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RESEARCH ARTICLE

Isolation and Identification of Keratinophilic Fungi from Soil of Gwalior Region and their Control by Methanolic Plant Extracts.

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ABSTRACT

Microorganism is ubiquition in nature. A large number of microbes are present in our environment. The human body occurs in dynamic equilibrium with these microbes .infection occurs when a microbe penetrate the body surface of tissues. In these it multiplies and the cumulation effect infects infections damage of disrupt tissues and organs and disease results. In the present study, we found that A. Fumigatus, T. mentagrophyte, T. rubrum. E. Floccosum and chrysosporium sp., A. Niger were the most prevalent keratinophilic fungi found in the soil of Gwalior region, which we have isolated. In vitero evalution was conducted for sensitivity testing with 5 different methanolic plant extracts for the inhibition of hyphal growth and spore formation in A. Fumigatus,, T. mentagrophyte, T. rubrum. E. Floccosum and chrysosporium sp. evalution antifungal activity was carried out by disc diffusion method and well diffusion method.

Plant secondary metabolites have been of interest to man for a lon time due to their pharmacological relevance. Higher and aeromatic plant have traditional been used in medicines due to their inhibitory effect on various microbes and they also have antifungal properties .most of their properties are due to essential oil product by their secondary metabolite.

Our study shows that fungal infection is common in human being. With the emergence of new effective system and tropical antifungal therapies. Tgere has been greater need search for alternative antifungal agent from microbes or plant. In our study it can be concluded that keratinophilicfungi occurs in the Gwalior region and we have used methanolic plant extracts against fungi. These extracts obtained from plant material [flowers, buds, leaves, twings, bark, herbs, wood, fruits and roots]. They can also be treated against fungi. In this way we have concluded that fresh methanolic plant extracts can be used us antifungal agent as they are found be effective against the test fungi. The ultimate conclusion of this study supports the traditional medicine use of different plant extracts in treating different infections caused by pathogenic fungi in gwalior either by using a single or combined extracts.

KEY WORDS: Isolation and identification of keratinophilic fungi from soil

INTRODUCTION:

Keratinophilic fungi are present in the environment with variable distribution patterns that depend on different factors, such as human and or animal presence, which are of fundamental importance. The potentially pathogenic keratinophilic fungi and allied geophilic dermatophytic species are widespread worldwide. Keratinophilic fungi include a variety of filamentous fungi mainly comprising hyphomycetes and several other taxonomic groups. Hyphomycetes include dermatophytes and a great variety of nondermatophytic filamentous fungi. Keratinolytic fungi occur in many natural and manmade habitats. These microorganisms exist incommunities together with keratinophilic fungi that have weaker affinity to keratin and utilize chiefly the products of its decomposition (*Dominik and Majchrowicz, 1964]* Keratinophilic fungi are an ecologically important group of fungi that decompose one of the most abundant and highly stable animal proteins on earth, keratin, which they use as a nutrient substrate for growth. The distributionof these fungi depends on different factors including the vitally important human and animal presence. Some of these fungi are well-known dermatophytes and are known to cause superficial cutaneous infections (dermatophytoses) of keratinized tissues (skin, hair and nails) of humans and animals. Mycotic infection is reported throughout the world and is extremely contagious. The occurrence of dermatophytes in soil was reported for the first time by Vanbreuseghem using the hair bait technique.

ECOLOGICAL ROLE:

The biological function of keratinolytic fungi in the soil is the degradation of keratinized materials such as hides, furs, claws, nails and horns of dead animals (*Box* 1).

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In the soil, these fungi live in their *teleomorphic* (=sexual) stages in the form of *cleistothecia*, whereas in keratinized material (host) they live in an *anamorphic* (=asexual) stage in which they develop only a very simple morphology. When there is ample keratin substrate available in soil, these fungi multiply by asexual means by producing enormous numbers of conidia (aleuroconidia, arthroconidia). When the keratin substrate is depleted, however, the fungi reproduce by sexual means and form characteristic fruiting bodies called ascomata.

COMMON HABITATS OF KERATINOLYTIC FUNGI:

Almost any place in nature where there is possibility of having keratin

- Cattle sheds
- Garbage
- Animal burrows
- Sewage
- Bird's nest
- Barber's hair dumping area
- Public places like parks, schools, marketplace, etc.
- Poultry sheds
- Herbivore or carnivore dung

With the invention of the technique of isolation of soil fungi , studies on keratinophilic fungi started in **1952** and soil proved to be natural reservoir of these fungi. Keratinophilic fungi also include Dermatophytes, which cause diseases of the skin and its appendages. Keratinophilic fungi have the unique ability to degrade keratinous substrates, e.g. horse hair, human hair , nail and peacock feather. The fungi which degrade these substrate completly are termed as keratinolytic. Several keratinolytic dermatophytes survive in the soil, in addition to their clinical habitat. Currently, almost all the habitats of the world have been surveyed for the presence of keratinophilic fungi. Most of these fungi belong to families *Arthrodermataceae* and*onygenaceae* , order Onygenales in ascomycetes. Most of the known fungi grow on higher plant or their remains, and surviv saprophytically. Keratinophilic fungi are natural colonize of keratinic substrates. Some are keratinolytic and play an important ecological role in decomposing a- keratins, the insoluble fibrous proteins. Because of the tight packing of their polypeptide chain in a- helix structure and their linkage by disulphide bridges, they are poorly degradable. Dermatophytes rae the keratinophilic fungi which causes infection called as *Dermatomycosis.* Dermatomycosis rae the mycotic diseases of skin caused by a few mycetes ; dermatophytes, and some opportunistic fungi as *Malassezia, Candida, Trichophyton, Rhodutorula, Cryptococcus or Aspergillus, Geotrichum, Alternaria* etc. Dermatophtes are a group of closely related filamentous fungi that invade keratinized tissue [skin, hair , nails] of human and other animals and produse infection called dermatophytosis or ringworm or "Tenia". The etiological agents of dermatophytosis are classified in three genera: *Microsporum, Trichophyton and Epidermatophyton* [Deuteromycetes]. On the basis of their primary habitat dermatophytes are divided in Anthrophilic dermatophytes (parasitic organisms that infect humans), Zoophilic dermatophytes (parasitic organisms that infect animals but also humans: agents of zoonosis) and Geophilic dermatophytes (saprobic fungi associated with keratinous material in soil). In the soil there are also structure associated with contagion, ["spore","arthroconidium"or "clamydospore"] of anthrophilic and zoophilic dermatophytes that may persist for years, in the environment , in hair or skin scales. Since on the skin of animals there are many saprobic organism [Malassezia] and many fungi may infect the fur , it is important to make an accurate diagnosis.

ETIOLOGY:

Dermatophyte is caused by fungi in yhe genera *Microsporum* and *Trichophyton.* These organisms called dematophytes are the pathogenic member of the keratinophilic [keratin digesting] soil fungi. *Microsporum* and *Trichophytona a*re human and animals pathogens. The dermatophytes were all formerly classified as members of the phylum *deuteromycota [fungi imperfecti].* Some are now known to reproduce sexually and have been reclassified in the phylum Ascomycota, family Arthrodermataceae. Each of these fungi now has two species names, one for the stage found in vertebrate hosts, and one for the form that grow in the environment[the perfect stage]. The dermatophytes have been classified into three ecological groups based on their habitat preferance – Geophilic, Zoophilic and anthrophilic.

GEOGRAPHIC DISTRIBUTION:

Dermatophytes grow best in warm and humid environments and are, therefore, more common in tropical and subtropical regions. The geographic distribution varies with the organism. *M.canis, M. Nanum T. Mentagrophyte, T. Verrucosum* and *T. Equinum* occur worldwide. *T. Simii [found in monkeys]* occurs only in *Asia* and *T. Mentagrophytes var. Erinacei* is limited to France, Great Britain, Italy and New Zealand.

TRANSMISSION:

Infection occurs by contact with arthrospore [asexual spores formed in the hyphae of the parasitic stage] or conidia [sexual or asexual spores formed in the "free living" environmental stage]. Infection usually begins

in a growing hair or a stratum corneam of the skin. Dermatophytes do not generally invade resting hairs, since the essential nutrients they need for growth are absent or limited. Hyphae spread in the hairs and keratinized skin, eventually developing infectious arthrospores

