Chapter 1

General Introduction
1. General Introduction

1.1 Medicinal Plants

Medicinal plants and their products constitute a treasury of immense value to humankind. Nearly 72,000 medicinal plants have been used in diverse human cultures (Schippmann et al., 2006). Medicinal plants are the source of a large number of essential drugs in Western medicine and are the basis of herbal medicine, which is not only the primary source of health care for most of the world’s population living in developing countries but also enjoys growing popularity in developed countries (Sucher and Carles, 2008). Herbal medicine has been enjoying renaissance among customers throughout the world. The World Health Organization estimates that 80% of the world’s population utilizes traditional medicines for healing and curing diseases. There is an increasing international market for medicinal plants for herbal medicine and pharmaceutical products. The natural medicines are much safer than synthetic drugs, have gained popularity in recent years, leading to a tremendous growth of phyto-pharmaceutical usage (Matthews et al., 2003; Ferreira-Machado et al., 2004).

Medicinal plants cover a wide range of plant taxa and closely related species. One such closely related species taxa which is commonly used in Indian traditional system of medicine is herbaceous species of Phyllanthus. Several species of this genus have long been used in traditional medicine, particularly in the Ayurvedic system of medicine, to treat, notably, kidney problems, urinary bladder disturbances, diabetes, pain, jaundice, gonorrhea, chronic dysentery, skin ulcers, and hepatitis B (Calixto et al., 1998; Duke’s Phytochemical and Ethnobotanical Databases, 2011).

1.2 Authentication of Medicinal Plants

Accurate and rapid authentication of plants and their respective adulterants is difficult to achieve at the scale of international trade in medicinal plants. Due to the complex nature and inherent variability of the chemical constituents of plant-based drugs, it is difficult to establish quality control parameters. Due to the popularity of herbal drugs globally, their adulteration/substitution aspects are gaining importance at the commercial level. Pharmaceutical companies are procuring materials from traders, who are getting these materials from untrained persons from rural and/or forest areas.
This has given rise to widespread adulteration/substitution, leading to poor quality of herbal formulations. Misidentification of herbs can be non-intentional (processed plant parts are inherently difficult to distinguish) or intentional (profit-driven merchants sometimes substitute expensive herbs with less-expensive look-alike ones) (Kiran et al., 2010). Adulteration can occur due to ignorance or intentional substitution with cheaper plant material and may cause damage to human body (Jordan et al., 2010). Therefore, authentication at various stages, from the harvesting of the plant material to the final product, is a need of the hour. However, there is no single authentication method that can be applied to every medicinal plant. The chain of custody must be formed by combining various technologies and practices that are needed.

The general approaches to herb identification are dependent on morphological (Carlsward et al., 1997), anatomical (Stern et al., 1994), chemical (Kite et al., 2009; Li et al., 2010) and molecular (Li et al., 2005) techniques. However, traditional taxonomic studies require experienced professional taxonomists. In the case where diagnostic morphological traits of the given specimen are lacking, it becomes difficult even for specialists to recognize a species correctly. Genetic analysis has a promising role in resolving disputes of taxonomic identities, relations and authentication of the species in question, as the genetic composition is unique for each species and is not affected by age, physiological conditions and environmental factors. It also helps in the identification of useful genotypes which are likely to improve efficacy of standard drug formulations; even the plant extract used in the herbal-drug formulations can be authenticated by DNA-based methods (Novak et al., 2007). Therefore, DNA-based methods have gained wide acceptance in quality control to authenticate crude materials.

1.3 Herbaceous *Phyllanthus* species

The genus *Phyllanthus* L. was first described by Linnaeus and belongs to the family Phyllanthaceae. It is one of the largest genera of flowering plants, with over 1200 species and accounts for more than half of the species in the family (Kathariarachchi et al., 2006). India has 53 species of *Phyllanthus*, of which 23 species are endemic. Among the 53 species of *Phyllanthus*, 37 are shrubs, 13 are herbs, and 3 are trees (Balakrishnan and Chakrabarty, 2007). Totally 24 species of the genus
Phyllanthus are recorded as medicinal plants by the ENVIS Center on Medicinal Plants in India (2010).


