4.1. Literature review

Fungi are extremely diversified group of eukaryotes and may be single celled or multinucleated organism. They are set for survival in virtually all kinds of environments, from soil to water to even air. Fungi maintain their submissive presence, and manifesting existence mainly by opportunistic infection in immuno-compromised hosts. Fungal infections are common in different parts of human body, which include superficial skin, hair, nail, vagina, or internal organs. Most common fungus causing infection are mainly ubiquitous colonizers like *Candida* sp., *Cryptococcus* sp. and *Aspergillus* sp. (Perfect and Casadevall, 2002; Denning and Hope, 2010). Global Action Fund for Fungal Infections (GAFFI, 2018) says over 300 million people of all ages suffer from a serious fungal infection every year globally, and over 1.5 million of them are estimated to die from fungal disease. More than 1 million eyes go blind yearly due to keratitis; fungal spores contributed reactive airway diseases is seen in over 10 million individuals, and almost one billion people get attacked by skin mycoses every year. More than 400,000 people develop pneumocystis pneumonia annually and most of them die without treatment. Every year around 220,000 new cases of cryptococcal meningitis are reported worldwide, causing 181,000 deaths concentrated only in sub-Saharan Africa (GAFFI, 2018).

In human, fungal infections are the 4^{th} common cause of life-threatening infection, as a co-factor in AIDS epidemic, cancer chemotherapy and organ or bone marrow transplantation (Pfaller and Diekema, 2007). AIDS patients, who are already immuno-compromised, are reported to be attacked by *Pneumocystis carinii* (pneumonia) and oesophageal candidiasis in 60% and 20% cases respectively (Moore and Chaisson, 1996). During the current COVID-19 pandemic, cases of fungal infection in COVID-19 infected patients are reported worldwide, which are most commonly caused by *Aspergillus* sp., *Candida* sp., *Cryptococcus neoformans* and *Mucoralis* order (Hoenigl et al., 2022, Chiurlo et al.,2021). The distribution of fungal infection, particularly mucormycosis (commonly known as 'black fungus disease') was found to affect India during second wave (Aranjani et al., 2021). Treatment with corticosteroids and

mechanical ventilation predispose COVID-19 patients to get infected by these opportunistic fungi (Kumar et al., 2021). The triad of uncontrolled diabetes, use of corticosteroids and SARS-CoV-2 was evident for the increase of maxillofacial mucormycoses (Al-Tawfiq et al., 2021).

Climatic diversity and the hot and humid weather of India is suitable for fungal infection. Candidiasis, aspergillosis, cryptococcosis andzygomycosis are common opportunistic fungal infections in India. Although there have been major advancements in drug development against fungal infection, the morbidity and mortality still remain high due to therapeutic limitations, ranging from diagnostic challenges, toxicity of available drugs, limited routes of drug administration, drug solubility, stability to drug resistance (Denning and Hope, 2010). Current treatment for fungal infection is based on antifungal drugs such as Ketoconazole, Fluconazole, Amphotericin-B, Clotrimazole etc., which are proved to be inefficient in some cases or often cause nephrotoxicity or hepatotoxicity (Zapata-Garrido et al., 2003). Moreover, some fungal species are reported to be resistant to commonly used drugs (Rodrigues and Nosanchuk, 2020). All these facts emphasize the need for novel antifungal agents with a broad spectrum of activities and fewer side effects.

4.1.1. Phylogeny and classification

Fungi are filamentous microscopic spore bearing organisms. They have cell wall made with chitin and cellulose and they lack chlorophyll. Heterotrophic nutrition using organic source of nutrients is a common feature of all types of fungi. Traditionally they are a member of plant kingdom but in recent time scientists constitute a separate fungi kingdom. Fungal life cycle comprises of sexual or teleomorph state and asexual or anamorph state. Some filamentous fungi predominantly or exclusively produce conidia, external asexual spore and they are termed as deuteromycetes. Some fungi, called allergen, produce air borne spores and are called ascomycetes (Simon-Nobbe et al., 2008).

