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Research Article

Microbiology for Health Care

Formulation of Anti-Fungal Cream to Treat Onychomycosis

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Abstract: Onychomycosis a common nail infection caused by a fungi affects the farmers of age group between 16-30 years. The main reason for farmers to acquire this infection is that they spend a lot of time in field doing various activities which includes land preparation, plowing, manuring, spraying pesticides, fertilizers, herbicides, sowing seeds, seed transplantation and harvesting etc is the main reason for them to acquire cutaneous mycoses. It can cause pain, discomfort and disfigurement and may produce serious physical and occupational limitations, as well as reducing quality of life. Onychomycosis can be initiated by three major groups of fungi: dermatophytes, yeasts, and non-dermatophytic filamentous fungi. It is a fungal infection of the nail unit, more common in toe nails than in fingernails. Up to 10% of cases of onychomycosis are caused by non-dermatophyte moulds and these are becoming more common worldwide. The aim of this study is to prepare and formulate an anti-fungal cream to treat onychomycosis infection among farmers. The methodology includes subjecting the collected samples for patch test followed by histological procedures and anti-fungal susceptibility test. An anti-fungal cream was formulated to treat this fungal nail disease. This formulation was prepared based on the traditional knowledge using different herbs. The prepared antifungal formulations showed good consistency and no evidence of phase separation during the study period.

Keywords: Onychomycosis, dermatophytes, antifungal, formulation, nail clippings

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1. INTRODUCTION

Rice farming is one of the major livelihoods in the southern part of Tamil Nadu State, India [1, 2]. Farmers are wide-open to various irritants like mud, cow dung, manures, fertilizers, herbicides, pesticides, dust, and soil contain substantial amounts of fungal spores [3,4]. Fungal infections affect the keratinized layers of the skin and its appendages[5,6]. Fungal infections caused by dermatophyte and nondermatophyte molds have been extensively reported to be a major health problem in India and all over the world [7, 8]. Onychomycosis is a predominant nail disease found among the farmers working in an agricultural set up [9,-11]. It is conventionally referred to as a non dermatophytic infection of the nail but is now used as a common term to denote any fungal nail infection[12;13]. This fungal infection accounts for 50% of all nail disorders with those affecting the toenails 25 times more than that of the fingernails.[14,15] The dermatophytes commonly associated with onychomycosis include *Trichophyton rubrum* and *Trichophyton mentagrophytes* with the former responsible for up to 90% of cases[16,17]. The moulds most often isolated from diseased nails are *Aspergillus spp., Fusarium spp, Scopulariopsis brevicaulis, Scytalidium dimidiatum, Scytalidium hyalinum, Onychocola canadensis*[18, 19]. The symptoms and signs of onychomycosis include discoloration of nail from white to black, green, and yellow, flaking and thickening of nail plate, change in the appearance of nail plate, nail separated from nail bed, broken and brittle nails, pain and so on[20]. Formulation is a term which is used to blend and mix several compounds that do not react with each other but provide a final product which interacts to treat the disease[21]. To treat onychomycosis, an anti-fungal cream was formulated in this present study[22]. This formulation was prepared based on the customary knowledge using different herbs[23,24]. The herbs used were *Calendula officinalis, Origanum vulgare*, *Allium sativum*, *Curcuma longa*, *Syzygium aromaticum*, *Melaleuca alternifolia* oil, *Cymbopogon* oil, *Ocimum tenuiflorum* and *Mentha Piperita* oil[25,26]. The aforementioned herbs have good anti-fungal, anti-inflammatory, anti-bacterial, antispasmodic and anti-viral properties[27,28]. Dermatophytes affecting the toenail cause changes in the features of the cells which can be revealed out using histological procedures[29,30]. The formulated antifungal cream prepared from this study can be a promising therapy to treat the onychomycosis in future with increased bioavailability and least toxicity in the skin due to its customary preparation using different herbs [31, 32].

2. MATERIALS AND METHODS

2.1 Collection Of Clinical Specimen

There were 13 male and 2 female subjects enrolled in the study yielding 15 pairs of nail samples. Ages varied from 20 to 62 years (mean 36, median 33, SD +/-11.4 years). The samples were collected from toe and finger nails. The abrasions were scrubbed with 70% alcohol with a sterilized scalpel[33]. A written consent was taken from the individuals for this study.

