SUMMARY

Title of thesis: "Antidermatophytic activity in Calotropis procera (Ait.) leaf extracts and its structural characterization"

In current scenario of medical and pharmaceutical advancement, microbes involve in the change of their metabolism and genetic structure to acquire resistance against the drugs used in the treatment of common infectious disease. These drug resistant candidates (microbes) are more pathogenic with high mortality rate and become a great challenge in the pharmaceutical and healthcare industry. To overcome microbial drug resistant, scientists are looking forward for the development of alternative and novel drugs. Natural sources such as plants, algae and animals provide an array of natural medicinal compounds for the treatment of various infectious diseases.

Despite significant progress in mycology therapeutics in the last decades, the urge to discover and to develop new, alternative or synergistic anti-fungal agents still remains. For centuries it has been known that the coarse shrub Calotropis procera is a very promising source of ascaricidal, schizonticidal, anti-bacterial, anthelmintic, insecticidal, anti-inflammatory, antidiarrhoeal, larvicidal and cytotoxic chemicals. Different compounds like norditerpenic esters, organic carbonates, the cysteine protease procerain, alkaloids, flavonoids, sterols as well as numerous types of cardenolides have provided this plant for centuries with scientists’ interest. The chemical class of cardenolides and their related bioactivity evaluation and structure activity relationship (SAR) studies pointed out their therapeutic utility and led to the discovery of promising drug components.
Plant *Calotropis procera* is economically being wild in nature and abundantly found in all parts of the country and is perennial in nature. It is traditionally used to cure a number of diseases such as fevers, rheumatism, indigestion, cough, cold, eczema, asthma, elephantiasis, nausea, vomiting and diarrhea. Recent realization that the plant already established for particular bio-efficacies, should be explored for other bonafied properties. This approach has prompted our interest to carry out the present piece of work with the following objectives:

- Isolation and identification of dermatophytes from samples of the infected patients of S.N. Medical College, Agra.
- Extraction of plant compounds in different solvents (Petroleum ether, Chloroform, Ethyl acetate and Ethyl alcohol)
- Assessment of anti-mycotic activity of the leaf extracts of *Calotropis procera* against species of dermatophytic fungi i.e. *Microsporum*, *Trichophyton* and *Aspergillus*.
- Isolation and characterization of the bioactive principles responsible for the anti-mycotic activity using various methods of structural elucidation.

In present investigation, the clinical samples were collected from patients, Skin Department, S.N. Medical College, Agra, from skin scrapings of the patients. The skin scrapings were collected on sterilized butter paper with the help of sterilized new blade from the centre or edge of the lesion, after cleaning the site with 70% alcohol. The skin scrapings were carefully transferred from butter paper to the petri-dishes containing SDA (Sabouraud’s Dextrose Agar) medium directly and incubated at 28±2°C. In Petri dish, when fungal colonies appeared on SDA medium, it was transferred to other dishes for the purification. Sub
culturing of respective colony was carried out to obtain pure culture. Then the pure cultures were morphologically identified with the help of ‘A Color Atlas of Pathogenic Fungi’. On the basis of their characteristics these isolates were identified as *Microsporum canis* and deposited to Institute of Microbial Technology (IMTECH), Microbial Type Culture Collection (MTCC) Chandigarh with the Accession no. of MTCC 3270. Other target fungal isolates *Microsporum fulvum, Trichophyton mentagrophytes, Aspergillus niger* and *Aspergillus fumigatus* were procured from Microbiology Lab., Botany Department, DEI, Agra.

The leaves of the selected plant used for the study were collected from the campus area of the DEI, Dayalbagh, Agra. The plant has been characterized by Taxonomy Division, Botanical Survey of India (BSI), Allahabad as *Calotropis procera* and the assigned Accession No. is 79385 (BSA). Dried and coarsely powdered plant material (200gm) was successively extracted with various solvents such as petroleum ether, chloroform, ethyl acetate and ethyl alcohol by using continuous hot extraction with soxhlet extractor, for 48 hours and extracts were evaporated at low temperature and reduced pressure to give a gummy solid residue. The extracts were weighed and the percent yield was calculated 2.75%, 3.75%, 1.88% and 4.33% for petroleum ether, chloroform, ethyl acetate and ethyl alcohol respectively. The extracts were dissolved in respective solvents in sterile test tubes to obtain concentrations of 1000-7000 ppm and were subjected to antifungal screening.

