2. REVIEW OF LITERATURE

*Phyllanthus niruri* L. is a valuable medicinal plant which has been used for centuries in ancient Hindu system of medicine *i.e.* ‘Ayurveda’ to cure gallstones, jaundice and diseases of urinogenital system. It is a subtropical plant of great value, which plays an important role in health improvement around the world. Every part of this plant has been investigated as a source of valuable compounds. The aim of the present study was to assess the genetic diversity by RAPD, phytochemistry, antibacterial activity, hepatoprotective property and *in vitro* response of this potential medicinal herb. As this medicinal plant is widely used in several health disorders, it has been subjected to intensive research. The earlier reports relevant to objectives of present context are reviewed and presented here.

2.1. Biogeography and Ecology

*Phyllanthus niruri* is a small plant widely distributed in tropical and subtropical regions of Central and South America, Asia including India and Indonesia, Africa and the West Indies (Mehrota *et al*., 1990; Eisei, 1995; Unander, 1995a; Calixto *et al*., 1998). This is a common weed which can be found along the roads, in valley, on the riverbanks and near lakes. It can grow well in moist, shady and sunny places (Cabieses, 1993; Nanden, 1998).

2.2. Taxonomy

This plant species belongs to Euphorbiaceae, a large family of upright or prostrate herbs or shrubs often with milky juice (Lewis, 1977). The plant genus *Phyllanthus* is a large one consisting 550 to 750 species under sub genera: *Botryanthus*, *Cicca*, *Conani*, *Emblica*, *Ericocus*, *Gomphidium*, *Isocladus*, *Kirganelia*, *Phyllanthodendron*, *Phyllanthus*, and *Xylophylla* (Unander *et al*., 1995b; Calixto *et al*., 1998).

The most common species of the genus *Phyllanthus* found in most of the West African countries including Nigeria are *Phyllanthus niruri* and *Phyllanthus amarus*. *P. niruri* and *P. amarus* are very closely related in appearance and in phytochemical structure. The major difference between these two is that *P. niruri* has
larger leaves and the plants as a whole is bigger when compared to *P. amarus*. Reorganization of the *Phyllanthus* genus has been however classified as *P. amarus* as type of *P. niruri* (Ekwenye and Njoku, 2006). *P. amarus* is usually misidentified with the closely related *P. niruri* in appearance, phytochemical structure and history of use (Morton, 1981).

### 2.3. Herbal medicines and their pharmacological uses

Traditional systems of medicine has been in vogue for centuries in all over the world. According to one estimate, 80% of the world population still depends on herbal products for their primary healthcare needs. The toxic side effect of the drugs of modern medicine and the lack of medicines for many chronic ailments has led to the reemergence of the herbal medicine, with possible treatments for many health problems. Consequently, the use of plant-based medicine has been increasing in all over the world (British Medical Association, 1993). Varieties of plants and growing conditions according to geographical origin often play a part in determining the quality and efficacy of these herbals (Kamboj, 2000). A rapid and accurate analytical technique is necessary to check if these factors cause wide difference in the samples and therefore their quality. Recently there is an increase in interest in the search of potential drugs of plant origin that are capable of minimizing the toxicity induced by chemotherapy to normal cells without compromising its anti-neoplastic activity. Traditional system of Indian medicine extensively used to derive some compounds in plants and formulations to modulate the immune system of the host. These herbal formulations were found to be either less toxic or non-toxic (Kamboj, 2000).

Different plant parts of *P. niruri* were ethnobotanically reported to have various therapeutic activities e.g. leaves as expectorant, diaphoretic and useful in strangury and sweats; the seeds as carminative, laxative, astringent to the bowels, tonic to the liver, diuretic, diaphoretic, useful in bronchitis, ear ache, griping, opthalmia and ascites (Kirtikar and Basu, 2001). An aqueous infusion of the whole plant, which is a typical preparation, is employed as a stomachic, aperitive, antispasmodic, diuretic, against constipation, fever including malaria, dysentery, gonorrhrea, syphilis, tuberculosis, cough, diarrhea and vaginitis (Paranjape, 2001). Fresh root is a remedy for jaundice. Leaves are stomachic. Milky juice is used as
application to offensive sore and a popular remedy against fever and infusion of young shoots is given in dysentery. This species is also used in stomach ailments such as dyspepsia, colic, dropsy, urinogential problems and also as external applications for edematous swelling and inflammation (Chopra et al., 1956; Calixto et al. 1998). Whole plants have been used in traditional medicine in Central and South America and Asia (including India and Indonesia) for the treatment of jaundice, asthma, hepatitis and malaria and for its diuretic, antiviral, and hypoglycemic properties (Mehrota et al., 1990; Eisei, 1995; Calixto et al., 1998).

2.3.1. Antiviral activity

Hepatitis B is one of the major diseases inflicting human population. Conventional treatment with interferon – alpha is very expensive and has many serious side effects. Alternative herbal medicine using extracts of Phyllanthus niruri and Phyllanthus urinaria has been reported to be effective against Hepatitis B and other viral infections (Meixa et al., 1995). The genus Phyllanthus has been intensively studied clinically for its antiviral effects. A systematic review of 22 randomized clinical trial showed that Phyllanthus species have positive effects on antiviral activity and on liver biochemistry in chronic hepatitis B virus infection (Calixto et al., 1998; Liu et al., 2001). Phyllanthus amarus Schum and Thonn is an another important medicinal plant species due to its antiviral properties and useful against hepatitis infection (Bratati et al., 1990; Joy and Kuttan, 1998; Raphael et al., 2002).

