STUDIES ON SOME PHYTOEXTRACTS FOR THEIR ANTIFUNGAL ACTIVITIES AGAINST *MICROSPORUM CANIS*

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Dermatophytes are responsible for a majority of superficial fungal infections. They are distinctive from other pathogenic fungi in their ability to use keratin as living. They can sustain in different ecosystems that added in their morphological and ecological range with high flexibility to changing environmental conditions. Lately, *Microsporum canis* has evolved as a resistant pathogen against several antifungals and has been in the focus of interest for causing outbreaks and epidemics in humans from domestic animals. The increasing evolution of resistance, therapeutic failures, and reduced number of available drugs stimulated to investigate therapeutic alternatives by using some commonly available plants empirically used for their antifungal properties. Likewise, the present study reports the *in vitro* antifungal efficacy of crude as well as fractionalized extracts with different solvents of *Ficus racemosa* leaves (L.), *Cassia fistula* L. and *Nerium oleander* L. Antifungal activity was evaluated according to Clinical Laboratory Standards Institute (CLSI) protocol by determination of Minimum Inhibitory Concentration (MIC's) values on a clinical isolate of *M. canis* that showed resistance to some commonly used antifungals. All crude phyto-extracts except *Nerium oleander* L. effectively inhibited the growth of *M. canis*. The methanolic extract of *Ficus racemosa* L. and *Cassia fistula* L. completely inhibited the fungal growth at a concentration of 2 µg/mL and 8 µg/mL respectively. These two phyto-extracts proved to have significant antifungal activity against common antifungal resistant *M. canis* isolate. Further studies with the identified plant parts through *in vivo* studies and pin-point isolation of the active compound may prove beneficial as an effective alternative therapy for the pathogen.

Key words: Antifungal resistance, *Ficus racemosa*, *Microsporum canis*, Phytoextracts

Dermatophytes are specialized pathogenic fungi having the ability to invade keratinized structures viz. cornified skin layers, hair, and nails causing a superficial cutaneous infection called dermatophytoses/ringworm (de Hoog *et al.*, 2017). They are distinctive from other pathogenic filamentous fungi in their ability to use keratin as their living. They can sustain in different

ecosystems that added in their morphological and ecological range as well as high flexibility to changed environmental conditions (Singh *et al.*, 2020). As per the new taxonomic tree, they are classified into nine clades based on multi-locus genetic analysis: *Trichophyton*, *Microsporum*, *Epidermophyton*, *Arthroderma*, *Nannizzia*, *Lopophyton*, *Paraphyton*, *Ctenomyces* and

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Guaromyces against only three pathogenic genera *Trichophyton*, *Microsporum* and *Epidermophyton* used to be considered earlier (de Hoog *et al.*, 2017). *Microsporum canis*, one of the zoonotic zoophilic dermatophyte can be isolated from dogs and cats with or without skin lesions (Singh *et al.*, 2018; Moriello, 2019) and has recently found to develop resistance against various antifungals like fluconazole, amphotericin-B and terbinafine (Aneke *et al.*, 2018). It produces arthroconidia which can remain infectious in the environment for as long as 12 to 24 months and this is the phase that plays crucial in the epidemiology of dermatophytoses through infection and re-infection (da-Cunha *et al.*, 2019).

Fungi are one of the most neglected pathogens as demonstrated by the fact that amphotericin B, a polyene antibiotic discovered as long ago as 1956, is still used as a “gold standard” for antifungal therapy (Faustino and Pinheiro, 2020). At the same time, the development of effective antifungal drugs is lagging so much that the future pipeline has been dry (Perfect, 2017). Over the last three decades, rampant uses of antifungals have led to the evolution of resistance and failures in the treatment which have accentuated the serious problem of antifungal resistance (Ortiz and Torres, 2018). Also, most of the clinically used antifungals have various drawbacks concerning toxicity, efficacy and cost (Graninger *et al.*, 2019).

In October 2013, World Health Organization (WHO) announced the 2014-2023 traditional medicine policy to offer guidance for countries to elaborate regulation, effective governance, and to integrate traditional medicine practices in modern medicine (WHO, 2013). Plant-derived bio-active constituents comprise safer and more effective substitutes for synthetically produced antimicrobial agents (Ndlovu *et al.*, 2019), and resistance against them is rarely observed (Warnke *et al.*, 2009). Also, the capacity of natural products to act on multiple cell targets is key to combat resistant microorganisms that do

not respond to conventional therapies (Lopes *et al.*, 2017). With this backdrop, the present study was aimed to determine the *in vitro* antifungal efficacy of crude and fractionalized purified extract of three empirically used plants namely *Ficus racemosa* L., *Cassia fistula* L. and *Nerium oleander* L. against common-antifungal-resistant *M. canis* isolated from asymptomatic pet kitten.

MATERIALS AND METHODS

Plant materials used: Leaves of the *Ficus racemosa*, *Cassia fistula*, and *Nerium oleander* plants were collected from Kolkata, West Bengal from a period of February 2020 to April 2020. Primarily, the plants which were reported to have some antibacterial or antifungal properties were selected. Secondly, their availability throughout the year was judged and parts having regeneration capacity to make the study sustainable were considered. Initially, the collected leaves were rinsed with distilled water to remove soil and other impurities and then dried in a cool and dark place for further processing.

