5. Discussion

5.1 Dermatophyte infections

Fungal skin diseases particularly dermatophytoses are serious concern throughout the world, as reported by various authors (Sharma and Borthakur, 2007; Prasad et al., 2005; Sen and Rasul, 2006). The Northeastern part of India is often considered as natural incubator for large number of pathogenic microorganisms, predominantly the dermatophyte species (Jaiswal, 2002; Sharma and Borthakur, 2007; Devi and Zamzachin, 2006; Das, 2003). Despite the stunning success of pharmaceutical industries in developing new antifungal drugs, finding novel and efficient antifungal agents for treatment of wide range of fungal diseases with particular reference to dermatophytoses is still a top priority.

5.2 Survey, collection of plants and their antidermatophytic activity

Effect of medicinal plants, either in the form of crude extracts or their active components, on pathogenic microorganisms is the subject of many recent scientific studies. Greater importance is being attached to the ethno medicinal uses by the local tribes / communities besides documented reports on medicinal plants (Sharma and Joshi, 2004; Das et al., 2008; Gajurel et al., 2001; Sener, 1994; Kato and Furlan, 2007; Zaveri et al., 2010). Svetaz et al., (2010) reported that selection of plants based on ethno pharmacological perspective enhanced the probability of success in new drug-discovery efforts. The findings of his investigation showed that the probability of detecting plants with antifungal properties was 40.3%, among the plants used in traditional medicine, while it was only 21.3% in case of randomly selected plants. Thus it is evident from the previous reports that plant products are the sources of
effective antimicrobial agents for microorganisms including dermatophytic fungi. The present study is an attempt to find out potent plants and their active components against important dermatophyte species.

An extensive survey was carried out in the foothills of Assam-Arunachal Pradesh border and its adjoining areas. It was observed that this region is an excellent reservoir of many plants of therapeutic importance, which is still unexplored. Many of these are used by the local people for treating various ailments. The present study is an effort to provide scientific evidence for their therapeutic activity in order to add value to these plant resources.

For extraction of active component, dried powder of plant material was preferred to avoid interference of water. The fresh material may affect solubility and subsequent separation of active compounds by solvent extraction method due to difference in water content of the sample. Besides, for large-scale extraction of active components, dried samples are more convenient than fresh plant materials.

A number of methods have been described for detecting antimicrobial activity of plant crude extracts or their active components. To determine the antimicrobial activity of natural products, both dilution and diffusion assays are widely used. Diffusion assays are not always suitable because it is impossible to measure the amount of the test substance diffused into the agar medium. On the other hand, dilution assays have one major advantage over diffusion assays, because the concentration of the test material in the medium is defined. Consequently, dilution assays are regarded as the method of choice to determine the MIC values (Hadacek and Greger, 2000). Considering this, the initial screening of the crude methanol extracts from the plant samples for possible antidermatophytic activity against T. mentagrophytes, T. rubrum, T. tonsurans, M.
fulvum and M. gypseum was carried out both by agar well diffusion and agar dilution methods. MIC values of the active extracts were determined only by agar dilution assay. Dermatophyte species namely T. mentagrophytes, T. rubrum, T. tonsurans, M. fulvum and M. gypseum, used in this study, were chosen primarily on the basis of their importance. These species were frequently isolated by many authors in various parts of the world and in most cases T. rubrum was found to be the most dominant species followed by T. mentagrophytes (Bindu and Pavithran, 2002; Grover and Roy, 2003; Straten et al., 2003; Prasad et al., 2005; Sen and Rasul, 2006; Nweze 2010).

In traditional medicines, water is used as extractant, but all the compounds can not be extracted with water i.e. in case the bioactive compounds are non-polar or medium polar, they can not be extracted by water. Organic solvents are certainly much better to extract maximum bioactive molecules. Although it is customary to use many solvents, but for preliminary screening only methanol was used, as large number of bioactive molecules can be extracted by this solvent.

