6.1. Literature review

In the humid and wet weather conditions of North Bengal, fungal infection is very common among local people. In general, fungal infections are the most common cause of many skin diseases in most of the developing countries. Opportunistic fungal infections, mainly resulting from the species of *Candida*, *Cryptococcus* and *Aspergillus* are life-threatening in immuno-compromised patients especially when affected with AIDS, cancer, or organ transplant. Resistance of many pathogens towards antifungal drugs is also a major factor that limits appropriate treatment. Therefore, it is necessary to search for more effective and less toxic novel antifungal agents from natural sources.

C. albicans is the most common causative agent of human infection among all *Candida* species, where infection ranges from mucosal surface to systemic organ (Miceli et al., 2011). Previous studies have shown that some plant extracts possess significant antifungal activities against different strains of *Candida* sp. but all are mainly screened *in vitro* (Soliman et al., 2017; Neto et al., 2017). The preliminary stage of clinical investigation for identification of biomolecules having antifungal activity is *in vitro* testing of natural materials which are mainly crude botanical extracts. However, this is not enough to invent new line of drugs because *in vitro* studies do not consider the individuals body system, organic metabolism or idiosyncrasies. Very few studies show antifungal activities of medicinal plant extracts on animal models. The latest advancements have focused primarily on mechanisms of action of natural compounds against opportunistic fungal pathogens at the genomic, molecular or proteomic levels (Martins et al., 2015).

6.1.1. *In vivo* evaluation of antifungal activity against candidiasis

In recent past, several studies on *in vivo* antifungal activities of medicinal plants evaluated on plant pathogens have been reported (Seepe et al., 2020; Dhar Purkayastha et al., 2018); but *in vivo* study on fungal pathogen of clinical importance still remains scarce. In this aspect, most of the *in-vivo* studies are concerned with candidiasis as this is the most frequently encountered fungal disease (Garber 2001). In a search for new antifungal treatments, the

mammalian animal models play a very important role for observing *in-vivo* efficacies. While mice are the most widely used models (Scorzoni et al. 2016), the ultimate choice of animal species along with the route of inoculation of the organism depends on the desired goal. In this review, the findings of few such studies are deliberated.

Zhang et al. (2005) studied the antifungal activity of eight steroid saponins isolated from n-butanol extract of *Tribulus terrestris* against six fluconazole resistant yeasts. *In vivo* testing of the purified saponin TTS-12, which showed maximum efficacy in preliminary tests was performed by the authors in a vaginal infection model, oestrogen-dependent rat vaginitis, developed with fluconazole-resistant *C. albicans*. The results showed remarkable therapeutic effect on candidal vaginitis upon vaginal administration of TTS-12. In another *in vivo* study, methanolic extract of *Syngonanthus nitens* scapes was tested in animal model (rat) infected by different *Candida* strains collected from patients with valvovaginal candidiasis. The treatment with cream containing the extract at different doses showed rapid clearance and eradication of vaginal fungal burden in experimental animals after 8 days of treatment (Araújo et al., 2013). Fabri et al. (2021) tested the antifungal activity of spilanthol, a bioactive alkylamide from the native Amazon plant species, *Acmella oleracea*. *In vivo* evaluation in an experimental vulvovaginal candidiasis model showed that infected rats treated with spilanthol recorded steady reduction of fungal burden when compared to the untreated animals.

The antifungal activity of aqueous extract of *Nigella sativa* seeds was studied on candidiasis in mice (Khan et al. 2003). Treatment of mice that were injected intravenously with *C. albicans* with the plant extract resulted in considerable inhibitory effect on the growth of the organism at the level of 5-fold decrease in cfu in kidneys, 8-fold in liver and 11-fold in spleen. In another study,antifungal activities of acetone extracts of leaves from *Combretum* species were tested against *C. albicans* and other pathogenic fungi associated with wound infections on immunocompromised Wistar rats (Masoko et al., 2010). The authors made wounds on the back of the rats and treatments were

