals, inter and intra-xylary phloem in the genus *Combretum*. In the present study except intra and inter-xylary phloem, all other diagnostic features were observed *C. albidum*.

**LEAF (CaL):** 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 are the major diagnostic characters (Sreedhar et al., 2013 in press).

Metcalfe and Chalk (1972) classified the diagnostic features of Leaf of Combrataceae; presence of glandular hairs, bicollateral vascular bundles, sclerenchymatous bundle sheath and cluster crystals of calcium oxalate were reported in the genus *Combretum*. Almost all the reported characters of the genus *Combretum* were observed in *C. albidum*.

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 (Bhattacharya & Zaman, 2009). To ensure reproducible quality of herbal products, proper control of genuine materials are utmost essential (Venkatesh et al., 2004). 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 (WHO, 2002). From the present pharmacognostic study major distinguishing features were identified and published, and that will be a parameter for the quality checking of the raw materials.
5.3. PHYSICO-CHEMICAL ANALYSIS

The physical constant evaluation of the powder is an important parameter in detecting adulteration or improper handling of drugs. The percentage of active chemical constituents in crude drugs is mentioned on air-dried basis. Therefore, the loss on drying of plant materials should be determined and the water content should also be controlled. The moisture content of dry powder of selected parts of C. albidum was studied. The value of loss on drying at 110\(^0\) showed the presence of moisture content in the sample, which is high in C1SB (11.30%) and low in C1SHW (7.27%). The earlier report suggest that moisture content upto 12%, which is not very high, hence it would discourage bacteria, fungi or yeast growth (Bhattacharya & Zaman, 2009). 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 (Musa et al., 2006). Low amount of total ash, acid insoluble ash and water soluble ash indicated that the inorganic matter and non-physiological matter such as silica is less in the samples. 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 ash contents showed the amount of inorganic matter present in the sample and the acid insoluble ash almost within 1%, which expresses low siliceous matter present in the sample. 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 alcohol and water were more in C2L (14.72%, 21.25% respectively) and less alcohol 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 values in all the solvents. Methanolic extractive value also reported more in leaf samples followed by heart wood and stem bark. The variation in extractable matter in various solvents is suggestive of the fact that the formation of the bioactive principle of the medicinal plants is influenced by number of intrinsic and extrinsic factors. High alcohol soluble and water soluble extractive values revealed the presence of polar substance like phenols, tannins and glycosides (Sharma et al., 2009).

5.4. PHYTOCHEMICAL STUDIES

The preliminary phytochemical screening of flower and fruit of C. albidum showed the presence of alkaloids, carbohydrate, proteins, steroids, cardiacglycoside, coumarins, tannins, anthraquinone glycoside, flavonoid and phenolics compounds. The leaf and stem showed the presence of alkaloids, carbohydrates & glycosides, saponin, phenolics & tannin, proteins, cardiac glycosides, flavonoids, quinine and coumarins (Bokhad et al., 2012). But in the case of stem bark and heart wood, no previous studies were reported. Phytochemical analysis on this plant is meagre and the author started the studies from 2009 onwards and made some publications related to the physicochemical standards, preliminary phytochemical investigation, chemical fingerprinting of sequential extracts of petroleum ether, ethyl acetate and methanol of stem bark (Sreedhar et al., 2012), heart wood (Sreedhar et al., 2013) and leaf (Sreedhar et al., in press).

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 (Manjunatha & Vidya, 2008; Das & Sarma, 2009; Absar et al., 2007). 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 bark 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. The presence of phenolic compounds, tannins and flavonoids have been associated with various degrees of anti-inflammatory, analgesic (Wang et al., 2008) and antioxidant activities (Molina et al., 2003, Gholivand et al., 2010). Therefore, the antioxidant, antimicrobial and hepatoprotective effects observed in this study may be due to the activity(s) of one or a combination of some of the classes of compounds present in different parts of C. albidum.