The antifungal activity of leaf extracts of plant *Calotropis procera* against the target fungi was studied in terms of **colony diameter**, **mycelial weight**, **sporulation** and **zone diameter** by disc diffusion method. Findings indicate that **ethanol leaf extract** is best inhibitory at **6000ppm** concentration followed by chloroform, ethyl acetate and petroleum ether. It was noticed that at higher concentration (7000ppm), percentage inhibition and zone diameter values were
reduced because a variety of biochemical processes occur within these organisms and the response may start to decrease in percent inhibition at higher concentrations.

**Reduction in colony diameter**

Findings in terms of colony diameter indicates that the maximum percentage inhibition in ethanol leaf extract were 94.62%, 74.96%, 68.70%, 87.35%, 76.01% for *M. canis, M. fulvum, T. mentagrophytes, A. niger* and *A. fumigatus* respectively at 6th day at test concentration of 6000ppm. The percent inhibitions at higher concentration (7000ppm) were 24.79%, 30.84%, 22.28%, 10.15% and 19.63% respectively at same incubation period against tested fungi. For chloroform leaf extract the maximum percentage inhibitions were 83.37%, 57.60%, 82.42% and 60.62% for *M. canis, M. fulvum, A. niger* and *T. mentagrophytes* at sixth day of 6000ppm concentration and 22.43%, 22.25%, 16.38% and 22.14% at 7000ppm concentration. For *A. fumigatus* maximum inhibition was 60.48% at 3rd day of 2000ppm concentration while the values dropped from 3000-7000ppm concentration.

The maximum inhibitions in ethyl acetate leaf extract were 50.01%, 43.70% and 58.97% for *M. canis, M. fulvum* and *T. mentagrophytes* after six days and for *A. niger* and *A. fumigatus* 74.19% and 69.12% after 3 days of 6000 ppm concentration while at 7000 ppm concentration values dropped as 08.95%, 26.54%, 18.13% and 28.21%, 08.78% respectively. For petroleum ether leaf extract, the maximum percentage inhibition against *M. canis* was 48.34%, for *M. fulvum* 37.25%, for *T. mentagrophytes* 49.36% with time exposure of six days and 17.58% and 40.54% for *A. niger* and *A. fumigatus* on exposure of three days at 6000ppm concentration. The percentage inhibitions at 7000ppm concentration were 16.68%, 14.04%, 15.16% for *M. canis, M. fulvum, T. mentagrophytes* and 09.35% for *A. fumigatus* and no inhibition at 7000 ppm for *A. niger*. 
Reduction in mycelial weight

The maximum percentage inhibition in terms of mycelial weight with ethanol leaf extract was 88.62%, 83.28%, 76.03%, 66.16%, 65.49%, for M. canis, A. niger, A. fumigatus, T. mentagrophytes and M. fulvum, respectively at 6th day and 6000ppm test concentration. At 7000 ppm concentration the percentage inhibition decreases as 27.86%, 59.20%, 18.85%, 20.49% and 31.12% at same incubation period. The chloroform leaf extract was effective against M.canis, M. fulvum, A. niger and T. mentagrophytes in terms of maximum percentage inhibition as 83.04%, 43.62%, 78.92% and 66.34% at sixth day of 6000ppm concentration while for A. fumigatus maximum inhibition was 67.57% at 3rd day incubation period of 2000ppm test concentration while percentage inhibition decreases from 3000-7000 ppm concentration. The drop down values in chloroform leaf extract was 21.40%, 26.58%, 17.24% and 19.70% for M.canis, M. fulvum, A. niger and T. mentagrophytes at 7000 ppm concentration at 6th day. The maximum inhibitions against fungi in ethyl acetate leaf extract were 48.89%, 43.84% and 59.10% for M. canis, M. fulvum and T. mentagrophytes at sixth day and 72.63% and 68.02% for A. niger and A.fumigatus at 3rd day of 6000 ppm concentration. The percent inhibitions at 7000 ppm concentration were 09.40%, 26.26% and 19.82% for M. canis, M. fulvum and T. mentagrophytes at sixth day and 23.53% & 13.29% for A. niger and A. fumigatus at 3rd day. Petroleum ether leaf extract shows 57.53%, 34.54% and 53.66% for M. canis, M. fulvum and T. mentagrophytes at 6th day and 13.06% and 41.99% for A. niger and A. fumigatus on exposure of three days of 6000 ppm concentration while at 7000 ppm concentration the percent inhibitions were 14.22%, 15.80%, 14.39% for M. canis, M. fulvum and T. mentagrophytes at 6th day and 01.58% and 11.44% for A. niger and A. fumigatus on exposure of three days.
Reduction in sporulation