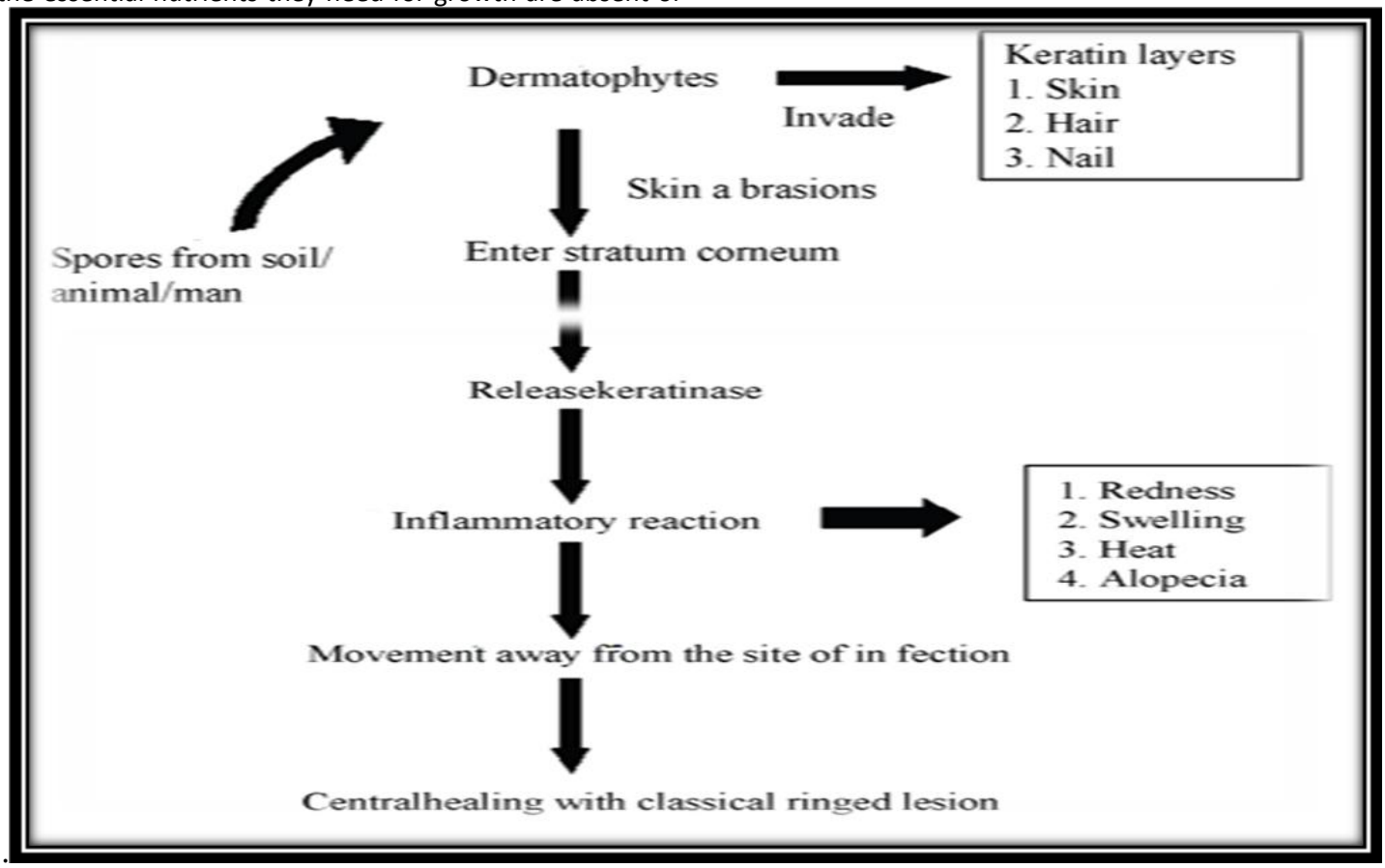


Figure No. 1: The schematic route of entry of dermatophytes into the host system and onset of immune response in the host in response to the pathogen entry.

PATHOGENESIS AND CLINICAL PRESENTATION:

The possible route of entry for the dermatophytes into the host body is injured skin, scars and burns. Infection is caused by arthrospores or conidia. Resting hairs lack the essential nutrient required for the growth of the organism. Hence these hairs are not invaded during the process of infection. The pathogen invades the uppermost, non-living, keratinized layer of the skin namely the stratum corneum, produces exo-enzyme keratinase and induces inflammatory reaction at the site of infection. The customary signs of inflammatory reactions such as redness (ruber), swelling (induration), heat and alopecia (loss of hair) are seen at the infection site. Inflammation causes the pathogen to move away from the site of infection and take residence at a new site. This movement of the organism away from the infection site produces the classical ringed lesion. The infections caused by dermatophytes are commonly referred to as "*tinea*" or "ring-worm" infections due to the characteristic ringed lesions. Based on the site of infection the tinea infections are referred to as *tinea capitis* (scalp), *tinea corporis* or *tinea circinata* (non-hairy, glaborous region of the body), *tinea pedis* ("Athletes' foot"; foot), *tinea ungium* ("*Onychomycosis*"; nail), *tinea mannum* (hands), *tinea barbae* ("Barbers' itch"; bearded region of face and neck), *tinea incognito* (steroid modified), *tinea imbricata* (modified form of*tinea corporis*), *tinea gladiatorium* (common among wrestlers') and *tinea cruris* ("Jocks' itch"; groin).

TYPES OF DERMATOPHYTOSIS:

On the basis of site of infection dermatophytosis is characterized as:

TINEA CAPITUS:

Tinea capitis, most often seen in children, is a dermatophyte infection of the hair and scalp. Tenia capitis begins with a small papule, which spread to form scaly, irregular or well-demarcated areas of alopecia. Most common agents: *T.tonsurans, M. audouinii and M. canis,* other agents: *T. mentagrophytes, T. verrucosum, M. gypseum* etc.

TINEA CORPORIS:

Tinea corporis, or ringworm, occurs on the trunk, extremities and face. It is characterized by single or multiple scaly annular lesions with a slightly elevated, scaly and erythematous edge. Most common agents: *T. rubrum, M. canis, M. tonsurans, T. verrucosum.* Other agents: *E.floccosum , M. audouinii, M. gypseum M. nanum, M. persicolor, T. equinum, T. mentagrophytes, T. raubitschekii, T. schoenleinii, T. violaceum.*

TINEA BARBAE:

Tinea barbae is an infection of the hairs and skin in the beard and moustache area, and is usually seen in men. The lesions may include scaling, follicular pustules and erythema. Most common agents: ***T. verrucosum.*** Other agents: *M.canis, T. mentagraphytes, T. rubrum T. violaceum.*

TINEA FACIEI:

Tinea faciei is seen on the nonbeared parts of the face. The lesions are usually pruritic; itching and and burning may become worse after exposure to sunlight. Most common agents: *T. tonsurans* in North America; *T.mentagrophytes* and *T. rubrum* in Asia.

TINEA CRURIS:

Tinea cruris is an infection of the groin, usually caused by anthrophilic dermatophytes. The symptoms include burning and pruritus. Most common agents*: E. Floccosum, T. rubrum*. Other agents*: M. nanum, T. mentagrophytes, T. raubitschekii.*

TINEA PEDIS & TINEA MANNUM:

Tenia pedis [Athlete's foot] is an infection of the foot, characterized by fissures, scales and maceration in the toe web, or scaling of the soles and lateral surface of the feet. Erythrema, vesicles, pustules and bullae may also be present. Most common agents: *T. rubrum T. mentagrophytes var interdigitale, E. Floccosum.* Other agenta: *M. persicolar, T.raubitschekii,T.violaceum*. other agents: *E. Floccosum, M.canis, M.gypseum, T. mentagrophytes.*

TINEA UNGUIUM:

Tinea unguium is a dermatophyte infection of the nail. It is characterized by thickened, discolored, broken and dystrophic nails. The nail plate may be separated from the nail bed. Most common agents: *T. rubrum, T. mentagrophytes var mentagrophytes.* Other agents: *E. Floccosum, T. tonsurans, T. violaceum*

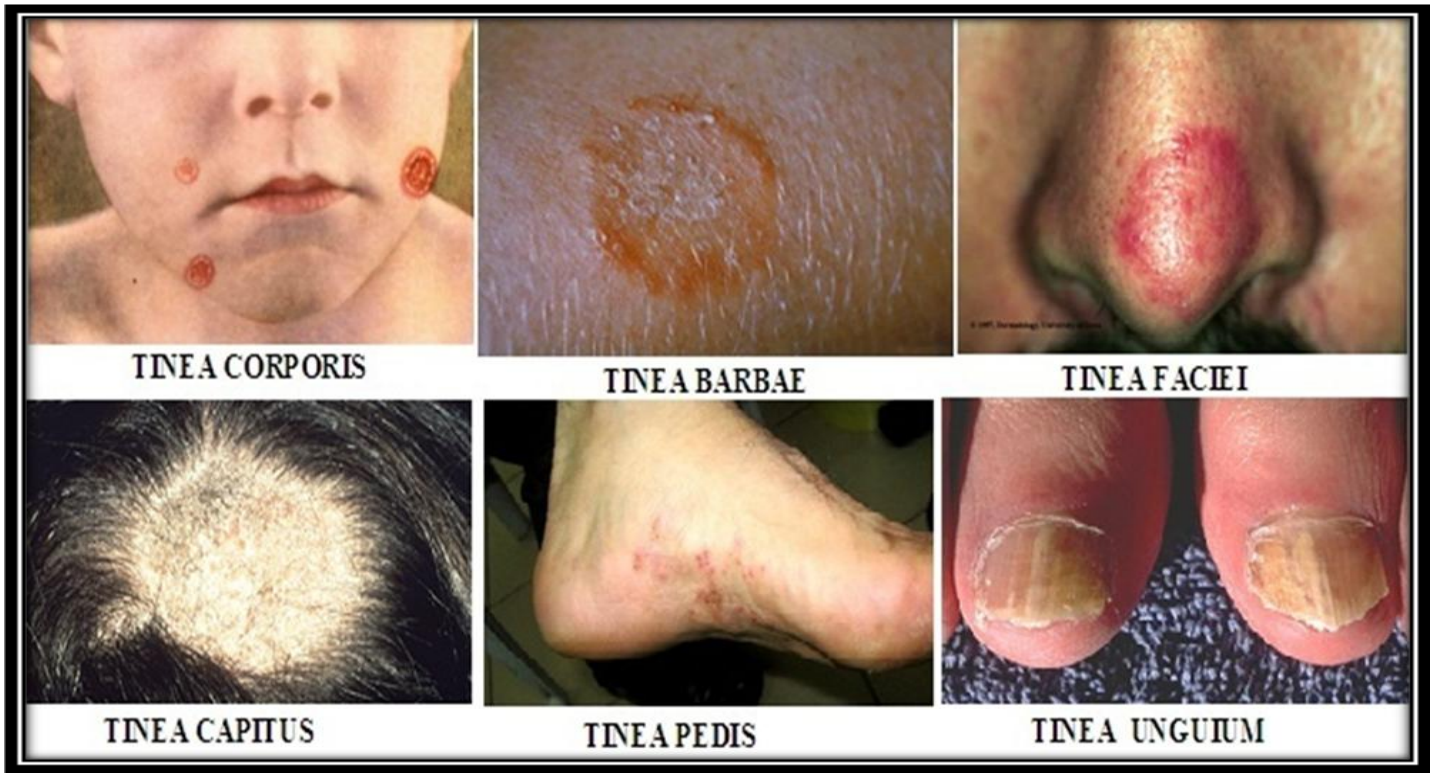


Figure No. 2: Types of *tinea unguium*

DERMATOPHYTIC FUNGI	YEAST	NON-DERMATOPHYTIC FUNGI
Epidermatophyton floccosum	*Candida albigans*	*Acermonium sp.*
Trycophyton concentric	*Candida famata*	*Aspergillus sp.*
Trycophyton mentagrophyte	*Candida guillermondii*	*Alternaria sp.*
Trycophyton minima	*Candida parapsilosis*	*Helmintosporium*
Trycophyton rubrum	*Candida tropicalis*	*Fusarium sp.*
Trycophyton shoenlinii	*Candida sake*	*Curvularia sp.*
Trycophyton soudanese		*Crptococcus sp.*
Trycophyton tonsurans		*Scedosporium sp.*
Trycophyton violaceum		

Table -1: Some Common Mycosis Causing Fungal Species

IMMUNITY BEHIND DERMATOPHYTIC INFECTION:

Host immune response to the invading pathogen is responsible for the clinical manifestations. The fungal pathogens induce both immediate hypersensitivity as well as cell mediated or delayed type hypersensitivity. Acquired resistance to the infection may also result from dermatophytic infection. The fungal growth is restricted by the inflammatory reactions produced as a result of infection with dermatophytes.

