Medicinal plants maintain the health and vitality of individuals and cure various diseases, including cancer without causing toxicity. With this view, the pharmacochemical characterization, GC-MS analysis, LC-MS analysis and the pharmacological potentials of the leaf and bark extracts of *H. mystax* have been studied.

**Pharmacochemical Characterization**

**Physicochemical constituents**

**Ash values**

The physical constant evaluation of the drug is an important parameter in detecting adulteration or improper handling of drugs (African Pharmacopoeia, 1986). Equally important in the evaluation of crude drug, is the determination of ash value and acid insoluble ash value. The total ash is particularly important in the evaluation of purity of drugs, i.e., the presence or absence of foreign organic matters such as metallic salts and/or silica (Musa *et al.* 2006).

The total ash values of leaf and bark of *H. mystax* are 9.34% and 10.21% respectively. These ash values are indicative of the impurities present in the drug. Since the ash values are constant for a given drug, these values are also one of the diagnostic parameters of the drug. In the present study, the total ash value is more for the bark than the leaf extracts of *H. mystax*. Both the samples have more water soluble ash than acid insoluble ash. These ash values are generally considered as the index of the purity as well as identity of the drug.
Fluorescence analysis

Many phytocompounds fluoresce when suitably illuminated. The fluorescent colour is specific for each compound. A non fluorescent compound may fluoresce if mixed with impurities that are fluorescent. The fluorescent method is adequately sensitive and enables the precise and accurate determination of the analyze over a satisfactory concentration range without several time consuming dilution steps prior to analysis of pharmaceutical samples (Pimenta et al. 2006).

The leaf powder of *H. mystax*, as such, fluoresced green under day light and short UV light and dark green in long UV light. The bark powder of *H. mystax*, as such, emitted green colour under day light and short UV light and dark green colour under long UV light.

Phytochemical studies

Presence or absence of certain important compounds in an extract is determined by colour reactions of the compounds with specific chemicals which act as dyes. This procedure is a simple preliminary pre-requisite before going for detailed phytochemical investigation. Various tests have been conducted qualitatively to find out the presence or absence of bioactive compounds. Phytochemical evaluation is one of the tools for the quality assessment, which includes preliminary phytochemical screening; chemo-profiling and marker compound analysis using modern analytical techniques. In the last two decades, HPTLC has emerged as an important tool for the qualitative, semi-quantitative and quantitative phytochemical analyses of herbal drugs and formulations. HPTLC method is fast, precise, sensitive and reproducible with good recoveries for standardization of herbal drugs.
The preliminary phytochemical screening of leaf and bark, methanol and ethanol extracts of *H. mystax* has revealed the presence of alkaloid, anthraquinone, catechin, coumarin, flavonoid, phenol, quinone, saponin, steroid, tannin, terpenoid, sugar, glycoside and xanthoprotein in them. HPTLC investigation also confirmed the presence of alkaloids, flavonoids, glycosides, saponins and steroids which could make the plants useful for treating different ailments and having a potential of providing useful drugs of human use. This is because; the pharmacological activity of any plant is usually traced to a particular compound.

Therapeutically terpenoids exert a wide spectrum of activities such as antiseptic, stimulant, diuretic, anthelmintic, analgesic and counter-irritant (Gokhale *et al.* 2003). Many tannin containing drugs are used in medicine as astringent. They are used in the treatment of burns as they precipitate the proteins of exposed tissues to form a protective covering (Handa and Kapoor, 1992). They are also medically used as healing agents in inflammation, leucorrhoea, gonorrhoea, burns and piles and as antidote (Ali, 1994).

Saponins, a group of natural products, occur in the investigated plant namely *H. mystax*. In plants, the presence of steroidal saponins like cardiac glycosides appear to be confined to many families and these saponins have great pharmaceutical importance because of their relationship to compounds such as the sex hormones, cortisones, diuretic steroids, vitamin D etc., (Evans and Saunders, 2001). A synthetic steroid by name sapogenin is prepared from plants and is used to treat a wide variety of diseases such as rheumatoid arthritis, collagen disorders, allergic and asthmatic conditions (Claus, 1956). Saponin reduces the uptake of certain nutrients including glucose and cholesterol at the gut through intra-luminal physicochemical interactions. Hence, it has been reported to
have hypocholesterolemic effect and thus may aid lessening metabolic burden that would have been placed in the liver (Price et al. 1987).

Several authors have reported that flavonoids, sterols/terpenoids and phenolic acids are known to be bioactive antidiabetic principles (Oliver-Bever, 1986; and Rhemann and Zaman, 1989). Flavonoids are phenolic substances isolated from a wide range of vascular plants, with over 8000 individual compounds known. They act in plants as antioxidants, antimicrobials, photoreceptors, visual attractors, feeding repellants and for light screening. Many studies have suggested that flavonoids exhibit biological activities, including antiallergenic, antiviral, antiinflammatory and vasodilating actions (Pietta, 2000). Flavonoids are known to regenerate the damaged beta cells in the alloxan induced diabetic rats (Chakravarthy et al. 1980). Flavonoids act as insulin secretagogues (Geetha et al. 1994). Most of the plants have been found to contain substances like glycosides, alkaloids, terpenoids, flavonoids etc., which are frequently implicated as having antidiabetic effects (Loew and Kaszkin, 2002).

To understand the nature of the fluorescence emission from these crude preparations under different conditions, the preliminary phytochemical analysis of these crude preparations were compared. The comparative analysis clearly showed a correlation between a compound present in it and their fluorescent behaviour under different conditions. The major bioactive compounds present in these crude preparations are the coumarins, flavonones, tannins, alkaloids and saponins. Coumarin especially hydroxyl amino acid derivatives like o-coumaric acid appears yellowish green in alkaline condition under short UV radiation. Flavonones which are light yellow in aqueous condition, under UV light, turns to bright yellow under alkaline conditions. Similarly the phytosterols, when treated with 50% H₂SO₄ show green fluorescence under UV light. Terpenoids,
especially sapogenins, exhibit yellow green fluorescence under short UV light (Horborne, 1976). Quinine, aconitin, berberin and emetin show specific colours of fluorescence (Aconitin - light blue; berberin - light yellow; emetin - orange). Fixed oils and fats fluoresce least, waxes more strongly and mineral salts most of all (Evans, 1996). Haydon (1975) studied the photophysical characters of coumarins. Hydroxy methyl coumarin fluoresced at 420 – 440 nm when observed in different solvents with increasing polarity (Chaltopudhyay et al. 2006). The fluorescence analysis of the crude drugs prepared from the leaf and bark of *H. mystax* exhibited clear fluorescence behaviours at different radiations which can be taken as standard fluorescence pattern.

