CHAPTER V

DISCUSSIONS
DISCUSSION

With the increased number of immuno compromised patients there has been a concomitant increase in patient morbidity and mortality due to the fungi. Patients with malignacies and chemotherapy induced neutropenia are commonly infected with candida. Patients with cell mediated immune dysfunctions such as acquired immune deficiency syndrome (AIDS) are susceptible to mucocutaneous candidiasis. Contributing factors such as broad-spectrum antibiotic use, intravenous catheterisation, malnutrition, hyperalimentation, multiple surgical procedures, trauma and steroids used either singly or in combination may also predispose patients to this invasive fungal disease (Stein and Sugar, 1989).

In yet another study, Daus and Hafez (1975), reported on the factors affecting the incidence and severity of vaginal candidosis, in pregnant and non-pregnant randomly selected patients. They reported 6 factors that seemed to be significantly associated with candidiasis, viz. drug addiction, obesity, oral contraceptive use, pregnancy, antibiotic therapy and diabetes mellitus.

Candida albicans is an opportunistic pathogen of human beings and other mammals. It has not been possible to apply the powerful methods of genetic analysis to understand its morphogenesis or pathogenesis, so as to combat its pathogenity, since it heritably and reversibly switches its cellular and colony morphologies. However, recombinant DNA techniques are increasingly being applied to C. albicans to solve many of the
unanswered questions of morphogenesis and pathogenesis. Though it would be highly satisfying to design drugs and combat this pathogen on the basis of the biochemistry or physiology of the organism. Our current status of knowledge on infectious diseases does not permit it. Facing the scientific progress in the field of candida and candidosis research in the age of HIV infection it seems rewarding to review the treatment of candidiasis.

Definitive diagnosis is often difficult to establish and usually requires invasive biopsy. Delay in the culture results due to the time required to process specimens and to allow the fungus to grow also contributes to the poor results of therapy. Biopsy of skin lesions represents a useful technique for making a diagnosis.

Recent advances in antifungal therapeutics promise to change the current approach to treatment for several of the mycoses. The availability of new oral azoles with wide spectra of activity has proved useful for prolonged oral therapy of these otherwise lethal mycoses.

Recent reviews on antifungal azoles have generated substantial interest as new therapeutic agents for many of the mycoses. Azoles are synthetic compounds with one or more 5-member rings where each ring contains either 2 nitrogens and known as imidazoles (ketoconazole) or 3 nitrogens and called triazoles. In addition to antifungal properties, the azoles have considerable biological versatility. The azoles are broad spectrum antifungal agents with activity against yeast, dimorphic and filamentous fungi.
The mode of action of azoles is widely accepted as involving the inhibition of the fungal cell membrane sterol and ergosterol. This inhibition is assumed to occur by the complexation of the azole antifungal agents with the heme moiety of cytochrome P-450, thus preventing lanosterol C-14 dimethylase from converting lanosterol to ergosterol (Vanden, 1987).

The imidazole antifungal agent ketoconazole and triazole antifungal agent fluconazole have a wide spectrum of action, and are more attractive as they allow oral therapy for systemic fungal infection (Batter et al., 1979). The disturbing feature of these drugs has been the high rate of recurrence of disease when the drug is stopped. In addition, there is some indication that the drug may not be effective in treating fungal infections in patients with impaired host response. The predominant factor that has stimulated the ongoing research in this area, today is the search for safer and more effective antifungal agents to combat the increasing incidence of systemic mycoses in immunologically compromised patients.

The establishment of imidazole and its family of derivatives as antifungal drugs and the search for newer drugs to combat systemic mycoses in immunocompromised conditions prompted us to explore this area of drug research and development, further.

Benzimidazoles and its derivatives have been established as antifungal drugs. Holt (1956), had observed that the substituted benzimidazoles have stronger antimycotic properties, when the substitution is in 2-position. Numerous
chemical modifications and substitutions in the benzimidazole molecule have led to the development of important newer drugs. However a survey of the literature showed that little or no information was available on 2-mercapto substituted benzimidazoles, nor its topological analogs benzoxazole and benzthiazole or their derivatives as antimycotic agents.

