INVITRO ANTIFUNGAL STUDY ON EXTRACTS OF A POLYHERBAL FORMULATION, "*DADRUHARA LEPA*" WITH SPECIAL REFERENCE TO RINGWORM (TINEA)

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***Abstract*- Ringworm (*Tinea*) is a most common dermatological problem affecting large number of population. *T.rubrum* and *T.verrucosum* is one of the most prevailing causative species. Tinea infection affects person's social & personal hygienic health. It is the most recurrent disease. There are many medicines indicated orally for this but the importance of external application of medicated paste (*lepa*) is specifically indicated. One of the most accepted clinical ancient text *Ashtang Hridaya* by *Acharya Vagbhatt* mentioned polyherbal combination *Dadruhara lepa* (DHL) for the management of *Dadru* (Ringworm). The effect of DHL on ringworm is the synergistic action of soluble alkaloids & other active components of mixture, therefore invitro study of aqueous & hydroalcoholic extracts of DHL against *T.rubrum* & *T.verrucosum* was carried out to evaluate the antifungal activity of the *DHL*. Both the extracts showed significant antifungal activity against the selected dermatophytes. Study was carried through Agar well diffusion method. The maximum inhibition zone against *T.rubrum* & *T.verrucosum* for the hydro-alcoholic extract at 40mg/ml was 36 mm & 40 mm respectively, whereas aqueous extract showed maximum inhibition zone of 24mm and 26mm at same concentration against both dermatophytes.**

Key words*: Ringworm, antifungal, *T.rubrum, T.verrucosum*. *Ayurvedic Lepa, Dadruhar Lepa

INTRODUCTION

Ringworm (*Tinea*) is a most common dermatological problem affecting large number of individuals. During the last decades, mycotic infections are increased to more than 20% - 25% of the world's population. Their etiological agents and predominating anatomical infection patterns vary with geographical location and environmental and cultural factors. Such fungi grow at surface temperatures of 25˚C - 28˚C with warm and humid conditions which is supported for infection on human skin. Infections by fungi are relatively common in tropical countries due to poor hygiene and poor medical care help to increase the epidemic spread of skin Mycoses.[1] The Indian subcontinent and close areas have a large number of ringworm infections throughout the country.[2] Several Indian mycologists have been reviewed and reported the mycosis in India.[3-9]

Ringworm is characterized by itchy red circular lesions on skin. Though the disease, 'Ringworm' is not a life threatening, it makes social embarrassing to person due to its appearance, severe itching disturbing routine and its nature susceptible to be chronic. In recent years there has been a considerable increase in the incidence of skin problems because of change in life style and use of incompatible foods & activities.[10]

Ayurveda, system of medicine based on equilibrium of body constitutions, maintaining and boost immune system of body to fight with external pathogens. Ring worm is compared with *Dadru* in Ayurveda. *Dadru* being one among *Kushtha roga* is also a *rasa, rakta* and *mamsapradoshaja vikara*.[11] It is usually caused by three types of *nidana sevana* i.e. *aharaja, viharaja* and *krimija*. Here *krimija (Raktaja krimi)* can be considered for the related fungi (dermatophytes) capable of causing skin changes of the type known as Tinea or Ringworm. *Dadru* presents clinically with the features of *kandu, raga, pidaka, daha, rukshta, udgata, mandala* etc.[12] which are very much similar with the features of Tinea like pruritis, erythema, vesicle, pustule etc.

The *Acharyas* in *Ayurveda* have mentioned three management regimes for skin diseases i.e. oral medication, external medication & invasive procedures (blood letting etc) for the management of skin diseases.[13] *Lepa kalpana* is a semi-solid preparation based on the principle of external medication. It is a supportive & effective route for drug application because it is applied directly over affected area.[14] The drugs are made into a fine powder. Before use on the body, it is mixed with some liquid medium indicated in each preparation and made into a soft paste.[15]

