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In-Vitro Antimicrobial Activity and Toxicological Aspects of a Polyherbal Oil Formulation: *Tuvaraka taila*

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ABSTRACT

The objective of the study was to test the polyherbal oil *Tuvaraka taila* (TT) for *in vitro* antimicrobial activity against Gram-negative and -positive bacteria, *Candida* species and dermatophytes and to evaluate its acute toxicological effects *in vivo*. The TT was found effective against all tested strains in agar well diffusion method. The minimum inhibitory concentration of TT ranged from 156 to 2500 µg/ml, the minimum bactericidal concentration ranged from 312 to 5000 µg/ml while the minimum fungicidal concentration ranged from 325 to 5000 µg/ml. The acute administration and dermal application of TT was devoid of toxicity in rats. TT may be a promising source in the search for new antifungal drugs due to its efficacy and low toxicity.

Keywords: Ayurveda, antibacterial, antidermatophytic, chaulmoogra oil
Abbreviations: TT, *Tuvaraka taila*

INTRODUCTION

Skin diseases like ringworm, leprosy, leucoderma and eczema are prominent problems in developed countries of tropical and subtropical region. Bacterial and fungal strains like *Candida*, *Microsporum*, and *Trycophytons* are the most common pathogens in human and animals. There has been constant search for biologically active compounds from natural sources due to a lack of efficacy, side effects and resistance offered by microorganisms to the existing drugs (Fontenelle *et al.* 2007; Sanguinetti *et al.* 2007; Jardim *et al.* 2008). Further, most antifungal and anti bacterial creams used are expensive. Therefore topical, traditional medicines, especially *taila* (a medicinal oil) preparations using local herbal material are very popular (Burdock and Carabin 2008; Cross *et al*. 2008).

Taila are polyherbal oil preparations. They form an important class of ayurvedic medicinal system. They were formulated with the main objective of incorporating the fat-soluble fractions of herbal drugs to a suitable oil base. There are number of marketed Ayurvedic *taila* which are used for treatment of skin diseases (Mukerjee 2002; Anonymous 1978). With an increasing number of people switching once again towards the alternative system of medicine, it is important to ensure that they get authentic medicines of recommended quality (Trease and Evan 2002; Fontenelle *et al.* 2007; Salman *et al.* 2008).

Chemical composition, toxicological aspects and antifungal activity of essential oil (EO) from *Lippia sidoides* Cham. was reported by Fontenelle *et al.* (2007). *In vitro* activity of *Citrus bergamia* (bergamot) EO against clinical isolates of dermatophytes was reported by Sanguinetti *et al.* (2007). Kan *et al.* (2006) reported chemical composition and antibacterial activity of *Satureja cuneifolia* EO. Antifungal activity and composition of EO of Brazilian *Chenopodium* was reported by Jardim *et al.* (2008). Salman *et al.* (2008) reported antimicrobial activity of *Nigellia sativa* seed oil. *In vitro* activity of tea tree EO against dermatophytes and filamentous fungi was carried out by Hammer *et al.* (2002).

Tuvaraka taila (TT) is a widely marketed Ayurvedic polyherbal oil used for various skin aliments like leucoderma, psoriasis, bacterial and fungal infections. TT is clear yellow-brown oil prepared by digestion of decoction of catechu bark (*Acacia catechu*; Family: Mimosacaae) and *chaulmoogra* seeds powder (*Hydnocarpus kurzii* (King) Warb.; Family: Flacourtiaceae) in chaulmoogra oil (oil of *H. kurzii*) as per classical Ayurvedic text (Sharma 1985; Anonymous 2000). The components of TT are reported to have wide range of antimicrobial activity (Jacobsen *et al.* 1973; Saini *et al.* 2008; Patel *et al.* 2009). But no scientific data on safety and efficacy of TT is available. In the present studies attempt was made to scientifically evaluate the *in vitro* antimicrobial activity of TT. Acute toxicity study was performed for TT.