The results of the preliminary evaluation indicated that some of the plants tested possessed high antidermatophytic activity. In agar well diffusion assay, methanol extracts from fifteen plant species exhibited broad spectrum of activity against all the test dermatophytes, producing zone of inhibition ranging from 9 to 42 mm at 20×10^4 μg ml^{-1} concentration. Except M. pudica, the remaining plant extracts that exhibited activity in agar well diffusion assay revealed their activity against all test dermatophytes in agar dilution assay (10×10^4 - 0.625×10^4 μg ml^{-1}) also. It proved the fact that plants are a vast reservoir of potential new drugs, as many of the tested extracts revealed antidermatophytic activity in the preliminary screening. Methanol extract of P. longum leaf was found to be the most active with lowest MIC value.
(1.25×10^4 μg ml⁻¹) and the zone of inhibition ranged in between 37 and 42 mm in diameter. The active extracts obtained from other plants i.e., A. vera, A. indica, C. sinensis, C. sophora, C. ternatea, L. plukentii, L. inermis, O. sanctum, O. basilicum, O. gratissimum, P. betleoides, S. melongena and V. negundo showed MIC values ranging from 10×10^{4} to 2.5×10^{4} μg ml⁻¹ and zone of inhibition ranged from 37 to 42 mm. Activity of clotrimazole at very low concentration (zone of inhibition 8-20 mm at 1×10^{2} μg ml⁻¹ concentration, MIC- 0.313×10^{3} μg ml⁻¹) as compared to the plant extracts may be attributed to its pure nature. Higher MICs of the crude extracts as compared to standard antifungal chemical observed in the present study may be because of crude extracts are mixture of active and non active compounds. Therefore even the MICs were high but considered as effective against dermatophytes in accordance with the report by Webster et al., (2008).

*Trichosporon beigelii* is an opportunistic fungus often associated with dermatophyte infections. Sensitivity of this species to methanol extracts of leaves of A. scholaris, Allamanda cathartica, C. sinensis, O. sanctum, O. gratissimum and Piper betle was evaluated employing agar well diffusion method. All test extracts except that of A. scholaris displayed varying degree of activity at 20×10^{4} μg ml⁻¹ concentration. However the composite form of all the extracts at equal proportion and at the same concentration (20×10^{4} μg ml⁻¹) exhibited better activity against the pathogen which may be due to synergistic action of different compounds present in the composite extract.

Although in the present study, methanol extract of *M. pudica* leaf was not found active in agar dilution assay within tested concentrations, the extract may exhibit activity at higher concentration. Traditionally the leaves and roots of *M. pudica* are used as
medicines by different tribes in Cachar district, Assam in various ailments (Das et al., 2008). Uses of *C. sophera* (seeds), *L. aspera* (Synonym- *L. plukentii* (leaves) and *O. sanctum* in treatment of skin diseases including dermatophytoses in veterinary practices were reported earlier (Sharma and Joshi, 2004). Traditional uses of *A. indica*, *C. ternatea*, *L. plukentii*, *O. sanctum* and *V. negundo* for treatment of various other diseases were reported (Das et al., 2008). *S. melongena* is another widely used plant in traditional medicine (Mutalik et al., 2003). Despite the wide dissemination of these medicinal plants in traditional medicine, their medicinal properties with respect to antidermatophytic activity have not yet been described. Previous study reported the strong antidermatophytic activity of hexane fraction of *O. gratissimum* leaves against *M. canis*, *M. gypseum*, *T. rubrum* and *T. mentagrophytes* causing 100% inhibition of growth of dermatophytes at a concentration of 125 μg ml\(^{-1}\) (Silva et al., 2005). The antidermatophytic activity of different species of *Ocimum* was also studied (Inouye et al., 2006; Nwosu and Okafor, 1995). Antifungal activity was reported previously for *A. indica* (Ghorbanian et al., 2007; Natarajan et al., 2003) and *S. melongena* (Das et al., 2010). Various pharmacological effects of *Camelia sinensis* (Bhatt et al., 2010), *A. cathartica* leaf extract (Masuduzzaman et al., 2008; Nayak et al., 2006) and *Vitex negundo* (Sahare et al., 2008) were reported earlier. Therapeutic uses of *P. longum*, *P. betle* and *P. nigrum* were mentioned in Ayurveda and other traditional system of medicines (Gajurel et al., 2001 and Zaveri, et al., 2010). *P. nigrum*, which was reported as traditional medicines (Parekh and Chanda, 2006), was not found active against dermatophytes in the present study. *Piper longum* is a highly valuable drug and is one of the essential ingredients in many ayurvedic formulations (Zaveri, et al., 2010). Different parts of *P. longum* such as essential oils, roots, fruits, whole plants
etc. possess medicinal properties. But there is no scientific report on antidermatophytic activity of *P. longum* leaves.