PREVENTION:

To prevent transmission, infected animals should be isolated untill the infection has resolved. Animals that have been in contact with the patient should also be checked for asymptomatic infections. Some veterinarians use antifungals prophylactically for in-contact animals. The premises should be cleaned [vacuumed] and disinfected to help prevent infections in other animals or humans.Rodent control can decrease to T. mentagrophytes. Access to infected soil should be prevented, particularly with geophillic species vaccines.

TREATMENT:

Animals often have self-limiting infections that resolve with in a few months, but treatment can speed recovery, decrease the spread of lesionson the animal, and decrease the risk of transmission. Treatment may include topicaln antifungal cream or shampoos, and systemic antifungals. Onycomycosis can be very difficult to cure; long term treatment or surgical declawing may be necessary. Animals should be isolated until the infection resolves. The environment and fomites should be cleaned to remove hair and skin flakes, and disinfected. Many drugs are liscensed for the treatment of dermatophytic infections such as Amphotericin B, Fluconazole, Itraconazole, Voriconazole,Terbinaffine etc. Amphotericin B deoxycholate [AmB-D;Fungizone] is polyene with a very broad spectrum of activity including most yeast and filamenyous fungi. Voriconazole is licensed for the treatment of infection due to *scedosporium sp and fusarium sp.* on the basis of several case reports. It is ineffective *in vitro* against isolates of Zygomycetes. Flucytosine [ancotil, valeant] is liscensed for use in the treatment of systemic fungal infection caused by sensitive organism. It has activity against *candida sp Cryptococcus sp* and some filamentous fungi. Intrinsic resistance in *candida sp* is uncommon [*ostrosky-zeichner et al.,2003]* Itraconazole, active against yeasts and mould, with the esception of *fusarium sp, scedosporium sp* and the zygomycetes *[Johnson et al.,1998].*But these antifungal agent causes many side effets in human beings for easy fevers, chills, nausea and vomiting & hypotension, gastrointestinal side effects [nausea & diarrhoea], hepatotoxicity and bone marrow suppression are reversible on discontinuation of the drug, headache, dizzinessraised hepatic transaminases, menstrual disorder, peripheral neuropathy and allergic reactions. Heart failure has been reported. In recent years there has been an increasing interest in the use of natural substances, and an increasing search for new antifungal compounds due to the lack of efficacy, side effects and or resistance associated with some of the existing drugs. Much attention paid to

plant derivedantifungal compound based on the knowledge that plants have their own defence system against fungal pathogen. Natural products obtained from many plants have been scientific interest. A few antifungal agents are available and licensed for use in veterinary practice or human being treatment. The use of systemic drugs is limited to treat man or animal due to their high toxicity and problems of residues in products intended for human consumption *(Araujo et al., 2009).* Different treatments have been recommended to control dermatophtes. In general, pharmacological treatment option include antifungal agents *[Aly, 1997; Agwa et al., 2000],* but recently the use of some natural plant products has been emerged to inhibit the causative organisms. These natural plants involve garlic, lemon grass, datura, acacia, a triplex, ginger, black seed, neem, basil, eucalyptus, alfalfa and basil *(Omarand Abd-El-Halim, 1992; Aly et al., 2000; Aly and Bafiel, 2008).* They are safe to human and the ecosystem than the chemical antifungal compounds, and can easily be used by the public Plant extract has been used traditionally to treat a number of infectious diseases including those caused by bacteria, fungi, protozoa and viruses *(Soylu et al., 2005; Yoshida et al., 2005; Nejad and Deokule, 2009).* A number of reports are available in vitro and in vivo efficacy of plant extract against plant and human pathogens causing fungal infections *(Natarajan et al., 2003).* The activity of plant extract against dermatophytosis i.e. the superficial infections of skin or keratinised tissue of man and animals can be very well visualized from the reports of Venugopal and Venugopal (1995). They reported the activity of plant extracts agains clinical isolates of dermatophytes which includes*Microsporum cannis, M, audouinii Trichophyton rubrum T mentagraphytes, T violaccum, Tsimii, T verrucosum T erinacci and Epidermophytn floccosum* by agar dilution technique. *Ocimum sanctum*is a great sacred medicinal plant in India.Basil has traditionally been used for head colds and as a cure for warts and worms, as an appetite stimulant, carminative, and diuretic. In addition, it has been used as a mouth wash and adstringent to cure inflammations in the mouth and throat. Methanolic extracts of basil have been used in creams to treat slowly healing wounds *(Wichtl 1989)*. Basil is more widely used as a medicinal herb in the Far East, especially in China and India. It was first described in a major Chinese herbal around A.D. 1060 and has since been used in China for spasms of the stomach and kidney ailments. The antifungal activity of *Ocimum* leaves, extracts, essential oils and their components is frequently studied, mostly in warm countries where the need for protection of plants and stored crops against fungi is of great importance. Also the effect of *Ocimum* oils against a number of dermatophytes has been studied. An ethanolic extract of *O.sanctum* was used to treat healthy ripe tomato fruits prior tom and after inoculation with *Aspergillus niger*in the presence of *Drosophila busckii.* The treatment kept the fruits free from rotting for 5 to 7 days *(Sinha and Saxena 1989).* The essential oil of *O.canum* was effective against damping-off disease causing fungi, *Pythiumaphanidermatum, P.debaryanum*and *Rhizoctonia solani. O.canum* could control damping-off disease of tomato up to 50% in soil infected with *P.aphanidermatum* and up to 43% in soil infected with *P.debaryanum.* The essential oil was not phytotoxic and showed superiority over commonly used synthetic fungicides such as Agrosan G.N. and Captan *(Pandey and Dubey 1992, 1994). Pandey and Dubey (1994)* determined the fungitoxic spectrum of *O.canum* oil and found 100% inhibition of the growth of the following fungi: *Fusariumoxysporum* f. sp. *ciceri, F.sesami, F.semitectum, Alternaria brassicae, A.solani,A.tenuissima, Cladosporium cladosporioides, Helminthosporium oryzae, Penicllium citrinum, Colktotrichum* sp. and *Drechslera auntii.* Exudates of *O.basilicum*decreased the population of various fungi, including*Aspergillus* spp. and *Fusarium* spp. in the phyllosphere of beans *(Afifi 1975).* Essential oils of *O.basilicum* were effective against*Trichophyton mentagrophytes, T.rubrum* and *T.verrucosum,* Neem (*Azadirachta indica*), a large tree of India, has been used for centuries in Asia as insecticides, fungicides, in popular medicine almost every part of this tree seeds, leaves, roots, bark, trunk and branches has multiple uses *(Chaturvedi et al., 2003]*. Some extracts from neem plant have been shown to be toxic to fungal pathogens, such as *Poria monticolad* infecting wood *(Dhyani et al., 2004),Aspergillus flavus* from soybean seeds *(Krishnamurthy et al., 2008), Pyricularia oryzae* infecting rice plant in field and the harvested rice *(Amadioha, 2000).* Clove has been used medicinally in the field of oriental herbal medicine and as a culinary spices. Plant's flower bud is used for both flavoring and from which the essential oil is extracted. Clove oil is reported to have strong antifungal activity against many fungal species. In this study we have evaluated antifungal potential of essential oil of Syzygium aromaticum against some common fungal pathogens of plants and animals namely Fusarium , Aspergillus sp., Mucor sp., Trichophyton rubrum and Microsporum gypseum. All fungal species were found to be inhibited by the oil when tested through agar well diffusion method. public places at Gulbarga, India. Mycopathologia 2005;159:13-21

Figure No.3:

MATERIAL AND METHODS:

MATERIALS:

1. STERILIZE GLASSWARE'S [conical flasks, beaker, measuring cylinder, petriplates, pipette, test tubes, slides etc.**]**

2. INSTRUMENTS-Hot air oven, Autoclave, Incubator, Laminar air flow

3. CULTURE / GROWTH MEDIA FOR KERATINOPHILIC FUNGI-

[A] SABAURAUD DEXTROSE AGAR MEDIA [SDA]:

Peptone: 10 gm.
Dextrose: 40 gm.
Agar: 15 gm.
Distilled water: 1000ml
PH: [5.6]

[B] MULLER HINTON AGAD MEDIA [MHA]:

Beef infusion: 30 gm
Casamino acids/acid hydrolysate of casein: 17.5gm
Starch: 1.5gm
Agar: 17 gm
Distilled water: 1000ml
pH: [7.4]

[C] CORN MEAL AGAR MEDIA [CMA]:

Cornmeal: 15gm.
Agar: 20 gm.
Distilled water: 1000ml.

4-REAGENT: LACTOPHENOL COTTON BLUE [LPCB] STAIN:

Lactic acid: 20 ml
Phenol crystals: 20 gm
Glycerol: 40 ml
Distilled water: 20 ml
Cotton blue: 02 ml

Add lactic acid and glycerol to the distilled water and mix thoroughly. Add phenol crystals and heat gently in hot water with frequent agitation until the crystals completly dissolve. Add the dye and mix thoroughly and store the stain in brown bottle

5-PREPARATION OF SWABS: The cotton swab was invented in the 1920 by a Polish-born American named Leo Gerstenzang.