2.2 Processing Of Specimen

Direct microscopy slides were prepared with 20% KOH for some portions of skin scrapings and nail clippings. The remaining portions and toe/finger web swabs were inoculated into duplicate plates containing potato dextrose agar (PDA) (Biotech) supplemented with 0.05 mg/mL chloramphenicol and 0.5 mg/mL cycloheximide. Another duplicate plate was inoculated with 0.05 mg/mL chloramphenicol excluding 0.5 mg/mL cycloheximide with the sample[34]. The plates were incubated at 27 °C.

2.3 Formulation And Preparation Of Antifungal Cream

Herbal plants like *Calendula officinalis, Origanum vulgare*, *Allium sativum*, *Curcuma longa*, *Syzygium aromaticum*, *Melaleuca alternifolia* oil, *Cymbopogon* oil, *Ocimum tenuiflorum* and *Mentha Piperita* oil were selected for their antifungal activity. All the herbals were weighed accurately & aqueous extraction had been done. The formulated cream was prepared in two different combinations by oil in water emulsion of 30% and 70% (Table 1). As the solution concentrates up to 30 ml, filtration was done. The emulsifier and other oil soluble components were dissolved in the oil phase and heated up to 80 °C[35]. After heating, the aqueous phase was added in portions to the oil phase with constant stirring until cream is formed, and cream was formulated having a mild lemon yellow color. Perfumes were added when the temperature is dropped to 45 °C ± 50 °C (table 2 and 3). The cream was evaluated for its thermal stability, physical properties like pH and test for microbial growth in formulated cream (table 4, 5 and 6).

2.4 Patch Test

A patch test is used to determine whether a specific substance causes allergic inflammation of a patient's skin. About 1-3 gm of cream to be tested was placed on a piece of fabric or funnel and applied to the infected part of the toenails. Control patches were also applied. The site of the patch is inspected after 24 hours. [36]

2.5 Histological Procedures

The samples were fixed in-between tissue holders and were labeled using a tag for identification. Then the sample with the tag is placed in a container with 10 % formalin solution. Then the sample was taken out and placed in a beaker containing 90% isopropyl alcohol to dehydrate the sample. Clearing was carried out to make the tissues more transparent and is usually achieved by placing the tissue in xylene for 30 minutes. The tissue holder containing the tissue was transferred to a beaker containing the molten paraffin wax, during the above process, the wax enters into the tissue causing the out flow of xylene (infiltration) and paraffin occupies all the tissue area (impregnation). The infiltrated tissue was taken out and placed inside the mold. The fresh molten paraffin is poured into the mold and the tissue is gently pressed and placed in an ice bath for faster hardening. Then with the help of Rotary microtome the sections were made. The sections were taken and subjected to dewaxing, during which the paraffin gets removed. The slide was placed in xylene solvent for 10 minutes for complete removal of wax [37]. The slide was then dipped in isopropyl alcohol twice with two shifts for dehydration. Finally, staining was carried out by hematoxylin and eosin dyes.

2.6 Antifungal Susceptibility Testing

Anti-fungal susceptibility testing was done following Kirby bauer method [38]. The fungal isolates from nail clippings were swabbed on Muller Hinton agar using sterile swabs. Holes

were drilled into the agar and filled with different concentrations of the herbal formulation aseptically. The plates were incubated at 25-30 °C for 48-72hours. The diameter of the zones of inhibition appearing around the holes was measured and recorded (table 7).