**GC–MS analysis**

Thirteen compounds were detected from the ethanol extract of leaf of *H. mystax*. The prevailing compounds in ethanol extract were Propane, 1, 1, 3-triethoxy- (2.49%), Dianhydromannitol (3.56%), 3,7,11,15-Tetramethyl-2-hexadecen-1-ol (2.85%), 1-Octadecyne (1.07%), Dibutyl phthalate (1.60%), n-Hexadecanoic acid, 13.52%, Hexadecanoic acid, ethyl ester (1.25%), 11,14,17-Eicosatrienoic acid, methyl ester (1.96%), Phytol (9.25%), 9,12-Octadecadienoic acid (Z,Z)- (3.20%), 1,2-Benzenedicarboxylic acid diisooctyl ester (48.75%), Squalene (6.41%) and Vitamin E (4.09%) respectively.

Twenty compounds were detected from the ethanol extract of bark of *H. mystax*. The results revealed that 2-Furancarboxaldehyde, 5- (hydroxymethyl)- was found as major compound (27.64%) followed by α-D-Glucopyranoside, methyl (15.00%), n-Hexadecanoic acid (14.69%), 9,12-Octadecadienoic acid (Z,Z)- (7.24%), Oleic Acid (7.03%), Benzaldehyde, 2-hydroxy-6-methyl- [Synonyms: 2,6-Cresotaldehyde] (6.79%), Benzofuran, 2,3-dihydro- [Synonyms: Coumaran] (5.25%), Octadecanoic acid (2.24%), Squalene (0.49%).
Among the identified phytochemicals, Tetradecanoic acid, n-Hexadecanoic acid and squalene have the property of antioxidant activity. 9, 12-Octadecadienoic acid (Z, Z) have the property of antiinflammatory and antiarthritic as reported by the earlier workers (Lalitha rani et al. 2009; Maruthupandian and Mohan, 2011b; Kala et al. 2011). Recently it has been found that squalene possesses chemopreventive activity against the colon carcinogenesis. (Rao et al. 1998). Lupeol have the property of anticancer, antiprotozoal, chemopreventive and antiinflammatory (Gallo and Sarachine, 2009). Lupeol significantly enhanced [-3H] glutamate uptake by astrocyte cultures and may play a role in treatment for neurodegenerative disorders (Martini et al. 2007). Stigmasterol is used as a precursor in the manufacture of semi synthetic progesterone, a valuable human hormone that plays an important physiological role in the regulatory and tissue rebuilding mechanisms related to estrogen effects, as well as acting as an intermediate in the biosynthesis of androgens, estrogens, and corticoids. It is also used as the precursor of vitamin D3 (Sundararaman and Djerassi, 1977, Kametani and Furuyama, 1987). Oleic acid may hinder the progression of adrenoleukodystrophy (ALD), a fatal disease that affects the brain and adrenal glands. Oleic acid also keeps cell membranes soft and fluid, allowing helpful antiinflammatory substances like omega-3 fatty acid to penetrate the cell membrane more easily and preventing the negative effects of bad cholesterol (Nicholas, 2002). Phytol, one of the major prevailing bioactive principles detected from leaf of H. mystax is also found to be effective at different stages of arthritis. It is found to give good preventive and therapeutic results against arthritis. The results show that reactive oxygen species-promoting substances such as phytol constitute a promising novel class of pharmaceuticals for the treatment of rheumatoid arthritis and possibly other chronic inflammatory diseases. (Ogunlesi et al. 2009).
Thus, GC-MS analysis is the first step towards understanding the nature of active principles in the medicinal plants and this type of study will be helpful for further detailed study. Further investigations in the pharmacological importance of leaf and bark of *H. mystax*, their diversity and detailed phytochemistry may add new knowledge to the informations in the traditional medical systems.

**LC – MS Analysis**

Twenty one and nineteen compounds and their molecular mass were identified in the leaf and bark of *H. mystax* respectively. Most of the identified phytochemicals are shown to have many pharmacological properties.

Numerous preclinical studies have shown that kaempferol and some glycosides of kaempferol have a wide range of pharmacological activities, including antioxidant, antiinflammatory, antimicrobial, anticancer, cardioprotective, neuroprotective, antidiabetic, antiosteoporotic, estrogenic/antiestrogenic, anxiolytic, analgesic and antiallergic activities. (Calderon-Montano, *et al.* 2011).

Taxifolin (bioflavonoid- dihydroquercetin) is the most promising natural antioxidant. The antioxidative activity of Taxifolin exceeds that of most common antioxidants such as tocopherols (vitamin E) and carotenoids (vitamin A). In addition, Taxifolin is much more resistant to the effects of oxidation and light. Health benefits of Taxifolin include capillary, liver, and radiation protection. By trapping free radicals, Taxifolin helps to protect the body against adverse environmental factors. Regular consumption of Taxifolin helps to sustain antioxidant reserves in the human body. Taxifolin is not mutagenic and low toxic compared to the related compound quercetin.
(Makena et al. 2009). It acts as a potential chemopreventive agent by regulating genes via an ARE-dependent mechanism. (Lee et al. 2007). Taxifolin has shown to inhibit the ovarian cancer cell growth in a dose-dependent manner. (Luo et al. 2008).

Carnosol, has been evaluated for anticancer property in prostate, breast, skin, leukemia, and colon cancer with promising results (Johnson, 2011). Naringenin has been shown to reduce hepatitis C virus production by infected hepatocytes (liver cells) in cell culture. This seems to be secondary to Naringenin's ability to inhibit the secretion of very-low-density lipoprotein by the cells. (Nahmias et al. 2008). Naringenin lowers the plasma and hepatic cholesterol concentrations by suppressing HMG-CoA reductase and ACAT in rats fed a high cholesterol diet. (Lee et al. 1999).