4.1.2. Infection caused by fungus

An impressive feature for survival of fungi is that, it is widespread. They survive everywhere, from air to water and soil, even as lichens inside Antarctic rocks. They can withstand a wide range of temperature. Another prominent factor for fungal growth is moisture. They can flourish well in hot and humid environment. The infection ranges from superficial skin to fatal systemic mycosis (Table 4.1). Fungal diseases have alarming impact on human health but remain neglected over the years. As it affects the poor population, it develops slowly and become worse progressively (if left untreated) and have long term consequences (Rodrigues and Nosanchuk, 2020).

Depending on degree of infected tissue the fungal infection may be classified into: a. Superficial or localized infection affecting skin, hair and nails, b. Subcutaneous tissue infection confined to dermis, c. Systemic infection involving deep internal organ, d. Opportunistic infection, where host is already immuno-compromised or immuno-deficient (Horner et al., 1995; Friedman et al., 1982; Salvaggio and Aukrust, 1981). Systemic fungal infection may be caused by either primary pathogenic fungi or by opportunistic fungi with marginal pathogenicity (Rodrigues and Nosanchuk, 2020). Sometimes central nervous system also gets infected by microorganisms as primary infection or uncommon ectopic site of infection. For example, *Aspergillus* sp. may create infection in brain abscess and gets in by inhalation. Some strains like *Candida* sp., *Mucor* sp., *Rhizopus* sp. also may attack CNS after entering through inhalation (Gilligan et al., 2014).

Table: 4.1. Common fungi infecting human and their clinical manifestations

Disease	Fungal Species	Common clinical manifestations of infection	Clinical status during infection	References
Aspergillosis	*Aspergillus* genera: *A. fumigatus, A. flavus, A. terreus, A.niger,*	Nodules, ulcers, skin necrosis, vesicles, indurated erythema, fever, chest pain, cough, hemoptysis, dyspnea, fatigue, shortness of breath, runny nose, headache, congestion, loss of smell, wheezing	People with weakened immune systems, lung diseases, COVID-19	Schmiedel and Zimmerli., 2016; Kniemeyer et al., 2011; Samson, 2007; Amin et al., 2021
Mucormycosis	*Mucorales* order: *Rhizopus* sp., *Mucor* sp., *Rhizomucor* sp, *Absidia* sp, *Lichtheimia* sp, *Apophysomyces* sp, *Cunninghamella* sp, *Saksenaea* sp,	Ecthyma gangrenosum-like lesions, muco-cutaneous ulceration and eschar, necrotic papulonodules, cellulitic plaques, necrotizing fasciitis, Rhino-cerebral: unilateral facial swelling, headache, congestion, fever, cough, nasal congestion, chest pain, shortness of breath, nausea, vomiting, gastrointestinal bleeding	Most common in diabetics, especially those with ketoacidosis, poorly controlled diabetes mellitus, hematopoietic stem cell transplant (HSCT) recipients and burn patients, COVID-19	Gnat et al, 2021, Gilligan et al., 2014; Amin et al., 2021
Candidiasis	*Candida albicans, C. glabrata, C.parapsilosis*, *C.tropicalis, C.auris*	Thrush or oropharyngeal candidiasis, vaginal infection, bloodstream infection, fever, chills, loss of taste, sore throat, Odynophagia	Use of catheters, intravascular or intracranial devices, solid organ transplantation, haematopoietic stem cell transplantation, COVID-19	Schmiedel and Zimmerli., 2016; Amin et al., 2021
Cryptococcosis	*Cryptococcus gattii*, *C. neoformans*	Cellulitis, ulcers, Pulmonary: fever, cough, fatigue, dyspnea, cryptococcal meningitis in HIV patients, Shortness of breath, headache, nausea, vomiting,confusion, chest pain, light sensitivity CNS: meningeal signs, focal neurological deficits	Tuberculosis, HIV/AIDS, Immunocompromised condition, COVID-19	Kohler et al., 2017; Miceli et al., 2011; Amin et al., 2021
Coccidioidomycosis (Valley Fever)	*Coccidioides immitis, C. posadasii*	Self-limiting mild flu-like in healthy people, severe disseminated infection	immunocompromised condition	Gnat et al., 2021