Saponins, which have cytotoxic effect, anti-inflammatory and considered as hemotoxic, it is also present in the stem bark, heart wood and leaf. Coumarins were present which is precursor for several anticoagulants. Tannins were present in all the selected parts of the plant, which have astringent and detergent properties and can be used against diarrhoea (Trease & Evnas, 2002; Bruneton, 1999). Coumarins and naturally occurring benzopyrene derivatives are hepatoprotective active compounds (Kostova et al., 2006), flavonoids, triterpenoids and tannins are well known for their hepatoprotective activities.
(Manjunatha et al., 2008; Das et al., 2009; Absar et al., 2007). Glycosides are present only on stem portion of C. albidum. There has been an assertion by Trease and Evans (2002) that naturally cardiac glycosides are used for treatment of various diseases associated with the heart such as in controlling supraventricular (atrial) cardiac arrhythmias, it also exert a slowing and strengthening effect on failing heart (Essiett et al., 2010). The presence of these compounds in C. albidum on could be the reason behind the use of this plant as hepatoprotective medicine by the tribals.

5.4.1. TLC comparison studies

Khatoon et al., (1993) used TLC finger printing technique and identify that the market samples 'Ratanjot' is derived from Arnebia nohilis. Asif & Shafiullah, (1993) analyzed 175 herbal drugs with infrared spectrum and evolved a method for checking the purity of herbal drugs. Quality standardisation of Medicinal Plants, Pharmacopoea of different countries are being used TLC as a major criteria for quality control parameter (Gupta et al., 2008). 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.

5.4.2. GC/MS analysis of essential oil of CaL

About 18 compounds were identified in leaf oil accounting for 89.21% of the total of which caryophyllene predominates in the oil with 21.58%. linalool, β-phellandrene and phytol were the other major compounds present in the oil.
analysed. It is the first report in this plant so far published to the best of my knowledge. Caryophyllene oxide, an oxygenated terpenoid, well known as preservative in food, drugs and cosmetics, has been tested in vitro as an antifungal against dermatophytes (Yang et al., 1999).

Linalool and linalyl acetate are the principal components of many essential oils known to possess several biological activities attributable to these monoterpenic compounds. It has evaluated individually the anti-inflammatory properties. Linalool and the corresponding acetate play a major role in the anti-inflammatory activity displayed by the essential oils containing them, and provide further evidence suggesting that linalool and linalyl acetate-producing species are potentially anti-inflammatory agents (Peana et al., 2002). The more pronounced effect of (-)-linalool on the writhing test with respect to the hot plate test is consistent with the observation that (-)-linalool possesses anti-inflammatory activity. Finally, the activation of opioidergic and cholinergic systems appears to play a crucial role in (-)-linalool-induced antinociception (Peana et al., 2003).

Several Linalool-producing species are used in traditional medical systems, including *Aeolanthus suaveolens* G. Dom (Labiatae) used as anticonvulsant in the Brazilian Amazon. Psychopharmacological in vivo evaluation of Linalool showed that this compound has dose-dependent marked sedative effects at the Central Nervous System, including hypnotic, anticonvulsant and hypothermic properties. Linalool has an inhibitory effect of on Glutamate binding in rat cortex. It is suggested that this neurochemical effect might be underlining Linalool psychopharmacological effects (Elaine et al., 1995).
The chemical composition of the essential oil of *Vitex doniana* Sweet leaves from Nigeria was investigated for the first time by GC/MS. A total of 12 compounds, representing 92.6% of the total oil were identified. The major constituents were $\beta$-phellandrene (31.3%), phytol (28.3%) and $\beta$-caryophyllene (12.6%). Other minor components were $\alpha$-caryophyllene (5.1%), caryophyllene oxide (3.6%), $\alpha$-pinene (3.4%) and bicyclogermacrene (2.5%). The essential oil exhibited antimicrobial activity against 3 of the tested microorganisms, *Proteus mirabilis*, *Bacillus subtilis* and *Candida albicans* (Oluwadayo *et al*., 2009).