The mercapto derivatives are of significance because the coenzyme A molecule is a mercaptol as it terminates in an-SH group. It is known that acetyl groups are covalently linked to the sulphur atom to form mercapto esters during the acetyl transfer reactions. In other words the form in which acetate is utilised in most of its biochemical reactions is the acetyl coenzyme A which is a mercapto ester. Further, it is also known that the acetyl group of the acetyl coenzyme A is transferred to oxaloacetate to yield a citrate during the citric acid cycle. These reactions are of importance for the oxidative degradation of carbohydrates and fatty acids in aerobic cells.

With these concepts in mind the synthesis characterisation and evaluation of 2-mercapto substituted topological analogs of benzimidazole, viz. benzoxazole and benzthiazoles and their derivatives was considered worthwhile in our quest for the development of new antifungals (Manfred, 1980).

These compounds were synthesised as described by Gilman Blatt (1971), and they are:

1) 2-mercapto benzoxazole
2) 2-carboxy methyl mercapto benzoxazole
3) 2-methyl mercapto benzoxazole
4) 2-mercapto benzoxazole ethyl acetate
5) 2-mercapto benzthiazole
6) 2-carboxy methyl mercapto benzthiazole
7) 2-methyl mercapto benzthiazole
8) 2-mercapto benzthiazole ethyl acetate
9) 2-mercapto benimidazole
10) 2-carboxy methyl mercapto benimidazole
11) 2-methyl mercapto benimidazole
12) 2-mercapto benimidazole ethyl acetate

The characterisation of these compounds was done with the help of IR and NMR spectra; and physical constants and elemental analysis. The compounds were then taken up for screening for their antimycotic properties as well as other pharmacological studies.

The antifungal studies involved the screening of all the twelve compounds for their antifungal activity against fourteen strains of C. albicans in vitro and also in vivo in mice.

The in vitro studies, using two fold serial dilution technique revealed that amongst the compounds tested, 2-mercapto benimidazole and 2-carboxy methyl mercapto benimidazole were the only two, that were effective against all the 14 strains of C. albicans against which they were tested, at minimal inhibitory concentration of 25 \( \mu \text{g/ml} \) - 50 \( \mu \text{g/ml} \) (of the broth) and were comparable to that of ketoconazole (MIC = 12.5 \( \mu \text{g} \) to 25 \( \mu \text{g/ml} \) of the broth). These results were also found to be statistically significant at 5% probability levels. Though 2-mercapto benoxazole and 2-mercapto benzthiazole did show antimycotic activity against certain strains of C. albicans, but
were found not to be significant. Hence, it was decided to only study 2-mercaptop benzimidazole and 2-carboxy methyl mercapto benzimidazole for their in vivo antifungal activity against *Candida albicans*.

The correlation of the results of in vitro systems with in vivo models is imperative as the in vitro assessment of drugs is markedly influenced by test conditions, media, pH, inoculum size, variability of testing procedures and end point determination.

The in vivo studies, of these compounds against *C. albicans*, with two different experimental models yielded promising results. Studies with experimental vaginal candidosis showed that both the compounds, 2-mercaptop benzimidazole and 2-carboxy methyl mercapto benzimidazole showed significant activity in controlling vaginal candidosis in mice as evidenced by the low incidence of colony forming units (3.30 x 10^2) and 4.60 x 10^1 respectively) when compared with the control group (7.48 x 10^5) and the positive control clotrimazole (1.5 x 10^2) on the 9th day post inoculation with 4% formulation mixture of the compounds and controls respectively. The statistical analysis of the data gives significant values at 5% probability levels.

In-vivo studies with experimental systemic candidosis once again revealed that both the drugs, 2-mercaptop benzimidazole and 2-carboxy methyl mercapto benzimidazole were able to effectively control systemic candidosis in mice as evidenced by their mortality rate (10% and 40% respectively at 50 μg/kg dose level) when compared with the solvent control.
group (100% mortality) and amorlifine which served as positive control (10% mortality). The cfu data from kidney homogenates obtained from the respective groups of animals once again support the above observations (2-mercapto benzimidazole - $4.2 \times 10^2$; 2-carboxy methyl mercapto benzimidazole - $2.1 \times 10^2$; amorlifine - $5.0 \times 10^1$; solvent control $4.0 \times 10^4$). The T/C% of the mean survival time of the treated (T) and control groups also concurs with the above results which were once again statistically significant.

These antifungal studies with the various synthetic compounds, gave two promising compounds that were able to effectively control the various strains of Candida albicans both in vitro and in vivo.