Disease	Fungal Species	Common clinical manifestations of infection	Clinical status during infection	References
Histoplasmosis	*Histoplasma capsulatum*	Self-limiting mild flu-like in healthy people,Pneumonia, immunocompromised condition	Healthy and immunocompromised condition	Gnat et al., 2021
Blastomycosis	*Blastomyces dermatitidis B. gilchristii*	Self-limiting mild flu-like in healthy people Pneumonia, immunocompromised condition	Healthy and immunocompromised condition	Kohler et al., 2017
Pneumocystis pneumonia	*Pneumocystis jirovecii*	Pneumonia	Immunocompromised individuals, especially with AIDS and Auto immune disease	Gnatet al., 2021; Chakrabarti, 2005
Necrotizing encephalitis, Rhinocerebralzygomycosis	*Mucor sp., Rhizopus sp.*	Aseptate hyphae in tissue, neural complications	Diabetics, neutropenic individuals	Gilligan et al., 2014; Firacative,2020
Dermatophyte infection	*Epidermophyton floccosum Microsporum canis, M. gypseum, M. audouinii, Trichophyton* sp.	Skin lesions, infection on keratinized tissue	Healthyor immunocompromised subject	Gilligan et al., 2014; Firacative,2020

4.1.3. Epidemiology

Systemic fungal infection has rapidly evolved in the last two decades. Medical advancement though improved general medication also led individuals at risk; such as people with indwelling catheter, patient in ICU, individual receiving immunosuppressive therapy or facing organ or stem cell transplantation, are prone to fungal infections. The most common fungal infection following organ transplantation is candidiasis (53%) followed by invasive aspergillosis (19%), cryptococcosis (8%), and zygomycosis (2%) (Pappas et al., 2010). Increase in size of population in general also causes increase in the size of immuno-compromised population, due to diseases such as blood cancer, bone marrow cancer and AIDS. Fungal infection is prevalent in patients treated with immunosuppressive drugs (Skalski et al., 2016; Wang et al., 2017). Massive population growth, rapid urbanisation and climatic change are increasing the risk of fungal infection day by day. Frequent natural calamities also contribute

to the changing epidemiology of fungal infection (Casadevall et al., 2019). Many fungi have developed the ability to grow and multiply within the host macrophage itself. In a healthy individual, at the site of infection, adaptive immunity cells form granulomas and later degenerate to scars and often calcify which may be seen after years on X-ray imaging. But patients with impaired immunity cannot control the infection and almost any organ can be affected by invading fungus (Wilson et al., 2002; Dasbach et al., 2000; Ben Ami et al., 2009; Rodrigues and Nosanchuk, 2020). There are some common fungi infecting HIV patients, imposing clear symptoms (Table 4.2). According to the Center for Disease Control and Prevention (CDC), fungi are among the leading causes of opportunistic infections. In fact, fungi cause most of the coinfections affecting patients with HIV/AIDS (CDC, 2017). Reports say, meningitis caused by the genus *Cryptococcus* is the second leading cause of death after tuberculosis in people living with HIV. Only in Latin America, approximately 30% of patients living with HIV/AIDS, die from histoplasmosis caused by *Histoplasma capsulatum*.

Table: 4.2. Common fungal co-infections in HIV patients and their areas of occurrence

