Chapter 6

Discussion
6. DISCUSSION

The present study revealed the potent anti-dermatophytic activity of few Indian medicinal plants against various species of dermatophytes which remains the major causative agents for superficial skin infection.

The advent of sophisticated life saving medical procedures, such as organ transplantation, intensive cytotoxic chemotherapy against cancer and the prevalence of HIV infection in recent years, has dramatically altered the immune status of the patient and consequently the incidence of dermatophytic infection has increased significantly. The main reason for the frequency of rise in serious fungal infections is due to the increased use of cytotoxic and immuno-suppressive drugs to treat both malignant and non-malignant diseases. The rise was also due to the increasing prevalence of infection due to human immuno-deficiency virus type I, and the widespread use of newer and more powerful antibacterial and antifungal agents (Rex et al., 1993).

Even though a variety of antifungal drugs are presently available, in many cases recurrent and chronic dermatophytosis has been reported. In some cases, the disease has got the tendency to recur at the same site or at
different sites with the discontinuation of treatment. More over, the existing antifungal drugs are toxic, expensive and has got fungistatic mechanism of action with development of resistance. This fact, coupled with the toxicity of many of these compounds and their prohibitive costs has been the reason for an extended search for newer drugs particularly from traditional system of medicine which could be less toxic, relatively cheap and with less undesired effect (Padma et al., 1998).

The predominant factor that has stimulated the search for safer and more effective antifungal agents has been the increasing incidence of systemic mycotic infections and the development of resistance by the organisms to the azole antifungals (Suresh et al., 1997). Response to the topical antifungal drugs was found to be variable depending upon the causative organism/species involved. The dermatophytes were thought to be uniformly sensitive to griseofulvin, but this was proved to be incorrect (Artis et al., 1981).

Thus to over come these limitations, it is imperative to conduct research for development of newer antifungal agents for which the medicinal plants present a promising future, since they are cost effective, efficacious, have a broad spectrum activity and may have less of side effects.
The literature survey revealed the therapeutic efficacy of many indigenous plants for a variety of diseases which has been widely documented in traditional medicinal literature (Satyavathi and Gupta, 1987). Many plants that are presently available in India have been reported by scientific investigators (Kirtikar and Basu, 1935; Chopra et al., 1956) to be effective against various skin diseases.

The use of medicinal plants in the treatment is an age old practice and the use of herbal medicines in India have been used to treat a variety of skin diseases and others ailments by the physicians of Ayurveda, Unani and Siddha (Nadkarni, 1976). A large portion of the world population, especially in developing countries depend on the traditional system of medicine for a variety of diseases. Several hundred genera are used medicinally, mainly as herbal preparations in the indigenous systems of medicine in different countries and are sources of potent and powerful drugs (Ahmad et al., 1998).

Many pharmaceutical products are of plant, bacterial or fungal origin, although synthetic organic compounds are substantially used, and plant products have proved their value in a broad range of human infections. Since the introduction of penicillin (from Penicillium notatum; Fleming, 1929) into
therapeutics more than 50 years ago, numerous drugs have been identified and developed from different plants (Smania et al., 1995).

With this background information and appreciating the knowledge of medicinal plants, the present study was carried out to determine the antidermatophytic activity of twelve commonly available Indian medicinal plants in vitro by broth dilution technique. The therapeutic efficacy of one of the plants compound was tested using guinea pig as an animal model and toxicity studies were done using Vero and Mc Coy cell lines.

The precise mode of extraction naturally depends on the texture and water content of the plant material being extracted and on the type of substances being isolated. The plant parts were shade dried since there is every liability of losing some unstable plant constituents in bright sunlight. Finely powdered substances absorb more solvent, hence the plants were coarsely powdered.

One of the most important and fundamental considerations in designing a phytochemical screening procedure is the selection of a proper solvent. Ethanol extracts of the plant exhibit a well marked antibacterial and antifungal activity (80%), and aqueous extracts are known to give only 20%
activity. Similarly the previous reports of Bhakuni et al. (1974) and Naqvi et al. (1976), indicate that the ethanol extracts are the most active extracts.

Several methods for preparing an initial extract of the plant material have been reported. Among the various methods that are available, 80% ethanol and methanol appears to be the best solvents (Farnsworth, 1966). The rational for adopting such an extraction procedure was based on the polarity of the solvents that would leach out compounds soluble in that particular solvent (Samy et al., 1998).