The screening of the plants in the present study demonstrated the importance of some plants such as *A. vera*, *A. indica*, *C. sinensis*, *C. sophera*, *C. ternatea*, *L. plukentii*, *L. inermis*, *O. sanctum*, *O. basillicum*, *O. gratissimum*, *P. betleoides*, *P. longum*, *S. melongena* and *V. negundo* against dermatophyte infections effectively. *P. longum* (leaf) was found most potent against the tested dermatophytes.

### 5.3 Solvent extraction of *P. longum* and evaluation for antidermatophytic activity

The highest antidermatophytic activity displayed by *P. longum* as well as wide availability of this plant prompted us to isolate and identify the active antidermatophytic component(s) present in *P. longum* leaves. The choice of solvents depends on ability of the solvents to extract the largest quantities of targeted materials. Leaves of *P. longum* was sequentially extracted with petroleum ether, chloroform, methanol and water in their increasing order of polarity in order to select the best solvent, for extraction of larger spectrum of its bioactive compounds and also to separate out the less effective or non effective compounds. The polarity of the solvent play an important role in the extraction of active plant extracts as shown by the higher yields obtained in water (13.0%), followed by methanol (4.5%), chloroform (3.2%) and petroleum ether (1.8%). Similar method for extraction of plant material with different solvents in increasing polarity was also used by other authors (Vries et al., 2005; Das et al., 2010).

Bioassay of each of the solvent extracts of *P. longum* using agar dilution method established the antidermatophytic activity of all solvent extracts. The results indicated
the differential activity among non polar, polar and highly polar solvent extracts. In
the present study chloroform and methanol extracts exhibited better efficacy. Using
similar study Vries et al., (2005) isolated five compounds from the ethanol fraction of
*Galenia africana* and established the antifungal activity of the compounds employing
agar well diffusion method. The potent activity of chloroform extract of *P. longum* in
inhibiting growth of some fungi was also reported by earlier workers (Ali et al., 2007;
Das et al., 2010). More or less similar observations were also made by other workers
too (Phongpaichit et al., 2005; Ali et al., 2007). Silva et al., (2005) reported higher
range of activity of hexane fraction of *O. gratissimum* leaves against *Microsporum
canis, M. gypseum, T. rubrum* and *T. mentagrophytes* as compared to its chloroform
fraction. Contrary to this, in our study the petroleum ether extract of *P. longum* in
which non polar compounds are extracted, was found comparatively less effective.
This difference may be due to various reasons like differences in plant constituents in
different plant species, time of sample collection or other geographical factors.

5.3.1 MIC of the chloroform and methanol extracts of *P. longum* leaf

The MIC values of chloroform and methanol extracts was found to be \(0.5 \times 10^4 \mu g \text{ ml}^{-1}\)
against all the test dermatophyte species. Owing to better efficacy, both chloroform
and methanol extracts were studied further to isolate the active component (s) through
chromatographic methods. Although water appeared as the best extractant (yield 13%)
in the present study, but due to its less efficacy it was not considered for further study.
This is in accordance with the observations recorded in case of other medicinal plants
by various authors (Kaushik and Goyal, 2008; Parekh and Chanda, 2007 and Sener,
1994; Lokhande et al., 2007).
5.3.2 Longevity and solubility of chloroform and methanol extracts of *P. longum* leaf

Longevity of the chloroform and methanol extracts at 4°C and at room temperature (25-34°C) was found to be 150 days and 90 days respectively. This information is essential for proper storage of plant samples used for bioassay study. The results of the solubility test would be useful in preparing test samples of chloroform and methanol extracts of *P. longum* for various biological experiments as well as formulation of herbal products. Solubility of the chloroform and methanol extracts was tested in water, coconut oil, DMSO, methanol, ethyl acetate and hexane. Chloroform extract was soluble in all the solvents tested except water. The methanol extract, however, was found soluble only in coconut oil, methanol and DMSO.