Fungal disease	Causative agent	Main epicenters	References
Cryptococcal meningitis	*Cryptococcus neoformans,* *Cryptococcus gatii*	Sub-Saharan Africa, Southeast Asia	Armstrong-James et al., 2014
Pneumocystis pneumonia	*Pneumocystis jirovecii*	Asia, Latin America, sub-Saharan Africa	Skalski and Limper, 2016
Disseminated histoplasmosis	*Histoplasma capsulatum*	North America, sub-Saharan Africa, Asia (parts of China, India, Thailand, and South Korea)	Wheat et al., 2016
Disseminated penicilliosis	*Penicillium sp.* (Mainly *Penicillium marneffei*)	Southeast Asia, mainly North Thailand	Armstrong-James et al., 2014
Aspergillosis	*Aspergillus sp.* (Prevalently *Aspergillus fumigatus*)	Worldwide	Patterson et al., 2016
Coccidioidomycosis	*Coccidioides sp. (C. immitis* and *C. posadasii)*	Semiarid regions of North and South America	Wang et al., 2017
Paracoccidioido Mycosis	*Paracoccidioides brasiliensis*	South and Central America (most common in parts of Brazil, Colombia, and Venezuela)	Wang et al., 2017
Disseminated Emmonsiosis, adiaspiromycosis	*Emmonsia pasteuriana* (for disseminated emmonsiosis). *Emmonsia parva* and *Emmonsi acrescens* (for adiaspiromycosis)	Italy, Spain, China, and India	Schwartz et. al., 2015; Wang et al., 2017

4.1.3.1. *Candida* sp.

Candida sp. is the most common fungus affecting human subjects, and candidiasis ranges from superficial invasion of mucosal surface to systemic organ infection (Miceli et al., 2011). All *Candida* species are of round or oval shaped budding yeast found single, in chain or forming cluster. After entering the host tissue, they form both pseudohypha and hyphae using chain of blastoconidia, which helps to invade the host defence system. They grow rapidly and form colonies. Healthy human tissues normally targeted by *Candida* sp. are, oral site, gastro intestinal tract and women genital organ like vagina (Soliman et al., 2017). In situation of impaired host defence or change in ecological niche, they become pathogenic (Miceli et al., 2011). The virulent attributes of *Candida* sp. are wide, like adherence to the host surface and enzyme production (phospolipase, proteinase). They form biofilms to penetrate the epithelium with ease and to adapt in stressful condition (Samaranayake and Samaranayake, 1994). Different strains of *Candida* sp. target different system or organ of host to infect. For example,*C. albicans* mainly causes invasive fungal infection on the mucosal membranes (Miceli et al., 2011). *C. parapsilosis* causes candidal endocarditis and onychomycoses (Ge et al., 2019). *C. krusei* (*Issatchenki aorientalis*) mainly attacks patient after stem cell or bone marrow transplant. Non-albican species (NAC) isolated between 1952 and 1992 were *C. glabrata, C. lusitaniae, C. lipolytica, C. guilliermondii, C. dubliniensis* etc. Population based study in USA shows that *C. albicans* is the most common species followed by *C. parapsilosis, C. tropicalis* and *C. glabrata*. Blood stream infection (BSI) is increasingly caused by NAC species mainly by *C. glabrata* in western world (Pfaller and Diekema, 2002). In India among the NAC species, *C. tropicalis* is predominant in all age groups (Ahmed et al., 2020).

4.1.4. Treatment available

There is no effective antifungal vaccine till date, the diagnostic tools are very limited and the therapeutic options are restricted to a few conventional drugs which are found to be either toxic or otherwise expensive. Most recently

approved antifungal agent is echinocandin, developed on 2002 (GAFFI, 2018). It shows that the drug development field is progressing at a very slow pace. Classical antifungal drug amphotericin B, discovered in 1955, still remain the first line medication for some fungal infection like cryptococcal meningitis. United State Food and Drug Administration (FDA) approved five categories of drugs to be used clinically for treating fungal infection from 1955 to 2010. They are polyenes, pyrimidine analogues, azoles, allylamine and echinocandins. Among these five categories of drugs, azoles are intensively studied and modified to increase the efficacy (Vandeputte et al., 2012) which act on fungal cell membranes and disrupt normal functioning. Echinocandins destruct fungal cell wall structure impairing the synthesis of β-1, 3-glucan. Fungal nucleic acid synthesis and mitotic division are inhibited by intracellularly activating antifungal drugs of pyrimidine analogue group (Lewis,2011). Deoxymulundocandin, a drug of echinocandin group, showed positive effect during*in vitro* and *in vivo* study on *Candida* and *Aspergillus* sp. (Mishra and Tiwari, 2011). Ambruticin is found effective in aspergillosis and coccidioidomycosis (Chiang et al., 2006).