The presence of various biotic compounds justifies the use of this plant for various ailments by traditional practitioners. However, isolation of individual phytochemical constituents and subjecting it to biological activity will definitely give fruitful results. It could be stated that *H. mystax* contains various biotic compounds. So it is recommended as a plant of phytopharmaceutical importance.

**Pharmacological studies**

**Antioxidant activity**

The systematic literature collection, pertaining to this investigation indicates that the plant phenolics constitute one of the major groups of compounds using as primary antioxidants or free radical scavengers. Flavonoids are the most diverse and widespread group of natural compounds and are likely to be the most important natural phenolics.
These compounds possess a broad spectrum of chemical and biological activities including radical scavenging activity.

Flavonoids are important secondary metabolites of plant modulating lipid peroxidation involved in atherogenesis, thrombosis and carcinogenesis. It has been confirmed that pharmacological effects of flavonoids is correlating with their antioxidant activity (Sri et al. 2006). Phenolic compounds are considered to be the most important antioxidants of plant materials. They contribute one of the major groups and compounds acting as primary antioxidants or free radical terminators. Antioxidant activity of phenolic compounds is based on their ability to donate hydrogen atoms to free radicals. In addition, they possess ideal structural properties for free radical scavenging properties (Sulaiman et al. 2011). The presence of these compounds such as phenolics and flavonoids in the leaf and bark extracts of *H. mystax* may give credence to its local usage for the management of oxidative stress induced ailments.

Free radicals and other reactive species are thought to play an important role in many human diseases. Radical scavenging activities are very important due to the deleterious role of free radicals in biological systems. Many secondary metabolites which include flavonoids, phenolic compounds, etc. serve as sources of antioxidants and do scavenging activity (Ghasemi et al. 2009; Doss et al. 2010). In this study, it is evident that the extracts of the study species, *H. mystax* possess effective antioxidant activity. This feature is perhaps due to the presence of respective phytochemicals like flavonoids, phenolics, etc. in this species (Anandakumar et al. 2009).
In vitro antioxidant activity of the petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of leaf and bark of *H. mystax* were investigated in the present study by DPPH, hydroxyl, superoxide and ABTS radical cation scavenging activities. These methods have proven the effectiveness of the extracts in comparison to that of the reference standard antioxidants, ascorbic acid and trolox.

DPPH assay is the most widely reported method for screening antioxidant activity of many plant drugs, based on the reduction of methanolic solution of colored free radical DPPH by free radical scavenger. The procedure involves measurement of decrease in absorbance of DPPH at its absorption maxima of 516nm, which is proportional to concentration of free radical scavenger added to DPPH reagent solution. DPPH is a stable, nitrogen-centered free radical which produces violet color in ethanol solution. It was reduced to a yellow colored product, diphenylpicrylhydrazine, with the addition of extracts of *H. mystax* in a concentration-dependent manner (Jothy *et al.* 2011). Among the solvent tested, Petroleum ether extracts of leaf and bark of *H. mystax* exhibited more DPPH radical scavenging activity.

Hydroxyl radicals are major active oxygen species causing lipid peroxidation and enormous biological damage. Hydroxyl radical scavenging capacity of extracts of *H. mystax* is directly related to its antioxidant activity. This method involves *in vitro* generation of hydroxyl radicals using Fe$^{3+}$/ascorbate/EDTA/H$_2$O$_2$ system using Fenton reaction. The oxygen derived hydroxyl radicals along with the added transition metal ion (Fe$^{2+}$) causes the degradation of deoxyribose into malondialdehyde which produces a pink chromogen with thiobarbituric acid (Battu *et al.* 2011). When *H. mystax* leaf and bark extracts were added to the reaction mixture, it removed the hydroxyl radicals from the
sugar and prevented the reaction. Among the solvent tested, methanol extract of leaf and petroleum ether extract of bark possessed more hydroxyl radical scavenging activity when compared with standard ascorbic acid.

Superoxide anion is also very harmful to cellular components and produced from molecular oxygen due to oxidative enzyme of body as well as via non-enzymatic reaction such as auto oxidation by catechocholamines (Naskar et al. 2010). The superoxide radicals generated from dissolved oxygen by PMS-NADH coupling can be measured by their ability to reduce NBT. The decrease in absorbance at 560nm, *H. mystax* leaf and bark extracts indicated the ability to quench superoxide radicals in the reaction mixture. The present study showed potent superoxide radical scavenging activity for *H. mystax* leaf and bark extracts. The leaf and bark ethanol extracts showed potent superoxide radical scavenging activity with IC$_{50}$ values 38.94µg/mL and 29.18µg/mL compared to ascorbic acid 22.93µg/mL and 25.13µg/mL respectively.

ABTS radical cation scavenging activity is relatively recent one, which involves a more drastic radical, chemically produced and is often used for screening complex antioxidant mixtures such as plant extracts, beverages and biological fluids. The ability in both the organic and aqueous media and the stability in a wide pH range raised the interest in the use of ABTS$^+$ for the estimation of antioxidant activity (Huang et al. 2011). The present study, leaf and bark methanol extracts of *H. mystax* were fast and effective scavengers of ABTS radical and this activity was higher than that of trolox standard. Proton radical scavenging is an important attribute of antioxidants. ABTS a protonated radical has characteristic absorbance maxima at 734nm which decreases with the scavenging of the proton radicals (Adedapo et al. 2009).
Several reports indicated that the reducing power of bioactive compounds was associated with antioxidant activity (Sidduraju et al. 2002). Therefore, it is necessary to determine the reducing power of phenolic constituents contained in the plant extracts to elucidate the relationship between their antioxidant effect and their reducing power. In the present study, increase in absorbance of the reaction mixture indicates the reductive capabilities of leaf and bark ethanol extracts of *H. mystax* in concentration dependent manner when compared to the standard ascorbic acid.