5.4 Phytochemical analysis

The active components of many plant derived drugs are secondary metabolites. Therefore, basic phytochemical analysis of the plant extract for its main bioactive components is vital to establish scientific rationale for its use as drug. Phytochemical analysis of the chloroform and methanol extracts of *P. longum* (leaf) revealed the presence of tannins and phenolic compounds. Saponins were found only in methanol extract. Alkaloids were not detected in any of the leaf extracts, which may be because of either due to low concentration or absence of alkaloids in the extracts tested. However, alkaloids such as piperine and pipernonaline were isolated from the fruits of *P. longum* (Lee et al., 2005; Yang et al., 2002; Lee et al., 2001). The findings of this work would be useful in selecting appropriate protocol for isolation and identification of bioactive compounds of interest.
5.5 Activity-guided fractionation of chloroform and methanol extracts of *P. longum*

Fractionation leading to isolation of active compounds should result higher activity than the original extract. This approach forms the basis of discovery of bioactive compounds from the naturally occurring sources and has led to discovery of many important drugs. The main difficulty in using natural products as sources for pharmaceutical leads is the separation of the plethora of compounds from the active compounds in crude extracts. The separation techniques leading to the isolation of bioactive compounds are crucial in the natural drug product discovery process (McRae et al., 2007).

A number of compounds have been isolated from different parts of *P. longum* through bioassay-guided isolation using silica gel column chromatography (Parmar et al., 1998; Lee et al., 2001; Yang et al., 2002; Lee et al., 2005) in the past. A piperidine alkaloid, piperine was isolated from the ethanol extract of *P. longum* fruits, which possessed antidepressant-like activity (Lee et al., 2005). Another fungicidal compound, pipernonaline, a piperidine alkaloid, was isolated from the hexane fraction of *P. longum* fruits (Lee et al., 2001). Pipernonaline, which was isolated from the hexane fraction of the methanol extract of *P. longum* fruit, was mosquito larvicidal in nature (Yang et al., 2002). Three biologically active compounds namely (+)-Asarinin, Guineensine and Retrofractamide A were isolated from petrol extract of air-dried stems and leaves of *P. longum* (Parmar et al., 1998). Prasad et al., (2005) reported the isolation of several compounds, particularly of biologically active amides and lignans and neolignans of five different structural types from 30 different *Piper* species and studied the potentiality of the chloroform extract of *P. longum* and the pure
compounds isolated from it as anti-inflammatory agents. This perhaps, was the largest number of compounds of this class reported from any genus. Most of the compounds are isolated from the fruits and other parts of *P. longum*. There are no reports on isolation of antidermatophytic compound from *P. longum*, particularly from leaves.

TLC and column chromatography are most widely used techniques for the separation and purification of many bioactive molecules. Plant materials with highly complex profiles of phytochemicals, isocratic separation cannot achieve satisfactory separation. Multiple mobile phases with increasing polarity are, therefore, useful for good separation. In the present study, activity-guided fractionation of the *P. longum* chloroform and methanol extracts using silica gel column chromatography resulted in the successful isolation and identification of the seven major antidermatophytic components. The antidermatophytic activity observed in isolated fractions / components was more pronounced than the crude methanol extract of *P. longum*. This could be due to presence of many undesirable compounds in the crude extract that got removed during fractionation. TLC was used for analyzing the isolated fractions and to select the mobile phase for column chromatography. In TLC fingerprint, petroleum ether: ethyl acetate (7:3) was found to be the most efficient mobile phase for chloroform extract. While hexane: chloroform (1:1) and hexane: ethyl acetate (4:6) were adjusted as the best mobile phase for methanol extract of *P. longum* leaves.