Findings indicate that the maximum percentage inhibition in ethanol leaf extract was 80.30%, 63.88%, 58.69%, 77.16%, and 69.52% for M. canis, M. fulvum, T. mentagrophytes, A. niger and A. fumigatus respectively at sixth day of 6000ppm concentration. At 7000 ppm concentration the percent inhibitions were 26.83%, 27.77%, 22.87%, 50.17% and 16.89% respectively. For chloroform leaf extract, the maximum percentage inhibition was 73.61%, 59.60%, 79.12% and 63.16% for M. canis, M. fulvum, A. niger and T. mentagrophytes at sixth day of 6000ppm test concentration while at 7000 ppm concentration the percent inhibition dropped down as 20.83%, 23.97%, 19.39%, and 20.76%. For A. fumigatus maximum inhibition was 59.40% at 3rd day of 2000ppm concentration and percent inhibition values dropped from 3000-7000 ppm concentration. The maximum percent inhibition against all the fungi in ethyl acetate leaf extract was noticed as 47.84%, 43.57% and 59.99% for M. canis, M. fulvum and T. mentagrophytes after six days while against A. niger and A. fumigatus it was 74.21% and 67.51% after 3 days of 6000 ppm concentration. At 7000 ppm concentration the percent inhibitions were noticed as 08.73%, 26.20%, 19.99% for M. canis, M. fulvum and T. mentagrophytes at 6th day and 25.87% and 12.54% against A. niger and A. fumigatus at 3rd day incubation period. For petroleum ether leaf extract the percentage inhibition for M. canis was 45.97%, for M. fulvum 40.64%, for T. mentagrophytes 53.87% at 6th day and 15.73% and 45.11% for A. niger and A. fumigatus on exposure of three days of 6000ppm concentration. The percent inhibitions noticed at 7000ppm concentration were 12.92%, 16.84% and 15.44% for M. canis, M. fulvum and T. mentagrophytes at 6th day and 02.24% & 11.80% for A. niger and A. fumigatus at 3rd day incubation period.
Paper Disc Diffusion method

The antifungal potency of different leaf extracts of *Calotropsis procera* against fungal species i.e. *M. canis, M. fulvum, T. mentagrophytes, A. niger* and *A. fumigatus* was also evaluated by the presence or absence of inhibition zones and zone diameters (mm). From the results it is evident that the **ethanolic extract** showed a maximum inhibitory zone 22.5 mm, 22.5mm, 21.75 mm, 19.13 mm and 18.5 mm respectively for *M. canis, M. fulvum, A. niger, T. mentagrophytes, and A. fumigatus* with 6000ppm concentration while the values dropped 14.5mm, 21.3 mm, 20.6 mm, absence of zone and 18.5 mm for **ethyl acetate** and 14.6 mm, 21.9 mm, 13.6mm, 11 mm and 11 mm for **chloroform leaf extracts** respectively when tested against the same organisms at same concentration. It was noticed that at higher concentration (7000 ppm) inhibition zone values were dropped down as 17.4, 11 and 11 mm for *M. canis*, 17.5, 11.25, and 17.5 mm for *M. fulvum* in ethanol, chloroform and ethyl acetate leaf extracts. Diameter of zone 15.25 mm and 10.75mm in ethyl alcohol and chloroform for *T. mentagrophytes* and absence of zone in ethyl acetate leaf extract, 13.5 mm and 13 mm for *A. niger* in ethyl alcohol and chloroform and no inhibitory zone in ethyl acetate leaf extract, 14 mm and 12 mm for *A. fumigatus* in ethanol and chloroform and absence of zone in ethyl acetate leaf extract. However, the petroleum ether extract was not effective against tested fungi. The antifungal potency of ethanol leaf extract of *Calotropsis procera* on tested fungi showed a larger diameter of clearance than that of other tested solvent extracts. The **MIC** (Minimum Inhibitory concentration) of ethanol leaf extract of *Calotropsis procera* was also observed. Moreover, the zone of inhibition achieved by *C. procera* leaf extract is comparable to that of standard drug Griseofulvin. Griseofulvin was effective against *M.fulvum* and showed a zone diameter of 15mm at same concentration while showed no activity against *M.canis* and *T. mentagrophytes.*
Statistical analysis

EC$_{50}$ values and other related parameters as EC$_{90}$, correlation coefficient were also calculated. EC$_{50}$ values of ethanol fractions for *M. canis*, and *A. niger* are 3232.03, 2117.93 & 3136.95 ppm and 4219.80, 5489.80 & 164077.19 ppm (for reduction in colony diameter); 4100.24, 2656.66 & 3944.90 ppm and 4931.47, 2222.87 & 40642.31 (for reduction in mycelial weight); 4521.93, 3334.36 & 5145.98 ppm and 5377.94, 4022.22 70456.22 ppm (for reduction in sporulation) at 3, 6 & 9$^{th}$ days of incubation period respectively. On the time scale, in each case the rate of inhibition increased from 3$^{rd}$ to 6$^{th}$ day and then started decreasing. These trends demonstrated that 6$^{th}$ day is the optimum time for maximum phytotoxicity caused, after that fungal colony appears to be acclimatized with the phytoextracts.