The present study reveals that the leaf and bark extracts of *H. mystax* exhibits satisfactory scavenging effect in all the radical scavenging assays. This is the first report on the antioxidant property of this plant. It is reported that phenolics and flavonoids are natural products which have been shown to possess various biological properties related to antioxidant mechanisms (Shirwaikan et al. 2004). Thus in the present study, the antioxidant potential of *H. mystax* may be attributed to the presence of flavonoids, phenolics and other constituents present in them.

**Anticancer activity**

The present investigation was carried out to evaluate the antitumour activity of leaf, bark and combined extracts of *H. mystax* in DAL tumor bearing mice. The leaf, bark (250mg/kg respectively) and combined (150 mg/kg leaf extract+150 mg/kg bark extract) ethanol extracts treated animals significantly inhibited the tumour volume and tumour (viable) cell count and brought back the haematological parameters to more or less normal levels.
In DAL tumor bearing mice, a regular rapid increase in ascetic tumour volume was observed. Ascetic fluid is the direct nutritional source for tumor cells and a rapid increase in ascetic fluid with tumour growth would be a means to meet the nutritional requirement of tumour cells (Prasad and Giri, 1994). Treatment with leaf, bark and combined ethanol extracts of *H. mystax* inhibited the tumor volume, viable tumor cell count and increased the life span of the tumor bearing mice. The reliable criteria for judging the value of any anticancer drug are the prolongation of the life span of animals (Clarkson and Burchenal, 1965). It may be concluded that leaf, bark and ethanol extracts of *H. mystax* by decreasing the nutritional fluid volume and arresting the tumour growth, increases the life span of DAL bearing mice. Thus, leaf, bark and combined ethanol extracts of *H. mystax* has antitumour activity against DAL bearing mice.

In cancer chemotherapy the major problems that are being encountered are of myelosuppression and anaemia (Price and Greenfield, 1958; Hogland, 1982). The anaemia encountered in tumour bearing mice is mainly due to reduction in RBC or haemoglobin percentage and this may occur either due to iron deficiency order to haemolytic or myelopathic conditions (Fenninger and Mider, 1954). Treatment with leaf and bark ethanol extracts of *H. mystax* brought back the haemoglobin (Hb) content, RBC and WBC count more or less to normal levels. This clearly indicates that ethanol extracts of leaf and bark of *H. mystax* possess protective action on the haemopoietic system.

The results of the present study demonstrates that the leaf, bark and combined ethanol extracts of *H. mystax* increased the life span of DAL tumor bearing mice, reduce tumour volume and improve the haematological parameters. The association between flavonoids and reduced cancer risk has been reported in previous studies that showed a decrease in cancer risk with consumption of vegetables and fruits rich with flavonoids.
(Ferguson et al. 2004; Park et al. 2008). The results of this study are accordance with this finding since the phytochemical screening showed the presence of flavonoids in the leaf and bark ethanol extracts of *H. mystax*. The anticancer activity of total flavonoids and alkaloids isolated from different plants were reported earlier (Vijayan et al. 2004; Park et al. 2008). Plants derived compounds have played an important role in the development of several clinical useful anticancer agents (Cragg and Newmann, 2006). Since the phytochemical screening of *H. mystax* leaf and bark showed the presence of alkaloids, flavonoids, terpenoids, steroids, saponins, glycosides and phenols, which could make the plants useful for treating anticancer drug. Further, the isolation of the compounds responsible for the activity has to be taken up which may result in a modern drug from these plants.

**Antidiabetic activity**

Diabetes is a major degenerative disease in the world today affecting at least 15 million people and having complications which induce hypertension, atherosclerosis and microcirculatory disorders (Ogbonnia et al. 2008 and Edem, 2009). Diabetes mellitus is also associated with long term complications, including retinopathy, nephropathy, neuropathy and angiopathy and several others (Krishova et al. 2008). Alloxan, a beta cytotoxin induces chemical diabetes (Alloxan diabetes) in a wide variety of animal species by damaging the insulin secreting pancreatic β-cells, resulting in a decrease in endogenous insulin release, which paves the way for the decreased utilization of glucose by the tissues (Bierman et al. 1975; Omamoto et al. 1981; Baynes, 1991; Saravanan and Pari, 2005 and Gurusamy et al. 2008). The prevention of diabetes is an urgent worldwide health concern. The period preceding the onset of type-2 diabetes is typically characterized by obesity and insulin resistance induced by over reacting and physical inactivity.
The ethanol extracts of leaf and bark of *H. mystax* were treated on alloxan induced diabetic rats (Group - III and Group - IV). The results based on biochemical parameters were compared with normal control rats (Group - I), diabetic control (Group II) and the positive control glibenclamide rats (Group - V) after fourteen days of treatment. After the alloxan induction, glucose, insulin, lipid profiles, protein and antioxidant were restored to control levels with the administration of the known drug glibenclamide and leaf and bark extracts of *H. mystax*. The results of the present study show the significant changes in biochemical parameters of the experimentally induced diabetes. Blood glucose, serum insulin, urea and creatinine levels of ethanol extracts and glibenclamide treated rats were compared with control. When compared to control rats, the administration of leaf and bark ethanol extracts of *H. mystax* decreases the blood glucose level whereas serum insulin level is increased in glibenclamide treated rats. The hypoglycemic activity of ethanol extract of *Butea monosperma* leaves was found to induce insulin release from pancreatic cells of diabetic rats (Sharma and Garg, 2009). Ahmed et al. (1991) fed the ethyl acetate-soluble fraction of an absolute ethanol extract of *Pterocarpus marsupium*, which significantly lowered blood sugar level with corresponding increase in insulin level in alloxan induced diabetic rats. It is evident from this study that there is an increase in insulin levels in diabetic rats treated with the extracts. Many plants have been studied for their hypoglycemic and insulin release stimulatory effects (Al-Hader *et al.* 1994; Hikino *et al.* 1989; Ivorra *et al.* 1989 and Morrison *et al.* 1985 and 1987).

Extensive research has been conducted in the last few decades on plants mentioned in ancient literature and used traditionally for antidiabetic activity. Grover *et al.* (2002) have reported 45 medicinal plants and their products that have been used in the Indian traditional system of medicine and shown experimental or clinical antidiabetic activity.
The most effective and commonly used antidiabetic plants are *Allium cepa, A. sativum, Aloe vera, Gymnema sylvestre, Syzygium cumini, Ficus benghalensis, Rubia cordifolia* and *Tinospora cordifolia* (Grover et al. 2002; Ziyyat et al. 1997 and Rao et al. 2005).

