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In vitro Evaluation of Some Herbal Compounds on Fungi Isolated from Clinical Cases in Animals and their Associated Environment

Bhoj R Singh*, Dharmendra K Sinha, Vinodh Kumar OR and Sakshi Dubey

Division of Epidemiology, ICAR-Indian Veterinary Research Institute, Izatnagar-243122, India

***Corresponding author:** Bhoj R Singh, 438-MLB, Division of Epidemiology, ICAR-Indian Veterinary Research Institute, Izatnagar-243122, India, Fax: +915812303284; E-mail: brs1762@yahoo.co.in

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Abstract

Background: Fungal infections are often difficult to cure in animals due to cost of treatment and emerging antifungal drug resistance. Herbal antifungals may be an alternate. Therefore, the objective of this study was to determine antifungal drug sensitivity pattern and activity of herbal antimicrobials on fungal isolates from clinical cases in animals and their associated environment.

Methods and Findings: This study was conducted on 69 isolates comprised of yeasts (49) and moulds (20) isolated from clinical cases of animal (26) and environmental (22) samples and one reference strain for determining antifungal efficacy of common antimycotic drugs (amphotericin B, miconazole, nystatin, clotrimazole, itraconazole, ketconazole and fluconazole) and herbal antimicrobials viz., ajowan (*Trachyspermum ammi*) seed oil, betel (*Piper betle*) leaf oil, guggul (*Commiphora mukul*) oil, thyme (*Thymus vulgaris*) oil, marjoram (*Origanum majorana*) essential oil, *Cinnamon* (*Cinnamomum verum*) bark oil, holy basil (*Ocimum sanctum*) oil, citral, cinnamledehyde, carvacrol, lemongrass (*Cymbopogon citrates*) oil, sandalwood (*Santalum album*) oil, methanolic extract of *Eupatorium odoratum* leaves, Methanolic extract of *Ageratum conizoides*, essential oil of *Zanthoxyllum rhetsa* seed carp, agarwood (*Aquilaria malaccensis*) oil, and patchouli (*Pogostemon cablin*) essential oil amphotericin B (100 units), miconazole (10 µg), nystatin (100 units), clotrimazole (10 µg), itraconazole (10 µg), ketconazole (30 µg) and fluconazole (10 µg). Most of the yeast isolates except six from soil (2) and water (4) were sensitive to nystatin. Amphotericin B inhibited almost two third of the yeast isolates and miconazole was the most effective (75%) imidazoles followed by clotrimazole (38.8%), itraconazole (32.65%), fluconazole (18.4%) and ketoconazole (12.25%). Among herbals, thyme oil, carvacrol, cinnamon oil and cinnamaledehyde inhibited the growth of all the yeast isolates tested with MIC ranging from 0.001 mg to 1.2 mg mL^{-1}. The next most effective herbal was holy basil oil (95.2%) followed by ajowan oil (87.75%), citral (87.75%) and lemongrass oil (77.75%). Lemongrass oil was equally antimycotic both on yeasts and moulds. The study revealed the promising antimycotic activity of herbs and prevalent antimycotic drug resistance among clinically important fungi in animals.

Conclusion: The fungi associated with animal clinical infections and their environment were often resistant to commonly used antifungal drugs but some of the herbals as cinnamon oil, thyme oil, ajowan oil and lemongrass oil as well as their active components have shown potential antifungal activity and hope for the future drug development.

Keywords: Antifungal drug resistance; Herbal antifungal; Carvacrol; Cinnamaldehyde; Citral; *Aspergillus*; *Candida*; Mycosis

Introduction

Emergence of drug resistance is a worldwide menace and is not limited to only bacteria but continuously being reported in ever increasing frequencies among other microbes including viruses, parasites and insects and pests and yeasts, moulds too [1]. Considering the emergence of resistance in microbes for conventional antimicrobials alternate sources are continuously under screening for antimicrobial potential including antibacterial [2] and antifungal [3] activities. The utility of antifungal drugs often depends on several factors like the site of infection, type of infection, drug toxicity, immune status and general condition of the patients. Under the stress, global climate change and increasing industrialization conditions burden of invasive fungal infections is consistently increasing specifically in individuals with compromised immunity [4] and the spectrum of fungal pathogens causing infections is also widening [5,6]. There are not many antifungal drug options for internal use, particularly for fungal infections resistant to two or more classes of antifungals [1,5]. In past some of the herbal compounds and herbal extracts have shown antifungal potential [3,7], however, needs more studies for exploring their clinical utility. The present study was undertaken to evaluate the antimycotic drug resistance pattern and effect of common herbal compounds and herbal extracts on fungal isolates from clinical cases in animals and their environment.