Similar observation was found in our study where we noted good anti dermatophytic activity with ethanol extracts when compared to other extracts that are used in the present study. Many factors affect the extractability which in turn increase/decrease the biological activity of the plants. Bose (1946) extracted Polystictus sanguineus with various solvents and observed that those extracts obtained by using ether, chloroform and ethyl acetate were active against Salmonella spp. but no activity was found for acetone extracts.

In general extracts obtained with less polar solvents (i.e. hexane, heptane, petroleum ether, ethyl ether, dichloromethane and butyl alcohol) have less inhibitory activity over the bacteria and fungi that is being tested.
In contrast to that, extracts obtained with more polar solvents (i.e. ethyl acetate, ethanol, methanol and acetone) showed an activity at very lower concentrations. In considering extracts that have antimicrobial properties, one of the factors that makes comparison of data from different sources difficult is the variation in testing techniques (Rios et al., 1988).

A great number of facts can influence the results of the antimicrobial testing - the extraction methods, inocula volume, culture medium composition, pH and incubation temperatures. The criteria for useful activity are inhibition of one or more organisms and reproducibility of results in atleast two successive series of the various testing procedures (Mitscher et al. 1972). Further more, the micro organisms used and the volume of the sample assayed can also interfere with the *in vitro* anti microbial testing procedure (Janssen et al., 1987).

The concentration of the plant extract also plays a major role in determining the antimicrobial efficacy of the plant extracts. According to Mitscher et al. (1972), crude plants extracts have to be tested at the concentrations of 1000 µg/ml and below, since a plant component which is not active atleast at 1000 µg/ml is unlikely to be of therapeutic value.
Diffusion methods are those which are most often employed in research in spite of certain difficulties, but they are models with a low credibility for samples that are difficult to diffuse in the media because there is no relation between diffusion power and antimicrobial activity. Pellecuer et al. (1976) showed that different results of the antimicrobial activity of the same plant was obtained when assayed using disc diffusion and tube dilutions method. When the sample was assayed by a tube dilution method, the plant extract was more active.

Gundidza (1987) had assayed the extracts of crescent polarity of Helinus integrifolius using the hole-plate diffusion and the tube - dilution methods. All the fractions exhibited activity against Candida albicans when the liquid dilution method was used. With the hole-plate diffusion method, none of the fractions exhibited activity against C.albicans. This is due to the fact that plant extracts frequently have low diffusion properties, while in the test tube dilution method, the samples are in direct contact with the test micro organism. Even if the extract has low solubility in water, at least the suspended particles will still be in contact with the test organism.

Antifungal susceptibility testing is of potential value as a means of predicting the therapeutic use of new antifungal agents and predicting the
likely outcome of therapy. Unfortunately, the current applicability of in vitro antifungal susceptibility tests is limited by inadequate standardisation and insufficient correlation of in vitro test results with clinical outcome. in vitro antimicrobial testing of plant extracts is an important technique to analyse the active principles, medicinal properties of various crude plant products and is an important prerequisite for further pharmacological analysis.

In vitro susceptibility test procedures with antifungal agents are influenced by a number of technical variables including inoculum size and preparation, medium composition and pH, duration and temperature of incubation and end point determination. Antifungal susceptibility testing is further complicated by problems unique to fungi which include slow growth rate and the ability of the certain (dimorphic) fungi to grow either as a unicellular (yeast) form which produces blastospores or as a hyphal (mold) form which may produce asexual spores depending on the conditions of pH, temperature and medium composition. Moreover, the basic properties of the antifungal agents themselves such as solubility, chemical stability and the tendency to produce partial inhibition of growth over a wide range of concentrations must be taken into account (Galgiani et al., 1992). The NCCLS (National committee for clinical laboratory standards) studies have identified a significant interest in antifungal susceptibility testing for clinical
purposes among hospital based laboratories in the USA. They found that the spectrophotometric method to be the most reproducible method of inoculum preparation, both within a given laboratory and among laboratories. The NCCLS multicentre studies have resulted in the development of spectrophotometric standardisation method, as the standard method for inoculum preparation.

Our present study also followed the spectrophotometric standardisation of inoculum preparation. Manavathu et al. (1996) reported from his studies that broth macrodilution is highly reliable, since it provided lower MIC values than the broth micro dilution which produced four fold higher MIC values. He suggested that the macrobroth dilution would be highly suitable for the determination of MIC’s of the antifungals. In our study we followed the same method as described by Manavathu et al. (1996).

