Research Article

MIC of A Siddha Formulation Kandhaga Rasayanam against Dermatophytes

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ABSTRACT

Dermatophytes are organisms responsible for ringworm infection (dermatophytosis) of skin. The three common genera are Trichophyton, Epidermophyton and Microsporum. Kandhaga Rasayanam is a Siddha herbomineral drug very effective in treating skin diseases. It is chosen from the classical Siddha textbook Siddha Vaidhya Thirattu. In Siddha system of medicine, "Kuttam" is a broad term used to indicate skin diseases. The symptoms of Padarthamarai kuttam, which is one of the type of 18 kuttas can be correlated to Tinea (Ring worm) infections. The aim of the study is to determine the Minimum Inhibitory Concentration of aqueous, ethanol and methanol extact of the drug Kandhaga Rasayanam against *Trichophyton rubrum* (MTCC no: 296), *Trichophyton mentagrophytes* (MTCC no: 8476) and *Epidermophyton floccosum* (MTCC no: 7880). The Minimum Inhibitory Concentration (MIC) was determined by broth dilution method. The readings were confirmed by microscopic examination and spot assay. The MIC for aqueous extract is 10mg/ml for *T. rubrum*, 20mg/ml for *T.mentagrophyes* and *E.floccosum*. The ethanol and methanol extracts inhibited the growth of all the three dermatophytes at 50mg/ml.

Keywords: Minimum Inhibitory Concentration, Dermatophytes, Siddha, Kandhaga Rasayanam, *T.rubrum*, *T.mentagrophytes*, *E.floccosum*.

INTRODUCTION

Dermatophytes are a group of closely related filamentous fungi that generally invades keratinized tissues such as skin, hair and nails. Superficial clinical manifestations caused by them are commonly called as ringworm infections. Dermatophytosis is caused by members of three genera, *Trichophyton, Microsporum* and *Epidermophyton*[1]. Worldwide prevalence rate of superficial fungal infections is found to be 20-25% and it is most prevalent in tropical and sub tropical countries like India[2]. In the recent past, the occurrence of such cases has increased alarmingly especially in immunocompromised patient groups including AIDS, diabetes, cancer etc. In severe cases, it may cause systemic skin infections on association with secondary bacterial infections[3]. The prevalence of humid weather, crowded population and poor hygiene are the possible predisposing factors.

Current treatment stratergies for these different forms of infections like tinea corporis, tinea pedis, capitis, barbae, cruris, manum and onychomycosis includes topical antifungal therapy for localized lesions and prolonged systemic therapy for extensive infections of scalp or nails[1]. High toxicity due to prolonged usage and emergence of antifungal resistance emphasize the urgent need for the development of new, safe and effective natural treatment alternatives[4, 5]. Plants are the best source for the identification of novel drug compounds. Low toxicity, easy availability, low cost and fewer side effects have made plant extracts and plant derived compounds as valuable source to treat wide range of infectious conditions[6]. About 2,500 plant species from India are known to have medicinal value and 80% of population from developing countries relies on traditional medicines from plants[7].

Siddha medicine is one of the most ancient systems of medical practice known to mankind. They treat infections with herbs, inorganic substances and animal products[7]. The medicinal herbs are used as decoctions, infusions, tinctures, and powders. Kandhaga Rasayanam is a classical Siddha herbomineral drug mentioned in the text Siddha Vaidhya Thirattu[8]. This drug contains herbs and mineral, sulphur (Kandhagam). Polyherbal preparations have diverse range of bioactive molecules and thus increase the antimicrobial spectrum. It is commonly used to treat skin diseases, urinary tract infections, diarrhea, venereal diseases and arthritis. However, there is only limited information in the literature on the antifungal activity of this herbomineral drug on dermatophytes. Hence, the purpose of this study was to evaluate in vitro antifungal properties of three extracts of the Kandhaga Rasayanam against three common dermatophyes namely *T.rubrum, T.mentagrophytes* and *E.floccosum.*

MATERIALS AND METHODS

The ingredients of the trial drug, Kandhaga Rasayanam were given below and it was prepared following good manufacturing practices. Prepared drug was tested free of microbial contamination.

