The spread of multidrug-resistant strains of fungus and the reduced number of drugs available makes it necessary to discover new classes of antifungal and compounds that inhibit these resistant mechanisms. This has led to a search for therapeutic alternatives, particularly among medicinal plants and compounds isolated from them used for their empirically antifungal properties. In these natural sources, a series of molecules with antifungal activity against different strains of fungus have been found, which are of great importance to humans and plants.

In the past few decades, a worldwide increase in the incidence of fungal infections has been observed as well as a rise in the resistance of some species of fungus to different fungicidal used in medicinal practice. Fungi are one of the most neglected pathogens, as demonstrated by the fact that the amphotericin B, a polyene antibiotic discovered as long ago as 1956, is still used as a “gold standard” for antifungal therapy. The last two decades have witnessed a dramatic rise in the incidence of life threatening systemic fungal infections. The challenge has been to develop effective strategies for the treatment of candidiasis and other fungal diseases, considering the increase in opportunistic fungal infections in human immunodeficiency virus-positive patients and in others who are immune-compromised due to cancer chemotherapy and the indiscriminate use of antibiotics. The majority of clinically used anti-fungals have various drawbacks in terms of toxicity, efficacy and cost, and their frequent use has led to the emergence of resistant strains. Additionally, in recent years public pressure to reduce the use of synthetic fungicides has increased. Hence, there is a great demand for novel antifungal belonging to a wide range of structural classes, selectively acting on new targets with fewer side effects. One approach might be the testing of plants traditionally used for their antifungal activities as potential sources for drug development. Medicinal plants are not only important to the millions of people for whom traditional
medicine is the only opportunity for health care and to those who use plants for various purposes in their daily lives, but also as a source of new pharmaceuticals. Natural products, either as pure compounds or as standardized plant extracts, provide unlimited opportunities for new drug leads because of the matched less availability of chemical diversity.

The plant world is a rich storehouse of bio chemicals that could be trapped for use of fungicides. Plants are the richest source of renewable bioactive organic chemicals. Many higher plants produce economically important organic compounds, pharmaceuticals and fungicides. In future, biologically active plant derived fungicides are expected to play an increasing significant role against mycotic infections. Considering this, in the present investigation four solvent (Petroleum ether, chloroform, ethyl acetate and ethyl alcohol) leaf extracts of C. procera were screened in vitro for antifungal activity against some human pathogenic fungi viz. Microsporum canis, M. fulvum, Trichophyton mentagrophytes, Aspergillus niger and A. fumigatus. The plant C. procera was selected based on the earlier reports in traditional medicine and random choosing. The antifungal potential of these leaf extracts has been confirmed by dry mycelial weight, sporulation, disc diffusion and poisoned food technique. The results of dry mycelial weight technique were further confirmed by the observation of poisoned food technique. The bioassay was performed to characterize the active constituent of ethanol leaf extract of C. procera that was found to be most active in the present study.

The results suggest that increasing concentration of extract may be necessary for antifungal activity. The effectiveness of plant extract was found to increase with increasing concentration, which is in agreement with the findings of Singh et al. (1980); Kumar and Chauhan (1992); Shrivastava and Shrivastava (1995); Ghawana (1997); Singh and Singh (1997); and Agarwal et al. (2004a). Considering this the antifungal activity was assayed at
various concentrations (1000-7000 ppm). Our results are contradictory to earlier reports showing that with increasing concentration up to 6000 ppm the percentage inhibition was increased but at maximum concentration 7000 ppm, there was found decline in percent inhibition in each case. The possible reason might be when any organism is exposed to the same treatment over and over, a variety of biochemical processes occur within this organism. These processes may keep the dose out of the cell, alter the target of the drug and the response may start to decrease at higher concentrations. The decrease in percent inhibition at 7000 ppm concentration may be due to the saturation. The present study also showed a significant effect of exposure time. It was observed that six day exposure was most effective; with increasing the period there was a subsequent decline in the inhibition rate. Surh and Neilsen (2003) discussed the reason, why the inhibition effect was lost with the increasing time period. It could be with the evaporation of volatile compounds. Agarwal et al. (2004 b) also noticed that with increased time period fungal colony appears to be acclimatized with phytoextracts.