The *C. albidum* have antibacterial, antioxidant and antifungal activity. The earlier report showed that the major compound present in it have antifungal antiinflammatory and antinoiceptive activities. The report presented here leads to activity based further research on this plant.

**5.4.3. Phytochemical characterisation of secondary metabolites**

The secondary metabolite produced by a fungus, or any other organism, to have a useful impact on human health, or provide agricultural benefits. The preliminary phytochemical screening of flower and fruit shows the presence of alkaloids, carbohydrate, proteins, cardiac glycoside, coumarins, tannins, anthraquinone glycoside flavonoid and phenolics compounds (Bokhad *et al*., 2012). The present author studied the secondary metabolites present in the leaf, stem bark and heart wood and reported as carbohydrate, proteins, cardiaclglycoside, saponins, coumarins, tannins, flavonoid & phenolics compounds. In which, tannins, favonoids and phenolics have many therapeutic activities. *C. albidum* an important tribal medicinal plant used for treating jaundice, hitherto unexplored for its chemical constituents and its pharmacological activity. 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, heartwood and leaf 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. The thin layer chromatography densitometry methods were found to be precise with RSD for intra-day 0.44, 0.31 and 0.21 and for inter-day 0.54, 0.98 and 0.20 for different concentrations of β-sitosterol, ursolic acid and gallic acid. Instrumental precision was 0.95, 1.02 and 0.85 (% RSD) for β-sitosterol, ursolic acid and gallic acid respectively. 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. The present findings provide certain evidence to the ethno medicinal property of *C. albidum* in treating against acute jaundice.

**5.4.4. PHARMACOLOGICAL ACTION OF ISOLATED COMPOUND**

**β-sitosterol**

Anti hepatotoxic, antitumor, anti-inflammatory (Shirwarkar *et al.*, 2004), antiarthritic (Agarwal & Rangari 2003), nutraceutical, chemopreventive agent (Surh, 2003), antimicrobial (Shai *et al.*, 2008), antiedemic, antihyperglycemic,
antioxalate, hypotensive and antiperoxidant activities (Sunitha et al., 2001). Hence one of the reasons behind the observed hepatoprotective activity of CaSB may be attributed to the presence of $\beta$-sitosterol. The percentage of $\beta$-sitosterol is almost same in leaf and heart wood in *C. albidum*

**Ursolic acid**

Both oleanolic acid and ursolic acid have been identified as active components in producing hepatoprotective effects (Max et al., 1986). Ursolic acid, the isomer of oleanolic acid, was also identified as an active hepatoprotective component in the preparation of *Sambucus chinesis* Lindl. (Max et al., 1986). 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 (Shukla et al., 1992, Max et al., 1986, Lin et al., 1988, Gan et al., 1988, Liu, 1995). From the present study, the presence of ursolic acid, a hepatoprotective agent in *C. albidum* may be the reason for the use of tribals against Jaundice by the tribals of Chinnar. The percentage of ursolic acid in leaf, bark and heart wood showed almost equal amount. This revealed that not only the stem bark, the other portion can also be used against Jaundice. A thorough pharmacology studies are required to strengthen the knowledge.

**Gallic acid**

Gallic acid (GA), a trihydroxybenzoic acid, possesses promising hepatoprotective effects (Rasool et al., 2010). The cause for the hepatoprotective activity of CaSB may be attributed to the presence of Gallic acid. From our study it is revealed that,
maximum amount of gallic acid was observed in leaf samples whereas in stem bark and heart wood it was almost same.

The presence of the isolated bioactive compounds indicates that the plant have high therapeutic potential. The biological activities of the compounds were already well known. The present findings provide certain positive evidence to substantiate the ethnomedicinal property of CaSB in treating acute jaundice. At the same time percentage wise these three compounds are also present in leaf and heart wood, which provides certain leads to use these parts also as hepatoprotective. 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 hepato protective agents proving its usage as hepato protective remedy by the tribals.

