Discussion
DISCUSSION

Chemical and pharmaceutical investigations have added a great deal of status to the use of medicinal plants by revealing the presence of active principles and their actions on human and animal systems. Investigations in the field of agrotechniques, pharmacognosy and pharmacology have supplied valuable information on medicinal plants with regard to their availability, botanical properties, method of cultivation, collection, storage and therapeutic uses. The knowledge and the use of medicinal plants are growing day by day and their continuous procurement alarms the pharmaceutical industry. Fear of deforestation and loss of natural resources, have provoked the eagerness to convert wild into domestic without affecting their potential property. There has been a tremendous upsurge in the demand for phytopharmaceutical, raw medicinal herbs and vegetable drugs of Indian origin from the western nations. There is also an increase in the domestic demand for raw materials used for perfumeries, pharmacies and biopesticidal units. The demand for traditional herbal use is also increasing day by day.

Since our country is said to be the proud possessor of an impressive medicinal heritage, India could become a potential supplier of phytopharmaceuticals, alkaloids and raw medicinal herbs for the emerging world market. Hence the cultivation, improvement and protection of medicinal plants assumes very important.
The systematic cultivation of few medicinal plants has been found to be of discouraging enterprice, mainly because of the uneconomical price they command. For example the sale price of *Phyllathus amarus* is as low as Rs. 10/Kg making it commercially unviable.

Hence, there is a need for enlighting the people that the cultivated product is going to be homogeneous in comparison to the unknown collection from natural resources, where there is chances for lot of variation. Besides, the problems of identification of medicinal plants have led to the use of adulterants. Physical verification is also difficult because the plant parts used in many cases like barks, roots, etc. show close similarity.

Keeping the above factors into mind the agrotechniques for cultivation of *P. amarus* has been taken in the present work. It is necessary to standardize the cultivation techniques, adaptable on an area wise or regional wise basis for important medicinal plants.

*Phyllanthus amarus* contain a novel antiviral activity against HBV and the related animal virus, and scored to be Indian gold in foreign market. Considering the growing global requirement for the miraculous plant of Indian origin that posses rich active principle, the cultivators should come forward to cultivate *P. amarus* in their fields.

Soil is the foremost important factor for cultivation of any kind of plants. In general, the growth of plants totally depend on the fertility and texture of soils (Tandon, 1999). Sandy loam and Red soils are the two major types available in most parts of Tamil Nadu and these soils are used for
extensive cultivation of agricultural crops. Therefore, these two types of soils were selected for conducting various experiments to develop agrotechniques for *P. amarus*. The soil analysis has indicated that the alkaline soil is also suitable for the growth of *P. amarus* provided the nutrients in the soil are to be maintained in required levels (Rakiyappan, 1995). It was interesting to note that the number of phyllanthoid branches on the main stem was relatively constant for the plants of a particular age, regardless of soil conditions. The plants seemed to respond to favourable conditions through compound branch growth rather than more rapid growth of the main stem. Plants under favourable condition were actually shorter, but had many more compound branches. According to Unander and Blumberg (1991) high soil fertility resulted in less apical dominance, increased numbers and length of compound branches in the species of *Phyllanthus*. The growth parameters such as height, number of branches or mean branch length were greatest at the soil pH of 7.3-7.5.

*Phyllanthus amarus* can be successfully propagated by seeds. Hence seed germination is regarded as a key characteristic for good growth (Angevine and Chalot, 1979; Mayer and Poljakoff-Mayber, 1989). Seed germination may differ between regions, due to local adaptations and to climate (Habeck, 1958; McNaughton, 1966; Winstead, 1971; Meyer and Monsen, 1991; Schutz and Milberg, 1997). Moreover, the percentage germination of seeds is quite variable and there may be different factors responsible for this.