The results revealed that the chloroform and ethyl alcohol leaf extracts were found to be highly effective against M. canis and A. niger. Literature survey reveals that ethyl alcohol and chloroform leaf extracts were reported to possess antifungal activity (Hassan et al., 2006, Kareem et al., 2008, Suverna and Patil, 2009, Vadlapudi and Naidu, 2009). Antifungal activity of ethyl alcohol and chloroform leaf extracts against M. canis has been reported for the first time in the present investigation.

The results revealed variations in the antifungal activity, it was evident from the data that these extracts were effective against all the target fungal species tested, where as petroleum ether and ethyl acetate extracts were not as much effective against all the tested fungi and showed least activity as compared to chloroform and ethyl alcohol leaf extracts.
The inhibitory activity of ethanol leaf extract against *M. canis* and *A. niger* were highly significant. Hence, ethanol extract was selected for further investigations for evaluating the structural characterization of the compound responsible for antifungal activity.

Inhibition of *Aspergillus niger* and *Trichphyton mentagrophytes* in petroleum ether, chloroform, ethanol leaf extracts has been demonstrated (Suverna and Patil, 2009, Kareem et al., 2008, Hassan et al., 2006). Most of the earlier workers have studied the antifungal activity of organic solvent extracts of *C. procera* mainly on species of *Aspergillus* and *Candida* in addition to human pathogenic and plant pathogenic fungi (Vadlapudi and Naidu, 2009, Suverna and Patil 2009, Kareem et al., 2008, Kuta, 2006, 2008, Hassan et al., 2006, Oladimeji et al., 2006, Sehgal et al., 2005, Oluma et al., 2002, Sharma and Trivedi 2002, Rai and Upadhyay, 1988a). In present investigation, the species of *Aspergillus* and *T. mentagrophytes* were also the test fungi selected for screening the antifungal potential of various solvent extracts. These are known to cause serious diseases as *Aspergillus niger* and *Aspergillus fumigatus* causes Aspergillosis and liver cancer while dermatophytes are responsible for skin and hair damage. Thus, the choice of the test fungi is appropriate considered to control the cutaneous and opportunistic diseases. The data collected from the present investigation clearly reveals that ethyl alcohol extract of *C. procera* leaves showed highly significant activity against *A. niger* and *M. canis*.

Sandhu and Arora (2000) in their review on plants as a source of antimicrobial agents mentioned that ethanol extract of roots of *Calotropis procera* exhibited maximum inhibitory activity against the tested pathogens. Kumari and Jariwala (1993) also reported that different inhibitory effect of different plant extracts on the growth of fungi could be due to the presence of different active components in different plant extracts. Agarwal et al. (2004 b) also mentioned that this increase in percent inhibition with increasing supplement of
extract indicates the presence of significant amount of chemical moiety responsible for exhibiting antifungal property in the foliar part of the plant. Studies of Tariq and Magee (1990) and Mushin et al. (2000) also provide evidence that the extracts suppresses or inhibits enzyme production by the fungi, which ultimately results in the change in cell wall permeability, disruption of the cytoplasm and ultimately lyses of spores. Further, Tariq and Magee (1990) and Rai and Vasantha (1995) also suggested that varying permeability of the mycelial and spore wall of different fungi in turn affect the binding of volatile components to the fungal wall or their ability to penetrate the microbial cells or restrict their movement to the protoplast. Singh and Agarwal (1988) suggested that this varying degree of activity might be due to the nature of the effective components and their capacity of diffusion into the medium. Shrivastava et al. (1997) mentioned that the pronounced inhibitory action of the extract might be due to the presence of certain components, which inhibit the keratinase. Differential inhibitory response may be due to the action of different inhibitory compounds on keratinase, which may interfere the activity of dermatophytic fungi. Mansfield (1983) also stated that plant composed of various phenolic components; having antifungal activities, prevent from fungal infection. Moreover, Iyer and Williamson (1991) opined that high phenol content probably has a direct relationship to the antimicrobial properties of the plants. Hence, such compounds released during extraction, inhibit fungal growth and invasion. Therefore, plant extracts having wide spectrum of fungi-toxic effects can be employed safely to prevent fungal attacks and invasions.