5.5. BIOLOGICAL ACTIVITIES

5.5.1. ANTIOXIDANT STUDIES

An antioxidant is a molecule that inhibits the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons or hydrogen from a substance to an oxidizing agent. Oxidation reactions can produce free radicals. Free radicals, which are generated in several biochemical reactions in the body, have been implicated as mediators of many diseases, including cancer, atherosclerosis diabetis mellitus, asthma, nephritis, liver diseases and heart diseases (Al-Dabbas et al., 2006; Tsao et al., 2004, Hayet et al., 2008, Maxwell et al., 1999), which are capable of binding to proteins, lipids or abstracting a hydrogen atom and unsaturated lipid, thus initiating lipid peroxidation. This process of lipid peroxidation can significantly damage hepatic plasma membranes
(Recknagel et al., 1989) and other organelle membrane. Although these 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 (Lim & Murtijaya, 2007; Scalbert et al., 2005; Gulcin et al., 2009). Antioxidants help to neutralise free radicals. Tannins are known as free radicals scavenger and could inhibit the lipid peroxidation of biomembranes caused by reactive free radicals and they could also be used as hepatoprotective substances against the CCl₄-intoxication (Bruneton, 1993). The leaves of C. glutinosum contain flavonoic heterosides, anthocyanosides (leucocyanidol and leucodelphinidol) and tannins (acid gallic, ellagic and ferulic) and these substances could explain the protective effect on the CCl₄ damage (Fortin et al., 1990). Flavonoides and anthocyanes are scavengers of free radicals. In fact they react with them and prevent damages related to their reaction with membrane phospholipids (Bruneton, 1993). The aqueous extract of C. dolichopentalum leaves possessed potent hepatoprotective activity against CCl₄ induced liver damage in rats (Kalu et al., 2011).

The majority of the antioxidant activity is due to the flavones, isoflavones, flavonoids, anthocyanin, coumarin lignans, catechins and isocatechins (Aqil et al., 2006). 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 IC₅₀ values. Most of the tested extracts showed scavenging activity above 80% with IC₅₀ values ranging between 28.4 and 48.3 µg/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 30.5µg/ml. IC$_{50}$ value obtained for standard quercetin was 8.1 and for gallic acid were 6.25.

The histochemical and preliminary Phytochemical screening revealed the presence and percentage of carbohydrates & glycosides, saponin, tannin, coumarins, steroids, flavonoid & phenolics compounds and triterpenoids. The isolated compounds of ursolic acid and gallic acid are reported to be natural antioxidants. Hence the increased level of free radicals scavenging activity is due to the presence of these antioxidants. In _C. albidum_, leaf showed maximum percentage of gallic acid compared to other two parts. 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 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 alkaloids, glycosides, tannins, and flavonoids in the crude methanolic extract. Such compounds are known to possess potent antioxidant activity (Lee _et al._., 2004). Some of these constituents, _i.e._, β-sitosterol, ursolic acid and gallic acid have already been isolated from this plant (Sreedhar _et al._., 2013). Hence, the observed antioxidant activity may be due to the presence of any of these constituents. The plant exhibited strong hepatoprotective, antibacterial, antifungal and several other activities. These properties
may be due to its antioxidant activity. The crude methanolic extract merits further experiments in vivo.