A significant elevation in serum constituents, urea and creatinine were observed in alloxan induced diabetic rats (Group - II), when compared to control rats. The leaf and bark ethanol extracts of *H. mystax* were administered orally to rats for fourteen days and this reversed the levels of urea and creatinine to near normal. The administration of glibenclamide also decreased the levels of urea and creatinine to some extent.

Alloxan is taken as an indication of an abnormal glomerular function where a single injection of cisplatin, at a dose of 5 mg/Kg body weight in rabbits, caused a marked reduction in the glomerular filtration rates, which was accompanied by an increase in the serum creatinine level, indicating the induction of acute renal failure. It is confirmed that there is a significant increase in serum creatinine in albino rats 14 days after alloxan administration. The present result shows that the treatment with leaf and bark ethanol extracts of *H. mystax* were effective in preventing alloxan induced increase in serum creatinine level when compared to the control.

It is very clear from the results presented in Table -22, a significant reduction in serum protein, albumin and globulin were observed in alloxan induced diabetic control rats (Group - II), when compared to normal control (Group - I) and glibenclamide treated rats (Group - VII). With the administration of leaf and bark ethanol extracts of *H. mystax* to the diabetic rats, protein, albumin and globulin levels were found to be restored to normal. These results were in accordance with the effect of *Eugenia singampatiana* and *Polygala rosmarinifolia* in diabetic rats (Kala et al. 2012 and Alagammal et al. 2012c). The increased levels of serum protein, albumin and globulin in alloxan induced diabetic
rats are presumed to be due to increased protein catabolism and gluconeogenesis during diabetes (Palanivel et al. 2001).

Alloxan has a profound effect on the activity of hepatic marker enzymes. The animals treated with alloxan developed hepatic damage which is evident from the increase in the enzyme activities. Pretreatment with the leaf and bark ethanol extracts of *H. mystax* and glibenclamide resulted in a decreased transaminase activity in alloxan treated rats. Chalasani et al. (2004) observed that the levels of serum AST and ALP increased as a result of metabolic changes in the liver due to administration of toxin, cirrhosis of the liver, hepatitis and liver cancer including diabetes. Similar elevated levels of serum SGPT and SGOT in alloxan induced diabetic rats were observed in the present study. It may be due to the leaking out of enzymes from the tissues and migrating into the circulation by the adverse effect of alloxan (Stanley et al. 1999). AST and ALP levels in serum were used as markers to assess the extent of liver damage in streptozotocin induced diabetic rats (Hwang et al. 2005).

This study revealed that the leaf and bark ethanol extracts of *H. mystax* regulated the activity of SGPT, SGOT and ALP in the liver of alloxan intoxicated rats. The effect of glibenclamide on the recovery of hepatic enzyme activity in serum was very similar to that of the earlier studies (Preethi and Kuttan, 2009 and Maruthupandian et al. 2010).

The levels of serum lipid profiles, total cholesterol (TC), triglycerides (TG), HDL-C, LDL-C, VLDL-C, PL and LDL/HDL in control and experimental animals were investigated in the present study. When compared to normal rats, the alloxan induced diabetic rats showed a significantly increased serum lipid profiles except HDL-C. The glibenclamide and the leaf and bark ethanol extracts of *H. mystax* treated rats showed a significant decrease in the content of lipid profiles comparing to diabetic control rats.
Similarly HDL-C level is decreased in alloxan induced diabetic rats when compared to normal control. With the administration of leaf and bark ethanol extracts of *H. mystax* and glibenclamide to the diabetic rats, HDL-C level was found to be restored to normal. The present study reveals that the levels of serum lipid profiles are usually raised in diabetic rats and such an elevation represents a risk factor for coronary heart diseases (Mironova *et al.* 2000). Lowering the serum lipid level through dietary or drug therapy seems to be associated with a decrease in the risk of cardio-vascular disease (Scott and Grundy, 1999).

During diabetes, there is an enhanced activity of the enzyme resulting in an increased lipolysis releasing more fatty acids into the circulation (Agarth *et al.* 1999). The increased fatty acid concentration also increases the β-oxidation of fatty acids, producing more acetyl Co-A and cholesterol during diabetes. In normal condition, insulin increases receptor-mediator removal of LDL-cholesterol and decreased activity of insulin, during diabetes, causes hypercholesterolemia. Hypercholesterolemia and hypertriglyceridemia have been reported to occur in diabetic rats (Mironova *et al.* 2000). The increased concentration of free fatty acid may be due to lipid break-down and this may cause increased generation of NADPH-dependent microsomal lipid peroxidation. Phospholipids are increased in alloxan induced diabetic rats. Phospholipids are present in cell membrane and make up vast majority of the surface lipoprotein forming a lipid bilayer that acts as an interface with both polar plasma environment and non-polar lipoprotein of lipoprotein core (Cohn and Roth, 1996). Increased phospholipids level in tissues was reported by Venkateswaran *et al.* (2002) and Pari and Satheesh (2004) in streptozotocin induced diabetic rats. Administration with the leaf and bark ethanol extracts of *H. mystax* and glibenclamide decreased the level of phospholipids.
The results of the present study showed increased lipid peroxidation (LPO) on serum, liver and kidney of alloxan induced diabetic rats. Earlier studies have confirmed that there is an increased lipid peroxidation in liver, kidney and brain of diabetic rats (Latha and Pari, 2003a and Ananthan et al. 2004). This may be due to relatively high concentration of early peroxidizable fatty acids contained by the tissues. In the present study, an increase in the levels of LPO was found and these levels were significantly reduced after the supplementation with the ethanol extracts of leaf and bark of *H. mystax* and glibenclamide. This indicates that leaf and bark extracts of *H. mystax* inhibit oxidative damage due to the anti-peroxidative effect of ingredients present in them. This could be correlated with the previous studies of Pari and Latha (2002) on *Cassia auriculata* flower, Prince and Menon (1998) and Prince *et al.* (2004) on *Syzygium cuminii*, Prince *et al.* (1999) on *Tinospora cordifolia* and Latha and Pari (2003b) on *Scoparia dulcis* indicating anti-peroxidative and antihyperlipidaemic effects in diabetic animals. Apart from the regulation of carbohydrate metabolism, insulin also plays an important role in the lipid metabolism. Insulin is a potent inhibitor of lipolysis, since it inhibits the activity of hormone sensitive lipase in adipose tissue and suppresses the release of free fatty acids (Loci *et al.* 1994).