6- STERILIZE SOIL SAMPLES:

7- HUMAN HAIR, HUMAN NAILS, HORSE HAIRS AND PEACOCK FEATHER:

8-COLLECTION OF PLANT MATERIAL: The collection of plant material [leaves of basil, neem, guava and pericarp of pomigranate and bud of clove] was done.Samples of five medicinal plants were brought to the laboratory and thoroughly washed in running tap water to remove debris and dust particles and then rinsed in distilled water.

Sr. No.	Plant Materials	Botanical Name	Family
1.	Leaves of basil	*Ocimum sanctum*	Lamiaceae
2.	Leaves of neem	*Azadirachta indica*	Meliaceae
3.	Leaves of guava	*Psidium guazava*	Myrtaceae
4.	Bud of clove	*Syzygium aromaticum*	Myrtaceae
5.	Pericarp of pomigranate	*Punica granatum*	Lythraceae

Table No. 2: List of medicinal plants used in the antifungal ssay.

METHODS:-

COLLECTION OF SAMPLES:

- In our dissertation work keratinophillic fungi were isolated from soil of Gwalior region.
- The sample were collected from soil by sterile brush.
- To reduce contamination brush, gloves, polythene, foil over sterilize in u.v. Sterilization.

HAIR AND NAIL BAITING:

- Half fill sterile Petri dishes with the soil samples.
- Spread short (2-3 cm) strands of sterilized defatted* human hair or horsehair and human nail over the surface of the soil.
- Add 10-15 ml of sterile water to the soil to facilitate germination of fungal spores. Some antibiotic [streptomycin & chloramphenicol] to prevent bacterial growth may also be added.
- Incubate the preparations at room temperature (20-25o C) in the dark, for 4-6 weeks. Examine the plates periodically for the development of mycelium using a Stereo binocular microscope
- Remove hairs with fungus growth or take inoculum and place it on plate of Sabouraud's dextrose agar.After one or more week, check the colonies and identify the fungus. Pure cultures can now be prepared.

Figure No.4: Positive soil plate with fungal growth on hair

PREPARATION OF THE METHANOLIC PLANT EXTRACTS:

For organic extraction, 10 grams of each sun-dried 5 medicinal plant material **[**leaves of *azadirachta indica, psidium guazava, ocimum sanctum,* pericarp of *punica granatum,* bud of *syzygium aromaticum**]*** were cut into small pieces and then macerated by blender 1–2 mm separately and the powder produced was blended with 100 ml of organic solvent [methanl] 1:10 w/v. Then, they were extracted under cold conditions for 24 h. The resultant extract was filtered through a glass wool filter and then rinsed with a small quantity (about 30 ml) of 96% ethyl alcohol. The extracts solutions were evaporated under reduced pressure at 40 °C. Subsequently, the extracts were diluted by distilled water and stored in the deep freezer at -10 °C and later lyophilized in a freeze dryer.

ISOLATION AND IDENTIFICATION:

[A] ISOLATION:

For fungal infection the sample is inoculated on SDA plates to which antibiotic [streptomycin & chloramphenicol] was added to inhibit baterial growth. The plates were incubated for 48-72 hrs at 27°C after which the colony were studied.

[B] IDENTIFICATION:

The colonies were identified by cultural morphology and microscopic morphology.

(1**) CULTURE MORPHOLOGY:**

The growth of fungi was observed by using following criteria:

- Age of culture
- Rate of culture
- General topology- flat or heaped
- Texture- yeast like powdery, granular, velvety, cottonyetc.
- Surface pigmentation
- Reverse pigmentation

(2**) MICROSCOPIC MORPHOLOGY:**

[A] Small part of fungal coloy was placed over a glass slide with platium fungi were idetify by wire needle and mouted in a drop of lactophenol cotton blue stain.

[B] Fungus seperated with needle.

[C] After putting cover slip followig study was done under microscope:-

- Cellular morphology-unicellular/hyphal, septate / asepted
- Branching or non branching
- General morphology
- True mycelium or pseudo mycelium
- Spore morphology- smooth/ rough, shape attachment
- Conidiophore /sporangiophore/ arthospore/ chlamydospore.

TEST ORGANISM:

Fungal strain isolated from the soil of different regions of gwalior which are *Tricophyton rubrum, Trichophyto mentagrophyte, chrysosporium sp., Epidermatophyton floccosum and Aspergillus fumigatus* were used & maintained in sabauraud's dextrose agar slants in department of microbiology, BIMR, Gwalior.

INOCULUM PREPARATION:

The mould inoculums preparation of conidial or sporangiophore suspension must be adjusted using a spectrophotometer with a test inoculum in the range 0.4×10.....to 5×10.....CFU/ml. The optical density (OD) at 530nm required is dependant on the conidial or sporangiophore size of the mould being tested i.e. for aspergillus sp. the OD= 0.09-0.11; for tween 80 was added as wetting agent to facilitate the preparation of inoculums.

ANTIFUNGAL ACTIVITY:

Antifungal activity of essential oils and 5 different methaolic plant extracts was tested using the agar well diffusion method. 20 ml of Muller hinton agar was taken into sterilized petridish and allowed to solidify. Afterwards 7mm well punched on plate with cork borar, 5 well punched in each plate. For our study different essential oils and different methanolic plant extracts were to know about the effect of it over the growth of the isolated keratinophilic fungus. 40µl of each of essential oils and plant extracts were transferred to each well for 24 hrs. and this process also checks the possible contamination during plating & loading. The MHA was taken seeded with test fungal strain by using sterile cotton swabswas dipped into the suspension [tween 80]. Pressed firmly against the inside wall of the tube just above the fluid level and rotate to remove excess liquid. The swabs were streaked over the entire surface of the medium three times. Rotating the plates approximately 60 degree after each application to ensure an even distribution of inoculums. Finally swabs was streaked all around the edge of the agar surface. All the dishes were than incubated at 28° c for 48 hrs.

RECORDING AND INTERPRETING OF RESULTS:

Diameter of zone of inhibition against test was measured [in mm] with antibiotic zone measuring scale [Hi media] on the under surface of the plate without opening lid and an average of independant determination was recorded.

RESULTS:

In the present study total 22 sample were collected from different soil samples of gwalior region and were further proceed with different keratin containing samplelike horse hair, human hair, human nail and peacock feather a positive diagnosis of keratinophilic fungi was obtained with some dermatophyte species like Epidermatophyton floccosum , Trycophyton mentagrophyte and T. rubrum , non dermatophyte like aspergillus fumigatus and Chrysosporium sp. etc. A total 5 methanolic plant extract were selected to determine antifungal activity. The individual extract was taken for this purpose to check its activity on fungus and to check whether they are more effective or less effective. The antifungal activity with varius magnitude. The zone of inhibition > 0mm diameter was taken as positive the sensitivity test for different fungi was carried out with different plant extracts. Out of all the methanolic plant extracts *Ocimum basilicum [basil], Azadirachta indica [neem], Punica granatum[pomegranate], Psidium guazava [guava] and Syzygium aromaticum [clove] extracts* have shown antifungal activity against T. mentagrophytes 10 , 5, 18, 7, 20 mm respectively, T. rubrum 19, 26, 5, 19 ,11mm respectively, epidermatophyton floccosum 5, 8, 23, 10, 19mm respectively, Aspergillus fumigatus 12, 13, 10 ,16, 7mm respectively and chrysosporium sp. 17,14,14,10,20mm respectivily. Therefore, the maximum antifungal was shown by Azadirachta indica [neem] on T. rubrum with zone 26mm and the minimum antifungal effect was shown by neem on T, mentagrophyte, basil on Epidermatophyton floccosum and punica granatum [pomegranate] on T. rubrumwith zone of 5 mm.

TRICOPHYTON MENTAGROPHYTES:

(A) CULTURAL MORPHOLOGY:

- Age of culture : 3-4 days
- Rate of growth : slow growth
- General topography : white floppy cottony type
- Texture : cottony
- Surface pigmentation : white
- Reverse pigmentatio : sometimes pinkish light to Yellow to reddish brown

(B) MICROSCOPIC MORPHOLOGY:

- Spiral hyphae are frequently present;
- Microconidia are round to pyriform in shape, unicellular, appear in closely re – branched clusters or along with otherwise undifferentiated hyphae, frequently numerous, however at times, may be present rarely in anthropophilic isolates ; and
- Macroconidia are often absent, but if present, mostly are club – shaped, with thin, smooth walls, are multi – septate, and solitary.

A

B

HEALTH EFFECTS:

Trichophyton mentagrophytes the anthropophilic type of isolates, are the frequent causative agents of chronic infection of the feet, the nails, and the groin. When infecting humans, the zoophilic isolates, such as *T. mentagrophytes* var. *mentagrophytes,* are more frequently associated with inflammatory lesions of the scalp, the glabrous skin, the nails, and the bear region.

TRYCOPHYTON RUBRUM:

A. CULTURAL MORPHOLOGY:

- Age of culture : 8- 10 days
- Rate of growth : Slow growth
- General topography : fluffy
- Texture : cottony
- Surface pigmentation : deep sometimes with pale yellow margine
- Reverse pigmentation : thin walled& wine red

B. MICROSCOPIC MORPHOLOGY :

- Presence of microconidia is numerous to rare, club – shaped to pyriform, may be found solitary along the hyphae or sometimes in clusters, and are unicellular; and
- Macroconidia are frequently absent; pencil – to cigar – shaped, and is multi - septate.

C. HEALTH EFFECTS:

Trichophyton rubrum is the most common agent of tinea of the feet, hands, nails, groin, and the glabrous skin, however, the scalp is rarely infected. Animals are very infrequently infected as well.

EPIDERMATOPHYTON FLOCCOSUM:

A. CULTURAL MORPHOLOGY:

- Age of culture : 8- 10 days
- Rate of growth : moderately growth
- General topography : felty & velvety
- Texture : flat and grainy
- Surface pigmentation : brownish yellow to olive gray or khaki
- Reverse pigmentation : orange to brown with yellow border

B. MICROSCOPIC MORPHOLOGY:

- Hyaline septate hyphae

- Microconidia are typically absent
- Macroconidia are club shaped with thin smooth walls, three to five celled, and may be solitary or in groups.