'Dadruhara Lepa' (DHL) is a poly herbal formulation of ten drugs i.e. *Chakramarda* (*Cassia tora* Linn), *Mulaka* (*Raphanus sativus* Linn), *Siddharthaka* (*Brassica compestris* Linn), *Haridra* (*Curcuma longa* Linn), *Shunthi* (*Zingeber officinale* Rosc), *Maricha* (*Piper nigrum* Linn), *Pippali* (*Piper longum* Linn), *Kushta* (*Saussurea lappa* Clarke), *Laaksha* (*Lacifera lacca*) , *Shriveshtaka* (*Pinus roxburgii* Sargent) along with *Takra* (butter milk). It is advised as an effective antifungal formulation for management of Ringworm and is therefore selected for this study.[16]

The ingredients of *Dadruhara lepa* (DHL) possess specific alkaloids and essential oils. *Laksha* (stick lac) contains Aleuritic acid (35 % of total resin) as the main constituent while shellolic acid and its isomers along with kerrolic acid and butolic acid are present in small extent.[17] *Shunthi* (Ginger) contains Gingerol Shagaol and essential oil (containing monoterpenes, sesquiterpenes, α and β-zingiberine) as main constituents.[18] *Maricha* (Black pepper) contains Piperine (5-10%), piperidine (5%), chavicine and essential oil as main constituents.[19] *Pippali* (Long pepper) contains Piperine (4-5%), volatile oil (0.7%).[20] *Chakramarda* (Foetid Cassia) contains Anthraquinoles and some fixed oils as chief constituents.[21] *Kutha* (Costus root) contains

alkaloid saussurine (0.5%), resinoids (6%), inulin (18%), essential oil (1.5%), fixed oil and traces of tannin and sugar.[22] *Mulaka beeja* (Radish seeds) contains Raphanin and Macrolysin some fixed oils and volatile oils.[23] *Haridra* (Turmeric) contains curcuminoids (6%), curcumin (50-60% of yellow colouring part), and essential oil.[24] *Gandhaviroja* (exudate of Pine tree) contains 1-α-pinene, 1-β-pinene, longifoline and other mono and sesquiterpenes.[25] *Siddharthaka* (Mustard seeds) contains glycosinolates, sinalbin, sitosterols, and glycerides of palmitic, stearic, linoleic acids.[26]

MATERIALS AND METHODS

1. Preparation of *Dadruhara lepa*:

The raw materials used for this formulation were procured from the local market and get authenticated in Department of Dravyaguna vigyana in Shubhdeep Ayurved Medical College Indore. Each of the ingredients (except *gandhaviroja* and *takra*) was sun dried and pulverized to fine powder (mesh size 120). All crude plant drugs were pulverised separately and then mixed together in equal ratio of 1:1 each (w/w). Later *Gandhaviroja* is added and mixture is triturated (*Bhavana*) with *Takra* (Butter milk) in an end runner until it becomes dry. It is shed dried until moisture is removed and again powdered and kept in air tight container.

Table No.1 – Contents of *Dadruhara lepa*

S.No.	Name of ingredient	Ayurvedic nane	Part Used	Amount
1.	Lacifera lacca	*Laksha*	Resin	100 gm
2.	*Zingeber officinale* Rosc.	*Shunthi*	Rhizome	100 gm
3.	*Piper nigrum* Linn	*Maricha*	Fruit	100 gm
4.	*Piper longum* Linn	*Pippali*	Fruit	100 gm
5.	*Cassia tora* Linn	*Chakramarda*	Seed	100 gm
6.	*Pinus roxburgii* Sargent	*Gandhaviroja*	Oleo-resin	100 gm
7.	*Saussurea lappa* Clarke	*Kutha*	Root	100 gm
8.	*Brassica compestris* Linn	*Siddharthaka*	Seed	100 gm
9.	*Curcuma longa* Linn	*Haridra*	Rhizome	100 gm
10	*Raphanus sativus* Linn	*Mulaka beeja*	Seed	100 gm
11.	Butter mik	*Takra*	-	Q.S.

2. Evaluation of Physical Parameters:

Physical parameters like total ash, acid insoluble ash, water soluble extractive values, alcohol soluble extractive values, and pH-value were carried out.[27] Thin layer chromatography (TLC) of aqueous and hydroalcoholic extracts was done for the phytochemical analysis.