Other Combretum species like C. racemosum leaves and root bark and C. celastroides subsp. laxiflorum leaves were used in Congolese traditional medicine for several therapeutic purposes, notably for the treatment of conditions consistent with hypertension. An in vivo study showed that methanolic extracts from leaf and stem bark have an antioxidant activity and an endothelium-dependent vasorelaxant effect. Leaf induces the vasorelaxant effect through the NO-cGMP pathway while bark extracts also act via a prostanoid pathway. Leaf extract demonstrated a modest but significant antihypertensive activity in SHR rats (Nsuadi et al., 2012). This reports says that leaves of C. racemosum and C. celastroides found in Africa having antioxidant activity and significant anti hypertensive activity. In the present study C.albidum leaf and stem bark also exhibiting high rate of antioxidant activity and the extract required further study to prove the anti hypertensive activity like the other species. Ramakrishna Rao et al., (2012) reported the antioxidant activity of leaf and bark extracts of wild versus cultivated varieties of C. albidum using TLC method collected from two districts namely Khurda and Baud of Orissa. Sixteen antioxidant bands were obtained in leaf extract of cultivated sample, while only eight were present in wild varieties. In case of bark samples, five antioxidant bands were obtained in cultivated sample where as 9 antioxidant bands were observed in wild variety.
5.5.2. ANTIMICROBIAL STUDIES

5.5.2.1. 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. In 1992, Alexander et al., found several antimicrobial compounds in 12 different African Combretum species. Martini and Eloff (1998) found evidence for at least 14 unidentified bacterial inhibitors from the leaves of C. erythrophyllum. Eloff (1999) quantified the antibacterial activities of the leaf extracts of 27 members of Combretaceae and Fyhrquist et al., (2002) found activity in extracts of the roots and stem bark of Combretum and Terminalia species used in Tanzania. The antibacterial properties of Combretum species are well investigated (Silva et al., 1996). C. imberbe has shown antimicrobial and anti-inflammatory activity (Angeh et al., 2007). Four pentacyclic triterpenes were isolated from C. imberbe, of which two are novel glycosidic derivatives of 1-α, 3-β, 23-trihydroxyolean-12-en-29-oic acid (hydroxyimberbic acid). Imberbic acid showing particularly potent activity against Mycobacterium fortuitum and Staphylococcus aureus (Katerere et al., 2003). C. molle has reported anthelmintic, antibacterial and antifungal activities (Ademola & Eloff, 2010; Asres et al., 2006). Antibacterial screening of medicinal plant extracts from Western Ghats by microtitreplate resazurin reduction assay showed no antibacterial activity of flowers of C. albidum against Staphylococcus aureus, Pseudomonos aeruginosa,
**Discussion**

*Bacillus subtilis* and *Escherichia coli* (Karuppusamy *et al.*, 2010). Ganesan (2007) reported the *C. albidum* bark is used to cure certain skin diseases by the traditional healers of Palapatty village of Tamil Nadu. So far no reports on *C. albidum* stem bark against antimicrobial activity. The author made an attempt to study the antimicrobial activity of water and methanol extracts of CaSB. The result showed significant antibacterial activity against *Escherichia coli*, *Klebsiella pneumonia*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Salmonella typhimurium* (Sreedhar *et al.*, 2013).

The study thus revealed the effectiveness of the tested plant extract against some pathogenic bacteria commonly associated with various human infections. CaSB can be used as a potential source for the development of a phytomedicine to act against infectious bacteria. It also gives positive correlation to the report of Ganesan (2009), that it is used against skin diseases by tribals. As the global scenario is now changing towards the use of non-toxic plant products having traditional medicinal use, development of modern drugs from plants should be emphasized for the control of infectious diseases.

5.5.2.2. Antifungal

West African Combretaceae extracts used in traditional medicine against several fungal species. The result of the phytochemical screening of these plant extracts showed that they were rich in tannins and saponins. Seven species of Combretaceae were investigated for their antifungal activity against the pathogenic fungi *Candida albicans*, *Epidermophyton floccosum*, *Microsporum gypseum*, *Trichophyton mentagrophytes* and *Trichophyton rubrum*. *Pteleopsis suberosa* and *Terminalia avicennioides* appear to be the most active plants in
Combretaceae (Baba-Moussa et al., 1999). Previous studies showed that extracts of South African *Terminalia* species (Masoko et al., 2005) and *Combretum* species (Masoko et al., 2007) have substantial antifungal activities, with MIC’s as low as 20µg/ml. These researchers suggested that the tannins and saponins, present in the plant extracts, may be responsible for the antifungal activity. Preliminary phytochemical screening revealed the presence of these compounds and it may be attributed to the antifungal activity of *C. albidum*. 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 *Candida albicans*. The extract also showed an efficient inhibition of the growth of *Aspergillus niger* and *Pencillum chrysogenum*. The results signify that the extracts possess almost same growth inhibitory activity with the standard antibiotics against all the tested organisms.