MATERIALS AND METHODS

Test material

Commercial marketed poly herbal oil formulation '*Tuvaraka taila'* manufactured by three different reputed Ayurvedic drug manufacturers (Bharadwaj Pharmaceuticals Ltd., Indore, Shree Baidyanath Ayurved Bhawan Pty. Ltd., Kolkata and Gangaram Mohandas Pharmaceuticals, Indore was collected in triplicate from retail pharmacies in Indore, Madhya Pradesh, India.

Chemicals

Gentamycin and Itraconazole were supplied as free samples by Zyg Pharmaceuticals Ltd, Indore Madhya Pradesh, India. Potato dextrose agar, Mueller–Hinton agar and Mueller–Hinton broth were purchased from Merck (Germany), and RPMI 1640 medium from Sigma (USA).

Test organisms

Bacterial and fungal strains selected for studies were obtained from the collection of Department of Microbiology (Choitthram Hospital and Medical Research Centre), Indore (Madhya Pradesh)

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Research Note

India. The test microorganisms used for the antimicrobial activity screening were Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228 and *Bacillus subtilis* ATCC 6633); Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Kebsiellia* sp. (clinical isolate) and *Pseudomonas aeruginosa* ATCC 27853) and dermatophytes (fungal) strains (*Candida albicans* ATCC 10231, *Candida tropicalis* ATCC 20336, *Trichophyton rubrum* ATCC 40051, *Trichophyton mentagrophytes* ATCC 9533, *Microsporum canis* ATCC 32903 and *Epidermophyton floccosum* ATCC 52066).

Animals

Albino rats of Wistar strain of either sex weighing between 150-200 g and inbred female New Zealand white rabbits weighing between 2.0-2.5 kg were used. They were housed in standard cages at room temperature (26 ± 2°C) and 44–56% relative humidity, under a light/dark cycle of 10/12 h, for 1 week before the experiments. Rats were provided with standard rodent pellet diet (Amrut, India) and water *ad libitum*. Rabbits were provided with rabbit feed (Amrut, India) and water *ad libitum*. The animals were deprived of food for 24 hrs before experimentation, but had free access to drinking water. All experiments were performed in the morning. The study was approved by the institutional ethical committee, (465/01/96/CPSCSEA) which follows the guidelines of CPSCEA (Committee for the Purpose of Control and Supervision of Experimental on Animals), which complies with international norms of INSA.

Antimicrobial testing

1. Preparation of bacterial and fungal inocula

Prior to testing, each bacterial isolate was subcultured overnight at 37°C in Muller Hinton agar slant. The slopes were flooded with 0.85% (w/v) saline and transferred to a sterile tube. The bacterial suspension concentration was set to 10^5 colony forming unit (CFU) per ml using 0.5 McFarland standard turbidity. Fungal isolates were subcultured on a potato dextrose agar (PDA) slant at 28°C for 2-10 days or until good conidiation was produced. For each dermatophytic isolate, a suspension of conidia was prepared in 0.85% (w/v) saline by swabbing the colony surface with a sterile swab. After the settling of the larger particles, conidia concentration of inoculum was set to 10^5 CFU/ml using 0.5 McFarland standard turbidity. 100 µl of the suspension of the tested microorganism containing 10^5 CFU/ml of bacterial, 10^5 CFU/ml of yeast was used for testing.