The seed germination study carried out under *in vitro* conditions has proved that moist Petriplate assay is the best method for *in vitro* evaluation of
seed germination. This could be correlated with the water holding capacity of
the filter paper and using this technique a rapid evaluation can be made to
select good seeds for other experiments. The sandy loam and red soils could
also provide sufficient moisture for the germination but counting of germinated
seedlings was much easier with filter paper technique. The rate of germination,
however, depends on the percentage of fully matured heavy seeds in a
particular lot. Fresh seeds collected from three months old plants and sown
immediately showed higher percentage of germination. The ripe seeds
collected, shade dried and stored in airtight tins retained their viability for
about six months. The viability of seeds dropped markedly with the increase in
the interval of time between collection and sowing. The germination rate of
seeds also differed under varying agroclimatic conditions. Use of fertilizers
enhances the rate of seed germination and seedling growth.

The highest germination was observed due to application of
vermicompost followed by FYM in both the soils. The FYM also supported
90% germination which was statistically on par with vermicompost treatment.
Considering the cost and non-availability of vermicompost in rural areas, FYM
can be used. Both the organic nutrients and soil conditions provided favourable
atmosphere for maximum seed germination. The organic matters increase the
water holding capacity, buffering and ion-exchanging capacities of soils,
besides, the increased microbial activity enhanced the nutrient cycling in soil.
The effective germination observed due to vermicompost and FYM application
is in support of the observation of Rakkiyappan (1995) who strongly
recommended that the soil organic matter play a vital role in enhancing the
crop productivity and considered as elixir of soil fertility. From the present results it is concluded that the fresh seeds with the above organic manure applied before sowing the seeds is the best method to get maximum seed germination.

Following the seed viability and nutrient requirement, seed rate is very important factor for seed germination and to calculate the number of healthy seedlings required for plating. Seed rate evaluation also would decide the ideal age of seedling for transplantation. In the present investigation, 250g seeds/ha was observed to be the best seed rate to get healthy seedlings with increased biomass, chlorophyll content, shoot and root lengths.

Though increased seed rate showed increased seedlings with the long roots, it could be due to competition of water and other nutrients due to over crowding. Short roots with 250g/ha seed rate is preferable for easy pulling of seedling for transplantation. The ideal age of seedlings was 30 days. Age of seedling for transplantation vary with different types of crops. Age of seedling is very important as delay in one day may even show greater variation in yield.

In the present investigation, nursery raising and transplantation was much superior to directly broadcasting the seeds in the main field. The reasons are many and are experimentally confirmed. Following this, spacing plays an important role for good healthy plants/crop and in the requirement of total number of seedling to plant hectare of land. Different spacing trials were carried out and spacing 20 x 30cm gave good crop growth.
High density of planting through narrow spacing proved to be superior and holds promise for enhancing compound branch production. This is mainly due to linear competition for soil moisture within row. Hence, a closer spacing with high plant population within rows would benefit economic utilization of water (Srinivasan, 1995).

The applications of both organic and inorganic fertilizers have gained importance in playing a vital role in the improved production of agricultural crops. The cultivation of potent herbs with standard practices is possible with the application of economically feasible biofertilizers and other inorganic fertilizers. These fertilizers have become popular among the farmers at present.

In practice, when the N is applied through inorganic fertilizers, about 50% is lost through fixation, volatalization and leaching. In sandy soils, the loss is even more which necessitate the application of high quantity of fertilizer for maximum production resulting in increased cost of cultivation. Hence, a judicial management practice is to be adopted to increase fertilizer efficiencies.

Experiments conducted in field by replenishing the nutrition has greatly influenced or supported the plants for the better production in term of the vegetative growth such as shoot and root lengths, number of compound and phyllanthoid branches, number of leaves per phyllanthoid branch, etc. These parameters have greatly been influenced with the treatments of FYM alone and FYM combined with Azospirillum and Phosphobacteria. A key and universally accepted concept is that the natural microbial population in soil or other living substrate are activated to grow around the developing plant roots, giving rise to
the so-called rhizosphere (Azcon-Aguilar and Barea, 1994). As rhizosphere develops at the root-soil interface, microorganisms interact with both plant roots and soil constituents, during which a communication among the different components could take place via chemical or biochemical signals, although physical interactions are possible. Inherent soil characteristics and the prevailing environmental conditions of the ecosystem (Bowen, 1980; Lynch, 1990) could modulate this phenomenon. These microbial biofertilizers increased the plant growth and ultimately crop yields by secreting growth promoting substances (Tandon 2002). Since, the entire plant is being utilized for the extraction of medicinal compound, the increased production of the plant in terms of biomass using cost effective and eco-friendly biofertilizer has provided the best means of cultivation.

