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Patho-Pause' in *Malassezia furfur*, a novel treatment approach for dandruff

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ABSTRACT

The present study describe the alteration in the virulence and drug resistance pattern in Malassezia furfur causing treatment resistant dandruff. Certain herbal preparations modify the above characteristics which is being thoroughly investigated in the present study where the herbal treatments to Malassezia furfur attenuated its ability to elicit certain specific inflammatory modulator release by keratinocytes which are responsible for hyper proliferation of keratinocytes, itching and inflammatory response. The present study provides the findings in detail.

Keywords: Virulence in Malassezia, treatment resistant dandruff, dandruff shampoo, herbal anti-dandruff

INTRODUCTION

A sudden and spontaneous spurt in cutaneous fungal infections have started to daunt the medical fraternity all over the world, post COVID.[1] Increased incidence of auto-immune (non-communicable) disease burden has significantly facilitated the fungal infection.[2] Many cutaneous disease causing fungi are showing drug resistance against most anti-fungal agents and their by exhibiting great resistance to treatment.[3] The cutaneous fungal diseases were once considered as meek and manageable disease though were a major public health[4] concern but now has turned into one of the diseases of perineal nature.

Among the various cutaneous mycoses, dandruff pose a major challenge with its high incidence and prevalence in population of all age and gender. The etiological cause of dandruff is by a lipophilic commensal flora of the scalp and skin-Malassezia furfur. Reason for the parasitic conversion of the commensal flora and thus causing dandruff to the host is less understood, however several prepondering factors have been identified.[5, 6]

The virulent species of Malassezia is known to induce several pro-inflammatory mediators released by keratinocytes such as IL-1β, IL-6, IL-8 and TNF-α.[7, 8, 9] Therefore, the drug used for the treatment if annul the ability of the organism from inducing the release of the above inflammatory mediators, such drug preparations may offer greater treatment success than targeting the organism as a whole as most present day anti-fungal drugs do. Malassezia furfur has already evolved as a commensal flora by developing extraordinary host-parasite relationship.

Steroidal preparations though may achieve the above benefit of pro-inflammatory suppression but such benefit is achieved through supressing the host response than the pathogen driven. In the present study, we have tried to mutate the virulence site of the organism which is further established by the inability of the organism to induce the release of pro-inflammatory mediators by keratinocytes.

We are the first to adopt the treatment strategy of virulence emasculation in Malassezia furfur for the treatment of dandruff.

Materials and methods

Five strains each of Malassezia furfur isolated from treatment resistant, active dandruff case as well as lab stored ones were used in the study. All the strains were maintained in Dixon agar.

Normal human epidermal keratinocytes (NHEK) was used for the present study. The cells were grown in basal media (KBM-2; 0.15 mM Ca2+) supplemented with bovine pituitary extract (7.5 mg per ml), human recombinant epidermal growth factor (0.1 ng per ml), insulin (5 mg per ml), hydrocortisone (0.5 mg per ml), epinephrine, transferrin, gentamicin (50 mg per ml), and amphotericin-B (50 ng per ml) at 37°C/5% CO2. The media was replaced every 2±3 d. When cells attained a confluence of 90% growth were disaggregated by trypsin 0.01%, whenever required.[10]

Co-culture of keratinocytes with Malassezia furfur

NHEK were grown with Malassezia furfur was by the method of Jiang et al (1996). Malassezia yeasts were harvested after 3 d of growth in Sabouraud's liquid medium containing 0.5% of Tween 40 at 37°C. These yeast cells were washed twice in phosphate-buffered saline. The yeast cells were re-suspended in serum-free keratinocyte growth medium, respectively. 1:1 ratio of yeast cells to keratinocytes was chosen for the co-culture experiment, and were grown in 96 well plate and incubate at 37°C, 5% CO2 for 16 h. After which Malassezia furfur was inoculated into the wells of the keratinocytes.[8] Cell viability was then checked by the Trypan blue exclusion tests. After 1± 24 h of co-culture, supernatants were obtained and kept at ±20°C until use for cytokine enzyme-linked immunosorbent assay (ELISA) for the following pro-inflammatory mediators IL-1β, IL-6, IL-8 and TNF-α

Pre-treatment of Malassezia furfur and co-culture experiment

Malassezia furfur culture were grown in sub-quarter lethal dose of a shampoo formulation for two generations and the strains thus obtained were tested for co-culture experiment with NHEK**.** Both strains isolated from treatment resistant active dandruff and lab maintained culture were used for the study. The co-culture experiment was performed as described above and the culture filtrate was centrifuged and tested by ELISA for the pro-inflammatory mediators.

Determination of Minimum Inhibitory Concentration (MIC)

Four of the five strains of drug resistant Malassezia furfur showed susceptibility to the test shampoo at 400 µg/ml while one of the five strains was susceptible at 350 µg/ml.

3/5 lab grown strains showed susceptibility at 150 µg/ml while 2/5 showed susceptibility at 200 µg/ml. Table- 1

Table – 1

Strain details	No. of isolates	Range of MIC in µg/ml shampoo formulation
Treatment resistant	5	350- 400
Lab grown	5	150- 200

1/5 treatment resistant strain of Malassezia furfur showed susceptibility to ketoconazole shampoo at 3 µg/ml, 2/5 showed susceptibility at 15 µg/ml, 2/5 strains showed susceptibility at 17 µg/ml.