administered topically using the extracts at 20% concentrations in aqueous cream. Their results showed that in the treated group, wound healing was evident as a rigid, dark, and thick crust formation after three days, but the lesion without treatment took longer time to heal. In a similar study, Sahgal et al. (2011) used crude methanolic seed extract of *Swietenia mahogany* against *C. albicans*. Here, experimental mice were infected with *C. albicans* suspension via injection at lateral tail vein and subsequently treated with 2.5 g/kg of seed extract by intraperitoneal injection after 24 hours. Their results showed significant reduction of the pathogen counts in both blood and kidney when compared to control group. In histopathological observations, the authors found that the treated kidney failed to exhibit the presence of either *C. albicans* or pseudomycelia. Similar study was performed by Jothy et al. (2012) with methanolic seed extract of the plant *Cassia fistula*. Mice were injected with *C. albicans* via intravenous route and treatment was performed with seed extract at 2.5 g/kg body weight administered intraperitoneally once daily for 3 days. Results revealed a six fold decrease in *C. albicans* burden in kidney and blood sample in the curative group mice when compared with those of the control group.

Dzoyem et al. (2014) studied the antifungal activity of methanol-dichloromethane extracts of twenty- one spices used in Cameroon. *In vivo* activity of *Olax subscorpioidea* extract which exhibited maximum activity during *in vitro* evaluation was tested in rat model of disseminated candidiasis caused by *Candida albicans*. The results revealed that oral administration to artificially infected rats at the dose of 2 g/kg of body weight recorded a significant reduction of cfu of *C. albicans* cells in the blood while a moderate decrease was observed in the kidney.

6.1.2. *In vivo* evaluation of antifungal activity against other superficial infections

Superficial fungal infections are found in the outermost layers of the skin, nails, hair and mucous membranes. There has been an increase in the incidence of these infections, mainly because of the increasing number of immunocompromised patients (Garber, 2001). The most common types of skin

diseases are caused by dermatophytes such as *Trichophyton* sp., *Microsporum* sp. and *Epidermophyton* spp. although non-dermatophyte yeasts such as *C. albicans* and other non-albicans *Candida* sp. along with the moulds, *Aspergillus* sp., *Fusarium* spp., *Acremonium* sp., *Scopulariopsis* sp. and *Scytalidium* sp. are also reported as causative agents in a growing number of cases (Tosti et al., 2000).

One of the past studies screened 50% ethanolic leaf extract of *Leptadenia reticulata* for antifungal activity against *Aspergillus flavus*. Here, wounds were made surgically in male albino rats on the posterior mid dorsal side of the animal and fresh spore suspension of *A. flavus* was introduced in the wounds by injection as a single dose. Treatment was done by application of cotton swab soaked in the plant extract on the wound at regular intervals of twice a day for 8 days which showed visible healing sign and the swab made from the healing wound region showed absence of the fungal hyphae (Sureshkumar, 2008).

Li et al. (2015) studied the antifungal activity of ellagic acid purified from the plant *Euphorbia humifusa*. Dermatophytosis was induced in guinea-pig infection model using suspension of *Trichophyton rubrum* as inoculum. Subsequently, the test compound was administered topically mixed with an ointment base once a day for 14 days. The treatment significantly enhanced the cure rate at all tested doses and microscopic examinations for the pathogen in the treated animals yielded negative result.

Garrido et al. (2015) tested efficacy of five Mexican traditional medicinal plants against human superficial mycoses causing fungus *in vivo*. Dermatophytosis was induced with *Trichophyton rubrum* in the sole of feet in mice to develop the tinea pedis model and subsequently from day 14 post-infection, the mice were treated intragastrically daily for seven days with 2.5 and 5 mg/kg of acetone extract of *Berberis hartwegiior* and *Zanthoxylum caribaeum* respectively. After 7 days of treatment with *B. hartwegii,* an improvement of100 with no signs of disease was observed. *Z. caribaeum treated* mice also showed significant improvement of disease condition.