Singh (1999) reported that the application of nitrogen in the form of FYM in a single dose during planting time significantly enhanced grain yield in Eleusine coracana. Husain et al., (2000) observed that the seed yield was influenced significantly with water management and nutrient applications. Significantly higher seed yield was recorded with two irrigations during branching and capsule initiation stage in conjunction with N\textsubscript{90} P\textsubscript{45} K\textsubscript{30}, which was at par to N\textsubscript{90} P\textsubscript{45} K\textsubscript{30} with one irrigation at capsule initiation stage. But use of FYM in combination with biofertilizer increased and accelerated the plant growth rate. It is always better to recommend well rotted FYM at the time of land preparation for best results.

Water management is an important factor which contributes on the good growth of the cultivated P. amarus. Though light showers claim the
germination and growth of *P. amarus*, for regular cultivation with a view to encourage the cultivation of *P. amarus* by the farmers, the irrigation method should be cost effective. Hence, experiments were conducted to promote the growth rate of *P. amarus* with different frequencies of irrigation. However, frequency and depth of irrigation depends upon climatic condition, soil type, organic matter content of soil and the plant type (Singh and Saran, 1993). In the present investigation, these factors were taken into account. The frequency or interval of irrigation is mostly based on the growth stages of crop. It should be irrigated at least every alternate day during summer and during rainy period the frequency of irrigation should be reduced. The vigorous growth of plant is between one month to two months after transplantation. Water logging is highly detrimental to *P. amarus*.

The crop being a small shrub to compete with weeds during its initial stages of growth, the land should be free from weeds. Weeds drain away about 30 to 40% of the plant nutrients from the soil and hinder intercultural operations along with reduction in vegetative growth (Singh et al., 2000) of the cultivated crop. Therefore, regular weeding has to be done for successful cultivation of any crop. In the present investigation, two hand weedings are recommended for the better growth of *P. amarus* as evidenced from the good growth. One more weeding can be taken up if the populations of unwanted weeds are high and if it interfere with pulling out of *P. amarus*.

Although *P. amarus* itself is a weed in other cultivated crops, it could be promoted round the year in all the seasons, June to November is the best and ideal periods for cultivation. In this season, *P. amarus* put forth excellent and
enormous vegetative growth, which ultimately reflected on biomass, seed yield and increased active principle.

The occurrence of powdery mildew caused by *Oidium phyllanthis* was first record on *P. amarus* during this investigation. Among four different periods, severe powdery mildew occurred during November to February. Powdery mildew spread fast due to the suitable and conducive weather prevailing during winter, which favours the primary spread and secondary spread of the pathogen. This disease is a major limiting factor for the successful cultivation of *P. amarus* during winter since defoliation of leaves will cause complete drying and crop loss. This prompted to find out an effective solution for the management of the disease using various chemical fungicides and biopesticides.

Two foliar spraying of Wanis (botanical fungicide), Propiconazole and Wetable sulphur was significantly effective in controlling the disease as compared to rest of the treatments. However, effectiveness of the above three treatments in controlling the powdery mildew was on par. Although Propiconazole found to be the best against *O. phyllanthis*, considering the bio-origin and eco-friendly characteristics, the Wanis, a bioproduct for fungal pathogens can be used instead of chemical fungicides, though there are many reports that EB inhibitors like propiconazole induces host resistance by altering the hormonal balance (Mathivanan, 1991).

The cultivation practices developed in the present study were demonstrated through on-farm trials in different fields located in various parts
of Tamil Nadu, Southern India. Results of these trials have revealed that the implementation of these practices gave better biomass and seed yields of *P. amarus*. In addition, this wild herb can be successfully cultivated at farmers level as commercial crop because the estimated net return/profit from the farmers’ fields is encouraging and it is also comparatively higher as compared to any other commercial crops, which are normally being cultivated in this state. The constant effort on conducting the demonstration trials at farmers’ fields created awareness among farming community, who are at present coming forward for the large-scale cultivation of this “wonder drug”, *P. amarus*.