Kadavul and Dixit (2009) reported that the people of Kalrayan and Shervarayan Hills of Eastern Ghats of Tamil Nadu used the wiry-stem, seed oil, root bark of *C. albidum* for eye problems, eczema and antimalarial. The present study
scientifically proven that the stem bark having both antibacterial and antifungal properties.

5.5.3. CYTOTOXICITY 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. Plants, vegetables and herbs used as food and in the folk treatment have been accepted currently as one of the main sources of drug discovery and development, but only a few of them have been scientifically investigated, especially regarding their toxic aspects (Pereira et al., 2010).

In the present study, cytotoxicity of the CA-H2O, CA-EtOH and CA-Hex were done in three different concentrations (200, 100 and 50 µg/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 100 µg/ml.

5.5.4. Anticancerous activity of methanolic extract on K562 cell lines

Fyhrquist et al., (2008) reported some of the Combretum species have shown anticancer activity against human cancer 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, 150 µg/ml) at 48 hrs duration. The three extracts in different concentrations showed
anticancerous activity as compare to the normal sample and IC\textsubscript{50} value observed in the concentration in between 75 to 100\(\mu\)g/ml.

**5.5.5. Hepatoprotective activity of extracts on Hep3B cell lines**

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.

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 CCl\(_4\) 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 (Wang *et al.*, 2009). The ability of a hepatoprotective drug to reduce
the injurious effects or to preserve the normal hepatic physiological mechanisms, which have been disturbed by a hepatotoxic agent is the index of its protective effect (Yadav & Dixit, 2003).

The selected species is pharmacologically unexplored but other four *Combretum* species from Africa has proved hepatoprotective activity. Three new triterpenes of *C. quadrangulare*, lupane type, 2α, 6β-dihydroxybetulinic acid and 6β-hydroxyhovenic acid, and an oleanane type, 6 β-hydroxyarjunic acid, together with several known compounds, have been isolated from the MeOH extract of the seeds and their hepatoprotective activities were tested for D-GalN/TNF-alpha-induced cell death in primary cultured mouse hepatocytes (Adnyana et al., 2001). The aqueous extract of *C. dolichopentalum* leaves possessed potent hepatoprotective activity against CCl₄ induced liver damage in rats (Kalu et al., 2011) and ethanol extract showed anti-ulcer activity (Asuzu & Onu, 1988). The leaves of *C. glutinosum* contain flavonoic hetersides, anthocyanosides (leuocyanidol and leucodelphinidol) and tannins (acid gallic, ellagic and ferulic) and these substances could explain the protective effect on the CCl₄ damage (Fortin et al., 1990). A new gallic acid derivative isolated from *C. quadrangulare* showed potent hepatoprotective activity (Adnyana et al., 2001). In *C. albidum* gallic acid was isolated from stem bark. The earlier report supports the hepatoprotective activity of gallic acid.

In this study, alcohol, hexane and water extract of CaSB exhibited significant hepatoprotective activity. The extract demonstrated a dose-dependent decrease in cell viability at concentrations ranging from 50 to 200µg/ml of ethanolic extract in comparison to silymarin., it showed nearly equipotent protective activity.
Moreover, *C. albidum* showed good antioxidant property as evident from the tests. The tested extracts showed scavenging activity above 80% with IC$_{50}$ values ranging between 28.4 and 48.3µg/ml and scavenging of free radicals DPPH. Leaf extract showed to have maximum DPPH scavenging activity followed by stem bark and heart wood. Among the extracts studied better activity respectively IC$_{50}$ of 28.4, 29.2 and 30.5µg/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.