Herbaceous Phyllanthus species have been used since ancient times in different systems of medicine, particularly for the treatment of liver disorders and urinary infection. Kannada name for herbaceous Phyllanthus species is ‘Nelanelli’ or ‘Kirunelli’ and in Sanskrit or Ayurveda it is known as ‘Bhumyamalaki’ or ‘Tamalaki’. The herbs known as ‘Bhumyamalaki’ in Indian literature refer to a complex group of *P. amarus*, *P. fraternus*, *P. debilis* and *P. urinaria*. The whole group forms a ‘niruri complex’ (Chaudhary and Rao, 2002). *P. maderaspatensis* and *P. virgatus* are also used as substitutes for ‘Bhumyamalaki’ (Chaudhary and Rao, 2002; Murthy, 1994; Yoganarasimhan, 1996). Although species of ‘niruri complex’ closely resemble each other, ethnomedical uses and some aspects of pharmacological activities among these species are different (Theerakulpisut *et al*., 2008).

1.4 Taxonomic incongruities in herbaceous Phyllanthus species

There remains a great deal of confusion even among scientists regarding plant identification and, in many cases, plant misidentification makes evaluation of published information difficult. Confusion exists in identification of herbaceous Phyllanthus species mainly due to referring them all with a common vernacular name, their similarity in gross morphology, close proximity in growth habitat and lack of guidelines to check the authenticity and quality of the medicinal plant sold (Dnyaneshwar *et al*., 2006).
Before the observation of Mitra and Jain (1987), there was confusion over the correct identity and even nomenclature of several taxa referred to under the name *P. niruri* L. in Indian floras and the whole group forms a taxonomic complex (Chaudhary and Rao, 2002). Realising this confusion, Mitra and Jain (1987) critically examined Indian materials of *Phyllanthus* and observed that the *P. niruri* described in Flora of British India (Hooker, 1887) is actually a mixture of three closely related but distinct species namely *P. amarus*, *P. debilis* and *P. fraternus*. Those reports from India on *P. niruri* are actually pertaining to investigations in any one member of this ‘niruri complex’ but not on *P. niruri* (Dnyaneshwar et al., 2006). Webster (1957) mentioned that true *P. niruri* is a native of New World and endemic to America and does not occur in India. However, there are many publications published even today from India bearing title on *P. niruri*. This makes evaluation of published information difficult.

Sasidharan (2004) indicated that *P. amarus* Schumach. & Thonn. and *P. fraternus* Webst. are the same species and both are found in South India. However, pharmacognostic studies have shown that *P. fraternus* is different from *P. amarus*. For example, phyllanthin and hypophyllanthin are present in *P. amarus* and not in *P. fraternus* (Khatoon et al., 2006). Therefore, *P. fraternus* could be a genetic variant or a different ecotype of *P. amarus*.

Chaudhary and Rao (2002) observed that the concepts and identification of various *Phyllanthus* species, particularly herbaceous ones, have been unclear mainly due to misidentification of specimens in several Indian herbaria. The distribution of some species has also not been shown correctly. Same thing has happened in floras of Karnataka state also. *P. asperulatus* Hutch. has been mentioned in flora of Bangalore (Ramaswamy and Razi, 1973) and Mysore (Rao and Razi, 1981). However, Chaudhary and Rao (2002) did not include this species in India. *P. debilis*, even though it has been reported from Gulbarga and Kodagu by Singh (1988) and Murthy and Yoganarasimhan (1990), Saldanha (1996) did not record this species from Karnataka region and expressed his doubts on the occurrence of this species from this region. Chaudhary and Rao (2002) mentioned its distribution only in coastal areas. *P. rotundifolius* generally grows near seashores in sandy soils. However, Kammathy et al. (1967) have reported it from Biligirirangana hills and Rao and Razi (1981) from Bedguli and Kethedevalagudi areas. Therefore, Chaudhary and Rao (2002) called for the confirmation of these reports. Even though *P. rheedei* reported from Dakshina...
Kannada (Saldanha, 1996), Bangalore (Ramawamy and Razi, 1973) and Mysore (Rao and Razi, 1981), Chaudhary and Rao (2002) did not include this species distribution in Karnataka. However, they observed morphological similarities between *P. scabrifolius*, *P. rheedei*, *P. kozhikodianus* and *P. tenellus*. Study by Gangopadhyay et al. (2004) found that *P. kozhikodianus* is distinct from *P. debilis* Hook. f. and is synonymous to *P. rheedei*. Therefore, doubts prevail on the taxonomic status of *P. kozhikodianus*, *P. rheedei*, *P. scabrifolius* and *P. tenellus*.