Among the twelve plants tested in vitro for their antidermatophytic activity, only 3 plants showed significant antidermatophytic activity in comparison with the other plants. We have chosen one out of these 3 plants, Plumbago zeylanica for further in vivo and cytotoxicity studies. Plumbagin extracted from root of this plant, was used because of its low MIC values against the various species of dermatophytes tested which ranged from 12.5-25 μg/ml.
In the present investigation, the leaf extract of *O.sanctum* and *Eucalyptus* spp. did not show any antidermatophytic activity upto a concentration of 2 mg/ml. Janssen *et al.* (1989) have reported the antimicrobial activities of essential oils of *Ocimum* species grown in Rwanda. We have used the cold extraction method. It is likely that the cold extraction method would not have extracted the active principles/ingredients present in *O.sanctum* leaves. The essential oil contains the active components such as thymol and eugenol which plays a major role in inhibiting the various bacterial and fungal strains. In our study we were not able to detect active inhibitory action of the extract on various species of dermatophytes. This was probably due to the fact that we did not test essential oils of *O.sanctum* and *Eucalyptus*.

Similarly, the leaf extract of *Eucalyptus* also did not show any activity upto a concentration of 2 mg/ml against the test organisms. Hajji *et al.* (1993) and Gundidza *et al.* (1993) have reported the antibacterial and antifungal activities of the essential oil of *Eucalyptus*. The organic extract of *O.sanctum* and *Eucalyptus* which we have used for the present study would not have contained the essential oil, which contain the active principles that is responsible for the inhibitory action. This would have been the reason for
the absence of antidermatophytic activity of the leaf extracts of both *Eucalyptus* spp. and *O.sanctum*.

No inhibition was observed upto a concentration of 2 mg/ml against the leaf extract of *Acalypha indica*. Alade and Irobi (1993) reported the antimicrobial activity of *Acalypha wilkesiana* leaf extract. MIC values of 2-8 mg/ml were obtained for the ethanol extract in the tests with the bacterial agents, whereas range of 0.25 - 16 mg/ml was recorded against the fungal isolates which included *Trichophyton rubrum* and *T.mentagrophytes*. We did not use such high concentrations in our study. The geo-climatic conditions and the habitat of the plant is known to play a major role in the formation of secondary metabolites.

Apisariyakul *et al.* (1995) studied the antifungal activity of turmeric oil extracted from *curcuma longa*. The results of their study showed that the isolates of dermatophytes were inhibited by turmeric oil at dilutions of 1:40 - 1:320. We have used the leaf extract of *C.longa*, which showed no activity upto 2 mg/ml concentration against the various dermatophytes species tested. But Apisariyakul *et al.* (1995) used the rhizome of turmeric (*C.longa*) from which they extracted turmeric oil. It is therefore clear that only turmeric oil
had antidermatophytic activity rather than the leaf extract. Hence, it clearly indicates the absence of the active principle in the leaf extract, which is present in the rhizone of *C. longa*. The ethanolic and ethyl acetate extract of *C. alata* were more active with an MIC ranging from 62.5 - 500 µg/ml for *T. rubrum* and *T. mentagrophytes*, and for *M. gypseum* and *E. floccosum* it ranged from 62.5 - 125 µg/ml. Our findings correlated well with the findings of Venugopal *et al.* (1993). The MIC’s of their study ranged from 10 to 100 mg/ml against seven species of dermatophytes tested.