The package of practices developed in the present study for successful cultivation of *P. amarus* is as follow:

- Seeds - 250g/ha
- Nursery bed size - 40 m²/ha
- Fertilizers - FYM 15t/ha (or) FYM + *Azospirillum* + Phosphobacteria (14t + 3 kg + 3kg/ha).
- Transplantation of seedling after 30 days in main field
- Spacing - 20cm between plants and 30 cm between ridges
- Alternate days irrigation during summer
- Two weedicings at 15 days interval after transplantation
- Harvesting - 60 days after transplantation
- Whole plant uprooted manually
- Shade drying (Seed collection and medicinal purpose)
- Biomass obtained (3.125 tons/ha)
- Stored up to 24 months
PHYTOCHEMISTRY

The aerial part of *P. amarus* is highly valuable in a number of countries for its curative properties and in India, the plant is often used by traditional medical practitioners for a variety of ailments including asthma, bronchial infection and diseases of the liver (Foo and Wong, 1992). Interest in *P. amarus* is increased by recent reports on the efficacy of the herb against Hepatitis B virus. All parts of the wonder plant are medicinally important. Hence, the agrotechnique developed for the cultivation of *P. amarus* is scored based on the chemical constituents, specifically phyllanthin content. The plant was assayed in various experiments conducted in the present investigation. There are many reports that the distribution of lignans in the leaves varied considerably with geographic location (Foo and Wong, 1992).

In different agroclimatic region of the country a number of geographical races exist. *P. amarus*, which collected from different localities (Madras India, Hainan and Henan Provinces – China) were assayed for phyllanthin (a bitter constituent) content and compared with the present cultivated race.

Many workers have reported that medicinal quality of *P. amarus* vary largely ranging from high activity to nil when the plants are collected from different geographical locations (Doshi et al., 1994; Milne et al., 1994; Wang et al., 1995).

In the present observations, the plants collected from natural resources at different localities showed less phyllanthin content when compared to the cultivated *P. amarus* using the agrotechnique developed here. Besides, the
application of FYM and FYM+Azospirillum+Phosphobacteria enhanced the active principle, phyllanthin. It is also confirmed in the present investigation that age of the plants plays a major role in the phyllanthin content. Ninety days old *P. amarus* in the present studies showed high phyllanthin content, than what is present in the wild plant as reported by Sane et al., (1997) and Murali et al., (2001). This increase in phyllanthin content can be attributed to the significant increase in compound branches, increased number of phyllanthoid branches, more number of leaves and chlorophyll content as compared to untreated control. The total chlorophyll content increased five times in FYM treated plants. Both HPLC and HPTLC confirmed the high content of phyllanthin in leaves.

It is also confirmed in the present studies that powdery mildew drastically reduced the phyllanthin content due to severe defoliation of leaves. The activity or quality of various medicinal or aromatic plants has long been known to be affected by environmental variables such as soil fertility, pH, moisture level, temperature, light quality and also by biotic stress like pests and diseases (Penka, 1978; Schmutterer and Zebity, 1983). Storage of the harvested plants powder is another important factor which shows variation in phyllanthin content. Freshly harvested leaves of *P. amarus* showed maximum phyllanthin content, which gradually reduces with increase in storage time. The phyllanthin content reduces by three folds after two years. The results in the present work clearly showed that *P. amarus* plants could easily be stored up to two years without much reduction in phyllanthin content. The analysis of adulterant plants, which are normally misidentified showed nil phyllanthin content. *P. amarus* could easily be differentiated from the adulterant plant due to its bitter taste of phyllanthin.
In the present investigation it is clearly indicated that phyllanthin content is enhanced due to the recommended agrotechniques to bring this wild plant or a weed, *P. amarus* to the farmers' field with high economic out turn. These agrotechniques should be practiced by the farmers under National co-ordinated trials to prove the efficacy of the cultivation techniques.

Following phytochemistry, pharmacognosy is another important area of research carried out to identify *P. amarus* from the substituted plants belong to the same genus.

