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Short Communication

EXPLORATION OF *IN VITRO* SYNERGISTIC ANTIFUNGAL POTENTIAL OF *FICUS RACEMOSA* AND *CASSIA FISTULA* L. AGAINST MULTI-DRUG RESISTANT *MICROSPORUM CANIS*

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ABSTRACT: *Microsporum canis* is a worldwide-diffused pathogenic dermatophyte that can cause outbreaks in both human and animal populations. Considerable upsurge in the incidence of multidrug-resistant *M. canis* has created the urgency to identify and develop novel antifungals to avoid therapeutic failures during treatment. The present study was designed to explore the synergistic antifungal potential of fractionalized extract with different solvents of *Ficus racemosa* and *Cassia fistula* Leaves (L.) against multidrug-resistant *M. canis* by broth micro-dilution assay. The mixture of the methanolic fraction of both the extracts showed a potent antifungal activity with a minimum inhibitory concentration (MIC) of 1 µg/ml as compared to their pure fractions. The results provided a scientific validation for a possible and novel antifungal agent which should further be explored for its safety by using suitable animal trials.

Key words: Antifungal resistance, *Cassia fistula*, *Ficus racemosa*, Herbal medicine, *Microsporum canis*.

Dermatophytes are specialized group of aerobic fungi which are ranked as one of the most common cutaneous mycoses all over the world (Narang *et al*. 2019). They use keratinized host structures such as hair, skin and nails as a growth substrate. They are also the only group of human-infecting fungi that have evolved into obligate infectious agents (Sherman *et al*. 2018, Singh *et al*. 2020a). Based on multi-locus genetic analysis they comprise of nine genera: *Trichophyton*, *Microsporum*, *Epidermophyton*, Arthroderma, *Nannizzia*, *Lopophyton*, *Paraphyton*, *Ctenomyces* and *Guarromyces* (de Hoog *et al*. 2017). *Microsporum canis*, a highly prevalent pathogen is a zoophilic dermatophyte that can cause mild to severe inflammatory reactions with circular and erythematous lesions called ringworm or dermatophytosis (Singh *et al*. 2018). Lately, *M. canis* has caused sporadic outbreaks both in animals as well as human population (Thakur and Kalsi 2019, Ovchinnikov *et al*. 2020) and has developed resistance to commonly used antifungals (Aneke *et al*. 2018, Kano *et al*. 2018).

Over the last three decades, the category of antifungal drugs has not evolved satisfactorily (Jha and Kumar 2019) and at the same time the development of effective antifungals is so scanty that the existing pipeline is evidently dry (Perfect 2017). Herbal medicine is the best alternative to treat drug resistant *Microsporum canis* and a wide resurgence have been seen as people choose them over modern drugs due to low side effects, less toxicity and eco-friendly nature (Sam 2019). Reports of having good antifungal property by *Ficus racemosa* and *Cassia fistula* plants have been studied by many researchers (Phongpaichit *et al*. 2004, Thendral and Lakshmi 2017). However, their additive and/or synergistic effect against *M. canis* has not been described. The aim of the present study was thus to explore the synergistic antifungal potential of *F. racemosa* and *C. fistula* Leaves (L.) against multidrug-resistant *M. canis*.

The study

Collection and Preparation of crude extract

Leaves of *Ficus racemosa* and *Cassia fistula* plants were collected in and around Kolkata, West Bengal, India. The plants were selected as they had good antifungal activity against many pathogenic fungi but probably no studies were conducted on drug-resistant *Microsporum canis* till date (Phongpaichit *et al*. 2004, Thendral and

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Lakshmi 2017). Also round the year these plants are available and no part of these are used by human for any other purposes. After thoroughly washing under running tap water, leaves were washed with double distilled water to remove any residual soil or other impurities. They were left to dry at room temperature in a dark place.

After complete drying, leaves were ground to powder form in a mechanical grinder. For hot crude extract, 100g of powder was extracted with 250 mL of ethyl alcohol in a soxhlet apparatus for 13-14 cycles at 40-45 °C and for the cold extraction the same amount was maintained in shaker incubator for 4 to 5 days. The final recovered extracts were passed through rotary evaporator to remove the solvent and the remaining extract was further dried under vacuum for a period of 2 to 3 days. The dried extracts were transferred to amber colored bottle and stored at 4°C for further study (Singh *et al.* 2020b). The antifungal sensitivity testing (AFST) of both the extracts of each plant was carried out using Clinical Standards laboratory Institute (CLSI) guidelines (NCCLS 2002).