Leaf extracts were separately subjected to preliminary phytochemical tests using standard methods. Ethanol leaf extract shows the presence of alkaloids, cardiac glycosides, flavonoids, tannins, steroids, saponins and terpenoids.

Activity guided chromatographic (Thin layer chromatography and column chromatography) fractionation of the ethanol crude extract has been carried out with a view to elucidate the structural determination of the bioactive component. Ethanol extract was subjected to column chromatographic separation and eluted with Chloroform: methanol (5: 1) solvent system. The recovered substance was monitored by thin layer chromatography (TLC) using solvent system [Chloroform: Ethyl acetate: Acetone 3: 1: 2]. The development of chromatogram in iodine chamber showed three spots of different Rf values (0.87, 0.8 and 0.23). The compounds were separated from the preparative TLC plate and the recovered
compound was screened for the antifungal bioassay against tested fungi. The compound was purified by re-crystallization. The compound did not show effective antifungal activity against *A. fumigatus*, *M. canis* and *A. niger*. The compound exhibited significant antidermatophytic activity against *T. mentagrophytes* and *M. fulvum* by paper disc diffusion technique.

The maximum zone diameter \(30.5 \text{ mm} \& \ 26.5 \text{ mm}\) was found at \(400 \text{ ppm}\) concentration against *T. mentagrophytes* and *M. fulvum* respectively while for *A. niger*, *A. fumigatus* and *M. canis* the values dropped to 17.75 mm, 16.5 mm and 15 mm. At 100 ppm concentration the zone diameter were minimum as 13 mm, 11 mm, 10.5 mm against *T. mentagrophytes*, *M. fulvum* and *A. fumigatus* respectively and absence of zone for *A. niger* and *M. canis*.

Gas Chromatography Mass Spectroscopy (GCMS) analysis of the crystalline compound was carried out in order to determine the structure of the bioactive constituent. The GCMS spectra of the fraction shows only two peaks with 93.85% and 6.15% area at retention time of 22.952 and 20.850 indicating the presence of *(E,E)-4,8,12-Trimethyl-3,7,11-Tridecatrien-1-ol* and *1,2-Benzenedicarboxylic acid*. The results indicates that *(E, E)-4, 8, 12-Trimethyl-3,7,11-Tridecatrien-1-ol* is the major compound (molecular formula \(C_{16}H_{28}O\)) responsible for antifungal activity and the another one is present as an impurity. The structure of the isolated antifungal compound on the basis of library ID is 

![Structure](image)
Identified pure compound and crude extract isolated from the wild and medicinal plant *Calotropis procera* possess antifungal substances. This compound could be considered strong candidate for the development of effective drug. However detailed study is required about the suitability of isolated compound before including in the list of therapeutic substances.

Another pure compound (EE91-9) showed significant antifungal activity against *A. niger* with the maximum zone diameter 19.50 mm at 400 ppm concentration. It also posses antifungal activity against *M. fulvum* and *M. canis* with inhibition zones of, 13.5 mm and 17.50 mm at 300ppm and 400 ppm respectively, the zone diameter was 15.75 mm for *T. mentagrophytes* and for *A. fumigates* 15.50 mm at 400 ppm concentration. Compound EE91-9 exhibited significant antifungal activity against *A. niger*.

This compound was directly analyzed by GC/MS. The results indicates that 1, 2-Benzenedicarboxylic Acid, Diisooctyl Ester is a compound with molecular formula $\text{C}_{24}\text{H}_{38}\text{O}_4$, responsible for antifungal activity. It is plasticizer compound. The structure of the isolated antifungal compound on the basis of library ID is

![Structure of 1, 2-Benzenedicarboxylic Acid, Diisooctyl Ester](image)

1, 2-Benzenedicarboxylic Acid, Diisooctyl Ester
To the best of our knowledge, based on the detailed medi-chemi-botanical survey of literature available, we report, first time, the occurrence of (E, E)-4,8,12-trimethyl-3,7,11-tridecatrien-1-ol and 1,2-Benzenedicarboxylic Acid, Diisoctyl Ester in the leaves of the plant Calotropis procera.