1.5 Molecular authentication of herbaceous *Phyllanthus* species

In recent years, efforts have been made to accurately identify medicinal plants used in raw drug trade to ensure the purity, quality and safety of drugs (Jayasinghe et al., 2009). Besides conventional methods including examination of wood anatomy and morpho-taxonomical keys, several-DNA-based methods have been developed for the identification of medicinal plants (Sucher and Carles, 2008). These are broadly categorized under (i) Hybridization-based (ii) Polymerase chain reaction (PCR)-based and (iii) Sequencing-based methods.

*Phyllanthus* species trade in India is mired by two problems: (1) taxonomic confusion among closely related species (Ganeshiah et al., 1998; Elvin-Lewis et al., 2002) and (2) the fact that many species in trade share a common vernacular name (Srirama et al., 2010). Consequently, it is not uncommon to find substantial species admixtures in trade samples (van Rhede, 1690; Dymock, 1883; Dymock et al., 1893; Kirtikar and Basu, 1935; Nadkarni, 1954; Ganeshaiah et al., 1998; Khatoon et al., 2006; Srirama et al., 2010). For example, although *P. amarus* is a predominant species in trade, it is often found mixed with several other *Phyllanthus* species, including *P. fraternus* and *P. maderaspatensis* (Khatoon et al., 2006; Ved and Goraya, 2008). Species admixtures may have significant implications on the quality and efficacy of the eventual phytomedicine made from these mixtures (Song et al., 2009).

Several related species of *P. amarus* (‘Bhumyamalaki’) occur in India that are widely used as medicinal herbs, though the efficacy of these species is not fully established. Further, since all these species are taxonomically closely related to *P. amarus*, they are often collected, marketed and used as *P. amarus*. The problem is further complicated as these species exhibit a lot of morphological diversity in different regions. *P. amarus* is closely related to *P. niruri*, *P. fraternus* and *P. debilis*.
morphologically, phytochemically and by use. In fact, because of the confusion, the species are often referred as the PAF (Phyllanthus-amarus-fraternus) complex (Ganeshaiah et al., 1998). *P. scabrifolius*, *P. rheedei*, *P. kozhikodianus* and *P. tenellus* are very similar in their gross morphology (Chaudhary and Rao, 2002).

Molecular markers are useful in authentication of species and to establish evolutionary linkages. DNA markers are reliable for informative polymorphisms as the genetic composition is unique for each species and is not affected by age, physiological status and environmental factors. Hence, DNA markers, such as PCR-restriction fragment length polymorphism (PCR-RFLP), amplification-refractory mutation system (ARMS), arbitrarily primed PCR (AP-PCR), random amplified polymorphic DNA (RAPD), sequence characterized amplified region (SCAR) and DNA sequencing, are useful for the authentication and standardization of medicinal plant species (Joshi et al., 2004). Sequence comparison of the internal transcribed spacer (ITS) region is widely used in taxonomy and molecular phylogeny because of high copy number, it is easy to amplify even from small quantities of DNA and has a high degree of variation even between closely related species. This can be explained by the relatively low evolutionary pressure acting on such nonfunctional sequences. It has proved to be useful for checking relationships among species and various genera in Asteraceae (Baldwin, 1993).