The levels of superoxide dismutase (SOD), catalase (CAT), glutathione peroxide (GPx) and reduced glutathione (GSH) in the serum, liver and kidney of the control and experimental rats were studied. A highly significant reduction in the activity of scavenging mitochondrial enzymes is observed in alloxan induced rats. These adverse changes could be reversed to near normal with the treatment of leaf and bark ethanol extracts of *H. mystax* and glibenclamide. The results were in accordance with the effect of *Polygala rosmarinifolia* (Nishanthini and Mohan, 2012).
Mitochondria are the energy reservoirs of the cell. The damage inflicted in mitochondria would ultimately result in the reduction of energy production and thereby leading to cell death (Sohal and Dubey, 1994). Sub-cellular membrane, associated with thiol bearing enzymes, represents sensitive sites for detoxification causing perpetuation of cellular function (Kyu and Byung, 1997). Reactive oxygen species can themselves reduce the activities of antioxidant defence mechanism. It was observed from the present study that the leaf and bark ethanol extracts of *H. mystax* enhanced mitochondrial enzymatic antioxidant activity and suppressed lipid peroxidation.

Free radical reacts with lipids causing peroxidation, resulting in the release of products such as malondialdehyde, hydroperoxide and hydroxyl radicals. The plant extracts have the capacity to scavenge free radicals directly or interfering with generation of free radicals (Reddy and Lokesh, 1992 and Dhuley *et al.* 1993). Thus, the inhibitory effects of these extracts on oxidative damage may be attributed to the suppression induced peroxidation (Selvendiran *et al.* 2004). It is well known that CAT, SOD and GPx play an important role, as protective enzymes, against free radical formation in tissues (Oberly and Buettner, 1974). Several investigators have reported that the reduced activities of CAT and SOD genes are induced by free radicals and also by certain humoral factors (Anderson *et al.* 1994 and Slaga, 1995). The present study indicates the reduction in the activity of SOD, CAT, GPx and GSH in alloxan induced diabetic rats (Group - II). These results reveal the protective role of these plant extracts in decreasing lipid peroxidation and by normalizing antioxidant system.

It is concluded that medicinal plants have been reported to possess antihyperglycemic activity. The preliminary investigation on the antidiabetic efficacy of
leaf and bark ethanol extracts of *H. mystax* will be significant to proceed further in this path for the isolation of active principles responsible for antidiabetic activity.

**Hepatoprotective Activity**

Liver is largest organ and it is target for toxicity because of its role in clearing and metabolizing chemicals through the process called detoxification (Larrey, 2003). Drug induced liver disorders occurred frequently can be life threatening and mimic all forms of liver diseases (Watkins and Seeff, 2006). CCl\textsubscript{4} is one of the most commonly used hepatotoxin. CCl\textsubscript{4} produces an experimental damage that histological resembles viral hepatitis. Toxicity begins with the change in endoplasmic reticulum, which results in the loss of metabolic enzymes located in the intracellular structures (Recknagel, 1983). The toxic metabolic, CCl\textsubscript{3} radical, is produced and further reacts with oxygen to give trichloromethyl peroxy radical. Cytochrome P\textsubscript{450} is the enzyme responsible for this conversion. Both the radicals can bind covalently to the macromolecules and induce peroxidative degradation of the membrane lipid of endoplasmic reticulum rich in polyunsaturated fatty acids (Recknagel, 1983). This leads to the formation of lipid peroxidases followed by pathological changes such as depression of protein synthesis, elevation levels of serum marker enzymes such as SGOT, SGPT and ALP, depletion of GPx, GRD, SOD and GAT and increase in lipid peroxidation.

In the present study, it was observed that; the rats treated with CCl\textsubscript{4} resulted in significant hepatic damage as shown by the elevated levels of serum markers. These changes in the marker levels will reflect in hepatic structural integrity. The rise in the SGOT is usually accompanied by an elevation in the level of SGPT, which play a vital role in the conversion of aminoacids to keto acids (Suky *et al.* 2011b). The leaf and bark
ethanol extracts of *H. mystax* at the doses of 150mg/kg and 250mg/kg significantly attenuated the elevated levels of the serum markers. The normalization of serum markers by ethanol extracts of *H. mystax* suggests that they are able to condition the hepatocytes so as to protect the membrane integrity against CCl$_4$ induced leakage of marker enzymes into the circulation. The above changes can be considered as an expression of the functional improvements of hepatocytes.

Alkaline phosphate concentration is related to the functioning of hepatocytes, high level of alkaline phosphatase in the blood serum is related to the increased synthesis of it by cells lining bile canaliculi usually in response to cholestasis and increased biliary pressure (Graw *et al.* 1999). Increased level was obtained after CCl$_4$ administration and it was brought to near normal level by leaf and bark treatment of *H. mystax*.

Protein metabolism is a major project of liver and a healthy functioning liver is required for the synthesis of the serum proteins. Hypoproteinemia is a feature of liver damage due to significant fall in protein synthesis. Albumin is decreased in chronic liver disease. Hypoproteinemic was observed after CCl$_4$ ingestion but the trend turns towards normal after treatment with leaf and bark extracts of *H. mystax*.

Bilirubin is a yellow pigment produced when heme is catabolised. Hepatocytes render bilirubin water soluble and therefore easily excretable by conjugating it with glucuronic acid prior to secreting it into bile by active transport. Hyperbilirubinemia may result from the production of more bilirubin then the liver can process, damage to the liver impairing its ability to excrete normal amount of bilirubin or obstruction of excretory ducts of the liver (Olaleye, 2010). Serum bilirubin is considered as one of the true test of
liver functions since it reflects the ability of the liver to take up and process bilirubin into bile. Elevated levels may indicate several illnesses. High levels of total bilirubin in CCl₄ treated rats may be due to CCl₄ toxicity. This may have resulted in hyperbilirubinemia. The significant reduction in the level of total bilirubin in the serum of H. mystax leaf and bark extracts treated rats suggested the hepatoprotective potential of extracts against CCl₄ intoxication.