Besides these two pure antifungal compounds other collected fractions which showed antifungal activity are CPR4, CPR9, EE73-1 and EE91-10.
CONCLUSIONS AND FUTURE PERSPECTIVES

Emergence of dreaded diseases like AIDS and cancer are responsible for increase in number of secondary infections generally caused by opportunistic fungi due to their immune-compromising capacity. The azoles and other antifungal drugs often fail to respond well to these infections. Therefore, there has been greater need to search for alternative antifungal agents from microbes or plants. *Calotropis procera* being a medicinal weed and also owing to presence of various phytochemical compounds in all plant part, may prove to be the best natural alternative antifungal drugs. Traditional utilization of this plant against skin infections provides evidence that they contain antifungal properties. The anti-mycotic nature of plant parts have been established by investigators of all over the world.

The present piece of work is aimed to evaluate antifungal properties of the leaves of plant *Calotropis procera* against *M. fulvum*, *M. canis*, *T. mentagrophytes*, *A. fumigatus* and *A. niger*. The foliar part of the plant has been extracted in different solvents of increasing polarity (Petroleum ether, Chloroform, Ethyl acetate and Ethyl alcohol). A detailed antifungal in terms of reduction in colony diameter, reduction in mycelial weight, reduction in sporulation and zone diameters by disc diffusion method. The ethyl alcohol extract proved to be most effective against all the microbes. The inhibitory activity of ethanol leaf extract against *M. canis* and *A. niger* were highly significant suggesting that ethanol leaf extract could be taken up for further investigations to exploit its antifungal potential.

Activity guided chromatographic (Thin layer chromatography and column chromatography) fractionation of the ethyl alcohol extract have been carried out with a view to elucidate the structural determination of the bioactive principle. Based on GC/MS analysis, (E, E)-4, 8, 12-Trimethyl-3, 7, 11-Trimethyltridecatrien-1-ol and 1, 2-Benzenedicarboxylic Acid,
Diisooctyl Ester have been ascertained bioactive principles for the observed antifungal activity in ethyl alcohol leaf extract.

The present study explores the toxicity of plant *Calotropis procera* as an antifungal agent against fungal infections. The study can prove as an asset for eco-friendly and sustainable management of fungal diseases.

**FUTURE PERSPECTIVES**

This research field is in its infancy. Although many interesting and potentially valuable bio-active products have been identified and characterized from *Calotropis procera*, there is much more to be discovered. In addition, there are many questions for which we have no answers currently, and which will need to be addressed before we can fully utilize the powerful synthetic potential of isolated compounds. Historically, the study of natural products has been the purview of botanists, but the recent discovery of interesting chemical compounds generated by plants has opened the field to structural chemists and biochemists. Real success in this area will probably only be achieved through an interdisciplinary approach, whereby the knowledge and insight of the botanist is combined with the technology and structural tools of the chemist.
SIGNIFICANCE OF WORK

The existing costly therapy of fungal infections does not bode well for the millions of individuals particularly in the developing world. The plant extracts are easily available secondary metabolites and are within the reach of needed down trodden and poor people. The need of the day is the demand of sincere efforts to identify the newer potentials of the plant kingdom. This study is likely to explore bio-efficacy of *Calotropis procera* as an antifungal agent against fungal infections. The study can be proved as an asset to the Eco-friendly and sustainable management of skin diseases problems resulting from modern life style. Further, it is necessary to unzip the mystery of proper mechanism of action of plant derived drugs on fungal pathogen as well as on hosts.

Tremendous therapeutical and commercial potential exists in the anti-mycotic agents of plants. But the need of the hour is to tap these valuable natural resources. Revitalization of natural curative power of plants will generate awareness among the people for utility of these plants. Eventually, there is a pressing need to search for more plants containing volatile substances which can be useful in combating mycotic infections. In 21st century, discovery of plant-based antifungal drugs might be a biotechnologically-driven process with increasing importance attached to discovery of drugs from plants.

**Popularity of herbal medicines:** The traditional medicine is largely gaining popularity over allopathic medicine because of the following reasons favourable to it:

1. Rising costs of medicinal care.
2. As these are from natural origin, so free from side effects.
3. Goes to root cause and removes it, so that the disease does not occur again.
4. Freedom from approaching various specialists.
5. Cure for many obstinate diseases.
6. Easy availability of drugs from natural sources