Attempts are being made to develop universal barcode regions for taxonomic identification of plants. DNA barcoding is a technique that provides rapid identification of species without using morphological cues. The method employs relatively small-standardized DNA fragments as tags to define or discover species. In plants, the mitochondrial genome evolves much more slowly than in animals. There is currently no consensus on which candidate markers comprise the best plant DNA barcoding region. However, DNA barcodes such as *rbcL*, *matK*, *psbA-trnH* and ITS have been proposed for the plant kingdom. Also very recently the chloroplast intergenic spacer (IGS) like *trnE-trnT*, *trnT-psbD*, *ndhF-rpl32* and *rpl14-rpl16* were also employed for discriminating the cultivar species (Selvaraj et al., 2013).

1.6 Phytochemistry of herbaceous *Phyllanthus* species

Because of the tremendous therapeutic potentials of the *Phyllanthus* species, numerous phytochemical and bioactivity studies have been carried out on these plants, resulting in the isolation and identification of various compounds, ranging from
alkaloids, coumarins, flavonoids, lignans, and terpenes (ISI Web of Knowledge, 2011). The major lignans of the genus namely, phyllanthin and hypophyllanthin, have been shown to be antihepatotoxic against carbon tetrachloride and galactosamine induced hepatotoxicity in primary cultured rat hepatocytes (Syamsunder et al., 1985). However, study by Khatoon et al. (2006) showed these compounds might not be exclusively responsible for hepatoprotective activity as these are present only in P. amarus, while P. fraternus and P. maderaspatensis also possess significant hepatoprotective activity.

Information on phytochemical investigations on about 35 Phyllanthus species is available to date. While some of the species like P. amarus and P. niuri have been studied extensively, investigations of a number of other species like P. acuminatus, P. arenarius and P. debilis are rather incomplete or partial. P. amarus, P. niruri, P. reticulatus, and P. urinaria have yielded most of the major classes of compounds. Triterpenes are widely distributed within this genus. The distribution of alkaloids and phenolics is also quite widespread. Many of the compounds isolated from this genus occur only in one species and therefore have limited chemotaxonomic value. However, there are a few compounds, especially alkaloids, lignans and tannins that occur in more than one species, might have some chemotaxonomic significance, at least at the family level (Nahar et al., 2011). The available phytochemical data on the genus Phyllanthus, with the exception of a few species, are rather incomprehensive, and many species demand further detailed studies because of their traditional medicinal uses. Also, less than 10% of the species have ever been phytochemically investigated. The structural diversity found among the Phyllanthus compounds is unique, and many compounds isolated from this genus so far are highly bioactive. Therefore, the genus Phyllanthus will continue to be one of the major plant sources of new chemical entities and possibly new drug molecules (Nahar et al., 2011).

1.7 Bioactive potential of herbaceous Phyllanthus species

Since ancient times, plants of the genus Phyllanthus have commonly been used in the treatment of jaundice and other liver diseases (Thyagarajan and Jayaram, 1992; Thyagarajan et al., 2002). Phyllanthus species are also used in Ayurvedic medicine in combination with other herbals to combat many disorders, including liver diseases (Wealth of India, 2003). Several other therapeutic properties have also been attributed to the genus Phyllanthus, such as antibacterial, antiparasitic, anticonceptive,
and antiviral activities (Raphael and Kuttan, 2003; Cimanga et al., 2004; Zhang et al., 2004; Melendez and Capriles, 2006; Rao et al., 2006). Crude extracts of different species have also been studied for their antioxidant and antigenotoxic effects as well as chondroprotective potential (Sanchez-Lamar et al., 1999; Ferrer et al., 2004; Lin et al., 2008; Londhe et al., 2008, 2009; Sumantran et al., 2008).

Biological activity is frequently correlated with taxonomy (Oliver-Bever, 1968; Gottlieb, 1982). Presumably, some of the species with in Phyllanthus genus possess greater level of biological activities than others. Unander et al. (1995) based on literature survey hypothesized that records for plants used against jaundice or hepatitis would be concentrated within the sub genus Phyllanthus, and this sub-genus should therefore receive top priority when budgeting finite resources for testing representative species against Hepatitis B Virus (HBV).