In the present study, methanolic leaf extracts of *R. serpentina* and *M. oleifera* was screened for antifungal activities *in vivo* using rats as model animal after getting significant results in the *in vitro* experiments with the same leaf extracts against *C. albicans*.

6.2. Materials and methods

6.2.1. Preparation of plant extracts

Leaf extract of plants were prepared following the procedure described in previous section (Section 3.2.1).

6.2.2. Effect of plant extracts *in vivo*

For *in vivo* experiment, male albino rats of similar weight (80-100 gm) and age groups (10-12 months) were taken. Altogether 15 animals divided into 3 groups were infected with *C. albicans*. Of these five infected rats were untreated and marked as control group. Rest of the 10 animals were treated with plant extracts.

6.2.2.1. Infection with *C. albicans*

A wound of skin depth (round, 1 cm diameter) was made with sterilized surgical blade at mid posterior dorsal side of each animal. The test fungal strain *C. albicans* was cultured in PDA medium and a cell suspension of$1x10^5$ cells/ml was used as inoculum. The prepared solution was applied superficially on wounds on the skin of test animals with cotton swab at a dose of 1ml in each case. It took 2 days to develop skin infection at the site of application of fungal spore suspension (Sureshkumar, 2008). The animal ethics committee of the institute (IICB, Jadavpur, Kolkata) approved all animal experiments.

6.2.2.2. Treatment with plant extract

Ten infected animals were treated with diluted plant extracts superficially on the wounds. One milligram of crude extract was dissolved in minimum volume of physiological saline (0.9% NaCl) and applied over the wound of each infected animal. Five infected rats were treated with *R. serpentina* leaf extract and remaining five were treated by *M. oleifera* leaf extract. This process was repeated in regular intervals of twice a day for next 8 days. The whole

experiment was performed thrice. All the animals were kept in uniform diet and environment throughout the period of experiment.

6.2.3. Recovery of pathogen from test animals

After 8 days of treatment with plant extracts, fungal loads on the wounds were estimated by standard Lacto-Phenol Cotton Blue (LPCB) and Gram staining methods. For this, sample was recovered from the dry wound using a sterile swab from both treated and control groups and smeared on the microscopic slides. The slides were then stained and finally the stained samples were observed under the microscope (Olympus).

For LPCB staining, one or two drops of the stain was added to cover the smear. One edge of the drop of stain was touched with a cover slip edge, and lowered gently, avoiding air bubbles to cover the liquid. The preparation was thus ready for examination (Procop, 2017).

Although Gram stain is useful in staining bacteria, certain fungi such as *Candida* and *Cryptococcus* are observed to be Gram positive yeasts. For Gram staining fungal strain, the smear collected from the wound was spread on glass slide and fixed and dried by rapidly passing the slide through the flame of spirit lamp (keeping the smear uppermost). The fixed smears were then covered with crystal violet stain for 30-60 seconds. The stain was rapidly washed off with clean water, and the smear was covered with Lugol's iodine for 30-60 seconds to form crystal violet-iodine complex. Next, iodine was washed off with clean water and decolorized rapidly (within few seconds) with acetone-alcohol, and again washed immediately with clean water. At last, the smear was counterstained with Safranin solution for 30 seconds. The stain was then washed off with clean water and examined under microscope (Procop, 2017).

6.2.4. Analysis of blood sample of test animals

After the period of treatment, on 9^{th} day of experiment, blood samples were collected from both control and treated rats. The blood samples were collected on glass vial containing EDTA (anticoagulant) and the total count (TC) of the leukocytes was determined. For finding the total leukocyte count,