Previous *in vivo* studies have been reported similar hepatoprotective properties of fruit peel and flowers' extracts of *P. granatum* against CCl$_4$, ferric nitritotriacetate (Fe-NTA), and TCA-induced hepatotoxicity. The extract showed protection against hepatic lipid peroxidation and preserved GSH levels and activities of antioxidant enzymes namely, catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR), and glutathione-S-transferase (GST). In total, these results suggest that hepatoprotection shown by the *P. granatum* extract may be due to its antioxidant properties (Chidambara *et al.*, 2002).

*Mangifera indica* and its components are commonly used in folk medicine for many curative effects. It possesses antioxidant, immunomodulatory, anti-mutagenic and anticancer properties. In this study, the possible hepatoprotective property of the bark extract of *M. indica* against t-BH-induced cytotoxicity. According to the results, *M. indica* at concentration ranging from 62.5 to 250µg/ml resulted in concentration-dependent protection of HepG2 cells, thereby indicating the opposing actions against toxic stimuli. However, on comparing with silymarin (IC$_{50}$ = 49.0µg/ml), it showed lesser efficiency, with 50% protection.
achieved at a concentration of 190.5µg/ml. Further, examined the antioxidant property of the extract in two different assay systems. In ORAC assay, the extract displayed a good activity with 8260 ± 32.53 µMTE/g. It showed efficient scavenging of ABTS and DPPH radicals with IC₅₀ values of 4.80µg/ml and 12.81µg/ml, respectively (Hiraganahalli et al., 2012). These results are in good agreement with present studies showing antioxidant and hepatoprotective activities of C. albidum.

Acacia catechu and its phytoconstituents possess widespread pharmacological properties namely hypoglycaemic, hepatoprotective, antipyretic, digestive, etc. In this study, the hepatoprotective property of heart wood extract of A. catechu against t-BH-induced cytotoxicity. The extract exhibited dose-dependent protection at concentrations ranging from 62.5 to 125µg/ml, 50% inhibition in t-BH induced toxicity was observed at 114.8µg/ml. However, in comparison to silymarin (IC₅₀ = 49.0µg/ml) the extract showed very less protection. Further, the extract was tested for its antioxidant property, where it showed maximum activity in ORAC assay with the highest Trolox value (41589 ± 151.30 µMTE/g). It also demonstrated a significant effect toward ABTS and DPPH free radicals. Additionally, the hepatoprotective role of A. catechu was thought to be due to the presence of tannins, cyanidanol, and quercetin (Rage et al., 1984; Rajnarayana et al., 2001). Hence, the probable mechanism by which A. catechu exerts its protective action is by minimizing the effects of free radicals, its antioxidant activity in association with the inhibition of lipid peroxidation.

In summary, it is well established that t-BH is metabolized by two distinct pathways in hepatocytes; one via cytochrome p-450, and other by glutathione
(GSH) peroxidase converting t-BH to t-butanol and oxidized GSH. These metabolic pathways increase cellular reactive metabolites which attack the membrane phospholipids, proteins, and nucleic acids. Thus, antioxidants that can inhibit free radical generation are important in terms of protecting the liver from chemical-induced damage by stabilizing the antioxidant systems in the cell (Rush et al., 1985). Our study clearly demonstrates that *C. albidum* possess a significant antioxidant and protective effect against cytotoxicity. In conclusion, the results of the present investigation infer that these plant extracts possess potent antioxidant and hepatoprotective property, the former being probably responsible for the latter. Thus, the extracts can be beneficial in treating liver damages caused due to chemical or xenobiotic exposure. The use of *C. albidum* stem bark against Jaundice by the Muthuvan tribes of Chinnar area could be substantiated by the present findings.

This contribution summarizes work done to date and identifies avenues for future research.