Lipid peroxidation has been postulated to the destructive process of liver injury due to CCl₄ administration. In the present study, the elevations in the levels of end products of lipid peroxidation in the liver of rat treated with CCl₄ were observed. The increase in malondialdehyde (MDA) levels in liver suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanisms to prevent formation of excessive free radicals. Treatment with leaf and bark ethanol extracts of H. mystax significantly reversed these changes. Hence, it may be possible that the mechanism of hepatoprotection by ethanol extracts of H. mystax is due to its antioxidant effect.

In the present investigations, CCl₄ intoxicated rats decreased the content of GPx and GRD in liver, whereas, treatment with leaf and bark ethanol extracts of H. mystax (150 and 250mg/kg) able to reverse such effects. Superoxide dismutase (SOD), a metallo protein is the most sensitive enzyme index in liver injury and one of the most important enzyme in the enzymatic antioxidant defense system. It scavenges the superoxide anion to form hydrogen peroxide and oxygen, hence diminishing the toxic effect caused by this radical (Curtis et al. 1972). In the present study, it was observed that the leaf and bark ethanol extracts of H. mystax significantly increased the SOD activity in CCl₄ intoxicated rats thereby diminished CCl₄ induced oxidative damage.
Catalase (CAT) is an enzymatic antioxidant widely distributed in all tissues and the highest activity is found in red cells and liver. Catalase is a heme protein, localized in the peroxisomes or the microperoxisomes. This enzyme catalyses the decomposition of H$_2$O$_2$ to water and oxygen and thus protects the cell from oxidative damage by H$_2$O$_2$ and OH. Therefore, the reduction in the activity of catalase may result in a number of deleterious effects due to accumulation of hydrogen peroxide (Chance et al. 1952). In the present study, treatment with leaf and bark ethanol extracts of *H. mystax* increased the level of catalase significantly in dose dependent manner and protected the liver from CCl$_4$ intoxication.

The results of this study demonstrate that the leaf and bark ethanol extracts of *H. mystax* have a potent hepatoprotective action against CCl$_4$ induced hepatic damage in rats. It’s mode in affording the hepatoprotective activity against CCl$_4$ induced liver damage may be due to cell membrane stabilization, hepatic cells regeneration and enhancement of antioxidant enzymes such as catalase, superoxide dismutase and glutathione peroxidase production. The hepatoprotective and antioxidant potential of the extracts could have been brought about by various phytochemical principles ie flavonoids, alkaloids, phenolics and tannins present in the leaf and bark of *H. mystax*. So results of this study demonstrated that *H. mystax* has significant protection on CCl$_4$ induced hepatotoxicity.

**Antifertility activity**

The results revealed a little change in the body weight of rats treated with the leaf and bark extracts of *H. mystax*, at doses of 150 and 250 mg/Kg body weight, for fourteen days. The weight of testis and other accessory sex organs was decreased significantly during the experiment. Among the accessory sex organs, a significant weight reduction
was noticed in the testis, caput and caudal epididymal segments and the weight reduction was dose dependent. Reduction in the weight of testis and other accessory sex organs might be due to low level of androgen, which was not enough to maintain the weight of gonads and accessories (Sharma and Jacob, 2001). It is known that the accessory sex organs viz., epididymis and vas deferens are androgen dependent target organs and manifest differential sensibility to androgens for maintenance of their structure and function. It is also known that any change in circulating androgens would affect the internal micro-environment of epididymis and thereby lead to the alternation in sperm motility and metabolism (Khan and Awasthy, 2003).

In the present study, the rats treated with leaf and bark ethanol extracts of *H. mystax* showed decreased sperm motility and sperm density in cauda and caput epididymal segments. Drastic effect on the nature of the normal sperms, in the caput and caudal region, was observed in *H. mystax* ethanol extracts treated rats. Further, tail region of the sperm was much affected in all the treated groups (Groups - II, III and IV) than the head regions. The development of normal and mature sperm is the key to optimum male fertility. The production of the sperm cells (spermatozoa) and testosterone in the testis are mainly regulated by the follicle stimulating hormone (FSH) and luteinizing hormone (LH), which are released from the anterior pituitary (Steinberger, 1971). FSH stimulates spermatogenesis in the sertoli cells, while LH stimulates the production of testosterone in the leydig cells of the testis (Kerr and Klester, 1975). Many studies on the testis of rat treated with plant extracts have also revealed the inhibitory activity on the proliferation of spermatogonia in mammals (Steinberger *et al.* 1964; Mancini *et al.* 1967 and Krueger *et al.* 1974). Spermatogenesis is therefore, a complicated process, covering proliferation of the spermatogonia, long-lasting process of the tissue meiosis and numerous changes in the
spermatids during their preformation (Steinberger, 1971 and Kerr and Klester, 1975). The results of the present study suggest that the leaf and bark ethanol extracts of *H. mystax* may affect the normal function of the sertoli and Leydig cells on continuous oral administration for fourteen days.

Sex cells can occur during the reproductive phase, mitotic division of the spermatogonia or during the maturation of the spermatozoa, thereby affecting the number and quality of the sperm cells produced in the testis. Among the leaf and bark ethanol extracts treated groups, group - III and group - V (250 mg/Kg body weight) produced a significant reduction in total sperm count and viable sperms. This may be as a result of the ability of the extract at the given doses, to either interfere with spermatogenetic process in the seminiferous tubules, epididymal functions or activities of testosterone on hypothalamic release factor and anterior pituitary secretion of gonadotropins which may result in alteration of spermatogenesis (Bowman and Rand, 1985; William, 2000). The presence of immature sperms was also observed in the experimental rats treated with 250 mg/Kg body weight of ethanol extracts of *H. mystax*. This suggests that the 250 mg/Kg body weight dose level could affect the maturation of the spermatozoa in the male rats, which might also be a contributory factor to the decrease in the mean total sperm count. The data generated in the present study, by and large, confirm to those already reported and studied with various plant extracts (Njar et al. 1995; Raji and Bolarinwa, 1997 and Parveen et al. 2002). The decrease in the caudal epididymal sperm count is a clear indication that the leaf and bark ethanol extracts of *H. mystax* can affect one or more aspects of spermatogenesis as well as spermiogenesis. Though a direct effect of ethanol extracts of *H. mystax* on the cellular mechanisms of spermatogenesis cannot be concluded,
it is likely that the impairment of the hormonal mechanisms concerned with the regulation of spermatogenesis may be the underlying cause.