**PHARMACOGNOSY**

Botanical identity of plant is the essential initial step in the pharmacological and pharmaceutical studies of the drugs. Uncertain identity, adulteration and substitution may largely deteriorate the pharmacognostic analysis of plants. Accurate identification of plants is possible when the specimens bear all floral parts necessary for identification. In the absence of such diagnostic parts, one has to seek other methods such as anatomy of the materials. Certain unique and specific anatomical features have proved to be helpful in the identity of fragmentary specimens. It is very much worthwhile to prepare a set of simple but reliable anatomical characters to serve as a ready reckoner whenever needed for identification of the plants. For many medicinal plants, no anatomical data worthy to be used for diagnostic purposes are available. Even a few studies seem to be based on wrong terminological concepts and poor observations. The proposed anatomical studies on *Phyllanthus amarus* vis-à-vis some coexisting weeds, often used for
adulteration or substitution, offer some genuine data for botanical standardization of *P. amarus* and other three related taxa.

Adulterants, alternatives, substitutes, misnomer and wrong-identities are some of the problems met with in the studies on the medicinal plants. These problems make an important as well as interesting area of study in the context of Indian System of Medicine. The unresolved taxonomic problems of medicinal plants trap the non-taxonomists in confused state. This is truer in the case of traditional medical practitioners, pharmacologists and pharmaceutical researchers. The problems associated with the nativity and identification of *P. amarus* remains still in controversial state. Its distinction from *P. niruri* is also elusive.

Three species of *Phyllanthus*, namely *P. debilis*, *P. simplex* and *P. maderaspatensis* were found to be coexisting along with *P. amarus*. There have been lots of controversies and ambiguity in taxonomic circumscriptions of these four species because of overlapping of certain exomorphic features.

In the present study, anatomy of different organs of the four taxa has been given more accents for distinguishing the species. These parameters will be supplementary for the floral and vegetative morphological characters. On the basis of data, an artificial dichotomous key is presented for identification of the four species. The similarities and comparisons of various anatomical features of *P. amarus*, *P. debilis*, *P. simplex* and *P. maderaspatensis* are given in the Table 17.

*Phyllanthus amarus* stands apart from the other co-existing species in having 5 perianth members in contrast to 6 in others. In branching pattern also
P. amarus is distinct from other members. P. amarus and P. debilis exhibit more overlapping features, while P. simplex and P. maderaspatensis resemble each other in certain essential characters. Ganeshiah et al., (1998) have tabulated differences in leaf morphology, perianth features, disc-nature and seed coat ornamentation among five species of Phyllanthus including P. amarus. These authors have also given the quantitative values for the above organs. Bagchi et al., (1992), though studied microscopic features for four species of Phyllanthus, their taxonomic key is mostly based on exomorphic features. In the present investigation, mostly microscopic features of P. amarus as compared to other species are provided for keys.