Preparation of plant extract (crude extract): The dried leaves of the selected plants were crushed in a grinder to make a powder. For hot crude extract, 100 g of dried powder was weighed and extracted with 250 mL of ethanol in a soxhlet apparatus at 40°C for 12-14 cycles and for cold crude extract the same amount was kept in shaker incubator for four to five days or until the extraction was completed (Lopez-Bascon and de-Castro, 2020). The final recovered extract was passed through a rotary evaporator to remove the solvent and the extract was dried under vacuum and stored in an amber-colored bottle at 4°C for further use. Both the extracts (hot and cold) of each plant were subjected to antifungal sensitivity testing (AFST) using the Clinical Standards Laboratory Institute (CLSI) guidelines (NCCLS, 2002).

Preparation of fractional extraction: Out of a total of six crude extracts, the best two crude extracts showing lowest MIC value were subjected to different solvent extractions as per the

phytochemical property of extract to isolate most suitable solvent fraction(s) containing bioactive components responsible for antifungal activity in a column chromatography viz. butanol, hexane, chloroform and methanol according to the protocol suggested by Coskun (2016).

Fungal isolate selection and inoculums preparation: The selected clinical isolate of *M. canis* (Fig. 1) detected from asymptomatic pet kitten in this present study, was confirmed by using both conventional cultural and molecular methods (Singh *et al.*, 2018). The isolate was also showing *in vitro* resistance to some common antifungals like amphotericin-B (MIC=32 µg/mL), fluconazole (MIC=64 µg/mL) and itraconazole (MIC=32 µg/mL). The gene sequence of the isolate has been submitted to the GeneBank® database under accession number MT270284.

The selected isolate was further sub-cultured on Potato Dextrose Agar (PDA) for 7-10 days at 28°C. To prepare the inoculum for the study, sterile normal saline solution (85%) was added to slants, and cultures were gently scrubbed with a sterile loop to dislodge conidia; thereafter vortexed and supernatant was collected. The optical density of the supernatant was read by double beamed UV spectrophotometer at 530 nm, adjusted to the transmittance of 60-70% and diluted with Roswell Park Memorial Institute (RPMI) 1640 medium to acquire final inoculums concentration of 0.4 to 5×10^4 cells/mL (Singh *et al.*, 2018).

Determination of Minimum inhibitory concentration (MIC): MIC values were determined by the broth micro-dilution assay method described by CLSI protocols approved standard M38-A (NCCLS, 2002). Dried extracts collected from the plants were diluted in 100% Dimethyl sulfoxide (DMSO) to the strength of 1 mg/mL to make a standard solution. In sterile microdilution plates (Tarsons®), columns 1 and 12 were filled with 200 µL each of plant extract and fungal inoculum to serve as a negative and positive growth control respectively. Columns 2 to 11 were filled with both fungal inoculum and serially diluted plant extract (100 µL each) so those final concentrations ranged from 64 to 0.125 µg/mL. The microdilution plates were then incubated at 28°C and were interpreted visually after 3 days of incubation. The well where the lowest concentration of the extract was completely inhibiting the visual growth of the dermatophyte was defined as the MIC value of that extract.

RESULTS

The MIC values of hot crude extract of leaves of *Ficus racemosa*, *Cassia fistula* and *Nerium oleander* were 4, 16 and 32 µg/mL respectively. None of the cold crude extracts showed an inhibitory effect against *M. canis* isolate (Table 1). The lowest MIC was seen in a methanolic fraction of hot crude extract of *Ficus racemosa* (FRM) and *Cassia fistula* (CFM) which was recorded as 2 and 8 µg/mL respectively (Fig. 2). The other fractions with butane (B), hexane (H) and chloroform (C) were also produced the weak antifungal effects.

Table 1. Showing Minimum inhibitory concentration values of selected crude phytoextracts (hot and cold) against *Microsporium canis* isolate

Selected phytoextracts	Crude extracts	Minimum inhibitory concentration (MIC) (µg/mL)
<i>Ficus racemosa</i> Leaves (L.)	Hot	4
	Cold	32
<i>Cassia Fistula</i> L.	Hot	16
	Cold	64
<i>Nerium oleander</i> L.	Hot	32
	Cold	64

Antifungal effect of phyto-extracts

Fig. 1. Obverse (A) and Reverse side (B) of *Microsporum canis* isolate on Sabouraud Dextrose Agar (SDA) plate

Fig. 2. Antifungal sensitivity testing (AFST) against hot crude extract *Ficus racemosa* (FR) and *Cassia fistula* (CF). (Starting two alphabets represent plant name and the last alphabet represent the fraction name; B-Butane, H-Hexane, M-Methanol, and C-Chloroform)

DISCUSSION

The present study revealed the methanolic extract of *Ficus racemosa* to have significant antifungal activity against *M. canis* isolate. This finding is in the same line as reported by Singh and Vidyasagar (2015) where antifungal activity (MIC=5 µg/mL) of the same fraction was observed against *T. rubrum* isolates. In a similar study by Phongpaichit *et al.* (2004) reported superior antifungal properties of the methanolic fraction of *Cassia fistula* L. against *T. rubrum* and *M. gypseum* isolates having a MIC of 8 and 4 µg/mL respectively. Again, Kadhim *et al.*, 2016 reported antifungal activity of methanolic fraction from fruits of *Cassia fistula* plant against *M. canis* isolates (MIC=4 µg/mL).

Based on the results obtained, it may be

concluded that the leaves of the plants' *Ficus racemosa* and *Cassia fistula* may prove useful as a new and potent source of the antifungal agent against the targeted dermatophyte. Further research with the identified plant parts through *in vivo* studies and pinpoint isolation of the active compound may prove beneficial to find out an effective alternative therapy for the pathogen.

Conflict of interest: The authors have no conflict of interest to declare.

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