2. Agar-well diffusion susceptibility test

The antibacterial susceptibility of TT was evaluated by the agar-well diffusion method. Petri dishes with a diameter of 15 cm were prepared with potato dextrose agar for fungal strains and Mueller Hinton agar culture for bacterial strains. Wells (6 mm in diameter) were then cut from the agar. The TT was weighed and diluted in dimethyl formamide to obtain the test concentrations of 25, 50, 75 and 100 mg/ml. Stock solutions of Gentamycin (1 mg/ml) and Itraconazole (0.5 mg/ml) were prepared in distilled water and tested as positive controls for bacterial and fungal strains respectively. Each bacterial and fungal suspension was inoculated onto the surface of the agar, from which 0.1 ml of inoculum was immediately and evenly distributed with a Drigalski spatula. These plates, after having been at 4°C for 2 hrs were incubated aerobically at 37°C for 24 hrs for bacteria and at 25°C for 24 hrs for yeast. All Petri dishes were examined for zones of growth inhibition and the diameters of these zones were measured in mm. Each experiment was repeated at least twice. A negative control was also prepared using the same solvent employed to dissolve the TT. Antimicrobial activity was evaluated by measuring the zone of inhibition against the tested microorganisms. The result recorded for each bioassay was the average of 3 tests. The results were expressed as mean ± SD of the diameter of the growth inhibition zones (mm).

3. Broth micro dilution method

The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) or minimum bactericidal concentration (MBC) for TT were determined by the broth microdilution method, MICs were determined using Mueller–Hinton broth for bacterial testing and for fungal testing, 3-(*N*-morpholino)propanesulfonic acid-buffered RPMI 1640 medium was used. The TT was 2-fold diluted in DMF to the highest concentration (5 mg/ml) to be tested. Then serial dilutions were made in a concentration range from 78 to 5000 µg/ml in nutrient broth. Gentamycin (7.8-500 µg/ml) was used as a positive control for bacterial strains and itraconazole (0.03 to 16 µg/ml) was used for fungal strains. The microdilution assay was performed in 96-well microdilution plates (Micro Test III™ Flexible Assay Plate; Falcon, Becton Dickinson and Co., CA). Growth and sterile control wells were included for each isolate tested. In brief, the wells of 96-well plates were dispensed with 95 µl of nutrient broth and 5 µl of the inocula. A 100 µl aliquot from the stock solutions of the TT initially prepared at the concentration of 5000 µg/ml was added to the first wells. Then 100 µl from their serial dilutions was transferred into six consecutive wells. The last well containing nutrient broth without compound and the same amount of inocula with previous wells on each strip was used as a negative control. The final volume in each well was 200 µl. The microplates were incubated at 37°C for 24 hrs for bacterial strains and 28°C for 2-8 days for fungal strains. All isolates were run in duplicate and repeated at least twice. MIC endpoints were read as the lowest concentration of antimicrobial that totally inhibited macroscopically visible growth of the inocula.

Toxicological studies

1. Acute oral toxicity studies

The effects of TT were observed after a single *per oral* administration to rats. Initially the study was carried out with one group consisting of three female animals at 200 mg of active ingredient per kg of body weight. Based on these observations the dose was increased to 2000 mg/kg body weight. All rats were subjected to a gross necropsy examination after fourteen days (OECD 2001).

2. Acute dermal toxicity studies

The effects of TT after a single dermal administration to rats were investigated. TT at a dose of 2000 mg/kg of body weight was administered once dermally on an area of 5 × 6 cm on the dorsal thoracic region of five male and five female rats and the duration of the exposure was 24 h. The animals were subjected to a necropsy including a gross pathological examination after 14 days (OECD 2001).

3. Dermal irritancy test

The effects of TT following a single application to the intact skin of rabbits were examined. The test substance (1.5 g) was spread on cellulose patches in a size of about 2.5 × 2.5 cm and was applied to the intact skin of each of three female white rabbits. At the end of the exposure period (4 h) the dressings and the patches were removed. The skin was examined for erythema/eschar and oedema as well as for other local alterations at 1, 24, 48 and 72 h after patch removal.

RESULTS

The main components of TT are chaulmoogra oil and acacia catechu bark. The antibacterial activity of TT has been attributed to these active components. Previous studies showed the antibacterial properties of both components of TT (Jacobsen *et al.* 1973; Saini *et al.* 2008; Patel *et al.* 2009).