The various other sperm abnormalities, like sluggish motility, coiled tail and sperm maturation are also due to the leaf and bark ethanol extracts of *H. mystax* toxicity. The hitherto unreported abnormal sperm morphology, coiled tail and malformed head could be attributed to both testicular and epididymal effects of *H. mystax* extracts. Coiling of the sperm tail is usually the product of abnormal axoneme and/or the outer dense fibril. The outcome of the present study affirms the male reproductive toxic effects of *H. mystax* extracts when applied as a therapeutic agent. Since male reproductive toxicology and male contraception are two sides of the same coin, the negative consequence of *H. mystax* leaf and bark extracts on the sperm may be taken as an advantage for further study. By the treatment employed in the study, no toxic effect was produced in the liver and kidney. It is because the liver and kidney are neither directly involved on the development nor functioning of the male reproductive system nor in the reproductive organs.

A significant decrease in the sperm density and motility was observed, in the present study, in the cauda epididymis of all the treated groups, which leads to proven in the impairment of fertility in all the treated groups. The results observed in this study also indicated that the treatment with the ethanol extract of leaf and bark of *H. mystax* to adult male rats reduces the number of female’s impregnation. In addition, the number of implantations and the number of viable foetuses were also decreased. This decrease in viable foetuses may be due to the decrease in sperm motility and sperm density observed in this study. This may be due to the effects of the given extracts on the enzymes involved in the oxidative phosphorylation process.
The present study revealed a decrease in the serum level of testosterone. This observation was similar to the earlier findings of Udoh and Kehinde, (1999); Udoh and Ekipeyong (2001) and Udoh et al. (2005a). The reduction in the serum level of testosterone could be probably due to the decrease of serum levels of LH / ICSH observed in this investigation. Leydig cells secrete testosterone by the stimulatory effect of LH (Udoh and Udoh 2005 and Udoh et al. 2005b). In males, the reduction of testosterone level may impair spermatogenesis and cause male infertility. The study also revealed a dose dependent increase in the serum estrogen level. This increase might probably be due to the conversion of testosterone to estrogen (Carr and Blackwell, 1993 and Chinoy and Padman, 1996).

Treatment with the leaf and bark ethanol extracts of *H. mystax* (150 and 250 mg/Kg body weight) was highly effective in producing reversible functional sterility. The drug treated male rats clearly indicated structural and functional alteration in testis, epididymis and seminal vesicle. Depletion of sperm count and sperm motility in the drug treated rats suggested alteration in sperm production in the testis and maturation in the epididymis. Changes in both sperm count and motility resulted in a partial infertility. This resulted in abnormal sperm function which ultimately gave rise to complete male sterility. Among the plant based contraceptives, inhibition of male fertility after administration of natural substances has been related to decreased spermatozoa density (Watcho et al. 2001). For male contraception, it is not necessary to stop spermatogenesis, but it is enough to eliminate the fertilizing ability of the spermatozoa by causing changes in the morphology or in the function of the sperm (Dwivedi et al. 1990).

The antifertility activity of *H. mystax* has been attributed to the action of various steroidal saponin. Saponins are important mainly because of their steroid structure. They
are precursors for the hemi-synthesis of birth control pills (with progesterone and estrogens) as well as similar hormones and corticosteroids (Crabbe, 1979). Recently, many laboratories are engaged in developing male contraceptives from plants (US National Academy of Sciences, 1992). Plant products, as contraceptives, will be more acceptable for economic reasons in terms of self reliance and the possible practicability for a male pill approach in countries where population pressure is high. Recently, extensive efforts have been made to study the antifertility drugs from plants (Handelsman, 1994; Khan and Awasthy, 2003 and Upadhyay et al. 1993). The present study showed that treatment with the leaf and bark extracts of *H. mystax* produced marked alterations in the male reproductive organs. Further studies are needed to prove whether the alterations are reversible or permanent after cessation of treatment and for understanding the exact mechanism.

**Antiinflammatory activity**

The present study clearly established the antiinflammatory activity of *H. mystax*. The leaf and bark extracts were found to inhibit the carrageenan-induced rat paw edema significantly, a test which has a significant predictive value for antiinflammatory agents acting by inhibiting the mediators of acute inflammation. Carrageenan induced inflammation is useful in detecting orally active antiinflammatory agents (DiRosa *et al.* 1971 and Ismail *et al.* 1997). The development of carrageenan induced edema is believed to be biphasic (Vinegar *et al.* 1969). The initial phase is attributed to the release of histamine and serotonin. The edema produced at the peak 3 h is thought to be due to the release of Kinin-like substances, especially bradykinin (Crunkhon and Meacock, 1971). The second phase of edema is due to the release of prostaglandins, protease and lysosomes.
and it is sensitive to most antiinflammatory drugs (Vinegar *et al.* 1969 and DiRosa *et al.* 1971).

Results of the present study suggest that the drugs under investigation predominantly inhibit the release of prostaglandin like substances. The leaf and bark extracts of *H. mystax* possessed varying degrees of antiinflammatory activity when tested at two different doses. The leaf and bark extracts of *H. mystax* at the dose of 500 mg/Kg showed high significant antiinflammatory activity at 3rd h, where they caused 83.91%, and 77.26% inhibition respectively, as compared to that of 10 mg/Kg of indomethacin (84.82%).

These investigations validate the use of leaf and bark of *Hugonia mystax* as a herbal drug and confirming their antioxidant, anticancer, antidiabetic, hepatoprotective, antifertility and antiinflammatory activities.