An aqueous extract of P. niruri was found to inhibit the hepatitis B virus (Thyagarajan et al., 1988) and also inhibits endogenous DNA polymerase of hepatitis B virus and binds to the surface antigen of hepatitis B virus in vitro (Venkateswaran et al., 1987). Aqueous extracts containing tannin, lignan and other isolated compounds from Phyllanthus species have been tested for their anti-HIV activity in vitro and in vivo. They inhibited the HIV-key enzymes like integrase, reverse transcriptase and protease (Thyagarajan et al., 1988; Calixto et al., 1998; Shead et al., 1992; Notka et al., 2004). P. amarus was also proved to be potential plant for the treatment of hepatitis B by suppressing the growth and replication of the virus (Mehrota et al., 1990, Yeh et al., 1993; Jayaram and Thyagarajan, 1998; Lee et al., 1996). The most recent research on P. niruri reveals that its isolated molecule niruriside’s antiviral activity extends to human immunodeficiency virus by inhibiting
the reverse transcriptase enzyme (Qian-Cutrone, 1996). Its antiviral activity extends to HIV-1 RT inhibition (Ogata et al., 1992, Naik and Juvekar, 2003). Nirtetralin and niranthin were tested against human hepatitis B virus in vitro (Huang et al., 2003).

Additional studies on callus and root extracts of different species of Phyllanthus have shown the presence of phyllemblin, a tannin which has antimicrobial activity, and the hydrolyzable tannins inhibited DNA polymerase and reverse transcriptase, of geraniin and its derivatives which showed high activity in the inhibitions of HIV reverse transcriptase and angiotensin-converting enzyme involved in diabetic complications (Ueno et al. 1988; Ogata et al. 1992; Unander, 1996). Recently, seven ellagitannins isolated from P. urinaria showed activity against Epstein-Barr virus DNA polymerase at a micromolar level, and the lignans phyllmyricin B and retrojusticidin B showed strong inhibition against HIV-RT. These results present an additional potential use of this herb against several DNA viruses including oncogenic Epstein-Barr virus and retroviruses human (Liu et al., 1999).

2.3.2. Antiplasmodial activity

In vitro antiplasmodial activity of this plant extract has been described by Tona et al. (2000). The in vitro and in vivo antiplasmodial activity of the ethanolic and dichloromethane extracts as well as the toxicity of the lyophilized aqueous extract of P. niruri have been previously reported by Tona et al. (1999; 2000).

2.3.2. Antidiabetic activity

In Brazil, infusion of leaves, stems and roots of Phyllanthus species has used in folk medicine for treating intestinal infections, diabetes and disturbances of the kidney (Calixto et al., 1998). An alcoholic extract of P. niruri was found to reduce significantly the blood sugar in normal rats and in alloxan diabetes rats, and indicates its potential antidiabetic action (Raphael et al., 2000). P. niruri extract also showed inhibitory activities against angiotensin converting enzyme (ACE) and aldose reductase (AR), which play a significant role in the reduction of aldose to alditol under abnormal conditions such as diabetes (Shimizu et al., 1989). P. niruri was also used as a hypoglycemic agent in traditional medicine to control non-insulin dependent Diabetis mellitus (Sivarajan and Balachandran, 1994).
2.3.3. Analgesic activity / Antinociceptive effects

In South India, an infusion of the leaves is given for headache (Kirtikar and Basu, 1987). An extract of the callus culture of *P. niruri* showed analgesic activity (Santos et al., 1994). Methanol and ethanol extracts of dried callus tissue of *P. niruri* administered intraperitonially (10 mg/kg) to mice and showed antinociceptive effects on 5 different models of nociception (Olive-Bever, 1986). Main compounds identified in the extracts of *P. niruri* like flavonoids, tannins, terpenes, sterols, alkaloids and phenols were found to be responsible for the antinociceptive activity (Santos et al., 1994; Catapan et al., 2000). Phytosterols, quercetin, gallic acid ethyl ester and geraniin were identified in *P. caroliniensis* and among them quercetin, gallic acid ethyl ester and some flavonoids were found to have antinociceptive action in mice (Filho et al., 1996).

2.3.4. Urolithiasis

Indigenous people of Amazon are calling this herb as ‘stone breaker’ and it has been used as an effective remedy to eliminate gallstone and kidney stones by them (Mello, 1980). *P. niruri* is used in Brazilian folk medicine for patients with urolithiasis (Paulino et al., 1996). Previous clinical studies demonstrated that *P. niruri* had no acute or chronic toxicity, and preliminary data suggested the effects, which promote stone elimination in stone-forming patients, as well as the normalization of calcium levels in hypercalciuric patients (Nishiura et al., 2004). Experimental studies had shown that *P. niruri* reduced the uptake of calcium oxalate crystals by MDCK cells, without evidence of cytotoxicity or biochemical alterations of the culture medium (Campos and Schor, 1999). Moreover, it prevented the growth of calculi in a model of CaOx-induced urolithiasis in rats (Freitas et al., 2002). It was also reported that with the CaOx crystallization process *in vitro* by reducing crystal growth and aggregation and favoured the formation of a less adherent dihydrate CaOx crystalline structure (Barros et al., 2003). Its role in urolithiasis was proved to inhibit the calcium oxalate endocytosis by renal tubular cells of experimental rats (Campos and Schor, 1999; Freitas et al., 2002).
2.3.5. Cardioprotective

The studies of the antioxidative and cytoprotective effects using H\textsubscript{2}C\textsubscript{2} cardiac myoblasts showed that *Phyllanthus urinaria* has a protective activity against doxorubicin cardiotoxicity. This protection was mediated through multiple pathways such as enhancement of survival factor through elevation of glutathione, activation of catalase/superoxide dismutase activity and inhibition of lipid peroxidation. This plant may serve as an alternative source of antioxidants for the prevention of doxorubicin cardiotoxicity (Chularojmontri *et al.*, 2005).