Dichotomous key for identification of four species of Phyllanthus

1. Seed surface with vertical, parallel bands of vermiform or bone shaped ridges, midrib not prominent.  
   2
1. Seed surface with reticulate, polyhedrall markings  
   Midrib prominently projection above and below the leaf surface  
   3
2. Pericarp thin, stem of the lateral branch circular in cross-section, growth rings distinct in stem and root, gelatine fibres are abundant in the secondary xylem, medullary pholem present in the stem  
   P. amarus
2. Pericarp fleshy and several layered, stem of the lateral branch transversely rhombic in sectional view, growth rings and gelatine fibres absent, medullary pholem absent.  
   P. debilis
3. Lateral branch with prominent conical lateral wings  
   P. simplex
3. Lateral branch wingless or with small less-prominent ridges  
   P. maderaspatensis
<table>
<thead>
<tr>
<th>S. No.</th>
<th>Microscopic features</th>
<th><em>P. amarus</em></th>
<th><em>P. debilis</em></th>
<th><em>P. simplex</em></th>
<th><em>P. maderaspatensis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Seed coat ornamentation</td>
<td>Vertical, parallel bands of vermiform ridges</td>
<td>Vertical, parallel bands of bone shaped ridges</td>
<td>Reticulate with circular pits at the junctions of the reticulations</td>
<td>Reticulations and minute pits within the reticulations</td>
</tr>
<tr>
<td>2</td>
<td>Pericarp</td>
<td>Membraneous, few layered</td>
<td>Freshly several layered</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Midrib</td>
<td>Not-prominent on both adaxial and abaxial sides</td>
<td>Not prominent on adaxial and abaxial sides. Druses present on the abaxial part of the phloem</td>
<td>Double conical projecting equally on the adaxial and abaxial sides. Palisade cells present on the adaxial part</td>
<td>As in <em>P. simplex</em></td>
</tr>
<tr>
<td>4</td>
<td>Petiole</td>
<td>Transectional outline circular</td>
<td>Transectional outline circular with less prominent wings</td>
<td>Transectional outline circular, not winged, outer ground tissue tanniniferous</td>
<td>Transectional outline circular with less prominent wings. Subepidermal cells tanniniferous</td>
</tr>
<tr>
<td>5</td>
<td>Lamina</td>
<td>Dorsiventral, mesomorphic, amphistomatic, anticlinal ways of the lower epidermis wavy</td>
<td>As in <em>P. amarus</em></td>
<td>As in <em>P. amarus</em></td>
<td>As in <em>P. amarus</em></td>
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</tr>
<tr>
<td>6</td>
<td>Stomata</td>
<td>Anomocytic</td>
<td>Anisocytic</td>
<td>Paracytic</td>
<td>Anisocytic</td>
</tr>
<tr>
<td>7</td>
<td>Stem of the lateral branch</td>
<td>Circular in transectional view, not winged</td>
<td>Transversely rhombic with two small lateral wings</td>
<td>Circular in transectional view with prominent conical wings</td>
<td>Circular with less prominent ridges, subepidermal cells tanniferous</td>
</tr>
<tr>
<td>8</td>
<td>Main stem</td>
<td>Medullary (pith) phloem present, growth rings distinct, gelatinous fibres abundant</td>
<td>Medullary phloem absent, growth rings and gelatinous fibres absent</td>
<td>-</td>
<td>As in lateral branches</td>
</tr>
<tr>
<td>9</td>
<td>Root</td>
<td>Periderm well developed, gelatinous fibres abundant, growth rings distinct</td>
<td>Periderm not distinct, secondary xylem solid and dense</td>
<td>Periderm thin but distinct, secondary xylem dense and solid</td>
<td>Periderm less distinct, gelatinous fibres and growth rings are absent</td>
</tr>
</tbody>
</table>
Summary
1. Highest in vitro germination of 93% for *P. amarus* seeds was observed in filter paper within 10 days.

2. Sandy loam soil favoured maximum germination of 90% on 12th day than the red soil.

3. Sandy loam soil amended with vermicompost supported highest in vivo seed germination of 95%.

4. About 250 g seeds are sufficient for obtaining seedlings for planting one-hectare area in main field.

5. Plant spacing of 20 cm x 30 cm was optimum for good vegetative growth of *P. amarus*.

6. Both sandy loam and red soils showed good growth and biomass, however, sandy loam soil gave best growth of herb.

7. FYM and FYM combined with *Azospirillum* and Phosphobacteria increased the growth parameters of *P. amarus*.

8. The optimum dosage for the fertilizers FYM and FYM combined with *Azospirillum* and Phosphobacteria were determined to be 15 tons/ha and 14 tons +3 kg +3 kg/ha, respectively.

9. Irrigation of two days intervals during summer is essential for the best growth of *P. amarus*.
10. Two-hand weeding could effectively allow the herb to put forth excellent vegetative growth.

11. The powdery mildew caused by *Oidium phyllanthi* is a limiting factor for the cultivation of *P. amarus* during winter.

12. The biopesticide, Wanis at 1000 mL/ha or Propiconazole at 250 mL/ha can be used for the effective management of powdery mildew.

13. Among the fertilizers, FYM treated plants showed two-fold increase in phyllanthin content.

14. High quantity of active ingredient, phyllanthin was estimated in three months old plants.

15. HPLC and HPTLC analyses in different plant parts have showed that the leaves contain the maximum phyllanthin content.

16. The plant materials can be stored up to 12 months without loosing the stability of phyllanthin.

17. Various anatomical features were studied for differentiating the four species of *Phyllanthus*.

18. A dichotomous key was developed for identifying the different species of *Phyllanthus* namely, *P. amarus*, *P. debilis*, *P. simplex* and *P. maderaspatensis* using the pharmacognostic studies.
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