The conventional drugs are found to be effective, but showed side effects in multiple occasions. Most effective and least toxic formulations like liposomal AmB are very expensive, more over it is available only in six out of 116 developing countries worldwide (GAFFI, 2018). Sometimes, it is found that if some low-cost antimetabolites (for e.g., 5-fluorocytosine) is combined with other approved antifungal drugs, it is beneficial for treating systematic mucosis. But the fact is, most of the drugs are not available in many countries, especially where the disease is most lethal. High price antifungal drugs face market failure in developing countries (Rodrigues and Nosanchuk, 2020). In the present days, the field of research too is lacking in developing new effective and low-cost antifungal agents. Sometimes proper diagnosis can improve the situation; for example, the symptoms of infection caused by *H. capsulatum* are similar to tuberculosis and in most of the cases, without proper diagnosis, the patients are treated for tuberculosis and as a result they usually die within 1-2

weeks out of drug toxicity (WHO, 2019). Considering these intrinsic and extrinsic difficulties, it is more realistic and impactful to focus on the available diagnosis and antifungal tests and use natural resources derived agents for treatment to minimise the cost and number of deaths. There are ongoing initiatives for developing antifungal vaccines, antifungal agents and diagnostic tools; but emergence of multi-resistant and largely unknown pathogens such as *C. auris* (Casadevall et al., 2019) is making the situation complex.

4.1.5. Antifungal drug resistance

Persistence of progressive infection caused by any pathogen despite of application of drugs is called drug resistance. Many synthetic antifungal therapeutic drugs are found to be resisted by some fungal pathogen. These fungal species are considered to inherit the resistance to those drugs (Sanglard, 2003). For example, *A. terreus* and *Fusarium* sp. are resistant to amphotericin ß; whereas *C. neoformans* and *Zygomycetes* sp. are resistant to caspofungin. Fluconazole resistance is seen in *Candida krusei*and *Aspergillus* sp. (Rogers, 2006). The multi-resistant pathogen *Candida auris* has emerged as a serious global threat to human health in the recent years, which, in immunocompromised patients, cause infections resistant to all major classes of antifungal drugs. Climate change and the widespread use of antifungal drugs are some of the determining factors for the emergence of the drug resistant strains of *Candida* (Clancy and Nguyen, 2017; Casadevall et al., 2019).

4.1.6. Prospect of medicinal plants as antifungal agents

Medicinal plants are proved to be a rich source of antimicrobial agents. From ancient times they are used to fight against different types of microbial pathogens by the indigenous people of countries like India. In the present time considering the toxic effect and resistant variety of pathogens, scientists concentrate on developing novel molecules from natural resources against these pathogens or to fight against diseases. A wide range of medicinal plants, plant parts, leaves, roots etc. are screened for antifungal activity with satisfactory result (Table 4.3). Scientists have isolated some compounds from

plant sources with antifungal activity (Cowan, 1999; Ahmad et al., 2006; Dzoyem et al., 2007; Endo et al., 2010; Hu et al., 2014; Sukieum et al., 2017).