Table No.2 – Physical parameters of *Dadruhara lepa* (DHL)

S.No	Physical Parameters	Values
1	pH of *Takra*	5
2	pH of prepared *Lepa*	4.81
3	Loss on drying at 105 ℃	5.2%
4	Total ash	8.05% w/w
5	Acid insoluble ash	42.43% w/w
6	Water soluble extractive value	24.5 %
7	Alcohol soluble extractive value	28.44 %
8	Organoleptic tests : ➢ Colour ➢ Odour ➢ Taste	 Greenish brown Characteristic Bitter pungent

Table No.3- TLC profile of DHL extracts

Extract	Solvent system	Spraying agent	Rf value
DHL (Aqueous)	Toluene : Ethyl Acetate (9 : 1)	Vanillin-Sulphuric acid	0.029, 0.074, 0.11, 0.149
	Chloroform : Methanol (19 : 1)	Vanillin-Sulphuric acid	0.03, 0.08, 0.1, 0.11, 0.166, 0.25, 0.33, 0.41
DHL (Hydro-alcoholic)	Toluene : Ethyl Acetate (9 : 1)	Vanillin-Sulphuric acid	0.151, 0.34, 0.71
	Chloroform : Methanol (19 : 1)	Vanillin-Sulphuric acid	0.22, 0.36, 0.55, 0.746

INVITRO ANTIFUNGAL STUDY:

Preparation of Plant extracts

To prepare the aqueous extract, 50gm of sample (*Dadruhara lepa*) powder was mixed with 200ml (four times) distilled deionised water & kept overnight. It was filtered using Whatmann filter paper no.1. Dried in water bath at 100ºC till all water gets evaporated and extract was obtained. Similar procedure was adopted for preparing the Hydro-alcoholic (40:60) extract by mixing 50 gm of sample in 300ml (six times) of solvent (Distilled water : Ethanol).

Microorganisms

The two most commonly prevailing species of Trichophytons i.e. *Trichophyton rubrum* & *Trichophyton verrucosum* were selected for the invitro antifungal study. Fungal strains of Trichophyton rubrum (NFCCI 2544) & Trichophyton verrucosum (NFCCI 3324) were procured from National Fungal Culture Collection of India (NFCCI-A National Facility, sponsored by DST, Govt. of India) Pune. The procured samples were sub-cultured and maintained in Sabouraud Dextrose Agar (HIMEDIA) Slants at 4ºC.

Preparation of culture media

It is prepared by suspending 65 grams of SDA (Himedia) in 1000 ml distilled water. Heated to boiling to dissolve the medium completely. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

Inoculum Preparation:

Isolates of *T.rubrum* and *T. verrucosum* were grown on SDA without antibiotics for 7 days. Colonies were covered with approximately 1ml of sterile 0.9% saline, 1 drop (approximately 0.01ml). The suspension of colonies was gently probed with the tip of a transfer pipette. The resulting mixture of conidia and hyphal fragments was withdrawn and transferred to sterile tube. Heavy particles were allowed to settle for 3 to 5 minutes, the upper homogenous suspension was transferred to a sterile tube, the cap was tightened and vortexed for 15 seconds. The turbidity was measured using a spectrophotometer at 1 McFarland standards.

Determination of Minimum Inhibitory Concentration (MIC)

MIC was determined according to the method described by Irobi *et al* [29]. The crude extracts were reconstituted with sterile distilled water and stock concentration of 80 mg/ml was made and MIC was determined by incorporating various concentrations of extract (40 mg/ml to 1.25 mg/ml) in Sabouraud Dextrose broth. 100 µl of fungal inoculum was added to each tube and incubated at room temperature for 21 days. The controls were also kept without any extract in culture media. The MIC was regarded as the lowest concentration of the extract that did not permit any visible growth after 7-21 days of inoculation.

Table No. 4 - Determination of MIC (from 40-1.25mg/ml)

Step	Intermediate Concentration (mg/mL)	Volume (ml)	Vol. of SDA (ml)	Final conc. (mg/ml)	Vol. of Inoculum (µl)
1	80	2	2	40	100
2	40	2	2	20	100
3	20	2	2	10	100
4	10	2	2	5	100
5	5	2	2	2.5	100
6	2.5	2	2	1.25	100

Table No. 5 - Antidermatophytic activity of both extracts of *Dadruhara lepa* powder