C. HEALTH EFFECTS:

Floccosum is one of the causative agents of cutaneous infection, dermatophytosis, in healthy individuals which infects the skin. Skin infection include the body surface (tinea corporis), groin (tinea cruris), feet (tinea pedis) and nails (onychomycosis). E. Floccosum has been reported in an immunocompromised patient with Behcet's syndrome, and can be transmitted by contact, particularly in common showers & gym facilities.

ASPERGILLUS FUMIGATUS:

A. CULTURAL MORPHOLOGY:

- Age of culture : 4- 5 days
- Rate of growth : rapid growth
- General topography : granular
- Texture : varies from wooly to cottony to granular
- Surface pigmentation : smokey gray- green
- Reverse pigmentation : yellow

B. MICROSCOPIC MORPHOLOGY:

- hyphae are septate and hyaline.
- conidiophores aresmooth- walled and terminate in dome- shaped vesicles.
- Conidia are round sub- globose.

C. HEALTH EFFECTS:

A. fumigatus is an occasionalcausative agents of aspergillosis in humans.cases of pulmonary , nasal, cerebral, bone ocular and organ infection have been reported especially among immunocompromised patient. B. fumigatus is also an agents of mycotic abortion in the cow and respiratory infections in fowl.

CHRYSOSPORIUM SP:

CULTURAL MORPHOLOGY:

- Age of culture : 3- 4 days
- Rate of growth : moderately growth
- General topography : wooly
- Texture : cottony & flat
- Surface pigmentation : white cream or yellow
- Reverse pigmentation : white to brown

B. MICROSCOPIC MORPHOLOGY:

- Chrysosporium produces hyphae, conodia and arthroconidia.
- Hyphae are septate while the conidia are hyaline, one- celled, smooth, or rough walled.

C. HEALTH EFFECTS:

Chrysosporium species may cause skin infection and onychomycosis in human and superficial infections. *Chrysosporium* spp. has occasionally been isolated from systemic infections in bone marrow transplant recipients and in patients with chronic granulomatous disease. The high mortality rate of systemic *Chrysosporium* infections is noteworthy.

Sr. No.	Date	Types of soil sample	Type of bait processed	Results
[1]	15-04-2012	Outdoor soil	Human hair Human nail Horse hair Peacock feather	No growth No Growth Fast growth Mild growth
[2]	16-04-2012	Outdoor soil	Human hair Human nail Horse hair Peacock feather	No growth No Growth White growth Slow growth
[3]	17-04-2012	Indoor soil	Human hair Human nail Horse hair Peacock feather	No growth Mild growth Fast growth No growth
[4]	19-04-2012	Indoor soil	Human hair Human nail Horse hair	Mild growth No growth Fast growth

			Peacock feather	No growth
[5]	22-04-2012	Indoor soil	Human hair Human nail Horse hair Peacock feather	White growth Slow growth Fast growth No growth
[6]	25-04-2012	Outdoor soil	Human hair Human nail Horse hair Peacock feather	mild growth slow growth fast growth slow growth
[7]	28-04-2012	Outdoor soil	Human hair Human nail Horse hair Peacock feather	No growth Mild growth Fast growth No growth
[8]	30-04-2012	Outdoor soil	Human hair Human nail Horse hair Peacock feather	No growth Mild growth Fast growth Slow growth
[9]	2-05-2012	Outdoor soil	Human hair Human nail Horse hair Peacock feather	Rare growth Slow growth Full growth Light growth
[10]	4-05-2012	Indoor soil	Human hair Human nail Horse hair Peacock feather	No growth Minor growth Fast growth Fast growth
[11]	7-05-2012	Outdoor soil	Human hair Human nail Horse hair Peacock feather	No growth Mild growth Fast growth No growth
[12]	9-05-2012	Outdoor soil	Human hair Human nail Horse hair Peacock feather	Mild Growth No growth Fast growth No growth
[13]	11-05-2012	Outdoor soil	Human hair Human nail Horse hair Peacock feather	Major growth No growth Fast growth Fast growth
[14]	14-05-2012	Outdoor soil	Human hair Human nail Horse hair Peacock feather	Growth No growth White growth Fast growth
[15]	16-05-2012	Indoor soil	Human hair Human nail Horse hair Peacock feather	No growth No growth Mild growth No growth
[16]	19-05-2012	Indoor soil	Human hair Human nail	No growth No growth

			Horse hair Peacock feather	Minor growth Mild growth
[17]	22-05-2012	Indoor soil	Human hair Human nail Horse hair Peacock feather	Mild growth No growth No growth Minor growth
[18]	25-05-2012	Indoor soil	Human hair Human nail Horse hair Peacock feather	Growth No growth Mild growth No growth

Table No.:3 List of isolation of keratinophilic fungi on the basis of indoor and outdoor soil sample of Gwalior region-

Sr. No.	Type of soil sample	Fungus growth transferr-ed on growth media	Cultural characterstics			Microscopic morphology	isolated keratinoph-ilic fungi
			Texture	Surface pigmentation	Reverse pigmentation		
[1]	Out-Door Soil	SDA	Powdery	White	Pale brownish-yellow	Ameroconidia, Pyriform to clavate and no macroconidia	*Chrysosporium sp.*
[2]	Out-door soil	SDA	Cottony & woolly	black	Pale or slightly yellow	Hyline, septate conidiophore, branching near the apex	*Gliocladium sp.*
[3]	Indoor soil	SDA	Velvety	Olivish brown	black	Blastic conidia, pigmanted and conidiophore: erect, and form tree like conidial structure	*Cladosporium sp.*
[4]	Indoor soil	SDA	Powdery	Olivish gold	Dark yellow	Long chain of conidia, unbranched,and oval, cylindrical, hyaline to lightly pigmented & smooth conidia	*Paecilomyces sp.*
[5]	Indoor soil	SDA	Often flat at first, becoming fluffy with age	White	unpigmented	Conidia are ameroconidia hyaline or pigmented, globose to cylindrical, and mostly aggregated in slimy heads at the apex of each phialide.	*Cephalospori-um sp.*

[6]	Out-door soil	SDA	Cottony	White with pale yellow margine	Thin walled	Multiseptate macroconidia	*Trycophyton rubrum*
[7]	Out-Door soil	SDA	Powdery	White	-	Hyphae are hyaline, septate, branched and break up into chains of hyaline, smooth, one-celled, subglobose to cylindrical arthroconidia	*Geotrichum sp.*
[8]	Out-Door soil	SDA	Fluffy	Olivish Grey	Dark black	Muriform , beaked conidia produced in acropetal chain.	*Alternaria sp,*
[9]	Out-Door soil	SDA	Suede-like to downy	Blackish-brown	Black	Conidia are pale brown, with phragmoconidia, cylindrical or slightly curved, with one of the central cells being larger and darker. Germination is bipolar .	*Curvularia sp.*
[10]	Indoor soil	SDA	Dense and granular	Globose, Dark black	thin walled	Conidiophores are smooth walled, hyaline or turning dark Conidial heads are biseriate with the phialides borne on brown, often septate metulae. Conidia are globose , dark brown to black and rough-walled.	*Aspergillus niger*
[11]	Out-door soil	SDA	Thin or floccose	Grey to dark brown	Brown and smooth	Dark holoblastic conidia	*Humicola sp.*
[12]	Out-door soil	SDA	Cottony	White	Sometime pinkishlight to yellow to reddish	Glabrous aerial mycellium, macroconidia are large, clavate and multiseptate	*T. mentagrophyte*
[13]	Out-door soil	SDA	granular	green	black	Septate and conidia present	*Aspergillus*

Table No.:4 frequencies of isolated fungi-

Sr. No.	Name of methanolic plantextract	Zone of inhibition of *T. mentagrophyte*
[1]	Ocimum basilicum	10 mm
[2]	Azadirachta indica	5mm
[3]	Punica granatum	18 mm
[4]	Psidium guazava	7mm
[5]	Syzygium aromaticum	20 mm
Sr. No.	**Name of methanolic plantextract**	**Zone of inhibition of *T. rubrum***
[1]	Ocimum basilicum	19 mm
[2]	Azadirachta indica	26 mm
[3]	Punica granatum	5 mm
[4]	Psidium guazava	19 mm
[5]	Syzygium aromaticum	11 mm
Sr. No.	**Name of methanolic plantextract**	**Zone of inhibition of *Epidermatophyton floccosum***
[1]	Ocimum basilicum	5 mm
[2]	Azadirachta indica	8mm
[3]	Punica granatum	23 mm
[4]	Psidium guazava	10 mm
[5]	Syzygium aromaticum	19 mm
Sr. No.	**Name of methanolic plantextract**	**Zone of inhibition of *Aspergillus fumigatus***
[1]	Ocimum basilicum	12 mm
[2]	Azadirachta indica	13 mm
[3]	Punica granatum	10 mm
[4]	Psidium guazava	16 mm
[5]	Syzygium aromaticum	7mm
Sr. No.	**Name of methanolic plantextract**	**Zone of inhibition of *Chrysosporium sp.***
[1]	Ocimum basilicum	17 mm
[2]	Azadirachta indica	14 mm
[3]	Punica granatum	14 mm
[4]	Psidium guazava	10 mm
[5]	Syzygium aromaticum	20 mm

Table No. 5: Antifungal effect of plant extracts on isolated test keratinophillic fungi-

Figure No. 5: Growth of keratinophilic fungi on horse hair / human hair/ human nail & peacock feather placed in soil of Gwalior region.