5.5.1 Column chromatography for isolation of antidermatophytic components from *P. longum* chloroform extract

Column chromatography, using petroleum ether-ethyl acetate-methanol as mobile phase yielded nine major column fractions. Bioassay results demonstrated the
promising activity of the column fraction 1, 4, 5 and 7 against *T. mentagrophytes* in agar well diffusion assay (Table 7 and Fig. 17). In order to isolate the active components from these active fractions, repeated column chromatography was performed using petroleum ether-dichloromethane-ethyl acetate-methanol as mobile phase. The thin layer chromatogram of column fraction-1 showed one spot when eluted with different mobile phases i.e., hexane: ethyl acetate (3:1) (Rf value 0.91) and hexane (100%) (Rf value 0.82) (Fig. 18). The Rf values provide corroborative evidence for the identity of a compound. If two or more compounds have the same number of spot in different mobile phases they are most likely, although not necessarily, the same compounds. HPLC chromatogram indicated that the fraction was a composite of at least two components. The GC-MS analysis of the fraction showed presence of four major components such as 2,4-di-tert-butylphenol, methyl 14-methylpentadecanoate, methyl linolenate and octadecanoic acid, methyl ester. It is difficult to attribute the activity of a complex mixture to a particular component. Nevertheless, it is reasonable to speculate that the activity of the fraction could be related to the presence of the major compounds. In most cases, the inhibitory effect has been attributed to the major compounds. (Deba et al., 2008). Earlier Inouye et al., (2006) tested seventy two essential oils and observed that the oils, containing phenol as a major constituent, possessed the highest activity against *T. mentagrophytes*. The antibacterial activity of phenol, carboxylic acid and ester was also observed by Roy et al., (2010). Some of the compounds identified in *P. longum* extract were also isolated previously from other medicinal plants. For example methyl 14-methylpentadecanoate, which is a sesquiterpene was first reported from ethanolic extracts of *Azadirachta indica* (pericarp) (Siddique et al., 2004). The presence of
octadecanoic acid, methyl ester in *Plumbago zeylanica* (roots) and *Ocimum basilicum* (leaves) was reported earlier (Ajayi et al., 2011; Dev et al., 2011).

From the results of the present investigation it could be inferred that the activity of the fraction was either due to single compound or due to the synergistic effect of different compounds present in the fraction. It could also be interpreted that the antidermatophytic activity might be due to the major component detected in the fraction isolated from chloroform extract of *P. longum* leaves. It was reported that in most cases, the inhibitory effect of the compound has been attributed to the most dominant components and not to the other minor compounds (Farag et al., 1989).

Column fraction-4 on second step chromatography gave rise to 72 sub fractions. The sub fraction-4, a deep orange coloured solid, was isolated with dichloromethane-ethyl acetate-methanol as eluent. The major compounds detected by GC-MS analysis were 2-benzenedicarboxylic acid, bis (2-ethylhexyl) ester, 2,2-dimethoxybutane and β-myrcene (Table..) apart from ten other compounds in traces. The most dominant compound, 1,2-benzenedicarboxylic acid, bis(2-ethylhexyl) ester (41.45%) identified in the *P. longum* leaf extract in the present study was previously isolated from rice (Var-Taichung Native -1) (Rimando et al., 2001). The presence of β-myrcene was also recorded in the essential oil extracted from *Artemisia herba* (Nezhadali et al., 2008).

In essential oils of lemon grass, β-myrcene was detected and it was found to possess analgesic property when tested on rodents (da-Silva, 1991). The presence of β-myrcene was also reported in leaves and flower of *Bidens pilosa* (Deba et al., 2008).

From column fraction-5, 24 sub fractions were isolated by repeating column chromatography. Sub fraction-10, sub fraction-17 and Sub fraction-24 were further purified. Sub fraction-10 was eluted with 40% petroleum ether in ethyl acetate. The
major compound (Fig. 26- TLC-8, 5a) present in the sub fraction-10 was isolated by recrystallization from methanol. Another pure component (sub fraction-17) was isolated with 10% petroleum ether in ethyl acetate as eluent (Fig. 26- TLC-9, 5b). Sub fraction-24 was eluted with 80-70% ethyl acetate in methanol. The black coloured crystal present in this sub fraction was isolated by recrystallization from petroleum ether (Fig. 26- TLC-10, 5c) GC-MS spectra of these three components (5a, 5b and 5c) did not match any spectra of the known compound when compared with the data on mass spectra of those compounds available in the NIST library (NIST/EPA/NIH mass spectral library). Probably the compounds in these three components i.e., 5a, 5b and 5c have not been reported yet. However to draw this conclusion more compound libraries will have to be consulted. All the components (5a, 5b and 5c) were retained for future studies.

A needle-shaped crystal, isolated from the column fraction-7, was analyzed using FT-IR, $^1$H NMR, and $^{13}$C NMR spectroscopic techniques. The IR spectrum revealed the presence of aromatic ring and primary amine as functional group in the component. $^1$H NMR and $^{13}$C NMR spectra showed the presence of aromatic protons and carbons along with presence of alkyl chain. Further, the component showed positive test for primary aliphatic amine. Hence it was predicted that the compound is an aryl substituted aliphatic primary amine. The antidermatophytic activity may be attributed to amino group attached to the aromatic ring. The antimicrobial activity of amines was also reported previously by Kabara et al., (1972).