2.3.6. Lipid lowering activity

Liver damage is followed by complex disturbances in the lipolytic activity of the vascular space which often appeared with hyperlipoproteinemia in patients (Vadivelu and Ramakrishnan, 1986). Abnormalities with lipid metabolism have been reported in cholesteosis (Seidel and Wall, 1983), alcoholism (Chander *et al.*, 1988) chemical intoxication (Dwivedi *et al.*, 1990) and hepatitis (Dudnik *et al.*, 2000). *P. niruri* was reported to possess lipid lowering activity (Khanna, 2002). In a 2002 study, Indian researchers reported that ‘chanca piedra’ increased bile acid (Khanna, 2002) secretion (demonstrated choleretic activity) and significantly lowered blood cholesterol levels in rats. Administration of alcoholic extracts of *P. niruri* in triton induced hyperlipidaemia rats, lowered the elevated level of low-density lipoprotein lipids (Chandra, 2000).

2.3.7. Antitumor and anticarcinogenic activity

3, 4-methylenedioxybenzyl-3, 4-dimethoxybenzylbutyrolactone from *P. niruri* has been reported to possess antitumor activity (Satyanarayana and Venkateswarlu, 1991). Antitumor and anticarcinogenic activities of *Phyllanthus amarus* have also been reported by Rajeshkumar *et al.* (2002). *P. amarus* extract administration has been shown to inhibit the liver tumour development induced by *N*-nitrosodiethylamine in rats and increased the life span of hepatocellular carcinoma harboring animals (Joy and Kuttan, 1998; Rajeshkumar and Kuttan, 2000). Free radicals, from both endogenous and exogenous sources, are implicated in the etiology of several degenerative diseases, such as coronary artery diseases, stroke, rheumatoid arthritis, diabetes and cancer (Halliwell *et al.*, 1992). Immunomodulating effects in
treatment of cancer by influencing the function and activity of the immune system has been reported (Ma’at, 2002). Lignans such as phyllanthin, hypophyllanthin, flavanoids, quercetin, astragalin, ellagitannins and hydrolysable tannins are shown to be present in this plant. Some of these compounds have been shown to have significant activity against experimental carcinogenesis (Calixto et al., 1998).

2.3.8. Anti-inflammatory activity

Aerial parts of *P. amarus* exhibited marked anti-inflammatory properties and suggest that these lignans are the main active principles responsible for the traditional application of this plant for the inflammatory complaints (Kassuya et al., 2005).

2.3.9. Pharmacognosy and Genetic transformation

Utility of *Phyllanthus* roots in traditional systems of treatment has been studied (Anonymous, 1969). But the number of studies into the medicinal potency of *Phyllanthus* roots has been limited. This may be due to constrains faced in the natural collection of roots which are much less in quantity compared to the aerial parts. To augment the availability of this plant organ as an alternative source of bioactive compound, root culture or hairy root culture may be ideal. Hairy roots, produced by genetic transformation through *Agrobacterium rhizogenes*, a soil bacterium, have proved to be more potent than the roots obtained by the conventional root culture method in respect of biomass production (Rhodes et al., 1987). Root culture, among all the techniques, has been used more frequently because, being organized structures, roots are more amenable to maintaining genetic stability even after a prolonged period of culture (Arid et al., 1988). During herbal drug market survey it was observed that *P. amarus*, *P. fraternus* and *P. maderaspatensis* are being sold under the trade name ‘Bhuiamlki’ in mixed form. Very little work on pharmacognostical studies on two species of *P. amarus* and *P. fraternus* is on record (De and Datta, 1990; Bagchi et al., 1992).

2.3.10. Antibacterial activity

The discovery, development and clinical use of antibiotics during the nineteenth century have substantially decreased public health hazards resulting from bacterial infections. However, there has been a parallel and alarming increase in bacterial resistance to existing chemotherapeutic agents as a result of their injudicious
use. In addition, antibiotics are occasionally associated with adverse effects to the host, including hypersensitivity, immune-suppression and allergic reactions (Ahmad et al., 1998)

These developmental demands that a renewed effort to be made to seek antibacterial agents effective against pathogenic bacteria resistant to current antibiotics. One possible strategy is the rational localization of bioactive products from folk medicines, with the hope that systematic screening of these will result in the discovery of novel effective compounds with potent and useful activities against microbes. There is an ever-increasing demand for plant-based therapeutics in both developing and developed countries due to a growing recognition that they are natural products, non-narcotic and no side effects in most cases and are easily available at affordable prices (Lewis and Elvin-Lewis, 1977; Bruneton, 1999).