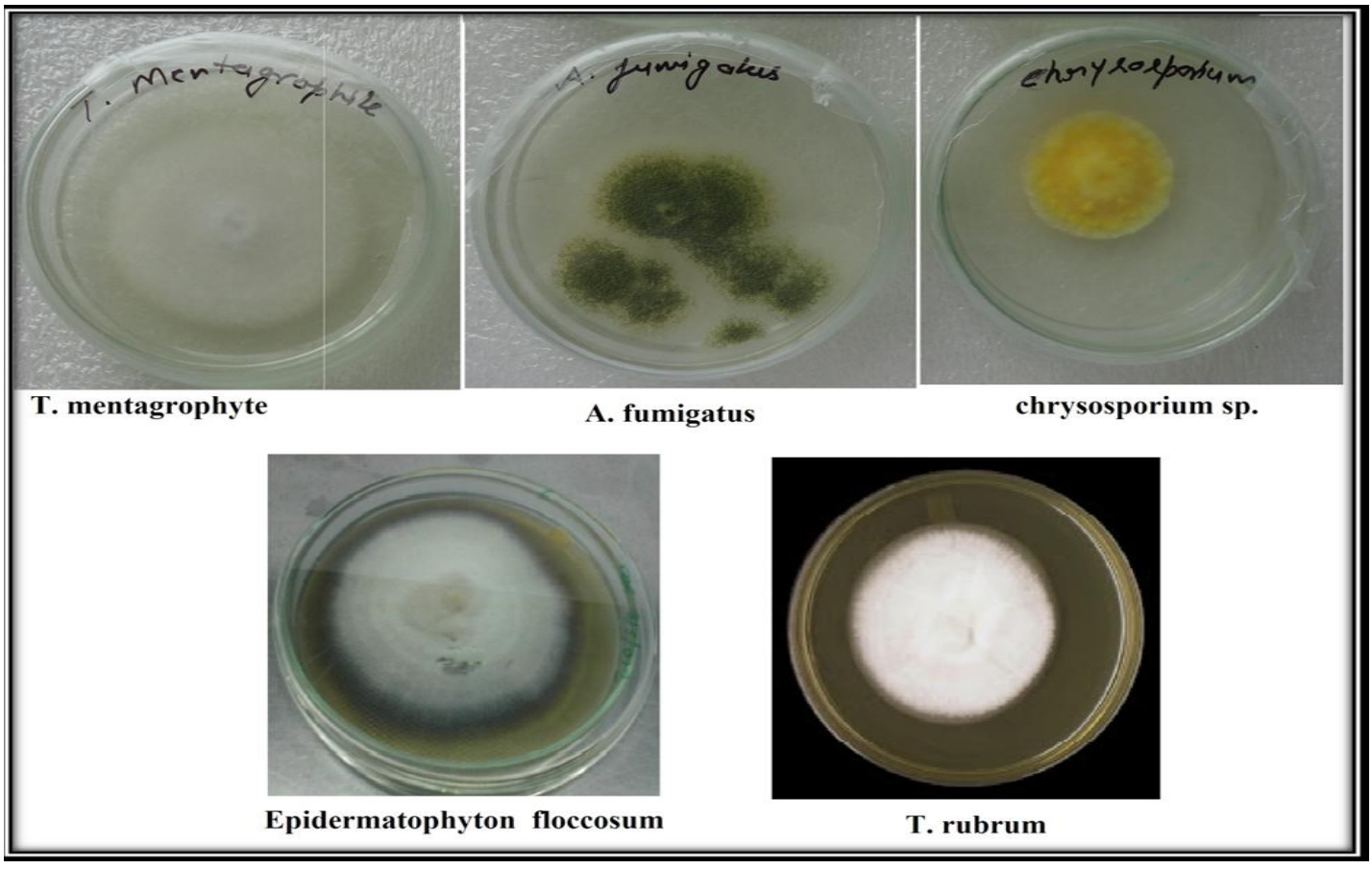


Figure No.6: Growth of isolates of different keratinophilic fungi on SDA media:-

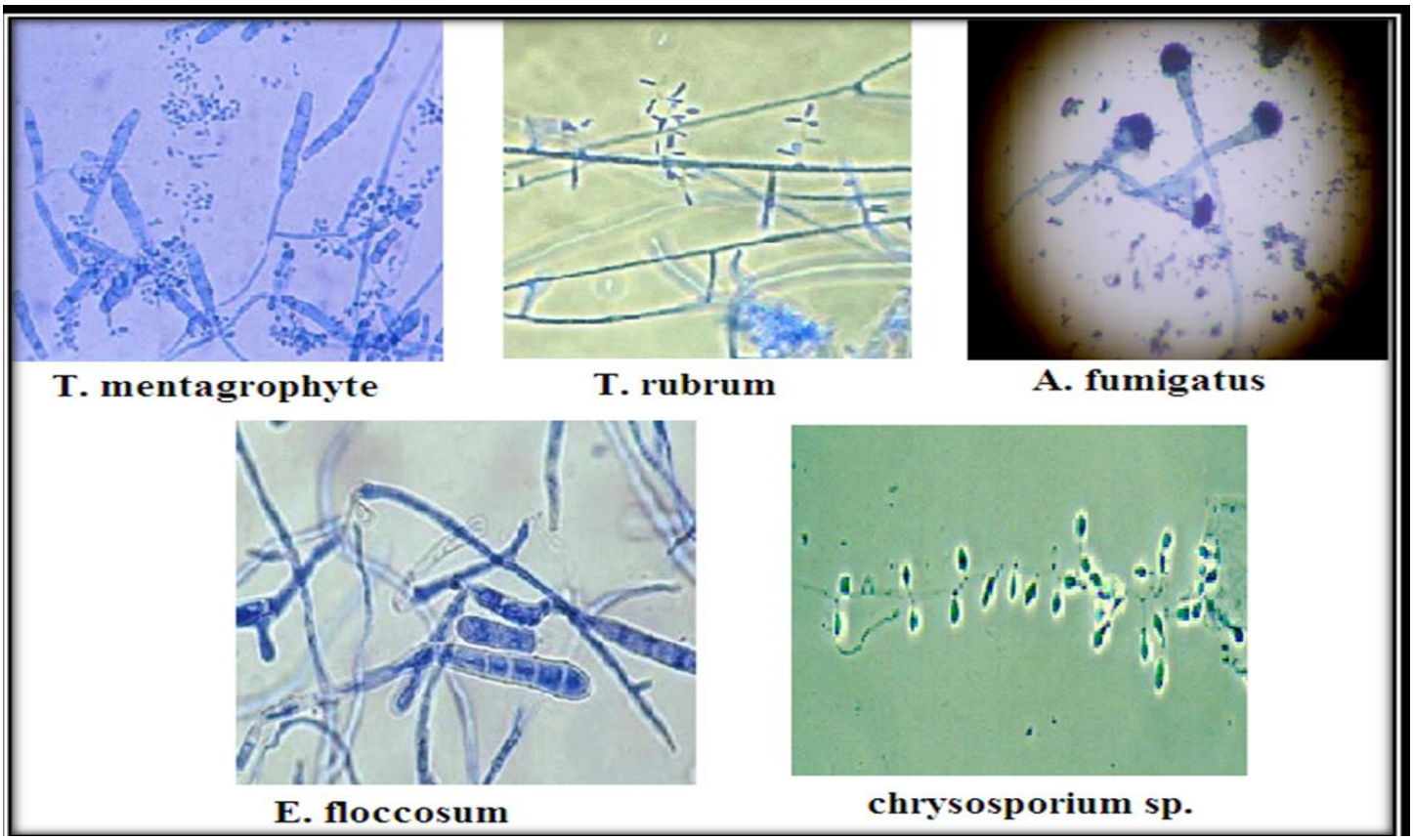


Figure No.7: Microscopic appearance of different fungal isolates:-