Darmodaron and Venkatraman (1994) studied the therapeutic efficacy of *Cassia alata* against pityriasis versicolor in human volunteers. They suggested the use of *C. alata* extract as an effective, reliable and safe herbal medicine for pityriasis versicolor. Ibrahim and Osman (1995) also reported the antimicrobial activity of *Cassia alata* from Malaysia. The report of their study revealed an MIC ranging from 62.5 mg/ml to 125 mg/ml against *T. mentagrophytes, T. rubrum* and *M. gypseum*. Our findings seem to agree with the previous report of Palanichamy and Nagarajan (1990) who also reported the antifungal activity of *C. alata* against dermatophytes.
The MIC$_{50}$ values of the ethanol and ethyl acetate extracts of *Lawsonia alba* was observed at 125 µg/ml for *T.mentagrophytes* and *T.ruhrum*, whereas the MIC$_{50}$ values for *M.gypseum* and *E.floccosum* were exhibited at 62.5 µg/ml and 125 µg/ml respectively. Our results correlated well with the findings of Venugopal *et al.* (1993), wherein they noticed the MIC values ranging between 1 to 100 mg/ml. Dixit *et al.* (1980) has reported an antifungal "Lawsone" from the leaves of *Lawsonia inermis* which was found to be fungicidal with broad spectrum activity. The growth of the dermatophytes were correspondingly decreased by increasing concentration of the extract. The results of our present study showed that the extract exhibited fungistatic activity at lower concentrations and fungicidal activity at higher concentration. Therefore, the MIC determination is important in giving a guideline to the choice of an appropriate and effective concentration of a therapeutic substance. However observations show that the aqueous extract was considered to be static in action while the ethanolic extract was found to have cidal property (Alade and Irobi, 1993). Similar observations were made by Olorundare *et al.* (1991) while studying the antibacterial activities of *Cassia alata*. The results of our present study revealed significant antidermatophytic activity of the leaf extract of *C.alata* and *Lalba*. 
Plumbagin extracted from the root of *Plumbago zeylanica* showed significant antidermatophytic activity. The MIC's of the extract ranged from 62.5 μg/ml to 125 μg/ml. It has been reported to have various therapeutic uses in the ayurvedic system of medicine. Plumbagin was known to possess significant antibacterial and antifungal properties (Krishnaswamy and Purushothaman, 1980). Farr *et al.* (1985) reported that Plumbagin induced repair mechanisms in *Escherichia coli*.

The MIC for the pure Plumbagin ranged from 12.5 μg/ml to 25 μg/ml for *T.mentagrophytes* and *T.rubrum*. The MIC's for *M.gypseum* and *E.floccosum* was observed at 12.5 μg/ml. In another study conducted by Durga *et al.* (1990), the MIC value for Plumbagin for *Escherichia coli* and *Staphylococcus aureus* was observed at 200 μg/ml and 20 μg/ml respectively. They also reported that the growth of the bacteria was completely prevented when the bacteria were grown in the medium containing antibiotic and Plumbagin together and this was attributed to the prevention of development of antibiotic resistance in bacteria. Plumbagin was found to have bactericidal properties as evidenced by a transfer of culture to drug free medium (Durga *et al.*, 1990).
Plumbagin has been reported to possess antitumor property. It is also reported to act against chemically induced fibrosarcoma in mice and against P388 leukaemia in vitro (Krishnaswamy and Purushothaman, 1980). The well characterised phytochemicals present in P.zeylanica are plumbagin, 3-Chloro plumbagin, 3 - 3’ biplumbagin and 6-6’ diplumbagin. Structurally Plumbagin is the simplest one being 5 - hydroxy - 2 - methyl 1 - 4 naphthaquinone. The result of our study well correlated with the findings of Krishnaswamy and Purushothaman (1980) wherein the antifungal activity was observed at 10 µg/ml for E.floccosum. Plumbagin present in P.zeylanica and P.rosea is prescribed for cancer treatment in the siddha system of medicine (Mudaliar, 1969).

The results obtained for Plumbagin extract were compared with that of the miconazole of SPIC pharma. The MIC values of miconazole (SPIC pharma) revealed the inhibitory action within a range of 1.56 - 3.12 µg/ml. Our results are in conformity with other investigators. Venugopal et al. (1993) have observed an MIC₉₀ for ketoconazole and miconazole at 2.5 µg/ml, whereas in our study MIC₉₀ for miconazole was observed at 1.56µg/ml. In another study Minocha et al. (1989) reported that miconazole inhibited 73.3% of Trichophyton spp. and it inhibited the various dermatophytes at a low concentration of 2 - 4 µg/ml. This was in agreement with our results obtained for miconazole. Our study reports emphasized that,
there was not much of a difference between the MIC values obtained for Plumbagin and miconazole (Gufic pharma). The MIC$_{50}$ and MIC$_{90}$ values were comparable to that of miconazole (SPIC pharma). The difference in MIC ranges obtained with miconazole from different sources (SPIC pharma & Gufic pharma) suggests a significant variation in individual preparation available commercially.

Dermatophytes are responsible for the majority of superficial fungal infections. Because arthrospores, the hyphal fragments of dermatophytes can remain viable in skin scales for years (Weitzman and Summerbell, 1995). Villars and Jones (1989) have investigated and reported that the use of fungicidal drug successfully prevented the relapse of the disease in the patients rather than fungistatic drugs. Plumbagin in our present investigation was proved to have fungicidal properties and the organism could not be recovered when it was subcultured on to the extract free media, after MIC determination.