A number of pathogens have developed resistance (Cohen, 1992; Gold and Moellering, 1996) to multiple antibiotics (Multiple Drug Resistance), threatening to develop complete immunity against all antimicrobial agents and therefore be untreatable. Thus, the search for novel antimicrobial agents is of the utmost importance. The increasing prevalence of multidrug resistant strains of bacteria and the recent appearance of strains with reduced susceptibility to antibiotics raises the specter of untreatable bacterial infections and adds urgency to the search for new infection-fighting strategies (Sieradski et al., 1999).

Plants have been used for centuries as remedy for human diseases because they contain components of therapeutic values (Kaushik, 1985). They are natural sources of antimicrobial agents primarily because of the large biodiversity of such organisms and the relatively large quantity of metabolites that can be extracted from them (Nostro et al., 2000). The systemic screening of antimicrobial plant extracts represents a continuous effort to find new compounds with the potential to act against multiresistant pathogenic bacteria and fungi. A special feature of higher angiospermic plants is their capacity to produce a large number of organic chemicals of high structural diversity. The so-called secondary metabolites (Evans et al., 1997), which are divided into different categories based on their mechanism of function like
chemotherapeutic, bacteriostatic, bactericidal and antimicrobial (Purohit and Mathur, 1999). The accumulation of phytochemicals in the plant cell cultures had been studied for more than thirty years and the generated knowledge had helped in realization of using cell cultures for production of desired phytochemicals (Castello et al., 2002). Plant-based remedies have been highlighted due to their fewer side effects in comparison to synthetic drugs and antibiotics. Successful transformation technology is thought to be one of the most reasonable approaches to enhance the production of secondary metabolites through genetic manipulation of biosynthetic pathway (Mann et al., 2000).

Several bacterial infections are associated with the risk of certain cancer, and viruses are now recognized as the second most important cause of human cancer. Many chemicals produced in plants are being examined for their potential to inhibit human pathogens (Mulligen et al., 1993). In recent years there has been a resurgence of interest in medicinal plants that are effective, safe and culturally acceptable as an alternative treatment for many human diseases (Atmani et al., 2003).

Antifungal and antibacterial properties were recorded in *P. urinaria* (Cruz et al., 1994), *P. fraternus* (Ramchandani and Chungalth, 1998), *P. embilica* (Jasril et al., 1999), antibacterial in *P. amarus* (Vinayagamoorthy, 1982; Verpoorte and Dihal, 1987; Kannan and Venkatakrishnan, 2002), *P. discoideus* (Mensah et al., 1990; Olukoya et al., 1993). The plant has also been reported to possess antifungal, antibacterial and antiviral activities (Verpoorte and Dihal, 1987).

Many countries have maintained research programs to screen traditional medicines for antimicrobial activity, as is the case of India (Ahamed et al., 1995; Valsara et al., 1997; Perumalsamy and Ignachimuthu, 2000; Ahmad and Beg, 2001; Kumar et al., 2006), Palestlin (Ali-Shtayeh et al., 1998; Essawi and Srour, 2000), Africa (Baba-Moussa et al., 1999), Italy (Panizzi et al., 1993), Cuba (Martinez et al., 1996), Honduras (Lentz et al., 1998), Jordan (Mahasneh et al., 1999), Indonesia (Goun et al., 2003), China (Janovska et al., 2003) and Brazilian south east region (Oliveira et al., 2007).

Alcoholic extracts of various medicinal plants such as *Adhatoda zeylanica* (George et al., 1947), *Emblica officinalis, Terminalia chebula, T. belerica, Plumbago*
zyzlanica and Holarrhena anidysentrica (Ahamed et al., 1995), Thymus vulgaris and T. origanum (Essawi and Srou, 2000), Cyperus rotundus (Puratchikody et al., 2001), Tulbagia violacea (Invernizzi, 2002), leaf of Aloe vera (Agarry et al., 2005), Andrographis paniculata (Xu et al., 2006) and R. communis (Al-zubaydi, 2009) showed a prominent antibacterial activity against deadly dangerous microorganisms.

Methanol extracts of various plants such as Euphobia hirta and Camellia sinensis (Vijaya et al., 1995), Evolvoulus alsinoides (Purohit et al., 1995); rhizome and leaves of Aristalochia paucinercis (Gadhi et al., 1999), Terminalia catappa, Swietenia mahagonii, Phyllanthus acuminatus, Ipomoea spp., Tylophora asthmatica and Hyptis brevipes have the antibacterial activities (Goun et al., 2003); and aerial parts of Anthemis tinctoria (Akgul and Saglikoglu, 2005), leaves of Cassia alata (Owoyale et al., 2005), Toddalia asiatica, Syzygium lineare, Acalypha fruticosa and Peltoporum pterocarpum (Duraipandiyan et al., 2006) also showed highly inhibitory effect against several pathogenic bacteria.