The Imidazoles have hitherto predominately yielded drugs that were either effective locally (isoconazole and econazole) or effective orally (ketoconazole and fluconazole). But the present studies have shown that both the compounds 2-mercapto benzimidazole and 2-carboxy methyl mercapto benzimidazole are effective both locally as well as systemically as evidenced by their effectively controlling experimental vaginal candidosis and systemic candidosis. With this new dimension a further study of the antifungal activity of these compound in immuno-compromised conditions would definitely be worthwhile.

Benzimidazoles are known to possess various pharmacological activities viz. antiinflammatory, antiviral and antihelmintic. It is imperative to understand the pharmacology of the drugs that are to be recommended for use. The outcome of
a therapy in a patient is probably the result of toxic and stimulatory effects on the parasite and host respectively. Moreover each effect is modified by interactions with serum lipoproteins, other serum proteins, and uptake into specific organs. In simple terms, it is important to know more about the action of these drugs at every level, so as to recognise and differentiate toxic and stimulatory effect on the host so as to avoid the former and exploit the latter to improve therapy.

In pursuit of the above objectives and to screen the synthetic compounds for their other probable beneficial actions they were screened for their other biological activities.

The antibacterial studies of the various synthetic compounds against various gram positive and gram negative bacilli and cocci viz. E.coli, S.aureus, P.auregenosa and B.subtilis, showed them to possess antibacterial activity which was dose dependent and statistically significant. Further studies relating to their development against pathogenic organisms may be of interest.

The various compounds, in general, depressed the central nervous system which was characterised by slight sedation subdual of reflexes loss of muscle tone; suggesting a probable tranquilising action.

The antipyretic activity of these drugs was significant as was evidenced by a peak fall in temperature within 30 min. of the administration of the drug.

2-methyl mercapto benzimidazole with its decreased gastric acid content, increased pH value and low ulcer scoring emerges as a potential antigastric ulcer drug and the action of
which is comparable to that of cimetidine and omeprazole that served as the positive control.

As already described it is also important to assess the toxic effects of a drug on the host probably due to their interaction with the various systems of the body. In an attempt to recommend the compounds 2-mercaptop benzimidazole and 2-carboxy methyl benzimidazole for their antifungal action and other beneficial pharmacological actions, they were subjected toxicological studies. These studies were carried out as already described and revealed that there were no significant variations observed in the most of the biochemical and hematological parameters studied.

Treatment withazole antimycotics has led to a marked improvement in the clinical condition of a number of patients suffering from chronic candidiasis. Prior to the introduction of ketoconazole, topical or parenteral administration of antifungals was required and even if clinical improvement could be obtained, it was often transient. In spite of this, Horsburgh and Kirkpatrick (1983) have described the isolation of strains C. albicans that appeared to be resistant to ketoconazole even after protracted treatment as well as to other azole antifungals both in vitro and in vivo. This report ofazole resistance developing in a patient during protracted treatment could be a cause for concern. It also remains to be seen, what place these new imidazoles and triazoles have in managing immuno compromised patients including those with acquired immune deficiency syndrome (AIDS).
The in vitro studies revealed that from amongst the various natural products tested, the volatile oil distillate of Santolina chamaecyparissus possessed significant antifungal activity against all the strains of C. albicans tested (MIC = 250 µg/ml - 500 µg/ml of the broth) thus becoming a potential candidate for in vivo studies.

The in vivo studies with the volatile oil distillate of Santolina chamaecyparissus against experimental vaginal candidosis revealed that the drug can effectively control the fungal disease and the activity was comparable to that of clotrimazole as evidenced by the number of colony forming units on the 9th day post inoculation in animals. (Santolina chamaecyparissus 4% - 1.0 x 10^3; clotrimazole - 1.5 x 10^2; solvent control 7.48 x 10^5). A statistical analysis of the data revealed significant antifungal activity of the said drug.

Not all natural drugs yield positive results in search for their medicinal value. In a study by Abraham et al., (1986), about 295 plant extracts showed various biological activities and only two of them showed antifungal activity. To discover a new drug costs several millions of dollars and several years of dedicated scientific research work, which may after some time be once again found to have developed resistance or be toxic in the parasite and host respectively.

The alternative systems of medicine such as Siddha, Unani and Ayurveda quote in their literature tens of thousands of formulae to cure thousands of diseases. These formulae have been in use for centuries by the practitioners of indigenous systems of medicine. The value of these medicines is looked down upon by the practitioners of modern medicine, since they lack
scientific validity. However these indigenous systems of medicine have come to rescue of modern system medicine, time and again, whenever there was a dearth of new drugs. Recently there has been a world wide interest in scientifically validating the therapeutic efficacy of traditional systems of medicine.