Interest in Phyllanthus genus has been stimulated by the reports of activity against Hepatitis B Virus (HBV) (Venkateswaran et al., 1987; Thyagarajan et al., 1988; Blumberg et al., 1989) and against cancer lines (Barclay and Perduce, 1976; Kupuchan et al., 1978; Pettit et al., 1983, 1990; Pettit and Schaufelberger, 1988). Studies suggest that Phyllanthus species may suppress the growth and replication of the virus (Yeh et al., 1993; Lee et al., 1996; Jayaram et al., 1997). In addition to helping the body fight hepatitis B, Phyllanthus may also support the overall health of the liver. However, in Thailand, a double-blind, placebo-controlled trial using Thai P. amarus failed to clear hepatitis B surface antigen (HBsAg) from asymptomatic carriers (Leelarasamee et al., 1990). Similarly, seroconversion was not seen in a trial conducted in New Zealand using material from Madras, standardized using geraniin-a hydrolysable tannin-as a marker for standardization (Milne et al., 1994). According to Premila (2006), despite the fact that there have been negative clinical trials, P. amarus must be considered a plant of potential use for viral hepatitis B. However, further work is required on choice of plant material, method of processing, dosage to be used, and period of treatment, especially because of the presence of ecotypes of P. amarus.

The hepatoprotective activity of Phyllanthus species is another important area of study which has been established with clinical research. The lignans phyllanthin and hypophysyllanthin have been shown to be hepatoprotective against carbon tetrachloride-induced hepatotoxicity in primary cultured hepatocytes (Syamsundar et al., 1985). However, study by Khatoon et al. (2006) showed these compounds might not be exclusively responsible for hepatoprotective activity as these are present only
in *Phyllanthus amarus* while *P. fraternus* and *P. maderaspatensis* also possess significant hepatoprotective activity. Further studies are needed to elaborate whether some other compounds are present in *Phyllanthus* species complex responsible for hepatoprotective activity.

Currently, researchers are concentrating on antioxidant activity of *Phyllanthus* species and they are suspecting this activity could be a possible mechanism for the hepatoprotective activity of *Phyllanthus* species (Promyothin *et al*., 2007; Naaz *et al*., 2007). So testing of herbaceous *Phyllanthus* species complex for both antioxidant and hepatoprotective activity at a time might prove this hypothesis.

### 1.8 Scope of the study

Confusion exists in the identification of herbaceous *Phyllanthus* species mainly due to referring them all with a common vernacular name, their similarity in gross morphology, close proximity in growth habitat, lack of guidelines to check the authenticity and quality of the medicinal plant sold and misidentification of specimens in several herbaria. Though much work has been done on the ‘Bhumyamalaki’ or ‘niruri’ complex, doubts prevail on the taxonomic status of *P. scabrifolius*, *P. rheedei* and *P. kozhikodianus*. The present study is aimed towards solving these confusions by combining conventional taxonomic methods with molecular techniques.

Most of the taxonomic keys are prepared exhaustively and serve the need of scientific community. Therefore, the present work is designed to develop a key for nonprofessional with minimal technical details mainly based on morphological characters especially on leaf shape.

Earlier phyllanthin and hypophyllanthin present in *Phyllanthus* species were thought to be responsible for hepatoprotective action. But recent studies indicate that these compounds may not be solely responsible for hepatoprotective activity as these are present only in *P. amarus*, while *P. fraternus* and *P. maderaspatensis* which do not have these compounds also possess significant hepatoprotective activity. Therefore, estimation of relative concentration of phyllanthin in herbaceous *Phyllanthus* species has been taken up.

Several studies on *Phyllanthus* species indicate that their antioxidant property could be the possible mechanism for their biological activity. Therefore, bioactive potential of herbaceous *Phyllanthus* species is targeted in the present study to
establish a possible link between antioxidant properties with biological activity such as hepatoprotection.

1.9 The objectives of the proposed work

1. Authentication of herbaceous *Phyllanthus* species grown in Karnataka based on morphological characters and molecular analysis.

2. Estimation of relative concentration of phyllanthin in herbaceous *Phyllanthus* species.

3. Bioactive potential of herbaceous *Phyllanthus* species.

4. Fractionation and identification of bioactive compound present in *Phyllanthus virgatus* extract.