The aqueous extracts of several medicinal plants such as Acalypha wilkesiana (Alade and Irobi, 1993), Lawsonia inermis, Eclipta alba, Nyctanthes arbour-tristis, Vinca rosea, Datura stramonium, Cleome gynandropsis and Ageratum conyzodies, Tridax procumbens, Cleome viscosae, Acalypa indica and Boerhaavia erecta (Perumalsamy et al., 1999), Cassia accidentalis and C. auriculata (Perumalsamy and Ignachimuthu, 2000), Azadirachta indica (leaf, stem and bark of neem) (Arora et al., 2005), Andrographis paniculata (Xu et al., 2006) and young stem, leaf and bark of neem (Azadirachta indica L.) (Ghangaonkar and Mukadam, 2006) were found to possess active principles against the growth of pathogenic microbes. The acetone plant extracts of Cyperus rotundus recorded high antibacterial activity (Puratchikody et al., 2001). Phenolic and flavonoid extracts of several medicinal plants showed antibacterial activity (Al-zubaydi, 2009).

2.3.1. Hepatoprotective activity

Drug-induced liver injury is a major health problem that challenges not only health care professionals but also the pharmaceutical industry and drug regulatory agencies. According to the United States Acute Liver Failure Study Group, drug-induced liver injury accounts for more than 50% of acute liver failure, including
hepatotoxicity caused by overdose of acetaminophen (39%) and idiosyncratic liver injury triggered by other drugs (13%) (Michael and Cynthia, 2005). Liver damage is generally associated with cellular necrosis, increase in tissue lipid peroxidation and depletion in the tissue GSH levels. In addition serum levels of many biochemical markers like SGOT, SGPT, triglycerides, cholesterol, bilirubin, alkaline phosphatase are elevated (Mascolo et al., 1998). Hepatotoxicity of CCl4 causes the formation of trichloromethyl and trichloromethyl peroxyl radicals, initiating lipid peroxidation and resulting in fibrosis and cell necrosis (Recknagel et al., 1989).

Herbal medicines have been used in the treatment of liver diseases for a long time. In many Asian countries, the species of Phyllanthus has long been used in folk medicine for liver protection (Gamble, 1956; Thyagarajan and Jayaram, 1992). A number of herbal preparations are available in the market (Dhiman and Chawla, 2005). P. niruri is used as one of the components of a multiherbal preparation for the treatment of liver ailments (Kapur et al., 1994). Among the herbal used for hepatoprotection, Phyllanthus niruri is a well-known hepatoprotective herbal plant. The aerial parts of P. niruri, known in Brazilian folk medicine as “quebra-pedra” (stone breaker), was widely used as a tea in the treatment of genitourinary and liver disorders (Venkateswaran et al., 1987; Santos, 1990). The hexane isolated fractions of P. niruri are reported to be hepatoprotective against carbon tetrachloride and galactosamine induced cytotoxicity in primary cultured rat hepatocytes (Shyamasundar, 1985).

Phyllanthus amarus (Euphorbiaceae) is widely used against various liver disorders (Bhattacharyya and Bhattacharya, 2001). It has been traditionally used in the treatment of a variety of ailments including hepatic disorders (Nadkarni, 1976; Kirtikar and Basu, 1993). This herb has a potent free radical scavenging activity and could scavenge superoxides and hydroxyl radicals and can inhibit lipid peroxides (Joy and Kuttan, 1995). Free radicals, from both endogenous and exogenous sources, are implicated in the etiology of several degenerative diseases, such as coronary artery diseases, stroke, rheumatoid arthritis, diabetes and cancer (Halliwell et al., 1992).
Mohammed Saleem et al. (2008) demonstrated the hepatoprotective effect of alcoholic and water extract of *Annona squamosa* (custard apple) in hepatotoxic animals with a view to explore its use for the treatment of hepatotoxicity in human. In the isoniazid with rifampicin induced hepatotoxic animals there was a significant decrease in total bilirubin accompanied by significant increase in the level of total protein. ALP, AST, ALT and γ-GT levels were decreased in treatment group as compared to the hepatotoxic group.

Hepatoprotective activity of methanol leaf extracts of *Orthosiphon stamineus* against paracetamol induced hepatotoxicity in rats was investigated by Maheshwari et al. (2008). Alteration in the levels of biochemical markers of hepatic damage like SGOT, SGPT, ALP and lipid peroxides was tested in both paracetamol treated and untreated groups. Paracetamol (2 g/kg) has enhanced the SGOT, SGPT, ALP and the lipid peroxides in liver. Treatment of methanolic extract of *O. stamineus* leaves (200 mg/kg) has brought back the altered levels of biochemical markers to the near normal levels in the dose dependent manner. The findings suggested that *O. stamineus* methanol leaf extract possessed a significant hepatoprotective activity.

Hepatoprotective ayurvedic medicine - a multi herbal preparation (HPN–12) containing *Glycyrrhiza glabra, Pichorhiza kurroa, Berberis aristata, Piper longum, Phyllanthus niruri, Solanum dulcamara, Zingiber officinale, Curculigo orchioides, Elettaria cardamomum, Tinospora cordifolia, Desmodium trifolium* and *Saccharum officinarum*, when orally administered to male albino rats at 1ml/100g body weight was found to be effective against liver damage (Latha and Rajesh, 1999).

Animals with carbon tetrachloride induced hepatopathy were treated with ‘catliv’ containing extracts of *Swertia chirata, Eclipta alba, Fumaria vaillanti, Picorrhiza kurroa, Andrographis paniculata* and *Phyllanthus niruri* at 25 ml orally, twice daily for six days starting at 48 hours after administration of carbon tetrachloride. On the basis of the result obtained it was concluded that the ingredients of catliv, effectively helped in the regeneration of hepatic cells in calves (Pradhan, 2001).
Herbal preparations containing Andrographis paniculata and Phyllanthus amarus for various liver disorders have been proved to have antihepatotoxic activity (Ram, 2001). CCl₄ induced hepatotoxicity in the liver of rats, as judged by the raised serum enzymes, glutamate oxaloacetate transaminase and glutamate pyruvate transaminase, was prevented by the pretreatment with the extracts of Phyllanthus niruri, demonstrating its hepatoprotective action (Harish and Shivanandappa, 2006).