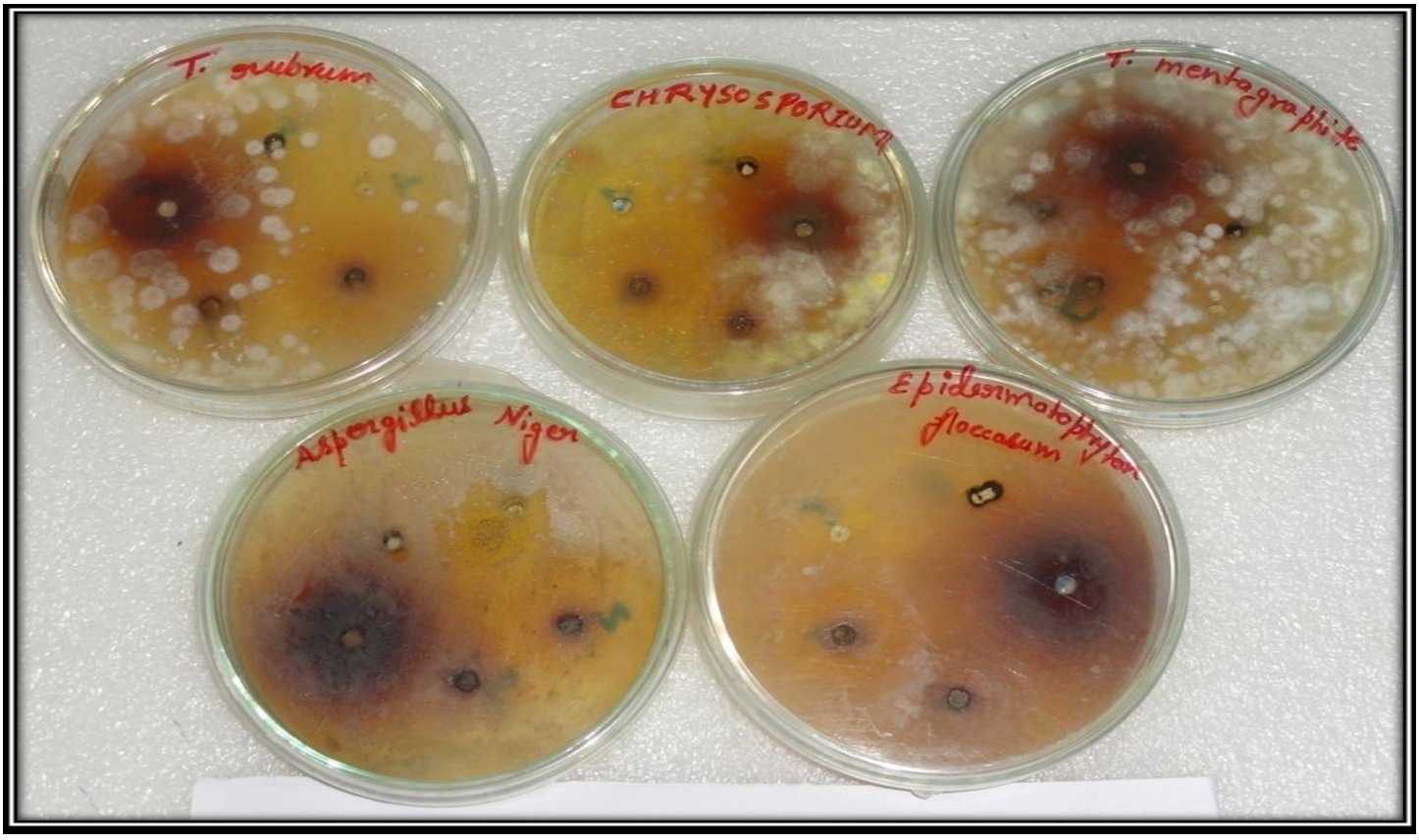
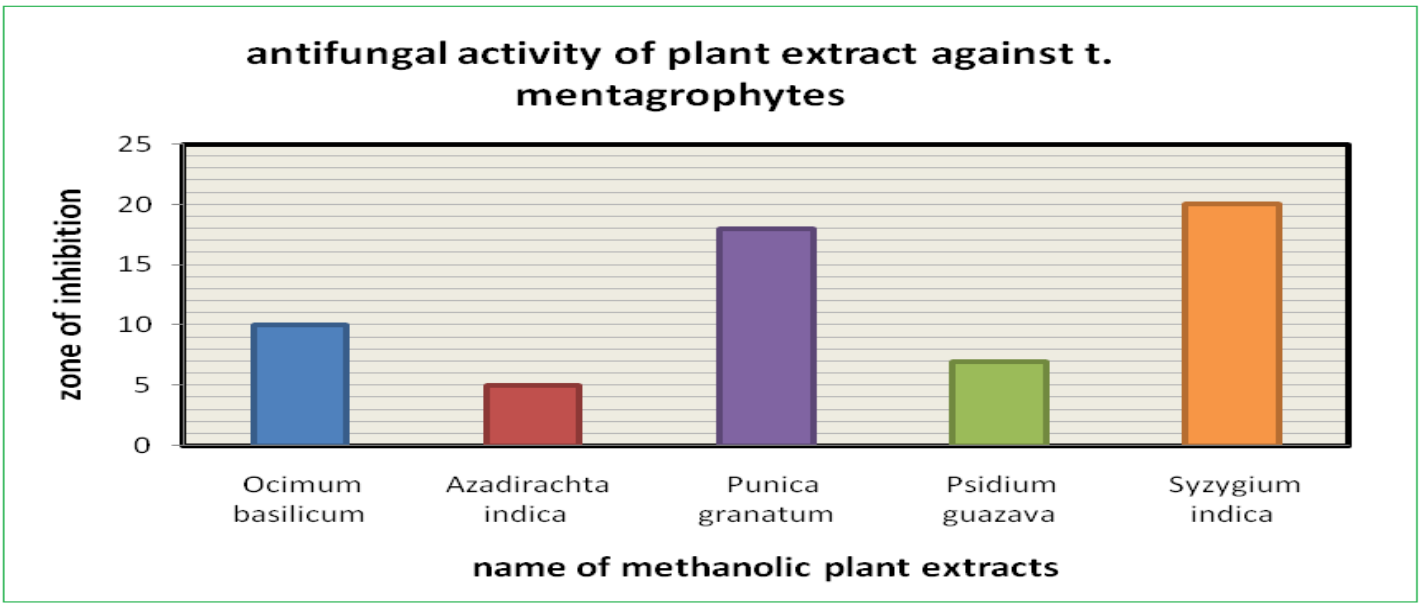
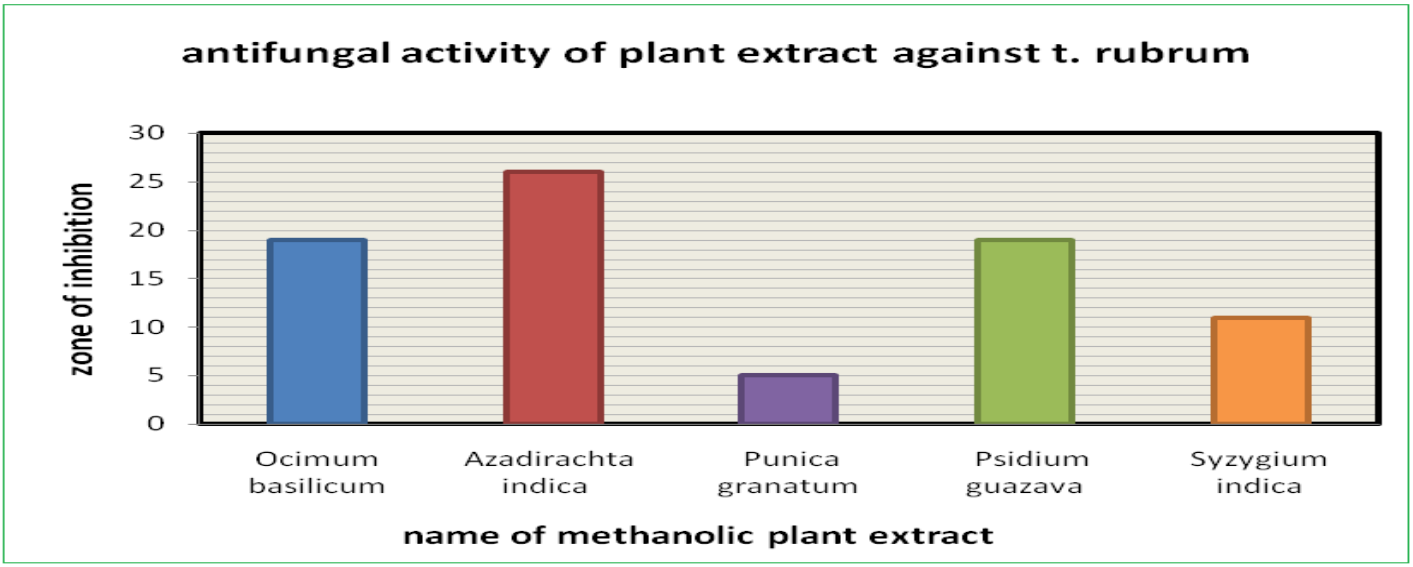
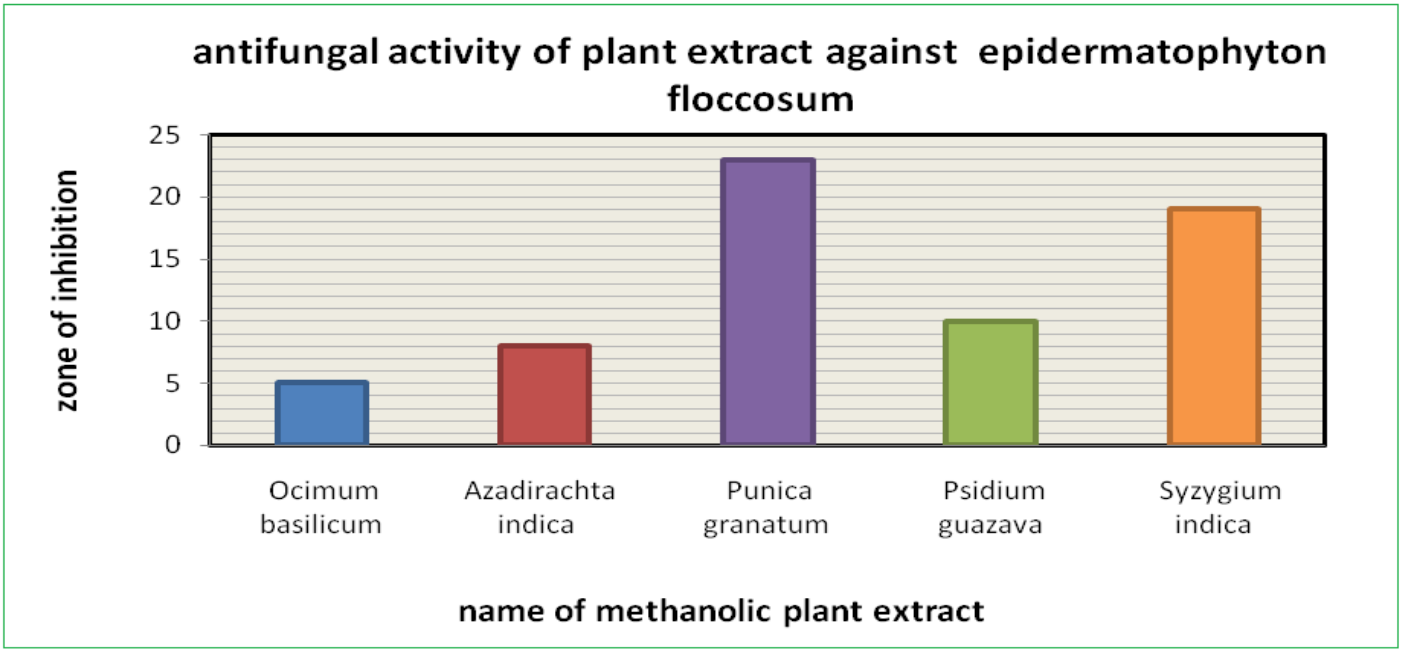
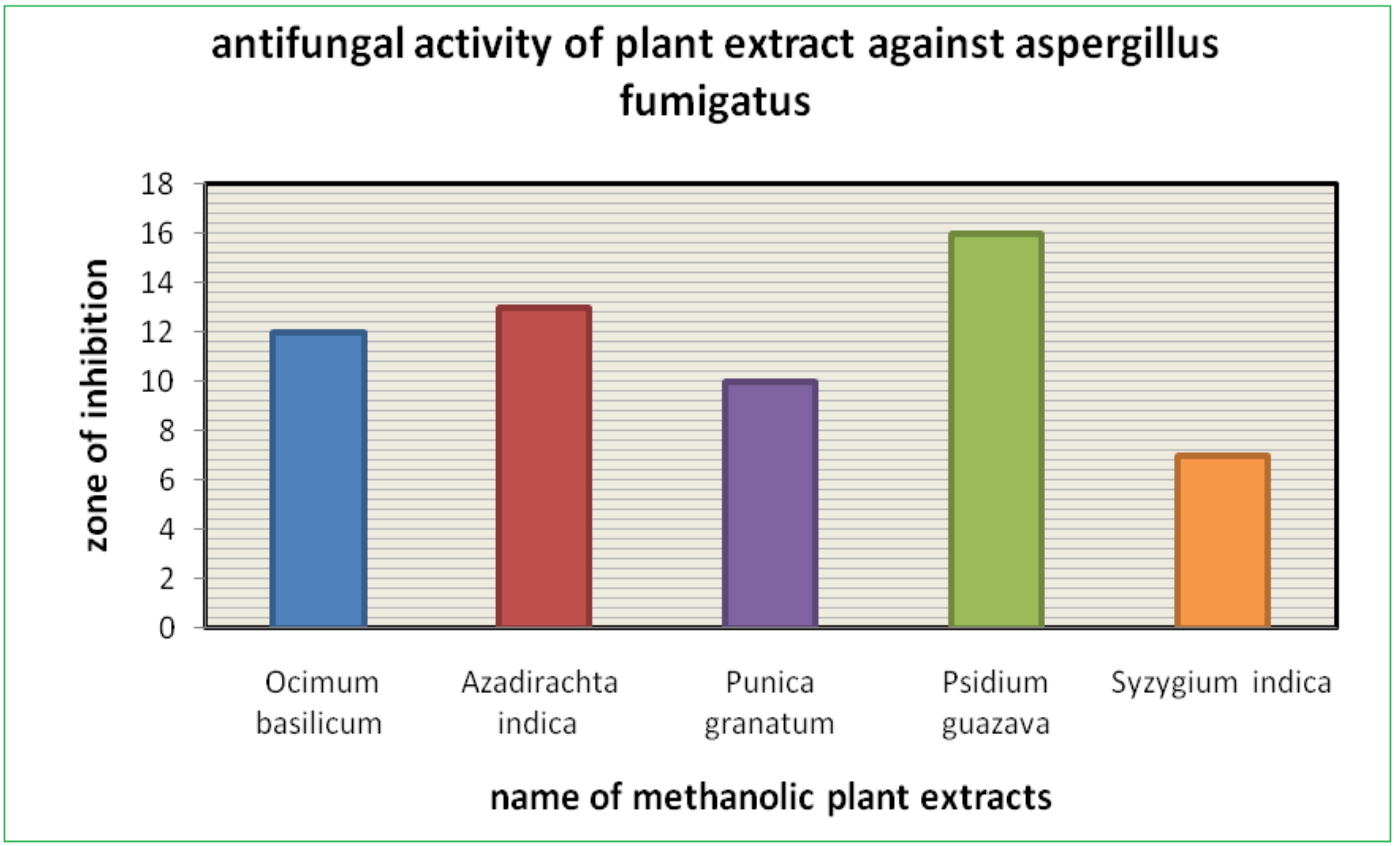