However, some reports are available on the antimicrobial activity of C. procera. Recently, the antimicrobial activity of stem, leaves and flowers of C. procera was checked in hexane, chloroform and methanol extract against Alternaria alternata, Aspergillus flavus, Aspergillus niger, Bipolaris bicolor, Curvularia lunata, Pencillium expansum, Pseudomonas marginales,
Rhizoctonia solani, Ustilago maydis by agar well diffusion method (Vadlapudi and Naidu, 2009). The antifungal activity of leaf extract (petroleum ether, chloroform, methanol and water) of C. procera (100 mg/ml concentration) against Candida albicans and A. niger has been assessed by well plate diffusion method (Suvarna and Patil, 2009). The antimicrobial activity (Parabia et al., 2009) of apical twig and latex of C. procera has been demonstrated. Several workers (Kareem et al., 2008; Kuta, 2006, 2008; Hassan et al., 2006; Kishore et al., 1997; Kumar and Chauhan, 1992; Rai and Upadhyay, 1988a) had reported the anti-dermatophytic activity of leaves, latex and stem bark of C. procera. Kareem et al. (2008) reported the antimicrobial effect of ethanol, aqueous and chloroform extracts of leaf and latex on six bacteria and three fungi namely A. flavus, A. niger and M. boulardii and one yeast C. albicans. The results of this study indicate that ethanol was the best extractive solvent followed by chloroform and water extracts. The MIC for the ethanol extract was between 5 to 20 mg/ml for fungi. Similarly, the strong inhibitory effect of aqueous extracts of C. procera stem, root and leaves has been demonstrated on the test microorganisms A. niger, Microsporum gypseum and Trichophyton rubrum (Hassan et al., 2006). Kuta (2006) observed the inhibitory effect of methanolic crude extract against M. canis and T. rubrum at 5.0 mg/ml concentration by well diffusion method. Kuta (2008) again observed inhibition of aqueous extract of stem bark of C. procera against Epidermophyton floccosum and Trichophyton gypseum in a concentration range of 1 to 5 mg/ml. Saxena (2004) has reported that effectiveness of different solvent extracts (Petroleum ether, acetone and ethyl alcohol) of leaves of C. procera on the basis of evaluated percentage inhibition in mycelial weight (biomass) and colony diameter of the target species (T. mentagrophytes). According to her observation, ethyl alcohol leaf extract proved to be most effective causing an inhibition rate of 87.7%, followed by acetone (66.1%) and petroleum ether (63.1%) at
4000 ppm concentration on 4th day exposure time. Similar was the trend followed in case of reduction in biomass or mycelia weight. Ethyl alcohol leaf extract showed better performance (82.05%), followed by acetone (79.5%) and least effective was petroleum ether (74.4%).

Rai and Upadhyay (1988 a) screened 19 medicinal plants against T. mentagrophytes and reported 50 to 75% inhibition in the growth of T. mentagrophytes with the treatment of leaf and stem extracts of C. procera. These observations, therefore, support the use of C. procera in herbal cure remedies. The demonstration of antifungal activity of C. procera leaf extract against fungal species may be an indicative of the presence of broad spectrum antibiotic compounds. The mechanism of action of the constituents of C. procera could be by inhibition of fungal cell wall, protein amino acid, sphingolipid biosynthesis and electron transport chain. However, it is important to note the crude extract of C. procera leaf, need to be further purified through bioactivity guided fractionation to isolate and identify the compound responsible for antifungal activity.