3. RESULTS AND DISCUSSION

The collected nail samples were inoculated in potato dextrose agar and observed microscopically. Colonies grown in potato dextrose agar are mostly flat to slightly raised, white to cream, suede-like to downy, with either no reverse pigment or a yellow-brown to wine-red reverse pigment which confirmed the *Trichophyton rubrum* infection in toenails[39] (figure 1).Mulvaney et al analysed a 45 year-old man with an eight-year history of discoloration of the nail plate Histopathology of the nail plate revealed numerous fungal elements arranged transversely and longitudinally, thus acid-Schiff (PAS) stain confirming endonyx onychomycosis [40]. In our study 13 male and 2 female subjects were taken yielding 15 pairs of nail samples. Davies et al discussed that onychomycosis infection, is a common fungal infection largely caused by dermatophyte fungi, such as *Trichophyton rubrum* or *Trichophyton mentagrophytes*, which affects a number of people. Treatment is either through oral antifungal medicines, or with topical antifungal treatments [41]. In this current study microscopic observation shows *Trichophyton rubrum* with slender clavate microconidia and cigar-shaped macroconidia [42] (figure 2). This current study observed three distinct histologic patterns of nail unit. The great toe nail shows xanthonychia and onycholysis infection. Histological findings of nail plate shows parakeratosis, fungus and neutrophils at the ventral aspect of the nail plate (figure 3). The second histological images of infected toenail shows white superficial onychomycosis with coexistent distal-lateral subungual pattern and shows thickening with subungual debris (figure 4). The third histological images of infected toenail show onychomycosis due to *Trichophyton rubrum*. The nail clip was stained with hematoxylin and eosin, which shows mostly spores along a few hyphae (figure 5). This work summarizes the practical approach, utility, and histologic findings of nail clippings in evaluation of onychomycosis, nail unit biopsy, melanonychia, hematoma of this obtained specimen. The uses of anti-fungal cream have been increased in many folds in personal care system [43]. The pH of the prepared cream was found to be around 6 which are suitable for topical application because the pH of the infected toenail is between 4.5 - 6. In this present study the prepared formulations showed good spread ability, no evidence of phase separation and good consistency during the study period (figure 6). The spreadability studies showed that formulations have better spreadability. Lim et al used Fractional carbon-dioxide laser therapy, combined with a topical antifungal agent, to treat onychomycosis[44].The stability studies of the various parameters like visual appearance, nature, pH of the formulations showed that there was no significant variation after two months of the study period and the results were summarized. The results of pH and spreadability are summarized in (table 8). The formulated cream shows no redness, edema, inflammation and irritation during patch test studies.

Table 1. Formula

Ingredients	Oil in water emulsion extract 30%	Oil in water emulsion extract 70 %
Calendula officinalis	0.50	1.45
Origanum vulgare	0.45	0.80
Allium sativum	0.40	0.60
Curcuma longa	0.40	0.40
Syzygium aromaticum	0.30	0.40
Melaleuca alternifolia oil	0.30	0.40
Ocimum tenuiflorum	0.37	1.08
Cymbopogon oil	0.20	0.40
Mentha Piperita oil	0.20	0.40

Table 2. Composition of cream

Ingredient	Composition	
	30%	70%
Composition of cream Extract	3gm	9gm
Bees wax	3gm	5gm
Heat paraffin	2gm	1.1gm
Petroleum jelly	1.2gm	0.5gm

Table 3. Quantitative standards

Parameter/ Drugs	Foreign matter (mg) % w/w	pH	Water soluble Extract % w/v
Calendula officinalis	0.2	5.59	15.47
Origanum vulgare	0.7	6.10	10.38
Allium sativum	0.2	6.55	18.83
Curcuma longa	0.6	5.71	12.7
Syzygium aromaticum	0.5	5.7	14.36
Melaleuca alternifolia oil	1.3	3.7	12.25

Ocimum tenuiflorum	0.05	4.63	5.8
Cymbopogon oil	0.3	6.2	7.9
Mentha Piperita oil	0.1	2-3	0.1

Table 4. Physical Properties of herbal Cream

S.No	Properties	30%	70%
1	Colour	Fenugreek Yellow	Fenugreek Yellow
2	Odour	Characteristic	Characteristic
3	Appearance	Semi - Solid	Semi - Solid

Table 5. Thermal stability and pH Determination

S.No	TEST	30%	70%
1.	Thermal stability (at RH 65% and 30 ± 40°C)	Stable, no oil separation	Stable, no oil separation
2.	pH (at 27°C ± 2°C)	6.02	5.59

Table 6. Accelerated Stability Studies

MONTHS/ TEST	Herbal Cream (30%)			Herbal Cream (70%)		
	Initial month	After - 1 month	After - 2 month	Initial month	After - 1 month	After - 2 month
Physical appearance	Semi solid	Semi solid	Semi solid	Semi solid	Semi solid	Semi solid
Texture	ok	Ok	Ok	ok	ok	ok
Colour	Fenugreek yellow	Fenugreek yellow	Fenugreek yellow	Fenugreek yellow	Fenugreek yellow	Fenugreek yellow
Odour	Characteristic	Characteristic	Characteristic	Characteristic	Characteristic	Characteristic
pH Value	5.7	5.8	5.8	6	6	6
Thermal stability	ok	Ok	Ok	ok	ok	ok
Degradation of product	nil	Nil	Nil	nil	nil	nil

Table 7. Antifungal Studies of *Trichophyton rubrum*

S.No	Test Organism	Zone of Inhibition (mm)				
		20µg/ml	40µg/ml	60µg/ml	Standard (Chloramphenicol)	Control (DMSO)
1.	*Trichophyton rubrum* (MTCC No. 296)	6.75±0.15	9±0.17	12±0.20	15±0.25	-