The semi-quantative agar well diffusion method is easy to perform, and is useful to detect the activity of antimicrobial agents on large numbers of cultures. It also gives an opportunity to evaluate the antibacterial activity to some extent without performing a MIC assay. The TT was effective

Table 1 Antimicrobial susceptibility testing of TT by agar well diffusion assay.

Strains	Growth inhibition zones (mm) ± S.D. (n = 3)					
	Tuvaraka taila (TT) (mg/ml)				Gentamycin (mg/ml)	Itraconazole (mg/ml)
	25	50	75	100	1	0.5
Gram-positive bacteria						
Staphylococcus epidermidis	0	7 ± 1.3	15 ± 2.1	35 ± 3.2	58 ± 4.2	-
Staphylococcus aureus	0	8 ± 1.5	17 ± 2.6	38 ± 3.6	55 ± 3.1	-
Bacillus subtilis	5 ± 0.8	10 ± 1.7	20 ± 3.4	45 ± 3.5	60 ± 3.8	-
Gram-negative bacteria						
Escherichia coli	0	9 ± 1.6	19 ± 1.7	32 ± 3.2	45 ± 3.7	-
Pseudomonas aeruginosa	0	7 ± 1.1	13 ± 1.5	27 ± 2.1	30 ± 2.9	-
Kebsiellia sp. (clinical isolate)	4 ± 1.2	11 ± 1.8	20 ± 1.9	44 ± 2.8	42 ± 2.5	-
Dermatophytes						
Candida albicans	0	6 ± 0.5	10 ± 1.1	16 ± 2.1	-	46 ± 2.6
Candida tropicalis	0	4 ± 0.8	8 ± 1.2	14 ± 1.4	-	44 ± 2.8
Trycophyton mentagrophytes	0	6 ± 0.9	14 ±.2	12 ± 1.1	-	52 ± 2.7
Trycophyton rubrum	0	7 ± 1.4	16 ± 1.2	24 ± 1.1	-	48 ± 3.2
Microsporum canis	3 ± 0.6	10 ± 1.5	22 ± 1.5	29 ± 1.4	-	55 ± 3.1
Epidermis flocossum	4 ± 0.3	9 ± 1.1	18 ±.7	26 ± 2.1	-	54 ± 3.5

Table 2 *In-vitro* antimicrobial activity of TT determined by broth microdilution method.

Strain	*Tuvarak taila* (TT) (µg/ml)		Gentamycin (µg/ml)		Itraconazole (µg/ml)	
	MIC[a]	MBC[b]/MFC[c]	MIC[a]	MBC[b]	MIC[a]	MFC[c]
Gram-positive bacteria						
Staphylococcus epidermidis	156	312	31.2	62.5	-	-
Staphylococcus aureus	156	312	31.2	62.5	-	-
Bacillus subtilis	156	312	15.6	31.2	-	-
Gram-negative bacteria						
Escherichia coli	625	1250	31.2	62.5	-	-
Pseudomonas aeruginosa	1250	2500	125	250	-	-
Kebsiellia sp. (clinical isolate)	156	312	31.2	62.5	-	-
Dermatophytes						
Candida albicans	1250	2500	-	-	1	2
Candida tropicalis	2500	5000	-	-	2	4
Trycophyton mentagrophytes	625	1250	-	-	0.125	0.250
Trycophyton rubrum	312	625	-	-	0.125	0.250
Microsporum canis	156	312	-	-	0.06	0.125
Epidermis flocossum	156	312	-	-	0.125	0.250