2/5 lab grown strains showed susceptibility at 0.04 µg/ml and 3/5 showed susceptibility at 1.2 µg/ml. Table -2

Table - 2

Strain details	No. of isolates	Range of MIC in µg/ml ketoconazole shampoo
Treatment resistant	5	3 - 17
Lab grown	5	0.04 – 1.2

Effect of Malassezia furfur in the release of inflammatory mediators by NHEK

The treatment resistant Malassezia furfur significantly increased the release of inflammatory mediatory by NHEK cells while the lab grown strain did not affect much as the value of the inflammatory mediators release were comparable with that of control that is untreated NHEK cells. Table 3

Table – 3

Treatment	**Inflammatory mediators/ pg/ml**			
	IL-1β	IL-6	IL-8	TNF-α
NHEK alone	9	16	64	11
NHEK+ treatment resistant *Malassezia*	28	39	80	34
NHEK+ lab grown Malassezia	18	21	65	20

Virulence modulation in Malassezia before and after treatment with ketoconazole shampoo
The pre-treatment of Malassezia furfur with ketoconazole shampoo did not alter their ability to induce the release of inflammatory mediators by NHEK cells irrespective of the strains being treatment resistant or lab grown. Table 4

Table -4

Treatment	**Inflammatory mediators/ pg/ml**			
	IL-1β	IL-6	IL-8	TNF-α
NHEK + Ketoconazole shampoo (2 µg/ml)	8	12	52	10
NHEK + ketoconazole shampoo treated treatment resistant *Malassezia (0.75 µg/ml)*	25	32	64	29
NHEK + ketoconazole shampoo treated lab grown Malassezia (0.02 µg/ml)	16	19	47	21

Virulence modulation in Malassezia before and after treatment with herbal shampoo at sub-quarter lethal dose
The herbal shampoo did not positively or negatively affected the release of pro-inflammatory mediators by NHEK cells. The pre-treatment of treatment resistant Malassezia furfur strains with herbal shampoo significantly reduced their ability to induce the release of inflammatory mediators by NHEK cells whereas such reduction was not seen with lab grown strains. Table 5

Table - 5

Treatment	**Inflammatory mediators/ pg/ml**			
	IL-1β	IL-6	IL-8	TNF-α
NHEK + herbal shampoo (20 µg/ml)	8	14	55	9
NHEK + herbal shampoo treated treatment resistant *Malassezia (88 µg/ml)*	18	19	42	18
NHEK + herbal shampoo treated lab grown Malassezia (38 µg/ml)	18	19	40	16

Discussion
Malassezia furfur is known to induce certain inflammatory mediators released by keratinocytes such as IL-1β, IL-6, IL-8 and TNF-α.[7, 8, 9] It has also been reported that the strain that show high ability to induce the inflammatory mediators is more virulent than the one which does not have such ability. We therefore hypothesized that by supressing such ability of Malassezia furfur could offer better treatment success to dandruff.

Our study has thrown a new light that the strains that exhibited high inflammatory mediators inducing ability by NHEK cells were also shown relatively high drug resistance to ketoconazole. Further all the above strains were isolated from known treatment resistant cases of dandruff where the problem has been already treated with multi-various treatment product such as ketoconazole/ Clotrimazole shampoo. So we presumed that the drug resistance and virulence at least in Malassezia may be linked.

When Malassezia strains were pre-exposed to sub-quarter lethal dose of the herbal shampoo formulation for two generations and so with ketoconazole and then the strains were co-cultured with keratinocytes to study their effect on inflammatory mediators release by NHEK.

The herbal shampoo treatment on Malassezia furfur had a significant effect on the treatment resistant strains by lowering the ability to induce the release of inflammatory mediators by keratinocytes. Whereas the herbal shampoo by itself did not alter the profile inflammatory mediators being released by keratinocytes. Since the lab grown strains showed poor ability in inducing inflammatory mediators release by keratinocytes the treatment of herbal shampoo did not alter such ability significantly. Similarly ketoconazole shampoo pre-treatment also did not affect the ability of the treatment resistant strains in inducing inflammatory mediators release by keratinocytes in the present study.

The above findings suggest that the herbal shampoo formulation has the ability to alter the virulence potential of the virulent strains isolated from treatment resistant dandruff cases. This may possibly also affect the drug resistance pattern of the strains. The strains that showed high ability to induce inflammatory mediators (treatment resistance isolates) showed relatively high resistance to ketoconazole clearly suggesting the possible link between drug resistance and virulence whereas such phenomenon was not observed among lab grown strains.

The herbal shampoo formulation (Lumina AD herbal shampoo) contains herbal actives such as Phylo nodiflora, Azadirachta indica, Allium cepa var. aggregatum, Cassia alata, Aloe vera, Hibiscus rosa-sinensis, Trigonella foenum-graecum, Indigofera tinctoria which are known to have multiple medicinal benefits including anti-fungal activity.

In the context of challenges faced in treating dandruff, our present findings assumes high significance as the virulence modulation resulting in reduced inflammatory mediators release is equivalent of steroidal effect but is specific to etiological agent and not affecting the host is the salient feature of the present study. Further the virulence alteration especially the inflammatory mediator inducing ability is also can be linked to drug resistance. Therefore the combination of herbal formulation may offer great promise to treatment of dandruff though our present findings may require more rigorous scientific validation.

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