Values are mean±SD;(n=6), ‾P<0.01 when compared with control

Table 8. Spreadability Test

Formulation	Time(sec)	Spreadability (g cm/sec)
30% cream	15	14.5
70% cream	15	13.6

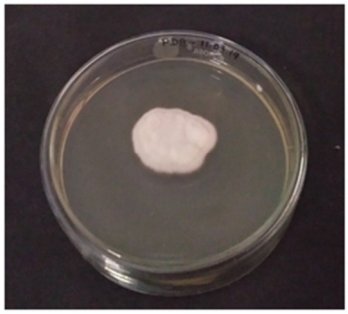

Fig 1. *Trichophyton rubrum* in potato dextrose agar

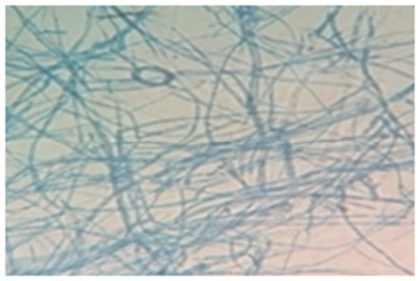

Fig 2. Microscopic examination of *Trichophyton rubrum* showing slender clavate microconidia and cigar-shaped macroconidia, some with terminal appendages

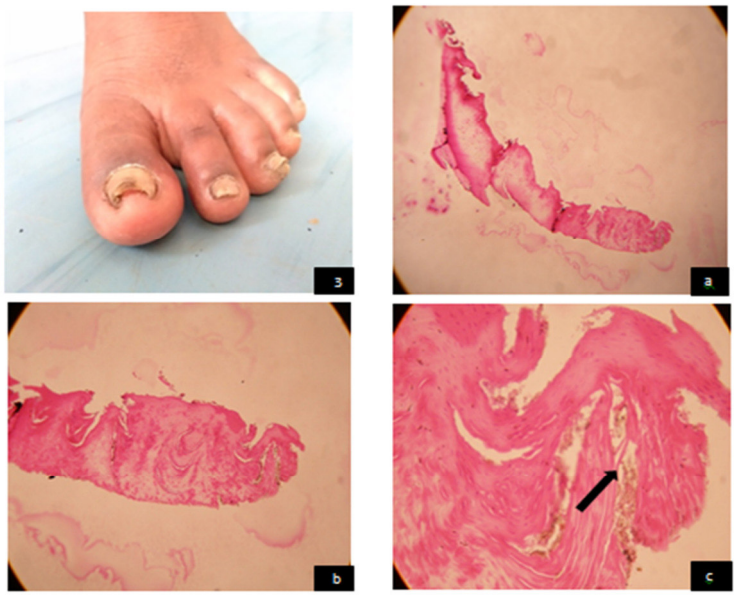

Fig 3. The great toenail showing xanth onychia and onycholysis. Figure 3a: At low magnification, the nail plate shows thickening and has subungual debris (hematoxylin and eosin, 4x). Figure 3b: At medium magnification the nail plate shows parakeratosis, fungus and neutrophils at the ventral aspect of the nail plate (hematoxylin and eosin, 40x). Figure 3c: High- magnification view of the nail plate shows many hyphal element within the nail plate (hematoxylin and eosin, 100x)