Materials and Methods

A total of 69 fungal (49 yeasts and 20 moulds) isolates (**Table 1**) available and isolated at Clinical Epidemiology Laboratory of Division of Epidemiology of the Institute were included in the

study. All isolates were identified at Mycology Laboratory of the Institute using growth and colony characteristics, morphology and sugar fermentation tests as per standard methods [8,9]. Isolates of yeasts (49) were tested both for antifungal drugs and herbal antimicrobials while isolates of moulds were tested for their sensitivity to selected herbal antimicrobials through disc diffusion method [10,11]. The tests were conducted using commercial discs for antifungal drugs (Hi-Media, Mumbai, India) and discs impregnated with 1 mg of a herbal antimicrobial. Herbal antimicrobials tested in the study were ajowan (*Trachyspermum ammi*) seed oil, betel (*Piper betle*) leaf oil, guggul (*Commiphora mukul*) oil, thyme (*Thymus vulgaris*) oil, marjoram (*Origanum majorana*) essential oil, Cinnamon (*Cinnamomum verum*) bark oil, and holy basil (*Ocimum sanctum*) oil, from Shubh Flavours and Frangrence Ltd, New Delhi; citral, cinnamledehyde and carvacrol from Sigma, USA; lemongrass (*Cymbopogon citrates*) oil, sandalwood (*Santalum album*) oil, methanolic extract of *Eupatorium odoratum* leaves, Methanolic extract of *Ageratum conizoides*, essential oil of *Zanthoxyllum rhetsa* seed carp, agarwood (*Aquilaria malaccensis*) oil, and patchouli (*Pogostemon cablin*) essential oil from Naga Fragrance Ltd, Dimapur, India. Individual antifungal drug loaded discs including amphotericin B (100 units), miconazole (10 µg), nystatin (100 units), clotrimazole (10 µg), itraconazole (10 µg), ketconazole (30 µg) and fluconazole (10 µg) were purchased from Hi-Media (Mumbai). Minimum inhibitory concentration (MIC) of carvacrol, cinnamaldehyde and citral was determined using agar well dilution method [12].

Table 1: Fungal isolates and their source.

Fungi	Number of isolates	Source (number of isolates)
Aspergillus flavus	6	Air of Dairy farm (6)
Aspergillus niger	10	Air of Dairy farm (5), Hands of animal handlers (5)
Penicillium spp.	3	Air of dairy farm (3)
Trichophyton equinum	1	Horse skin infection (1)
Candida albicans	4	Balenoposthitis in bull (1), Droppings of birds perching in dairy farm (3)
Candida famata	2	Pond water (1), Wound in an elephant (1)
Candida kefyr	1	Pond water (1)
Candida krusei	2	Droppings of birds perching in a dairy farm (1), Hands of animal handlers (1)
Candida pseudotropicalis	4	Pond water (4)
Candida spp.	17	Metritis (6), Balenoposthitis (6), skin infections (5)
Candida tropicalis	10	Pond water (10)
Geotrichum candidum	1	Soil of dairy farm (1)
Rhodotorulla glutinus	1	Soil of dairy farm (1)
Trichosporon spp.	7	Pig skin swabs (5), Mastitis in a cow (1), Reference strain (1)

Results

Except for an isolate of *Trichcophyton equinum* isolated from ringworm case of a horse all other isolates of moulds (*Aspergillus flavus,* 6, *A. niger,* 10 and *Penicillium spp.*, 3) were isolated either from air samples of a dairy farm (14) or from hand swabs of dairy farm workers (5). Most of the moulds were resistant (**Table 2**) to patchouli oil (PO), guggul oil (GO), carvacrol, methanolic extract of *Eupatorium odoratum* leaves (MEUO), sandalwood (*Santalum album*) oil (SWO) and essential oil of *Zanthoxyllum rhetsa* seed carp (ZREO), however, lemongrass oil (LGO), agarwood oil (AWO) and methanolic extract of *Ageratum conizoides* (MEAC) inhibited ≥ 70% of the moulds.