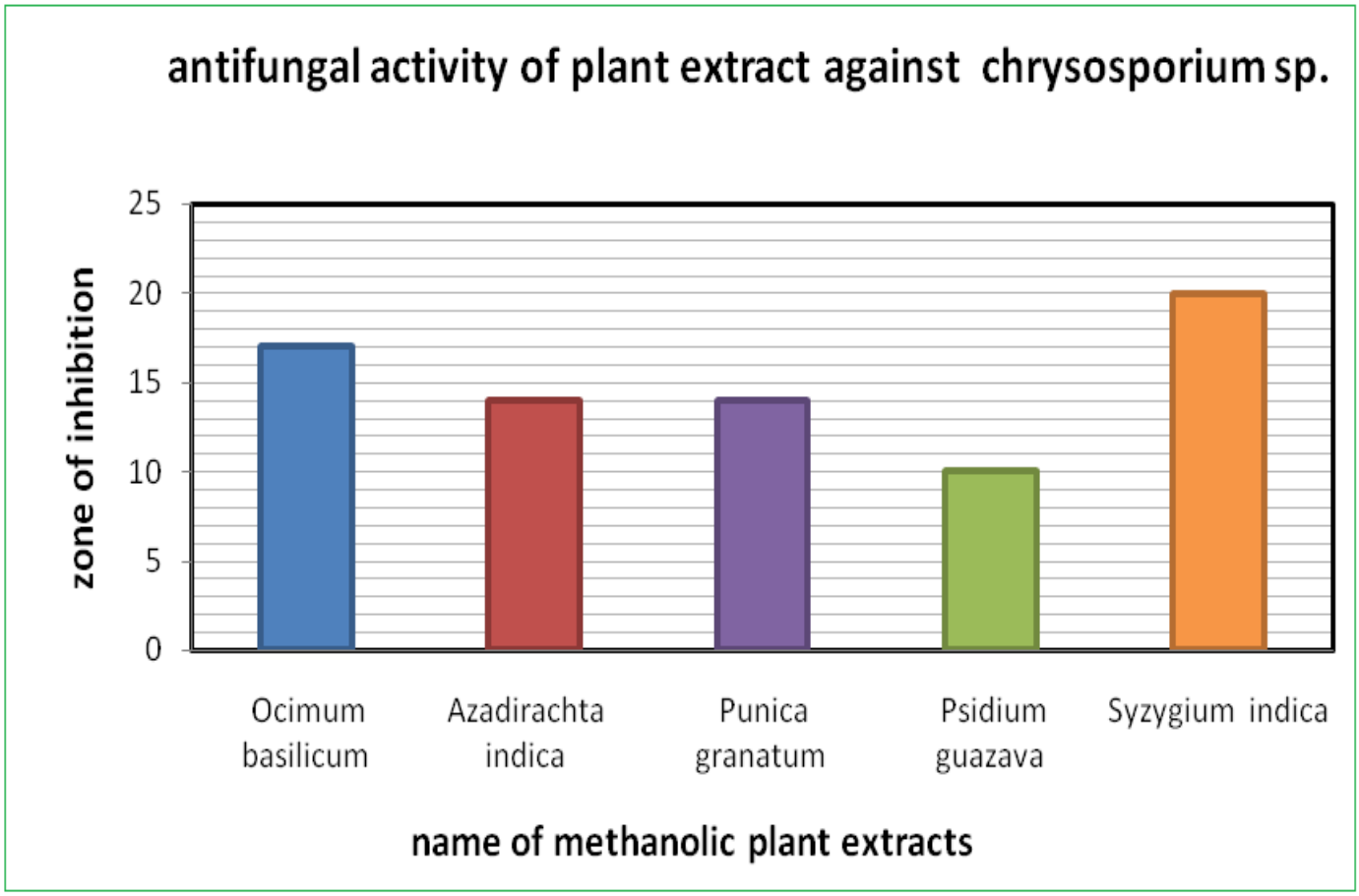
Figure No.8: Antifungal activity of different methanolic plant extracts on different isolated fungi:-


antifungal activity of plant extract against t. mentagrophytes
zone of inhibition
25
20
15
10
5
0
Ocimum basilicum
Azadirachta indica
Punica granatum
Psidium guazava
Syzygium indica
name of methanolic plant extracts


antifungal activity of plant extract against t. rubrum
zone of inhibition
30
25
20
15
10
5
0
Ocimum basilicum
Azadirachta indica
Punica granatum
Psidium guazava
Syzygium indica
name of methanolic plant extract


antifungal activity of plant extract against epidermatophyton floccosum
zone of inhibition
25
20
15
10
5
0
Ocimum basilicum
Azadirachta indica
Punica granatum
Psidium guazava
Syzygium indica
name of methanolic plant extract


antifungal activity of plant extract against aspergillus fumigatus
zone of inhibition
18
16
14
12
10
8
6
4
2
0
Ocimum basilicum
Azadirachta indica
Punica granatum
Psidium guazava
Syzygium indica
name of methanolic plant extracts


antifungal activity of plant extract against chrysosporium sp.
zone of inhibition
25
20
15
10
5
0
Ocimum basilicum
Azadirachta indica
Punica granatum
Psidium guazava
Syzygium indica
name of methanolic plant extracts

DISCUSSION:

Many investigations were carried out to discover plant products that inhibit the fungi like *Trichophyton rubrum* and *Microsporum canis.* These two species cause common infections in humans which are difficult to control effectively. Hence, plant products that inhibit their growth without harming the host represent potential therapeutic agent. Keratinophilic fungi are the fungi which utilize keratin which mainly grow on skin , nails, hairs etc.they may be Dermatophytes like *Trycophyton* and *Epidermatophyton* which cause skin infection etc and may be non- dermatophytes like Aspergillus niger , trycophyton sp. etc. Dermatophytes cause dermatophytosis which is a chronic infection of the nails, hair and skin. Now days considerd a serious problem for public health, In view of its high occurrence in the worldwide population (Elewski,et,al.,1998). Although this disorderis not serious in terms of mortality or physical or psychological sequelae, It has significant clinical consequence given its infection nature esthetic consequence, chronicity and therapeutic difficulties. The prevalence is probably higher than is currently thought as the difficulty in clinical mycological diagnosis, inappropriate collection of material for analysis as well as ineffective treatment make it hard to ascertain the true profile such dermatomycosis. In recent years, there has been an increasing search for new antifungal compound due to lack of efficacy, side effects and or resistance associate with some of the existing drugs. To fight against keratinophilic dermatophytic fungi in our present study we have tested combination of some essential oils and some methanolic plant extracts against them which are proved to be effective as plant essential oils area potentially useful source of antimicrobial compounds. Essential oils and their constituents have a long history of application as antimicrobial agents. Essential oils are often fungi static rather than fungicidal this means that they stop the growth of the fungi while it is ex-posed to the oils.

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