[a] minimum inhibitory concentration
[b] minimum bactericidal concentration
[c] minimum fungicidal concentration

against all tested strains in the agar-well diffusion susceptibility tests

The antibacterial activity of the TT was examined against a broad spectrum of microorganisms in the present study. The results (**Table 1**) showed that, activity against several Gram-positive (*S. aureus*, *B. subtilis*, *S. lutea*), Gram-negative (*P. aeruginosa*, *E. coli*, *Klebsellia* spp.) bacteria, dermatophytes (*M. canis*, *T. mentagrophytes*, *T. rubrum*) and *Candida* species was observed with TT. The TT induced a significant growth inhibition zone (45 ± 3.5 mm) at the higher concentration (100 mg/ml) against Gram-positive bacteria and 44 ± 2.8 mm against Gram-negative bacteria. For dermatophytes the maximal inhibition of fungal growth induced by TT was 29 ± 1.4 mm, at the higher dose used (100 mg/ml). The positive control, gentamycin induced a significant growth inhibition zone (60 ± 3.8 mm) against bacteria and itraconazole induced a significant growth inhibition zone (55 ± 3.1 mm) against dermatophytes and *Candida* species. The inhibition produced by TT was comparable to the standard drugs at $P < 0.001$. By the broth microdilution method, MICs ranged from 156 to 2500 µg/ml and MBCs/ MFCs ranged from 312 to 5000 µg/ml (**Table 2**). This suggests that the TT represents a useful source of mixtures of antibacterial compounds that may exhibit potential for use as medicine.

The oral and dermal LD_{50} of TT in rats was found to be above 2000 mg of oil/kg of body weight. After a single oral administration of TT at a dose of 2000 mg/kg of body weight to female rats, all animals survived and no toxic symptoms were evident. A single dermal application of TT at a dose of 2000 mg/kg of body weight revealed no toxic effects of the test substance. All animals survived until the scheduled termination of the study. Body weight and body weight gain were inconspicuous during the whole study in all rats, and all animals were normal at the terminal necropsy. In the acute dermal irritation/corrosion study with rabbits, no general toxic effects of TT were observed and all exposed skin sites were found to be normal at each examination term.

DISCUSSION

Traditional herbal preparations are a potentially useful source of antimicrobial compounds (Fontenelle *et al.* 2007; Sanguinetti *et al.* 2007; Jardim *et al.* 2008). This study was an attempt to evaluate the poly herbal oil preparation '*Tuvaraka taila*' (TT) for antibacterial activity. TT can be an excellent candidate for the treatment of many infectious diseases, including mycosis, due to the increasing development of antimicrobial resistance as well as the appearance of undesirable effects of some antifungal agents (Sharma 1985; Anonymous 2000). Early reports on chaulmoogra oil and *Acacia catechu* revealed their antimicrobial action. There are reports on the highest and broadest activity against bacteria and fungi, including yeasts, dermatophytes and non-dermatophytic fungi (Jacobsen *et al.* 1973; Saini *et al.* 2008; Patel *et al.* 2009). High catechin content in TT was reported by Dubey *et al*. (2009). The present study showed

that TT was quite effective against bacteria and, the most common species of dermatophytes that cause superficial fungal infection.

The antimicrobial activity of TT was also evident in the agar-well diffusion and broth microdilution studies. As there was a good correlation between the MICs, MFCs and the agar-well diffusion values of the formulation, it may be concluded that the antimicrobial activity of *taila* preparation could be preliminarily investigated by the agar-well diffusion test for rapid screening (Fontenelle *et al.* 2007).

TT showed a low acute oral and dermal toxicity with an LD_{50} > 2000 mg/kg of body weight (a concentration high above the therapeutic dose) and was not irritating to the skin. Further toxicity studies including acute eye toxicity, skin sensitization, mutagenicity and chronic exposure are needed to determine the complete toxicity profile of TT (Craiga *et al*. 2004; Buxbaum *et al*. 2006; Burdock and Carabin 2008).

Owing to its broad spectrum of antimicrobial effect *in vitro*, and low toxicity, TT proved to be a promising source as a new antimicrobial drug. However, specific pharmacological approaches will be needed in future clinical trials to validate its use as a phytotherapeutic product (Burdock and Carabin 2008; Cross *et al*. 2008).

The preliminary results of the present study demonstrated its broad antimicrobial properties, which make TT a promising tool for topical application in the prophylaxis and treatment of bacterial and fungal infections.

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