A perusal of the literature of Siddha system of medicine revealed that there are several drugs available for the treatment of fungal diseases. Anandan et al., (1991), in a pilot study with three Siddha drugs against different types dermatomycoses have reported significant activity. Another drug 'chirattai tailam' used in the Siddha system of medicine is reported by Mudaliar (1969), to cure several skin diseases.

Hence the screening of Siddha drugs for their antifungal activity and providing the necessary scientific validation was considered worthwhile and the following drugs were screened for their antifungal activity against the various strains of C.albicans.

1) Palakarai parpam
2) Nandhi mezhugu
3) Vaan mezhugu
4) Iedi Vallaathi mezhugu
5) Erasa kenth Mezhugu
6) Sivanar amirtham
7) Parangi pattai chooranam

The results of the in vitro studies with these drugs gave four promising drugs for use against candidal infections which are given below in the order of their efficacy, which were also statistically significant.
The azole are also recognised only as static antifungal agents and accordingly, host immuno competency is required to offset relapse after therapy. While resistance to azoles has not become a problem, presently, when detected they seem to be cross-resistant to all the azoles tested so far. (Rogers and Galgiani, 1986; Dupont et al., 1988; Hughes et al., 1988).

As already indicated, the predominant factor that has stimulated the ongoing research for safer and more effective antifungal agents has been the increasing incidence of systemic mycoses in immunologically compromised patients and the unfortunate incidence of certain strains of C. albicans becoming resistant to azole antifungals. Hence there is a need to develop wider variety of antifungal agents useful in the treatment of fungal diseases. The search for naturally occurring compounds with antifungal activity has become quite intense due to above reasons.

In the quest for searching new antifungal drugs among the medicinal plants a study of the literature on medicinal plants and their biological activities gave several leads, of which the following plants evinced our interest and were taken up for study in vitro and in vivo against C. albicans.

1) Santolina chamamaecryparissus
2) Araucaria bidwilli
3) Ailanthus excelsa
4) Pristimera grahami

The antifungal activity of the various extracts obtained from the above plant materials, after preliminary phytochemical investigation, was studied in vitro and in vivo for their efficacy against various strains of Candida albicans.
It was the purpose of this thesis to study the experimental approaches to the discovery of antifungal drugs from amongst synthetic compounds, natural products from medicinal plants and the traditional system of medicine for antifungal activity, especially against the various strains of C. albicans.

Our studies have indeed given promising leads to drugs in each area of research undertaken by us. They are once again highlighted here for information.

Search for antifungal drugs from synthetic drugs yielded.
1) 2-mercapto benzimidazole
2) 2-carboxy methyl mercapto benzimidazole

Search from the medicinal plants for antifungals gave us
1) Santolina chamaecyparissus (volatile oil)

Search from the Traditional - Siddha - system of medicines validated the use following drugs as antifungals.
1) Nandhi mezhugu
2) Vaan mezhugu
3) Erasa kenthi mezhugu
4) Parangi pattai chooranam

All the above drugs have been found to be effective as antifungals against all the 14 strains of C. albicans tested by us in vitro which showed minimum inhibitory concentration of the drugs in the order of,

- Synthetic drugs - 25 µg - 50 µg/ml of broth
- Natural products - 250 µg/ml of broth
- Siddha drugs - 250 µg - 500 µg/ml of broth
Without correlation of in vitro systems with in vivo models the studies remain incomplete. A comparison of the in vivo activity of the synthetic compounds and the natural products against experimental vaginal candidosis on the 9th day post inoculation shows that 2-carboxy methyl mercapto benzimidazole followed by 2-mercapto benzimidazole and the volatile oil distillate of Santolina chamaecyparissus as evidenced by their cfu's of $4.60 \times 10^1$, $3.3 \times 10^2$ and $1 \times 10^3$ respectively were effective in controlling the fungal disease.

However before recommending them as potential antifungal drugs for clinical studies it is necessary to study how these antifungals work, the molecular or cellular basis of the action of these agents, and their mechanism of action at every level; studied with appropriate in vitro models and compared with in vivo results for positive correlations. Better and more reproducible susceptibility tests that can help to determine appropriate dosage and more information of pharmacokinetics would help in utilizing these antifungals according to best possible schedules.