2.4. RAPD analysis

Different methods have been used to assess the diversity of plant breeding materials. This information can be obtained by studying pedigrees and determining the points of origin of the breeding germplasm. However, reliable and detailed pedigree or accession records are not always available. Morphological traits are also commonly used to determine relationships but they do not provide good estimates of genetic distance because they are influenced by the environment and they are not variable enough to adequately characterize genetic differences among elite genotypes (Smith and Smith, 1992).

Correct genotype identification of the plant material, therefore, remains important for protection of both the public health and industry. Chemoprofiling and morphological evaluation are routinely used for identification of the plants. Chemical complexity and lack of therapeutic marker(s) are some of the limitations associated with chemical approach while subjective bias in morphological evaluation limits the use.

Molecular biology offers various techniques that can be applied for plant identification (Techen et al., 2004). Genetic polymorphism in medicinal plants has been widely studied which helps in distinguishing plants at inter- and/or intra-species level (Joshi et al., 2004). Among others the assessment of genetic diversity of the germplasm has been used by plant breeders for numerous reasons like selection of parents, germplasm management and germplasm protection (Lee, 1995).

In the last decade, molecular markers such as RFLP, RAPD, SCAR and AFLP have been used to assess the genetic variation at the DNA level, allowing an
estimation of the degree of relatedness between individuals without the influence of environmental factors (Miller and Tanksley, 1990; Pandian et al., 2000). Molecular phylogenetic studies have substantially increased the understanding of the systematics of Euphorbiaceae sensu lato (s.l.) (Samuel et al., 2005). Molecular markers have also been used to quantify genetic diversity in plants (Clegg, 1990). The advantages of using molecular markers are that they allow direct comparisons of genetic similarity to be made at the DNA level (Newbury and Ford-Lloyd, 1993), they are not affected by plant development, they are not modified by the environment and also they are very abundant (Novy et al., 1994).

Factors such as speed, efficiency and amenability to automation which make RAPD analysis is the most suitable method for effective germplasm management with respect to estimating diversity, monitoring genetic erosion and removing duplicates from germplasm collections (Virk et al., 1995). Two major factors may be responsible for this variation are the difficulties in maintaining homogeneity in harvesting the P. amarus population from a plethora of closely resembled Phyllanthus species and the climatic variations resulting in biological differences in plants occurring at various geographic regions (Lee et al., 1996). Information about genetic relationships among accessions within and between species has been used in several important applications and also in plant improvement (Thormann et al., 1994).

A collection of P. amarus was made from various parts of India to determine the extent of genetic variability using analysis at DNA level. RAPD profiling of 33 collections from different locations, covering states of Tamil Nadu, Karnataka, Maharashtra, Gujarat, Assam, West Bengal, Tripura, Uttar Pradesh, Punjab and Haryana was generated (Jain et al., 2003). Analysis through UPGMA revealed up to 65% variation among these accessions. However, intra-population variation was found to be much larger in the accession from the southern part of the country. Nevertheless, interpopulation variation also overlaps in the phylogenetic clustering, which is understandable from the natural dissemination of this plant species as a weed that has spread across the geographical boundaries.
Negi et al. (2000) used RFLP analysis to find out the relationship among collection of *W. somnifera* from the mountain regions of Jammu and Kashmir (Kashmiri type) and those from the plains of central parts of the India (Nagori type). The cluster analysis separated *W. somnifera* into three sub classes corresponding to Kashmir and Nagori groups and act an intermediate type. The RFLP profile of Kashmir individuals was distinct from that of the Nagori and Kashmir individuals, even though it was identified as a Kashmir morphotype. Furthermore, a low level of variation was observed within populations, but high level of polymorphism was observed between Nagori and Kashmiri populations.

Date palm (*Phoenix dactylifera* L.) varieties were fingerprinted using fourteen random primers to detect the DNA polymorphism. Primer OPC-02 revealed a 1400 bp fragment amplified in ‘Bugal White’ (salinity tolerant) and ‘Khlas’ which is known to be drought tolerant. Primer OPD-02 distinguished ‘Bugal White’, which proved to be salinity tolerant, with a DNA fragment of about 1200 bp. Primer OPD-02 amplified a 1600 bp fragment in ‘Khashkar’, ‘Bugal White’, ‘Shaham’ and ‘Khlas’ (Kurup et al., 2009).

Inter and intra specific variation of two ginseng species *Panax ginseng* and *P. quinquefolius* was studied by Artyukova et al. (2004) and estimated by studying 159 RAPD and 39 allozyme loci. Gene diversity in the total *P. ginseng* sample was comparable with the mean expected heterozygosity of herbaceous plants. This suggests that wild *P. ginseng* plants in various areas of the currently fragmented natural habitat and cultivated plants of different origin have retained a significant proportion of their gene pool. The mean heterozygosity calculated per polymorphic locus for the RAPD phenotypes is similar to that of the allozyme loci and may be helpful in estimating gene diversity in populations of rare and endangered plant species.