Kandhagam (Sulphur) 350 grams

Amukkara kizhangu (*Withania somnifera*.Dunal,) 175 grams

Parangi chakkai (*Smilax china* Linn.) 70 grams

Kadukkai (*Terminalia chebula*. Retz.) 35 grams

Nellikai (*Phyllanthus emblica* Linn.) 35 grams

Thandrikkai(*Terminalia bellerica* Roxb.) 35 grams

Chukku (*Zingiber officinale*.Roscoe.) 35 grams

Thippili moolam (root of *Piper longum*.Linn.) 35 grams

Milagu (*Piper nigrum*.Linn.) 35 grams

Vaividangam(*Embelia ribes*,Burm.) 35 grams

Ealam(*Elataria cardamomum*.Linn.) 35 grams

Kirambu (*Cinnamomum zeylanicum*.Breyn.) 35 grams

Chandhanam (*Santalum album*,Linn) 35 grams

Kadalai (*Cicer arietinum*,Linn.) 35 grams

Senkottai(*Semecarpus anacardium.* Linn.) 35 grams

Chithiramoola verpattai(root bark of *Plumbago zeylanica*,Linn.) 35 grams

Sugar, Honey and Ghee. As required

Preparation of extract from the drug

One gram of the herbal medicine was extracted at 50-60°C with 1 L of solvents with different polarity like methanol, ethanol and water in a Soxhlet apparatus. The medicine was extracted until it becomes a clear solution. Extracts in different solvents were concentrated under reduced pressure in rotary evaporator. The extract was gumaceous in nature, hence final products of all three extracts was freeze dried using lyophilizer to get a fine powder and stored at 4°C until further investigation.

Test organisms

Three standard strains of dermatophytes namely *Epidermophyton floccosum* (MTCC 7880), *Tricophyton rubrum* (MTCC 296) and *Trichophyton mentagrophytes* (MTCC 8476) obtained from Microbial Type Culture Collection, Chandigarh, India were used in the study. All the standard cultures were revived as per the instructions given in the website (http://mtcc.imtech.res.in). Briefly, fungal suspension was prepared by adding 0.5 ml of sterile Sabouraud's dextrose broth (SDB, Hi-media laboratories, Mumbai, India) to each of the lyophilized vial. After twenty minutes, few drops of the suspension were inoculated onto Sabouraud's dextrose agar (SDA, Hi-media laboratories, Mumbai, India) plates and incubated at room temperature for 14-21 days. Dermatophytes were identified by their respective colony morphology on SDA plates and further confirmed by microscopy using slide culture technique. Remaining suspension was preserved at -80°C for later use.

Antifungal susceptibility testing

The *in-vitro* anti-dermatophytic activity of aqueous, ethanol and methanol based crude extracts of Kandhaga Rasayanam were analyzed using broth micro dilution method as per protocol M38-A2 of the Clinical and Laboratory Standards Institute (CLSI) for filamentous fungi with some modifications[9]. All the laboratory experiments were carried out in Dr. ALM PG Institute of Basic Medical Science, Madras University, Taramani campus, Chennai.

Inoculum preparation

Prior to testing, all strains were sub cultured on SDA slants and incubated at room temperature for 14-21 days[10]. Colonies were covered with 5 ml of SDB broth and suspension containing a mixture of conidia and hyphal elements was prepared by scraping and macerating the agar surface with inoculation needle/loop. The contents were transferred to a sterile 5ml centrifuge tube and filtered using sterile Whatman® Quantitative filter paper, grade 40 (Sigma Aldrich Pvt Ltd, USA). The concentration of microconidia in the suspension was adjusted to 0.5-5 X 10^6 spores/ml using SDB via spectrophotometer.

Drug preparation

Two grams of powdered form of aqueous, ethanol and methanol extracts of the drug was dissolved in 2 ml of sterile RPMI 1640 medium with L-glutamine; without sodium bicarbonate (Sigma Aldrich Pvt Ltd, USA) buffered to pH 7.0 using Morpholinepropan-sulfonic acid (MOPS, Sigma Aldrich Pvt Ltd, USA). Herbomineral drug was tested in series of two-fold dilutions at concentrations ranging from 0.78 mg/ml – 200 mg/ml for both solvent extracts and from 0.62 mg/ml-20 mg/ml for aqueous extract. Fluconazole (Pfizer Ltd, USA) was used as positive drug control at a concentration of 10µg/ml. All dilutions were carried out using sterile RPMI 1640 medium.

Test procedure

The anti-dermatophytic activity of aqueous, ethanol and methanol based crude extracts of Kandhaga Rasayanam were analyzed using broth dilution method as per Irobi et al[11]. Various dilutions of test drug were added to tubes containing standardized inoculum (100 µl). Uninoculated medium was used as negative control; the growth control tube contained only inoculum. Fluconazole was used as a known positive control. All the tubes were incubated at room temperature. The turbidity in the tubes was recorded after one week for *T.rubrum* and *T.mentagrophytes* and after 14 days for *E. floccosum*. The MIC value of a drug was defined as the lowest concentration at which no growth is visible in the tubes when detected visually. This was further confirmed by

microscopic examination using slide culture technique and spot assay on SDA plates. For the spot assay, 10µl samples from all optically clear tubes (visible growth inhibition) plus the growth control tube were sub cultured on SDA plates. The plates were incubated and checked for growth periodically.