a sample of blood was diluted with Turk's fluid (1.5 ml glacial acetic acid, 1.5 ml of 1% gentian violet solution and 100 ml distilled water) which destroyed the RBC and stained the nuclei of the leukocytes to make them visible. Initially 1 ml of Turk's fluid was taken in a watch glass, the counting chamber was placed on a microscope stage containing 16 WBC squares. A WBC pipette was filled with the blood sample up to the mark 0.5 and Turk's fluid was sucked to the mark of 11, and the blood and fluid were mixed thoroughly. That gave the dilution of ratio 1:20. In another pipette, blood was drawn up to mark of 1 followed by Turk's fluid up to mark of 11. After discarding first two drops of fluid from the pipette, the chambers on its both sides were charged. The cells were allowed to settle for the next 3 to 4 minutes and the chambers were transferred under the microscope, first under low magnification and then switched to high magnification. The leukocytes were seen as round shaped cells with clear unstained cytoplasm and deep blue-violet nuclei. WBCs were counted in six squares marked in the counting chamber, where cells were in four groups of sixteen squares, i.e., in a total of 64 squares. In a counting chamber, the volume of a square is 1/160 mm^3, therefore the volume of 64 squares is 4/10 mm^3. Thus, the total volume of diluted blood, in which WBCs were counted, was 4/10 mm^3. By multiplying the number of WBC count by 10/4, the WBC count in 1 mm^3 diluted blood was found. The blood was diluted 20 times, so 1 mm^3 of undiluted blood from the first pipette contained A x 10/4 x 20=A x 50 WBCs (A=WBC count). In case of second pipette, it was A x 10/4 x 10=A x 25. Two counts were taken for comparison and confirming the accuracy (Procop, 2017).

6.2.5. Data analysis

Total count of WBC (mean $\pm$ SD) were analysed by student "t" test.

6.3. Results

The wounds caused by the infection of *C. albicans* were treated with leaf extracts of *R. serpentina* and *M. oleifera*; this caused healing of infection which was reflected by the reduction of fungal load in the samples collected from treated rats. The observation was confirmed by LPCB and Gram staining

methods. On the other hand, in infected but untreated rats, a significant presence of *C. albicans* was observed in samples collected from the wound after 8 days (Table 6.1).

The process of recovery from infection is faster in *R. serpentina* treated animals in comparison to *M. oleifera* treated group. On the fourth day of treatment with *R. serpentina* leaf extract, the scar on skin of the experimental animal totally disappeared; but in case of *M. oleifera,* few signs of infection were still present (Fig: 6.1). On the eighth day of treatment, the body fur was regained at the site of the wound in *R. serpentina* treated group, but very little skin hair was observed in case of *M. oleifera* treated animals.

In both *R. serpentina* and *M. oleifera* treated rats, there was a significant increase in mean WBC count in comparison to untreated group indicating that both the plant leaf extracts have a protective role in reducing fungal burden in the animals. (Table: 6.2).

Table: 6.1. Treatment of skin lesion induced by *C. albicans* using plant extracts.

Treatment	Occurrence of *C. albicans* at different days during treatment			Nature of wound at different days during treatment		
	0 d	**4 d**	**8 d**	**0 d**	**4 d**	**8 d**
untreated control	+++	+++	+++	Severe	Severe	Severe
Infected and treated with *R.serpentina* leaf extract	+++	+	-	Severe	Superficial	Absent
Infected and treated with *M. oleifera* leaf extract	+++	++	+	Severe	Moderate	Superficial