Yeasts could be isolated both from cases of clinical illness (25), wallowing pond waters (16), dairy farm soil (2), droppings of the birds perching in dairy farm (4) and one was on the hands of animal handler and a strain was a reference strain in the laboratory (**Table 1**). Isolates of *Candida tropicalis*, *C. psudotropicalis* and *C. kefyr* were isolated exclusively from pond water while yeasts from soil belonged to *Geotrichum candidum* and *Rhodotorula glutinous* species only. Except *C. albicans* associated with balenoposthitis in a bull and *C. famata* isolated from infected would of an elephant, yeasts associated with disease conditions either of *Candida* or *Trichsporon* genus could not be identified to species level due to limitations of the laboratory.

Most of the yeast isolates except six from soil (2) and water (4) were sensitive to nystatin. Amphotericin B inhibited almost two third of the strains and most ($p \leq 0.05$) of the resistant strains belonged to *Candida* species isolated from clinical cases. However, for other antifungal drugs, there was no significant difference in sensitivity pattern with respect to the clinical or environmental source of isolation. Among the imidazole group of antifungal antibiotics, miconazole was the most effective (75%) followed by clotrimazole (38.8%), itraconazole (32.65%), fluconazole (18.4%) and ketoconazole (12.25%).

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Herbal antimicrobials including thyme oil, carvacrol (an active ingredient of thyme oil), cinnamon oil and cinnamaldehyde (active ingredient of cinnamon oil) inhibited the growth of all the yeast isolates tested in the study (**Table 2**) with MIC ranging from 0.001 to 1.2 mg mL^{-1}. The MIC of carvacrol was the maximum (1.2 mg mL^{-1}) for *C. albicans* isolates from an animal handler and a *Candida* isolate from a case of metritis in cow while it was the minimum for an isolate of *Candida spp.* isolated from urinary tract infection in a dog (0.001 mg mL^{-1}). The MIC of cinnamaldehyde was the maximum (1 mg mL^{-1}) for *C. krusei* isolates from an animal handler and was the minimum for an isolate of *Candida spp.* isolated from urinary tract infection in a dog (0.001 mg mL^{-1}). Other effective herbal antimicrobials were holy basil oil (95.2%), ajowan oil (87.75%), citral (87.75%) and lemongrass oil (77.75%). The MIC of citral for sensitive yeast isolates ranged from 0.01 to 1.2 mg mL^{-1} while for those designated as resistant it was between >1.2 mg mL^{-1} to 25.6 mg/mL. The MIC of citral was the maximum for *Trichosporon* isolates from clinical cases and minimum for *C. tropicalis* isolates from pond water.

Table 2: Resistance patterns of fungal isolates tested against antifungal drugs and herbal antimicrobials.

Antimicrobials tested	Molds (20)	Resistant (%)	Yeasts (49)	Resistant (%)
Ajowan (*Trachyspermum ammi*) seed oil	NT	NT	49	6 (12.25)
Betel (*Piper betle*) leaf oil			49	18 (36.73)
Guggul (*Commiphora mukul*) oil	20	16 (80.00)	49	37 (75.51)
Carvacrol	20	16 (80.00)	49	0 (0.00)
Thyme (*Thymus vulgaris*) oil	NT	NT	49	0 (0.00)
Marjoram (*Origanum majorana*) essential oil	NT	NT	49	25 (51.02)
Cinnamon (*Cinnamomum verum*) bark oil	NT	NT	49	0 (0.00)
Holy basil (*Ocimum sanctum*) oil	NT	NT	49	2 (4.80)
Cinnamledehyde	NT	NT	49	0 (0.00)
Citral	NT	NT	49	6 (12.25)
Lemongrass (*Cymbopogon citrates*) oil	20	6 (30.00)	49	11 (22.45)
Sandalwood (*Santalum album*) oil	20	11 (55.00)	49	37 (75.51)
Methanolic extract of *Eupatorium odoratum* leaves	20	16 (80.00)	49	38 (77.55)
Methanolic extract of *Ageratum conizoides*	20	7 (35.00)	49	30 (61.22)
Essential oil of *Zanthoxyllum rhetsa* seed carp	20	17 (85.00)	49	38 (77.55)
Agarwood (*Aquilaria malaccensis*) oil	20	7 (35.00)	49	45 (91.84)
Patchouli (*Pogostemon cablin*) essential oil	20	19 (95.00)	49	24 (48.98)
Amphotericin B (100 units)	NT	NT	49	16 (32.65)
Miconazole (10 µg)	NT	NT	49	12 (24.49)
Nystatin (100 units)	NT	NT	49	6 (12.25)
Clotrimazole (10 µg)	NT	NT	49	30 (61.22)
Itraconazole (10 µg)	NT	NT	49	33 (67.35)
Ketconazole (30 µg)	NT	NT	49	43(87.75)
Fluconazole (10 µg)	NT	NT	49	40 (81.63)
NT, not tested				