Preparation of fractional extract

From a total of four crude extracts, the best two crude extracts which revealed lowest Minimum Inhibitory Concentration (MIC) values were subjected for fractional extraction in a column chromatography. Solvents used were butanol, hexane, chloroform and methanol to isolate the most suitable solvent fraction(s) containing biologically active compounds responsible for the antifungal property (Singh *et al.* 2020b).

Fungal isolate and inoculum preparation

The selected dermatophyte (*M. canis*) was a clinical isolate obtained from the hair sample from an asymptomatic pet kitten. The isolate has been confirmed both conventionally and molecularly and has shown a high *in vitro* resistance to Amphotericin-B, Fluconazole and Itraconazole. Confirmation through sequencing of the internal transcribed spacer (ITS) region of the ribosomal RNA (rRNA) was done and the gene sequence was submitted to GenBank database under accession number MT270284.

To induce sporulation the selected isolate was sub-cultured on Potato dextrose agar (PDA) at 28°C for 10 days. Sterile normal saline solution (0.85% Sodium chloride) was poured into slants and culture was scrubbed to remove the aerial conidia. The supernatant was collected in a fresh tube after vigorous vortexing the sample at room temperature. The optical density of the supernatant was read by double beamed UV spectrophotometer adjusted at 530 nm and diluted with Roswell Park Memorial Institute (RPMI) 1640 medium to acquire final inoculum concentration of 1 to 5×10^4 cells/mL (NCCLS 2002).

Assessment of the antifungal potential

Antifungal potential was determined by broth micro-dilution assay method suggested by CLSI approved standard M38-A (NCCLS 2002). The dried extracts were diluted in dimethyl sulfoxide (100%) to get a standard solution of 1 mg/ml. Column 1 and 12 in a sterile micro dilution plates (Tarsons®) were filled with 200µL 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 (100µL each) to obtain concentrations ranged from 64 to 0.125 µg/µL Plates were interpreted visually after incubation at 28°C for 3 days. For synergistic effect, the plant extracts were mixed together in the ratio of 50:50 and the same protocol was performed. The MIC value was determined as lowest concentration of the extract, which completely inhibited the visual growth of the selected dermatophyte on third day of incubation.

Discussion

In the present study, MIC values of the hot crude extracts of *Ficus racemosa* L. and *Cassia fistula* L. were 4 and 16µg/mL respectively while no cold crude extracts showed any inhibitory effects against multi drug-resistant *M. canis*. After fractional extraction of the hot crude extracts of both the isolates the lowest MIC values were 2 and 8µg/µL respectively which were obtained from methanol extraction. The remaining other fractions with butane, chloroform and hexane produced low antifungal activity. The synergistic effect of methanolic fractionalized extracts of both the plants (50:50) revealed a MIC of 1µg/µL (Table 1). This result was interesting from the point of view of obtaining a maximum antifungal potential of both the extracts. Several researchers have reported the antifungal effect of medicinal plants like *Ficus racemosa L.* and *Cassia fistula* L. on dermatophytes (Gupta *et al*. 2017, Niranjan 2017). This may be the first study reporting the synergistic antifungal potential of the selected plants against multi drug-resistant *M. canis*. The possible synergistic action could be due to the different bio-active components found in these plant extracts which acted on different molecular targets against the selected isolate. The use of different plant extracts also reported to cause lower side effects in comparison to that when used singly (Politi *et al*. 2016).

Table 1. **Minimum Inhibitory concentration (MIC) values of selected phytoextracts with their method of extraction against multi drug-resistant *Microsporum canis* isolate.**

Selected Phytoextracts	Method of extraction	Minimum Inhibitory concentration (MIC) (µg/mL)
Ficus racemosa Leaves (L.)	Hot crude extract	4
	Cold crude extract	32
Cassia fistula L.	Hot crude extract	16
	Cold crude extract	64
Ficus racemosa hot crude extract	Fractionalized methanolic extract	2
Cassia fistula hot crude extract	Fractionalized methanolic extract	8
Synergistic effect of fractionalized methanolic extracts of *Ficus racemosa* and *Cassia fistula* hot crude extracts		1

Conclusion

Multiple-herbal preparations containing various bio-active derivatives have different mechanism of actions which complement in achieving a dose reduction in the overall combination and reducing the toxicity of a single plant. In addition, the synergism produces a higher antifungal potential making the composition potent in treating resistant microbial infections. The result of the present study is a good example of this hypothesis. Further investigation on the isolated bio-active compounds, synergistic effect on other dermatophytes and *in vivo* safety trials to obtain possible novel antifungal agents are warranted.

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