+++ High occurrence, ++Moderate occurrence, +Low occurrence, -Absence

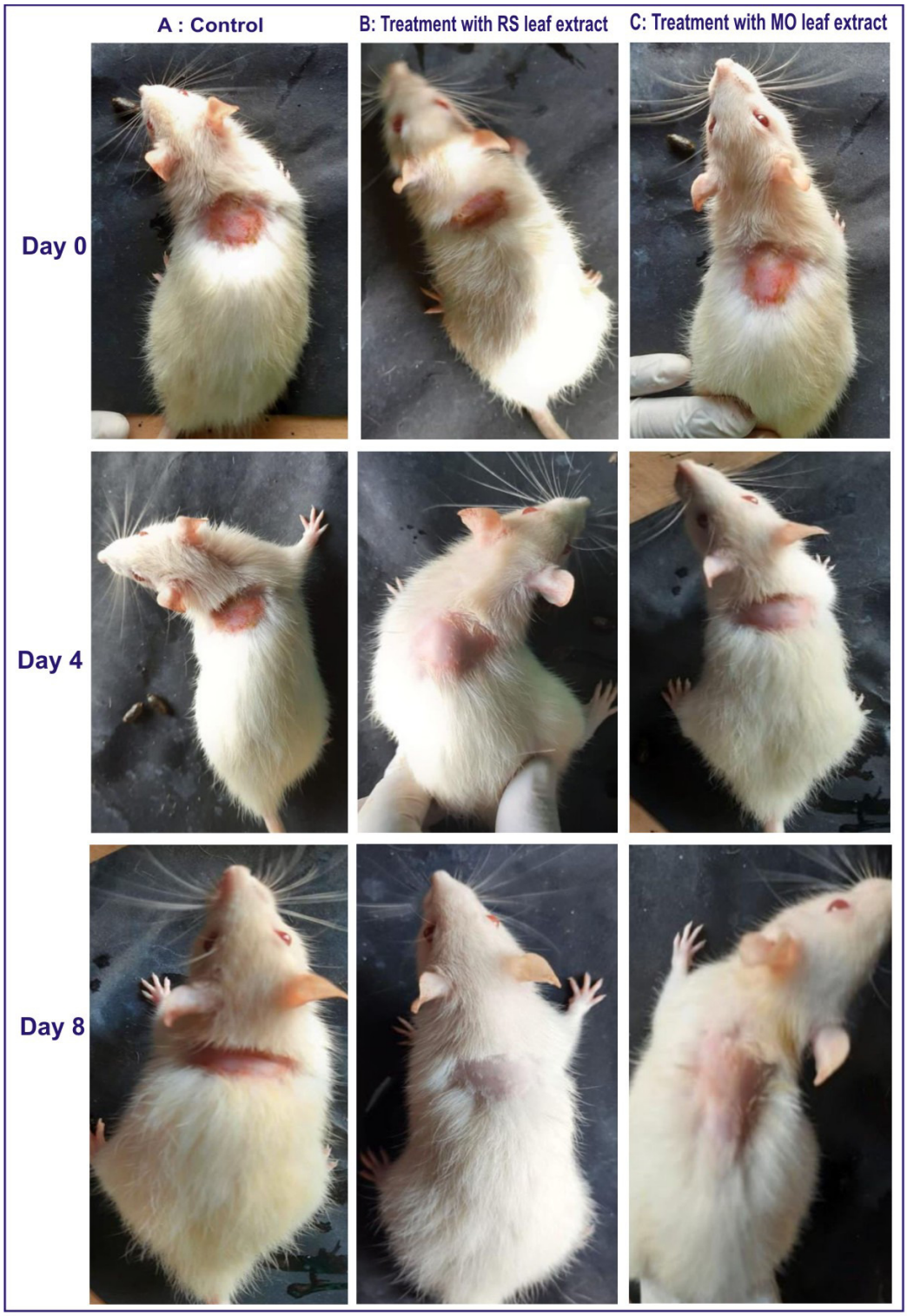

Figure: 6.1. Effect of plant extracts on superficial infections by *C.albicans* on experimental animals: A. Control group experimentally infected with *C.albicans*. B. Infected animals treated with leaf extracts of *R. serpentina* (RS) and C. Infected animals treated with leaf extracts of *M. oleifera* (MO)

Table: 6.2. Total leukocyte count in blood samples of experimental rats infected with *C. albicans* and treated with plant extracts.

Sample	White blood cell count[#] Cells/mm^3
Infected but untreated rat	7634±141
Infected rats treated with *R. serpentina* leaf extract	8735±130
Infected rats treated with *M. oleifera* leaf extract	8642±122

[#] (average no. in 5 rats± SD)