5.5.1.1 MIC of the isolated components

All the isolated components (1, 4, 5a, 5b, 5c and 7) were found to be potentially active against *T. mentagrophytes* and *T. rubrum*. The MIC values of the isolated components
ranged in between $0.625 \times 10^3$ and $2.5 \times 10^3 \, \mu g \, ml^{-1}$ whereas MIC values of standard antifungal agents griseofulvin and clotrimazole was $0.313 \times 10^3 \, \mu g \, ml^{-1}$ against *T. mentagrophytes* and *T. rubrum*. Highest antidermatophytic activity was displayed by the active component-4 and 7 with the least MIC value ($0.625 \times 10^3 \, \mu g \, ml^{-1}$ concentration).

### 5.5.2 Column chromatography for isolation of active components from the methanol extract

Two promising column fractions were obtained from the methanol extract of *P. longum* leaf, using mobile phase hexane: chloroform (40:60) and hexane: ethyl acetate (70:30 – 60:40). The MIC was recorded as $1.25 \times 10^3 \, \mu g \, ml^{-1}$ and $2.5 \times 10^3 \, \mu g \, ml^{-1}$ respectively against *T. mentagrophytes* and *T. rubrum*. Considering the potent activity, it is considered necessary to carry out further studies, in order to isolate and characterize the active compound (s) present in these fractions.

From the findings of the present investigation, it can be inferred that *P. longum* is a highly potent antidermatophytic plant. The isolated fractions, from the *P. longum* leaf showed more pronounced antidermatophytic activity than the crude extract. As the crude extract is a mixture of all these constituents, the purity and the concentration of the isolated constituents exerted better activity profile than crude extract. The basis of varying degree of sensitivity was due to intrinsic tolerance of the dermatophytes and the difference in chemical nature of the constituents. The results highlighted the possibility in finding novel compounds and hence it should be the best target for further research for the development of broad spectrum antidermatophytic agents. The findings of the present study validated the use of *P. longum* in treatment of skin
infections which is amongst the various traditional uses of *P. longum* such as treatment of skin infections, chronic bronchitis, cough and cold, anti-dote in snake biting and scorpion sting.

5.6 Authentication of *P. longum* by DNA fingerprinting

Accurate identification of medicinal plants, which are the starting materials in herbal drug technology, is of utmost importance to avoid misidentification of the targeted plant and to detect the adulterants. Great range of variability in *Piper* species makes it difficult to identify the species using conventional method. Application of various DNA based tools to authenticate medicinal plants has been described (Srivastava and Mishra, 2009; Zhang et al., 2007; Bala, 2007). Jain et al., (2007) reported that fruiting spikes of *Piper chaba* is used to adulterate *P. longum* while *P. nigrum* is adulterated with *Carrica papaya* seeds and fruits of *Embelia ribes* and *Lantana camara*. Considerable works on molecular fingerprinting of *Piper* species from South India have been carried out (Pradeepkumar et al., 2001, 2003; Sen et al., 2010). So far there are no reports on molecular study of the medicinally important *Piper* species of Northeast India. However, one report on RAPD analysis of three female varieties of *P. longum* (Varieties - Viscum, Calicut and Assam) indicated that all the varieties were genetically different. Viscum and Calicut varieties were genetically closer to each other than being closer to the Assam variety (Philip et al., 2000). The published reports indicated the importance DNA fingerprinting for proper identification of targeted plants to ensure the quality, efficacy and safety of herbal products.

In view of this, DNA fingerprinting using RAPD technique was performed to authenticate the targeted *P. longum* and to rule out the possibility of collecting cryptic
species. The findings of the study on RAPD analysis by PCR of genomic DNA of *Piper* samples showed high of genetic variations among different *Piper* species. RAPD fragments showing fixed frequencies in *P. longum* (Fig 39, Lane 1, 2, 16 and 17) with OPN-5 primer can be used as species-specific marker. Further analysis with inclusion of large number of samples and more primers would be useful to determine the genetic variations in this species and accurate identification of *P. longum.*