In any drug screening procedure, it is necessary to conduct safety studies of the drug in the in vivo systems. In general, animal models correlate better with clinical efficacy than do in vitro methods (Hare and Loebenberg, 1988). Ideally, an effective animal model should involve a simple technique for induction of infection (Rinaldi, 1992). We have chosen the guinea pig for our in vivo studies, since it is easier to handle and it appears to be a better system for studying the therapeutic efficacy of medicinal plants.
We have used a standardised inoculum of *T. mentagrophytes* in our *in vivo* study for experimental induction of dermatophytosis in guinea pigs. This experimental model with *T. mentagrophytes* seemed to be an ideal system for investigating the progress of infectivity of dermatophyte lesions as well as antifungal therapy (Chittasobhon and Smith, 1979).

Plumbagin (0.25%) was used in treating the experimentally induced dermatophytosis and its effect was compared with that of clotrimazole in controlling the dermatophytic lesions. Topical application of (0.25%) Plumbagin extract in experimental lesions in guinea pigs showed symptomatic and mycologic cure. Clearance of erythema, crust and hair regrowth started on the 10th day of treatment. In the case of clotrimazole treated group, similar results was observed as that of Plumbagin and hair regrowth started on the 8th day of treatment. We could not recover the organisms in culture in both Plumbagin and clotrimazole treated group. Whereas in the case of untreated group KOH positivity was observed till 27th day of inoculation. Hair regrowth was observed on the 31st day of inoculation.
The results of the in vivo study clearly proved the efficacy of Plumbagin extract in vivo as an antidermatophytic agent. Its efficacy was similar to that of the commercial antifungal agent clotrimazole.

Our studies correlated with the investigation of Apisariyakul et al. (1995). They studied the inhibitory activity of turmeric oil in Trichophyton induced dermatophytosis and its result was compared with that of the control drug canesten. From their studies, they observed the reduction of erythema and scales, on the sixth day in both turmeric oil and canesten treated group. The findings were similar to our observations. In another study Suresh et al. (1997) used clotrimazole (2%) cream in controlling experimentally induced vaginal candidiasis in mice and also in controlling antymycotic activity in guinea pigs.

Subsequent to the in vitro and in vivo studies using Plumbagin extract, we have also done cytotoxicity studies using Vero and Mc Coy cell lines to prove that they were not toxic. Before proceeding for in vivo trials it is a fundamental procedure to determine the cytotoxicity of any natural product in an established cell line.
From our results, it is clear that Plumbagin extract were not cytotoxic to the Vero and Mc Coy cells. No observable cytotoxic effect was seen when it was analysed in vitro in both Vero and Mc Coy cells at concentrations ranging from 2 mg/ml to 31.25 μg/ml. The LD$_{50}$ of Plumbagin in mice was approximately 10 mg/kg body weight (Kavimani et al., 1996). The ED$_{50}$ of Plumbagin for fibrosarcoma in rats is found to be observed at 0.75 mg/kg body weight, and at 4 mg/kg against lymphocyte leukaemia (Krishnaswamy and Purushothaman, 1980).

Subacute toxicity of Plumbagin conducted in Wister rats for two weeks and six weeks at 4 mg/kg body weight orally showed no histopathological changes in any vital organs. There was no toxic effect of Plumbagin in any vital organs such as liver, uterus etc. The bio-chemical picture of the drug also showed no adverse effect on any vital organ. No mortality was observed in either two weeks or six weeks (Purushothaman et al., 1984). In our cytotoxic studies also we didn’t observe any cytotoxic effect in both Vero and Mc Coy cells upto 2 mg/ml concentration.

Practitioners of indigenous medicine have used Plumbagin orally to cure cancer patients. But in our study, we have used the extract only for topical application.
Hence, we suggest that Plumbagin could be used successfully for treating ringworm infections, since it is easily available, possesses fungicidal activity and has no ill effects. Plumbagin was seen to exhibit fungicidal properties against several species of the dermatophytes. Therefore, our studies highlights the possibility of using this plant compound in traditional medicine for the treatment of various tinea infections caused by dermatophytes. Nevertheless, more clinical trials on the effects of Plumbagin in treating dermatophytosis is essential.