Conc. in mg/ml	Growth in Aqueous extract		Growth in hydro-alcoholic extract	
	T.rubrum	*T.verrucosum*	*T.rubrum*	*T.verrucosum*
1.25	+	+	+	+
2.5	+	+	+	-
5	+	-	-	-
10	-	-	-	-
20	-	-	-	-
40	-	-	-	-
Control	+	+	+	+

Key: + = presence of growth, - = Absence of growth

In-vitro assay with Agar Cup Diffusion Technique

The in-vitro antifungal screening is done by Agar cup diffusion method.[28] 200µl of fungal suspension is uniformly spread over solidified Sabouraud Dextrose Agar (SDA) plates with the help of a sterilized spreader. Four wells of 6 mm diameter were made in the centre of four quadrants of these agar plates with the help of a sterile cork borer. Each of the wells were then filled with the respective (one with aqueous extract & one with hydro-alcoholic extract) test extracts at different concentration ranging from 40mg/ml to 5mg/ml and allowed to diffuse at room temperature for an hour. Then the plates are incubated at 28±2ºC for 72-96 hours to 2 weeks depending on the growth rate of the test pathogen. The antifungal activities of the extracts were determined by measuring the diameter of the inhibition zone around the well that was filled with the extracts.

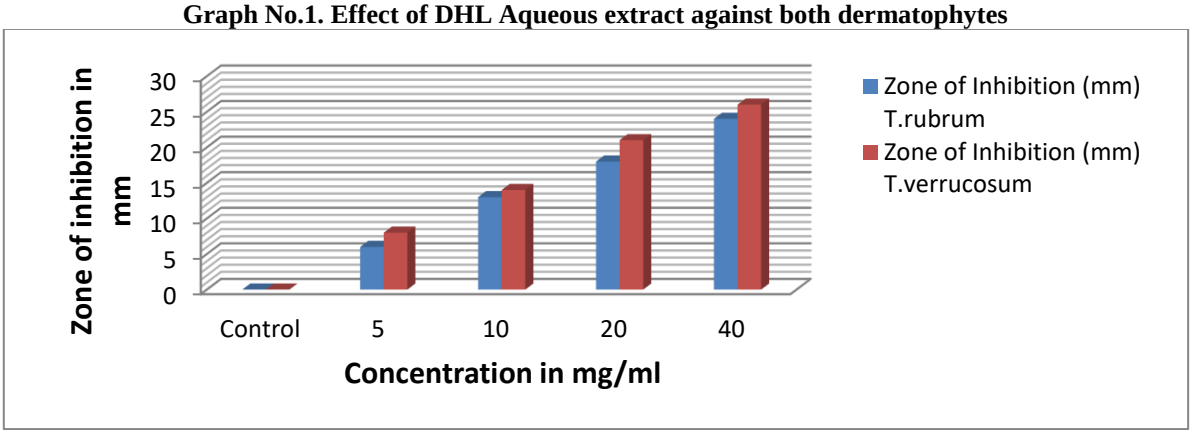
Table No.7 - Zone of Inhibition at different concentrations.

Extract Conc. in mg/ml	Zone of Inhibition (mm) Aqueous Extract		Zone of Inhibition (mm) Hydroalcoholic Extract	
	T.rubrum	*T.verrucosum*	*T.rubrum*	*T.verrucosum*
5	06	08	10	12
10	13	14	18	19
20	18	21	25	28
40	24	26	36	40
Control	0	0	0	0

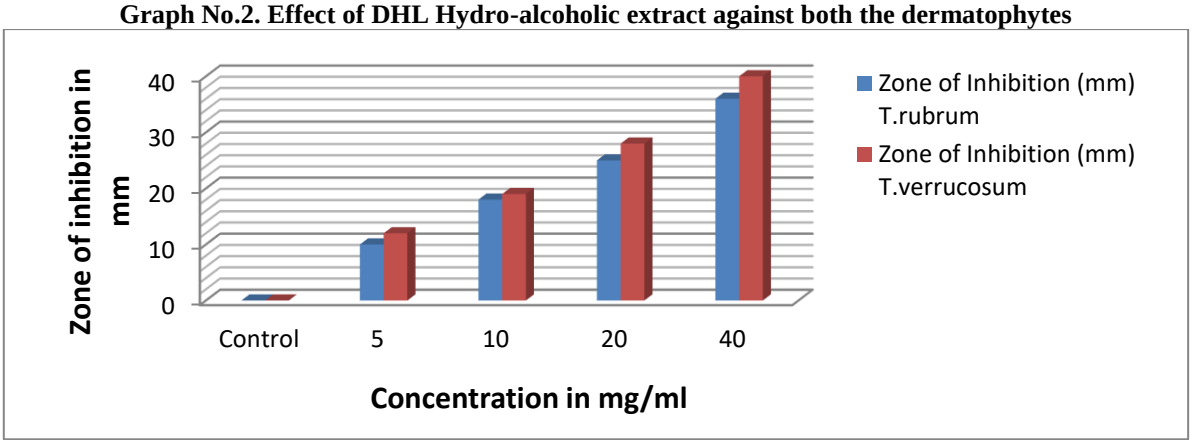
OBSERVATION AND RESULT:

The maximum inhibition zone of 36mm was observed against *T.rubrum* for the hydro-alcoholic extract at 40mg/ml while aqueous extract showed maximum inhibition zone of 24mm at 40mg/ml. The maximum inhibition zone against *T.verrucosum* for hydro-alcoholic extract was 40mm and 26mm for aqueous extract at 40mg/ml respectively (table No.6 & 7). The aqueous and hydro-alcoholic extracts were found to be effective against the tested dermatophytes in the Agar cup diffusion technique evident from the zone of inhibition they have produced (Table No.7). The MIC value for hydro-alcoholic extract ranged between 2.5mg/ml to 5mg/ml against *T.rubrum* and between 1.25 mg/ml to 2.5 mg/ml against *T.verrucosum* and for aqueous extract the MIC value ranged between 5mg/ml to 10mg/ml against *T.rubrum* and between 2.5 mg/ml to 5mg/ml against *T.verrucosum* (Table No.6). The control did not produce any inhibitory activity against the organisms.

Graph No.1. Effect of DHL Aqueous extract against both dermatophytes



Graph No.2. Effect of DHL Hydro-alcoholic extract against both the dermatophytes



DISCUSSION:

Dadruhara lepa is a polyherbal formulation enriched with many antifungal phytochemicals. The *Dadruhara lepa* extracts contain natural antimicrobial substances such as alkaloids, flavones (flavonoids, flavonols, quinones), lectins, polypeptides, phenolics, polyphenols, tannins and terpenoids which are known for their antimicrobial properties.[30-46]

Most plant derived medicines have been developed on the basis of traditional knowledge in the health care and in many cases there is a correlation between the indication of pure substances and those of respective crude extracts used in traditional medicine. The above mentioned drugs have shown antifungal activities against dermatophytes but no study has been carried out on the effect of these herbal drugs in combination. The results of the present study justifies that the Aqueous and hydro-alcoholic extracts of *Dadruhara lepa* showed good antifungal activity against *T.rubrum* and *T.verrucosum*.

Dadru (Ringworm) is a *kaphaja twak vikara* having *kapha dosha* dominating symptoms like itching, discolouration, inflammation etc. and the drugs present in *Dadruhara lepa* mostly have *Katu, tikta, laghu, ruksha* and *Ushna* properties which pacifies the dominating *kapha dosha*, hence it can act as a good antifungal medication for *Dadru* or Ringworm.

When a *Lepa* is applied over the surface of skin opposite to the direction of hairs on it, through a proper base, the active principles of the ingredients of Lepa are released into that base. After that, this combination enters the *Romkupa* & further gets absorbed through the *Swedavahi Srotas* & *Siramukh* it does the Cutaneous Biotransformation and which will pacify the *Doshas* and leads to breaking of *Samprapti*.

CONCLUSION:

The study was aimed at finding a good antifungal formulation for Tinea or Ringworm infections. It is seen that the antifungal activity of the extracts was concentration dependent; more zone of inhibition was seen in higher concentration of extracts, also more Rf values in the TLC for both the extracts in the Chloroform: Methanol (19:1) solvent system indicates more soluble active ingredients in the solvent. The result of the present study showed that *Dadruhara lepa* possesses antifungal property, the hydro-alcoholic extract showed better efficacy than the aqueous extract. This study will make track for evidence based and rational Ayurvedic medication against the cutaneous fungal infection.

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