The interactions between these factors can lead to complex genetic structures within populations, which are often difficult to resolve. The use of biochemical and molecular markers can enhance the understanding of such complexities (Dawson et al., 1993). The assumption that forms the basis for such analysis is that genetic structure as measured by neutral and selective genes reflects both deterministic and
stochastic evolutionary processes. Several reviews have described a detailed range of molecular markers useful for assessing plant genetic diversity (Rafalski and Tingey, 1993; Staub et al., 1996). Molecular markers are the powerful tool for rapid and efficient assess of genetic variability and have been used in germplasm banks and breeding programs of various crop species (Rafalski and Tingey, 1993).

2.5. Phytochemistry

Plants produce a great number of secondary metabolites, many of them with antibacterial and antifungal activity. Well-known examples of these compounds include flavonoids, phenols and phenolic glycosides, unsaturated lactones, sulphur compounds, saponins, cyanogenic glycosides and glucosinolates (Gomez et al., 1990; Bennett and Wallsgrove, 1994; Grayer and Harborne, 1994; Osbourne, 1996). The accumulation of phytochemicals in plant cell cultures has been studied for more than thirty years, and the generated knowledge has helped in the realization of using cell cultures for production of the desired phytochemicals (Castello et al., 2002). It is well known that the plant species synthesize and accumulate various secondary metabolites belonging to different phytochemical groups. In intact plants, the formation of these metabolites is regulated in a coordinated fashion. Differentiation of plant cells or tissues during development is implied in this process. On the other hand, plant cell cultures are widely used for the comparison of biological activities of extracts, fractions or isolated compounds from the intact plant material to that of cultured plant material obtained in some experimental conditions (Santos et al., 1994; Sokmen et al., 1999).

A great variety of species belonged to the genus Phyllanthus have been phytochemically investigated and several molecules were isolated and identified. Although most of these compounds are chemically known, their pharmacological properties remain, in general, undetermined. Constituents such as alkaloids, flavonoids, lignans, tannins, phenols and terpenes have been identified. However, the composition of the aqueous extract, as used for medicinal purposes, has not been adequately studied. Although the specific compounds have not been precisely defined, some research results credit the therapeutic action on urinary tract stones to the phenols (Ishmaru et al., 1992; Calixto et al., 1998). A wide range of plant species

Phytochemical, pharmacological, in vitro propagation and molecular studies on Phyllanthus niruri L. 22
belonged to the genus *Phyllanthus* has been phytochemically investigated. Among the studied species, *P. niruri*, *P. urinaria*, *P. emblica*, *P. flexuosus*, *P. amarus*, and *P. sellowianus* have received the most phytochemical and biological attention (Calixto *et al*., 1998).

The complexity of the mixture of compounds and the presence of several compounds in small concentrations can make the isolation and identification of these substances present in this genus is very laborious. Different environmental conditions can also affect the chemical constitution of the plants, and differing interpretation of the spectral data of the complex structures has been reported to result in considerable confusion (Khan *et al*., 2010). The choice of solvent in the isolation of compounds has proved to be crucial, because the use of ethanol or methanol may lead to the production of artefacts, e.g. ethyl gallates or methyl gallates, during the extraction process (Calixto *et al*., 1998). Callus extracts of *P. niruri*, *P. tenellus* and *P. urinaria* have the main compounds identified in the extracts were flavonoids, tannins and phenols (Santos *et al*., 1994). The accumulation of phytochemicals in plant cell cultures has been studied for more than thirty years, and the generated knowledge has helped in the realization of using cell cultures for production of the desired phytochemicals (Castello *et al*., 2002). Recently, seven ellagitannins isolated from *P. urinaria* such as phyllmyricin-B and retrojusticidin-B etc., (Liu *et al*., 1999). Callus cultures are also initiated for analytic and quantitative comparative studies of secondary metabolites synthesis between the intact plant material and callus extracts (Bahorun *et al*., 1994; El-Bahr *et al*., 1997; Rady and Nazif, 1997; Balz *et al*., 1999; Zhentian *et al*., 1999).

Currently the various applications of genetic engineering are implemented in medicinal plants to increase the production of secondary metabolites (Nisha *et al*., 2003). The aerial parts of *P. niruri* have been reported to contain alkaloids, flavonoids, phenols, coumarins, tannins, terpenoids and lignans. Several of these isolated compounds have been tested for their pharmacological activities (Ishimaru *et al*., 1992; Calixto *et al*., 1998; Huang *et al*., 2003; Naik and Juvekar; 2003). Lignans from this plant have been studied most intensively; 17 different lignans have been found so far. Several of these lignans were tested for cytotoxicity and other biological
activities in vitro. The lignans were found to enhance the cytotoxic response mediators by vinblastine with multidrug resistant cultured cells (Somanabandhu et al., 1993). The lignans such as niranthin, phyltetralin and nirtetralin isolated from aerial parts of P. amarus and suggest that these lignans are the main active principles responsible for the traditional applications (Kassuya et al., 2005). Phyllanthin and hypophyllanthin were protective against carbon tetrachloride- and galactosamine-induced cytotoxicity in primary cultured rat hepatocytes (Syamasundar et al., 1985). Several compounds isolated from P. urinaria are known to have pharmacological effects, especially rutin, β-amyrin, ellagic acid, geraniin, quercetin and β-sitosterol (Calixto et al., 1998). Filho et al. (1996) isolated several compounds such as alkaloids, tannins, flavonoids, lignans, phenols and terpenes and identified in various species of Phyllanthus.