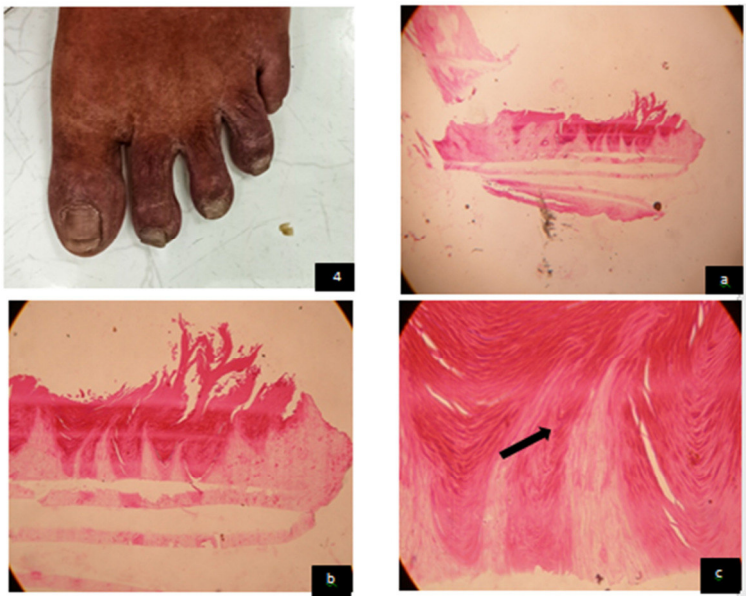

Fig 4. White superficial onychomycosis with coexistent distal-lateral subungual pattern. Figure 4a: At low magnification, the nail plate shows thickening and has subungual debris (hematoxylin and eosin, 4x). Figure 4b: At medium magnification, the nail plate shows parakeratosis,fungus and neutrophils at the ventral aspect of the nail plate (hematoxylin and eosin, 40x).Figure 4c: High- magnification view of Sagittal section of onychomycosis showing onycholysis with extensive subungual hyperkeratosis (hematoxylin and eosin, 100x).

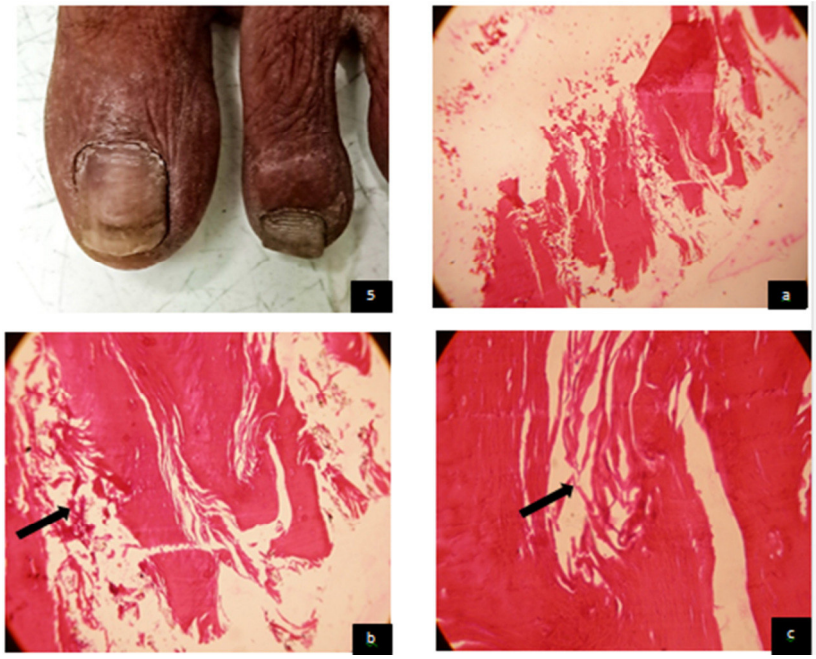

Fig 5. Onychomycosis due to T. rubrum affecting all the toenails. Figure 5a: A nail clip stained with hematoxylin and eosin. The bottom part is the ventral part facing the nail bed. Figure 5b: (hematoxylin and eosin) staining of a nail clip showing mostly spores along a few hyphae. Figure 5c: Nail clips showing (A) globules of plasma and parakeratosis (hematoxylin and eosin)

Fig 6. The antifungal cream formulation

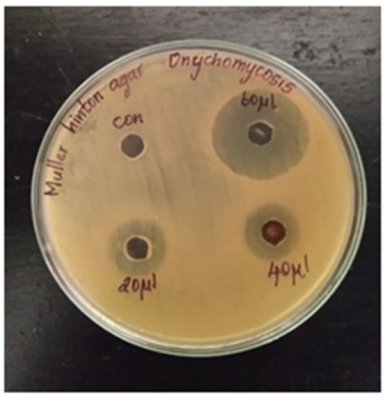

Fig 7. Well diffusion plate

These formulations are safe to use for skin and toenails. The formulated creams were tested for its antifungal activity by culturing it in agar medium. The plates were incubated for 24 hours at 37°C and it showed to have antimicrobial properties as compared to the standard (Figure 7) The prepared antifungal cream will be considered as an alternative therapeutic option in the near future for patients who are affected by onychomycosis infection.

4. CONCLUSION

The prepared antifungal cream formulation showed good consistency and no evidence of phase separation during the study period.

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6. AUTHORS CONTRIBUTION STATEMENT

Dr. P.F.Steffi, Ms.M.Pragathi and Ms.P.F.Mishel formulated the the cream, collected and analyzed the data, and wrote the manuscript. Dr. B.Thamaraiselvi and Dr. S.Reshma supervised all phases of this study and contributed significantly to the manuscript.

7. CONFLICTS OF INTEREST

Conflict of interest declared none.

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