Table: 4.3. Medicinal plants and identified antifungal compounds

Scientific name	Common name	Class of Compound	Compound	References
Allium cepa	Onion	Sulfoxide	Allicin	Cowan,1999
Allium sativum	Garlic	Sulfoxide, terpenoids	Allicin, ajoene	Ahmad et al., 2006
Arnica montana	Mountain tobacco	Lactones	Helanins	Cowan,1999
Camellia sinensis	Green tea	Flavonoid	Catechin	Ahmad et al., 2006
Clausena excavata	Agnijol	Coumarin	Excavarin-A	Kumar et al., 2012
Croton cajucara	Chandra Sacaca	Essential oil	Linalool	Ahmad et al., 2006
Diospyros crassiflora	African ebony	Naphthoquinone	Plumbagin	Dzoyem et al., 2007
Gloriosa superba	Glory lily	Alkaloid	Colchicine	Cowan,1999
Humulus lupulus	Hops	Phenolic acids	Lupulone,humulone	Cowan,1999
Malus sylvestris	Apple	Flavonoid Derivative	Phloretin	Ahmad et al., 2006
Mentha piperita	Peppermint	Terpenoid	Menthol	Ahmad et al., 2006
Moringa oleifera	Drum stick	Protein	Mo-CBP2	Neto et al., 2017
Papaver somniferum	Poppy	Alkaloids	Opium	Cowan,1999
Piper betel	Betel pepper	Essential oils	Catechols, eugenol	Ahmad et al., 2006
Piper nigrum	Black pepper	Alkaloid	Piperine	Ahmad et al., 2006
Podocarpus nagi	Tree bard	Lactone	Negilactone	Ahmad et al., 2006
Punica granatum	Pomegranate peel	Polyphenols	Punicalagin	Endo et al., 2010;
Ranunculus bulbosus	Buttercup	Lactone	Protoanemonin	Cowan,1999
Rauvolfia serpentina	Rauvolfia	Alkaloid	Reserpine	Cowan,1999
Satureja Montana	Winter savory	Terpenoid	Carvacrol	Ahmad et al., 2006
Syzygium aromaticum	Clove	Terpenoid	Eugenol	Cowan,1999
Toddalia asiatica	Orange climber	Coumarin Alkaloid	8S-10-O demethylbocconoline	Sukieum et al., 2017
Vinca minor	Periwinkle	Alkaloid	Reserpine	Ahmad et al., 2006
Withania somnifera	Ashwagandha	Lactone	Withafarin A	Cowan,1999

To fulfil the demand of antifungal drugs which improve patient's recovery rate and shortens treatment time, there is no better way than exploration of vast natural resources. Our ancient therapeutic history and current research have given evidence that plant extracts exhibit antimicrobial and antifungal activities. The present study screens four such well known medicinal plants for antifungal activity.

4.2. Materials and methods

4.2.1. Plant materials

Leaves of all the four plants, *R. serpentina, M. oleifera, N. arbor-tristis, C. excavata* were collected from road side and forest areas of Jalpaiguri district of sub-Himalayan West Bengal.

4.2.2. Extraction and purification of excavarin-A from *C. excavata* leaves

Crude extracts from fresh leaves of each of the four plants were prepared as described in previous chapter (section 3.2.1). Purification of the antifungal compound excavarin-A was done from *C. excavata* leaves following the method of Kumar et al. (2012). The dry powdered leaves (200 mg) were extracted in dichloromethane in Soxhlet apparatus at 35^0C for 24 h and then concentrated under vacuum to obtain a brown sticky solid (14g). This crude extract was further subjected to silica gel column chromatography. Elution with n-hexane, n-hexane: dichloromethane (3:1, 1:1, 1:3), dichloromethane, ethyl acetate: dichloromethane (3:1, 1:1, 1:3), ethyl acetate, methanol: ethyl acetate (3:1, 1:1, 1:3) and methanol gave thirteen fractions (F1 to F13). Based on activity tested through bioautography on TLC plates, fraction F7 (2 g) was rechromatographed on a silica gel column, eluting with petroleum ether (200 ml) and ethyl acetate: hexane (2%, 5%, 10%, 15%, 20%, 25%, 30% and 35%) by which a total of 8 fractions (f1 to f8) were collected. The bioactive fraction f7 and f8 were fractionated into 40 sub-fractions (Sf1 to Sf40). Six sub-fractions (Sf30–Sf35) were pooled and crystallized with hexane–ethyl acetate to give the purified compound (100mg). Colourless needle shaped crystals of excavarin-A, a γ-lactone coumarin, was obtained whose identity was confirmed after being

analysed by UV–Vis, IR and NMR (1H- and 13C-) spectroscopy. The excavarin -A was also tested for antifungal activity by agar cup bioassay and MIC was also determined.