2.6. Tissue Culture

Tissue culture technology is a powerful tool for the conservation and rapid multiplication of many threatened plant species (Fay, 1992). It has been particularly useful for the conservation and rapid propagation of valuable, rare and endangered medicinal species. The P. niruri is widely used in traditional medicine by simple cultivation or collection from the wild (Unander et al., 1995a).

The conventional method of propagation of these species is through seeds. However, poor germination potential restricts their multiplication. Micropropagation technique offers an alternative method for cloning these plants (Unander, 1991; Santos et al., 1994). In recent years, there has been an increased interest in in vitro culture techniques which offer a viable tool for mass multiplication and germplasm conservation of rare, endangered and threatened medicinal plants (Sahoo and Chand, 1998; Ajithkumar and Seeini, 1998). Commercial exploitation and elimination of natural habitats consequent to urbanization have led to gradual extinction of several medicinal plants. Micropropagation is an effective approach to conserve such germplasms. Further, genetic improvement is another approach to augment drug-yielding capacity of the plant (Tejavathi and Shailaja, 1999).

Few studies are available on the tissue culture of Phyllanthus spp. on callus cultures of P. emblica, P. urinaria, P. amarus, P. abnormis, P. caroliniensis, P.
tenellus, and *P. niruri* and on transformed root cultures of *P. niruri* (Khanna and Nag, 1973; Unander, 1991; Ishimaru *et al.*, 1992; Santos *et al.*, 1994). Direct regeneration has already been achieved (Johnson, 2006). However, the establishment of a micropropagation protocol for *P. niruri* constitutes a useful tool for large scale plant production, assuring continuous availability of plant material appropriate for the study of factors that influence the production of the target secondary metabolites as well as for strategies of *in vitro* culture to increase the yield of these active principles accumulated in cultures of *P. niruri*. A way of obtaining genuine crude drug is being limited due by large-scale destruction of natural habitat due to population pressure and overexploitation, which have become a major threat to important bioresources of *P. niruri* (Sangeeta and Buragohain, 2005). Considerable efforts have been made for *in vitro* plantlet regeneration of *P. amarus* from shoot tips and nodal and internodal segments (Bhattacharya and Bhattacharya, 2001; Ghanti *et al.*, 2004).

Sivanesan (2007) compared different media (MS, SH and B5) for the shoot multiplication from the shoot tip explants of mature plants of *W. somnifera*. MS medium was found superior to SH and B5 medium. Similar observation was made in *Eclipta alba* (Baskaran and Jayabalan, 2005). Liang and Keng (2006) developed a protocol for a rapid production of *P. niruri* plantlets using nodal segments. Rapid and efficient propagation of *P. niruri* using shoot tip culture for providing a better source for continuous supply of plants in the manufacturing of drugs (Karthikeyan *et al.*, 2007). Kalidass and Mohan (2009) developed an efficient micropropagation protocol for the medicinal plant *P. urinaria* Linn. using nodal segment for axillary shoot proliferation.

The regenerated shoots were found to produce flower buds after 6 weeks of culture in the medium supplemented with KIN (0.5 to 4 mg/l) and IAA (0.1 mg/l) in *Withania somnifera* (Saritha and Naidu, 2007). Similar observation was made in *Ocimum sanctum* (Karthikeyan *et al.*, 2009). Rooting of regenerated shoots of *Physalis peruviana* was occurred on hormone free MS medium (Jayasree *et al.*, 2005). Profuse rooting (46.8 per shoot) was induced by IBA (2.0 mg/l) with a root length of 19.7 cm in *Adhatoda vasica* (Sangeetha and Buragohain, 2005). Highest frequency of rooting (85%) was obtained in both apical and axillary bud derived shoots of *Heliotropium indicum* in half strength MS medium supplemented with IBA.
(0.1 mg/l) (Senthilkumar and Rao, 2007). About 90% of rooting was achieved in *Withania somnifera* on MS medium with IBA (3.6 µmol) (Ashutosh et al., 2004). Micropropagated plants of *Withania somnifera* were hardened in half strength MS medium and then established in sand and soil (1:1) mixture (Ujjwala Supe et al., 2006). Rooted plants of *Phyllanthus amarus* were hardened on MS basal liquid medium added sterile soil + vermiculite (1:1). The survival rate of plantlets in the field was found to be very high (85%) (Ghanti et al., 2004). A maximum of 60% survival rate was noticed in *Macrotyloma uniflorum* on a mixture of soil, sand and manure (1:1:1 ratio) (Tejavathi et al., 2010). Rooted shoots of *W. somnifera* were hardened successfully in garden soil - vermicompost (3:1 w/w) under a glass house (Sabir et al., 2008).

Duangporn and Siripong (2009) investigated the effects of various combinations of auxin and cytokinin on the callus growth and accumulation of Phyllanthusol-A on *P. acidus*. A few reports are available for phytochemical analysis in *in vitro* derived cultures. Based on this back ground information, the present study was initiated on calli production and organogenesis in *P. niruri*. Therefore, the development of an *in vitro* protocol is of critical importance as it will provide plants that can be used for reintroduction in their natural habitats and for further chemical analytical and pharmacological studies.