Further, comparison of data in this study is quite problematic because the composition of different extracts varies accordingly along with climatic and environmental conditions (Janssen et al., 1987 and Sivropoulov et al., 1995). Secondly, the protocol used to assess the anti-microbial activity and choice of test organism varies with different publications (Janssen et al., 1987). Kumar and Chauhan (1992) also agreed to the opinion of Agarwal (1988) that this variation may be due to seasonal change in the properties of phyto-chemicals of plants.

In present study, phytochemical analysis suggest that the presence of biologically active compounds alkaloids, flavonoids, cardiac glycosides, tannins, terpenoids, steroids, saponins in the plant extract could be correlated to the antifungal effects of substances known to possess antimicrobial properties as shown by Tschesche (1971), Scalbert (1991)
and Favel et al. (1994). Thus, this is probable that these molecules are the principle antifungal agents in the leaves extract of *Calotropis procera*.

In this study, the ethanol extract of *C. procera* exhibited good bioactive properties. It is significantly inhibited the growth of dermatophytes (*M. fulvum, M.canis, T. mentagrophytes*) and also inhibited the growth of *A. niger* and *A. fumigatus*. So, ethanol leaf extract has been selected for its structural elucidation of the compound responsible for antifungal activity.

Although a number of compounds have been reported from the leaf extracts of *C. procera* like calotropin (Hesse and Richander, 1936), Uscharin amyrin esters, uscharidin, calotoxin and calactin (Hesse et al., 1950), Tarxsterol isovalerate (Anjaneyulu et al., 1968), D-glucose, D- arabinose, D- glucosamine and α-rhamnose (Qudrat-i-Khuda et al., 1969), α- amyrin, β-amyrin, β-sitosterol (Saber et al., 1969), Asclepin (Singh and Rastogi, 1972), Calotropin and Calotropagenin (Malik and Chughtai, 1979) but there is no report for antifungal activity.

In our practical session of structural characterization, we have found that out of 140 fractions collected from column chromatography a total of six fractions were found to have antifungal activity. From these six bioactive fractions two of them were in their pure form and showed single spot when run on TLC plate. GC/MS properties of other remaining fractions were also determined. Purity of these fractions was not possible because of their very small quantity.

Interpretation of mass spectrum (GC-MS) was conducted using the database of Wiley8 Library having more than 328385 patterns. The Name, Molecular weight and Structure of the components of the test materials were ascertained.
The first pure crystalline compound (CPR 8) identified as (E,E)-4,8,12-Trimethyl-3,7,11-Tridecatriene-1-ol was used to perform GC-MS technique for structural elucidation. This compound has shown significant antifungal activity against *T. mentagrophytes* and *M. fulvum* and also showed activity against *M. canis, A. niger* and *A. fumigatus*. Chemically, it is a triterpenoid. This compound was reported in literature as chemically synthesized from (±)-ambrox (*Barrero et al., 1996*) and there is no any report found for natural occurrence of this compound from plants. Ambrox is very much similar to “Ambergris” a metabolite of sperm whales (*Physeter macrocephalus* L.) which accumulates as concretions in the gut of the animal (*Ohloff, 1982*). It was synthesized chemically because its constituents are more important commercial substitutes. Naturally this compound is present in *C. procera* leaves extract and we have first time reported this compound as an antifungal one.

The second identified pure crystalline antifungal compound EE91-9 identified as 1, 2-Benzenedicarboxylic Acid, Diisooctyl Ester is also first time reported from the leaves of *C. procera*. It is a plasticizer compound and is used as an antimicrobial and antifouling agent (*Merlin et al., 2009*). As most of the compound was used to perform biological activity, so various techniques for structural elucidation could not be performed except GC-MS analysis. This compound showed good activity against *A. niger* and *A. fumigatus* as compared to dermatophytes.

The other collected fractions (CPR-4, CPR-9, EE73-1 and EE91-10) were firstly checked for their antifungal activity and then directly analysed through GC-MS and found to possess a number of compounds present. The possible reason of showing activity of fractions could be the synergistic effect of compounds present in the bioactive fractions.

The present study supports the view that ethno-medicinal plants might be useful as anti-fungal agents resulting in the development of novel drugs through ethno-pharmacology.