4.2.3. Test organism

The antifungal activity of the plant leaf extract was screened against the fungal strain *C.albicans* (MTCC183) obtained from Institute of Microbial Technology (IMTECH), Chandigarh, India.

4.2.4. Agar cup bioassay

The agar cup bio assay was performed to evaluate the antifungal activity of the selected crudeplant extracts and the purified compound excavarin-A following the method of Saha et al.(2005b). First potato dextrose agar (PDA) medium was autoclaved at 121°C for 15 minutes, then cooled to 45°C. Subsequently 1ml of pure fungal suspension ($1x10^5$cells/ml) of the test pathogen (*C. albicans*) was mixed with 19 ml molten medium. Then the mixture was poured into sterile Petri plates of 9 cm diameter. After solidification of the medium, agar cups were prepared with sterile cork-borers (4 mm diameter) in PDA plate, which were seeded with cell suspension of the test fungi. For screening the plant leaf extracts for their antifungal activities, different concentration of the methanolic extracts were placed (50 µl) into each well and the plates were incubated at 28^0C for 48-72 hours. Antifungal activity was determined by measuring the zones of inhibition of fungal growth around the wells. Measurement was done by using centimetre scale. The experiments were done in triplicate in complete aseptic condition and the mean value was noted.

4.2.5. Minimum inhibitory concentration (MIC)

Minimum inhibitory concentrations of the four medicinal plants and the isolated compound were tested against *C. albicans* by using the 96-well micro-titer plate assay following the method of Kumar et al. (2012). The crude leaf extracts and active constituent of *C. excavata*, excavarin-A, were serially

double diluted in methanol (5, 2.5, 1.25, 0.625, 0.3125, 0.156, 0.078, 0.039 mg/ml) and 50 µl of each of the different concentrations were poured into the wells of the micro-titer plate. The antifungal antibiotic nystatin was used for comparison. When the solvent was evaporated, a mixture (100 µl) of sterile PDB and fungal inoculums were loaded in each well. For positive control, only inoculum and PDB was taken. A negative control set was taken which contained the test compound and PDB only. The covered plates were incubated in a growth chamber at 28°C. Fungal growth was monitored after 48 h by measuring absorbance at 600 nm using a microtiter-plate reader (Mios Junior, Merck). A zero-hour reading was taken as blank. MIC was considered as the lowest concentration that did not record any growth.

4.3. Results

Methanolic leaf extract of *R. serpentina* of different doses were applied against pure cell suspension of the test pathogen (*C. albicans*) and after the period of incubation zone of inhibition (ZOI) was measured. Table 4.4 shows the ZOI (in cm) for three different concentrations of leaf extracts of four plants and also for excavarin-A. The ZOI was high in the case of *R. serpentina* leafextract. It was 4.87 cm at the concentration of 100 mg/ml. At the same concentration ZOI for *M. oleifera, N. arbor-tristis, C. excavata* were 3.2cm, 1.6cm and 2.4cm respectively. Excavarin-A emerged as most effective antifungal agent with a ZOI value of 4.6 cm at a concentration of only 10mg/ml.

The least MIC value was recorded for excavarin-A (0.078 mg/ml) followed by *R. serpentina* (0.156 mg/ml) (Table 4.5). Results indicated that both *R. serpentina* leaf extract and the molecule excavarin-A have antifungal activities close to known antifungal antibiotic nystatin (0.039 mg/ml).

Table: 4.4. Antifungal activities of crude methanolic leaf extracts of tested plants and the purified compound excavarin-A against *C. albicans*

Tested plant species	Zone of inhibition (cm) at different concentrations			
	100 mg/ml	10 mg/ml	1 mg/ml	0.1 mg/ml
R. serpentina	4.87	3.17	1.27	Not tested
M. oleifera	3.2	1.9	0.73	Not tested
N. arbor-tristis	1.6	0.8	0	Not tested
C. excavata	2.4	1.12	0.41	Not tested
Excavarin-A	Not tested	4.6	2.9	1.13

Table: 4.5. Minimum inhibitory concentration (MIC) of crude methanolic leaf extracts of tested plants and the purified compound excavarin-A against *C. albicans*