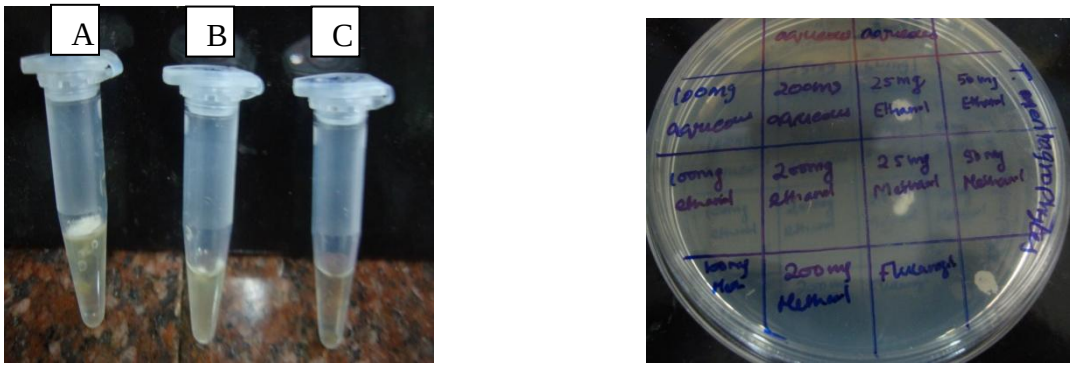
RESULTS AND DISCUSSION

Preliminary antimicrobial assessment was carried out for crude extracts of Kandhaga Rasayanam against dermatophytes, *Trichophyton rubrum, Trichophyton mentagrophyes and Epidermophyton floccosum*. Antimicrobial activity was evaluated using broth dilution method as per standard guidelines. MIC of the crude extract against pathogens is reciprocal to the amount of antimicrobials present in the crude extracts. Minimum inhibitory concentration of aqueous and solvent based crude extracts of Kandhaga Rasayanam against dermatophytes was found to be within the range of 10 and 50 mg/ml.

Aqueous extract of Kandhaga Rasyanam inhibited *T.rubrum* at 10 mg/ml; whereas *T.mentagrophytes* and *E.flocosum* at 20mg/ml concentration. Methanol, ethanol extracts of Kandhaga Rasayanam inhibited all the dermatophytes at the concentration of 50mg/ml concentration. Inhibition zone produced by positive control, fluconazole (10µg/ml) was satisfactory. The MIC of the aqueous, ethanol and methanol extracts of Kandhaga Rasayanam against *T.rubrum, T.mentagrophytes* and *E.floccosum* were tabulated in Table 1. Inhibition exhibited by positive control, fluconazole (10µg/ml) was satisfactory.

Table 1: MIC of the aqueous, ethanol and methanol extracts of Kandhaga Rasayanam

Type of extract	MIC (mg/ml) of dermatophytes strains used in the study		
	T.rubrum	***T.mentagrophytes***	***E.floccosum***
Aqueous extract	10	20	20
Methanol extract	50	50	50
Ethanol extract	50	50	50



i) Broth dilution method ii) spot assay

Figure 1: Determination of minimum inhibitory concentrations (MICs) of Kandhaga Rasyanam by micro-broth dilution method against different dermatophyte isolates. i) MIC by broth dilution method, a) growth control tube b) methanol extract of *T.mentagrophytes* at 25 mg/ml c) methanol extract of *T.mentagrophytes* at 50 mg/ml. ii) Confirmation of MIC values by spot assay

Skin diseases are unique as recurrence is common and they carry a high level of morbidity than mortality. Medicines which are economical, highly available, without side effects is need of the hour. For that case, various traditional Siddha medicines are being tested against pathogens causing superficial infections. However, such studies are limited against fungal pathogens because of the difficulty in handling filamentous fungi and lack of standard guidelines. We attempted to do antifungal testing against Kandhaga Rasyanam based on M38-A2 CLSI guidelines but with some modifications like inoculum size and incubation temperature and time.

Antifungal activities of the herbomineral drug, Kandhaga Rasyanam against dermatophytes have not been previously demonstrated in vitro. The data from this pilot study clearly suggests that standard strains of dermatophytes are susceptible to this drug. All the three strains showed similar inhibitory effect. Results of this *in-vitro* study also reveal that all the three extract forms of Kandhaga Rasyanam have antidermatophytic activity ranging from 10 mg/ml to 50mg/ml. The results were compared with standard antifungal drug, fluconazole. These variations in antifungal activity could be due to the differences in the secondary metabolites of the herbs used in the drug or solvent used for extraction. However, various other researchers have showed antidermatophytic activity of plants, herbs at similar concentrations[12, 13].

Sulphur is generally excellent rejuvenator and along with other herbs incorporated; this drug serves as a potent antifungal agent especially against dermatophytes. Literature also suggested various other medicines such as *Semecarpus anacardium, Santalum album, Plumbago zeylanic, Smilax china, Elatteria cardamomum, Piper nigrum* for fungal infections. With the help of modern research methods, effect of many traditional medicine preparations documented many years back by Siddhars are accredited.

CONCLUSION

The present study revealed the *in-vitro* inhibitory effect of various extracts of Kandhaga Rasayanam available in the traditional system of medicine against most common pathogenic species of three main genera of dermatophytes causing skin infections. However, further studies are needed to better evaluate the potential effectiveness of the crude extracts as the antifungal agents.

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