6.4. Discussion

As eukaryotic pathogens, human fungal diseases differ from other infections fundamentally. Fungi share many similarities with their host cells, this impairs the activity of antifungal compounds. A single fungal pathogen can infect a wide range of cell types and multiple tissues in the same patient especially if the host is immune compromised in any way (Rodrigues and Nosanchuk, 2020).The Global Action Fund for Fungal Infections has highlighted the devastating impact of focal fungal diseases in patients in advanced stage of AIDS(GAFFI,2018). In addition, patients on anticancer therapies and other immunosuppressive medications are affected, which have contributed to the excess number of deaths due to fungal diseases worldwide. The Center for Disease Control and Prevention (CDC) and the World Health Organization (WHO) have emphasized on this fact in their reports (CDC, 2017; WHO, 2020b). Despite this fact, fungal diseases have been continually neglected over the years and persistence of its neglected status is caused by failures in science, market, and public health. The financial support for fungal disease research is incredibly low, drug development field is progressing at a very slow pace. Classical antifungal drugs Amphotericin ß discovered in 1955, still remain the 1st line medication for fungal infection. The affordable conventional formulations include significant side effects. On the other hand, most effective and least toxic formulation like liposomal Amß, is available at a very high cost.

As the deadliest fungal infections affect mainly the neglected and poorest populations of the world, it faces market failure in the form of lack of interest from the pharmaceutical sector in the development of medicines, vaccines, and diagnostic tests for human fungal diseases or for drug commercialization (Rodrigues and Nosanchuk, 2020). In this scenario, it is the need of the hour to unturn the rubble from natural resources in search of new therapeutic formulation which is effective, low cost and devoid of any side effect.

In the last two decades, phytotherapeutic agents derived from natural resources with antimicrobial and antifungal potentiality, has grabbed the attention of scientific researchers (Martins et al., 2014). Various *in vitro* studies have been conducted to screen the antifungal activities of different medicinal plants. But plant-derived bioactive molecules show synergic, antagonistic and polyvalent relationship when they enter into human body. Their mode of action, structural and functional conformations are also modulated due to organic metabolism or in complex environment of the host body (Martins et al., 2015). Thus, *in vitro* screening in laboratory set up is not enough to establish the activity of any plant extract or any biomolecule derived from medicinal plants; and so, *in vivo* screening and clinical trials are needed (Sealbert et al., 2000; Alviano et al., 2009). Some studies are reported in past showing antifungal activities of medicinal plants *in vivo* (Masoko et al., 2010; Araújo et al., 2013; Sahgal et al., 2011; Jothy et al., 2012; Garrido et al., 2015). The present study intended to further extend the positive findings of *in vitro* screening in animal model.

A very simple *in vivo* experiment was designed where skin infection was developed in experimental animals with *C. albicans,* and then effectiveness of the plant extracts was observed by superficial application. Although the study was very basic in nature, it showed visible results. Superficial infections on experimental animals (male albino rats) were treated with diluted methanolic leaf extract of *R. serpentina* and *M. oleifera*, twice a day for the next 8 days, and the control group remained untreated. Standard LPCB and Gram staining showed significant recovery which were also visible prominently with the

naked eye. *R. serpentina* showed better healing in comparison to *M. oleifera,* but both the plant extracts reduced skin infections when compared to the untreated group. On the fourth day of treatment, the scar completely faded out in *R. serpentina* treated animals, and on 8th day the skin hairs reappeared. In case of *M. oleifera* treated group of animals, on the 8th day of treatment, the scar disappeared totally but very little amount of skin hair was observed at the site of wound. Haematological analysis of blood samples from animals of both treated and untreated groups showed significant results.

Total count of WBC had increased in both cases of treated animals. This finding was in agreement with previous work described by Suresh Kumar (2008). As a part of host's innate immune system, neutrophil, leukocytes or polymorphonuclear leukocytes (PMNLs) are known to function as major phagocytic cells, which cause elimination of the fungi during fungal infection (Demirezen et al., 2015). The elevation in total count of WBC which was found by haematological analysis of blood samples of treated animals, suggests that the leaf extracts of both the plants have protective roles in improving host defence to counter fungal attack. From the findings of the present work, it is evident that methanolic leaf extracts of *R. serpentina* and *M. oleifera* possess antifungal activities against *C. albicans*. The active principles responsible for the antifungal activities, and their mode of action may be explored through further research.