Studied extracts sample	MIC (mg/ml)
R. serpentina	0.156
M. oleifera	0.313
N. arbor-tristis	2.5
C. excavata	0.625
Excavarin-A	0.078
Nystatin	0.039

4.4. Discussion

The present study screened crude leaf extracts of medicinal plants for antifungal activities *in vitro*. *C. albicans* was used as test pathogen as it is the most common opportunistic fungal strain that may cause epidermal as well as potentially life threatening invasive-systematic infection, especially in immuno-compromised subjects or in immuno-suppressed patients. Commonly used antifungal drugs have toxic side-effects, moreover cases of drug resistance are increasing sharply, especially with long-term usage of the drug (Whaley et al., 2017). This creates an urgent need for development of new line

of treatment to fight against this noxious human pathogen, for which herbal remedy may be a good option.

Several medicinal plants have shown promising activities against *C. albicans in vitro* (Duarte et al., 2005; Soliman et al., 2017). In a recent study a protein molecule isolated from seeds of *M. oleifera*, named Mo-CBP2 was shown to possess antifungal activity against *C. albicans* (Neto et al., 2017). In the same year, extract from aerial parts of seven known medicinal plants were reported to have anticandidal activities (Soliman et al., 2017). Santos and Pereira (2018) enlisted several Brazilian traditional medicinal plants which were found to be effective against different strains of *Candida* sp. collected from mouth of infected population. Reports say that phytocompounds are potentially effective antifungal agents or they may work as synergistic agents with antifungal drugs (e.g., Fluconazole) particularly against *Candida* sp. (Lu et al., 2017). Plants having high flavonoid content showed good antimicrobial activity, probably due to their ability to complex with extracellular or soluble proteins of microbial cell walls (Marjorie, 1996; Tsao and Yang, 2003).

In the present work, leaf extracts of all four plants had shown good flavonoid content in phytochemical analysis, so they were further screened for antifungal activity. Excavarin-A emerged as most effective antifungal agent, which is in agreement with the previous finding of Kumar et al. (2012) who had isolated the novel molecule excavarin-A from leaf extract of *C. excavata* and screened it for antifungal activities. In our study, the crude methanolic leaf extract of *C. excavata* was also found to be antifungal when tested against *C. albicans*, *in vitro*. Leaf extract of *C. excavata* has been reported as potent therapeutic agent having wound healing and antioxidant activities *in vivo* (Albaayit et al., 2015). Antibacterial activity of ethanolic extract from the leaves of four Rutaceae species, including *C. excavata*, was reported in a recent study (Van et al., 2020).

The MIC value was lowest for excavarin-A followed by *R. serpentina*. There are previous reports on screening of extracts of different plant parts of

R. serpentina for antimicrobial activities (Negi et al., 2014). Elizabeth (2017) found significant antifungal activity of crude extract of *R. serpentina* when tested against human pathogen *C. albicans*. Our study also showed that the antifungal activities of *R. serpentina* leaf extract and excavarin-A were close to the activity of the antifungal antibiotic nystatin. Therefore, these may be potent candidates for effective antifungal agents of natural origin.

M. oleifera is a well-known traditional medicinal plant in Asia, it has also been a part of food habit in India. We found methanolic leaf extract of the plant had good antifungal activity against *C. albicans*. Similar trend was observed in some previous reports which screened different parts of *M. oleifera* for antimicrobial activities (Kalpana et al., 2013; Zaffer et al., 2015; Neto et al., 2017). Reports say extracts of different parts of *N. arbor-tristis* were screened for *in vitro* antifungal activity and showed significantly positive results previously. Different extract of bark of *N. arbor-tristis* was found active inhibitor of *C. albicans* and *Aspergillus fumigates* when compared with ketoconazole as standard antifungal drug (Sharma et al., 2013). Shrivastava et al.(2018) reported antifungal activities of different parts of *N. arbor-tristis,* which is in accordance with our findings. Further work is needed for isolation of biomolecules responsible for the antifungal property of these tested leaf extracts.