Discussion

The most effective antifungal drugs on yeasts in the study were nystatin, miconazole and amphotericin B while other antibiotics could inhibit <50% of the isolates tested. The commonly used antifungal drugs for internal and external use include clotrimazole, econazole, miconazole, terbinafine, fluconazole, ketoconazole, amphotericin B and luliconazole [13]. Nystatin and amphotericin B is polyene group drugs, but nystatin, due to its severe side effects, cannot be used parenterally [14]. Probably the limited use of nystatin in therapy might be one of the causes for the sensitivity of the most of

yeast isolates from clinical cases. Amphotericin B, the 3rd most effective drugs in the study, failed to inhibit about a third of the yeast strain tested in the study. Amphotericin B, is considered to be one of the best drugs for treatment of mycosis due to its wide spectrum of activity [15] but the resistance to this valuable antifungal has been reported to be emerging [16]. Among the imidazole group, antifungal drugs miconazole was the most effective and the least effective was fluconazole. It might be due to limited use of the first and common use of the latter in the treatment of mycosis [13] leading to the emergence of resistance [16] for the latter, as reported for the commonly used antibiotics for treatment of infections caused by bacteria [17]. Resistance to fluconazole in more than 80% isolates of potentially pathogenic yeast is alarming and may jeopardise the utility of this most commonly used antifungal drug for treatment of mycosis.

Observing promising antimycotic activity of cinnamon oil (active ingredient cinnamaldehyde) and thyme oil (active ingredient carvacrol) against potentially pathogenic yeasts may be important for future antimycotic drug development. Though lot many herbal antimicrobials have been identified to act on fungi [3] little is known about the antimycotic activity of cinnamon and thyme oils. Ajowan oil (also containing carvacrol as an active ingredient) also inhibited growth of 87.75% yeasts similar to citral. Citral is an active antimicrobial in lemongrass oil which has been reported earlier as an effective antimycotic equally effective on yeasts and moulds [7]. Antimycotic activity of lemongrass oil on yeasts and moulds in the study are in concurrence to earlier observations [7]. Though many of the herbal antimicrobial compounds were not much active against yeasts and moulds (**Table 2**), promising antimycotic activity of cinnamon oil, thyme oil, ajowan oil, holy basil oil and lemongrass oil and their active ingredients may be hope for future antimycotic drug development. However, to conclude about increasing antimycotic drug resistance and potential of herbal antimycotics study on more number of clinical and environmental isolates of yeasts and moulds is warranted.

Despite the fact of promising antimycotic activity of cinnamon oil, thyme oil, ajowan oil, holy basil oil and lemongrass oil and their active ingredients, the biggest question is how to use the herbals in antimycotic therapies? Herbal ingredients being irritant and sometimes toxic and allergic in therapeutically effective dose are often avoided in conventional therapy [18]. However, there are several herbal antifungal creams and lotions in market based on Chinese ethnic and Indian Ayurvedic knowledge incorporating essential oils of herbs and this study provides a scientific base for the formulation of better antimycotic ointments. Inhalation of essential oils in steam may be an effective alternative for systemic administration or internal use. However, organized clinical trials are the only answer to determine the routes of administration and therapeutic efficacy. Most of the systemic mycotic infections are airborne and efficacy of herbal essential oil has been documented for indoor air fungal decontaminants [19]. There are already several essential oil nebulizers for different purposes but their utility as antimycotic treatment can be established through conduction of systematically organized studies.

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Competing Interests

The authors have no conflicts of interest.

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