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RESEARCH ARTICLE

Anti-yeast efficacy of *Cinnamomum verum* extracts on dermatologically prevalent yeast *Malassezia furfur*

Sreelatha G.L.[1], Lakshmeesha T. R.[1], Sharath Kumar L.[2], Soumya K.[1], Sharmila T.[1]*

1. Department of Microbiology and Biotechnology, Bangalore University, Bangalore-560 056.

2. Phytochemistry, Research and Development, The Himalaya Drug Company, Makali, Bangalore- 562 123.

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Corresponding Author

Dr. Sharmila. T
dr.sharmilathirumale@yahoo.com

Abstract

Malassezia furfur, a lipophilic, saprophytic yeast causes superficial skin diseases such as dandruff, pityriasis versicolor, folliculitis, seborrhoeic dermatitis and some forms of atopic dermatitis. The present study was aimed to investigate anti-yeast potential of *Cinnamomum verum* bark extracts. The powdered plant material was extracted with water and also successively extracted with hexane, chloroform and methanol, using the soxhlet apparatus. The anti-yeast activity was screened by disc diffusion assay and MIC by microdilution method. The results revealed that hexane extract of *C. verum* exhibited significant anti-yeast activity with Minimum Inhibitory Concentration (MIC) of 0.195 mg mL^{-1}. Qualitative phytochemical tests of *C. verum* bark extracts was done using standard protocols and demonstrated the presence of phytocompounds such as alkaloids, carbohydrates, flavonoids, glycosides, naphthoquinones, phytosteroids, and terpenoids. Subsequently, high performance thin layer chromatographic analysis (HPTLC), followed by scanning of the spots at 254 nm and 366 nm using a UV detection mode and derivatization method was made to establish the phytochemical profile of *C. verum*. The results substantiate that hexane extract of *C. verum* exhibited significant activity to be used as an antimicrobial agent.

INTRODUCTION

Superficial fungal infections are widespread throughout the world. Most of these infections are contagious and are caused by dermatophytic moulds (anthropophilic or zoophilic species) (Cabanes, 2000). A second group of superficial infections are caused by yeasts *viz*., *Candida* species (candidiasis or thrush) and *Malassezia furfur* (pityriasis simplex and pityriasis versicolor) (Ingham and Cunningham, 1993). Yeasts of genus *Malassezia* (synonym: *Pityrosporum*) are obligatory or non obligatory lipophilic, normal flora which are found in regions of the body that are rich in sebaceous lipids, such as the head, trunk and upper back (Gaitanis et al., 2012). The main focus of the study is on the species *Malassezia furfur*, which was known to be present in various hosts and body sites. The cause of transformation of the yeast phase of *M. furfur* to the mycelial one responsible for pathogenicity is presumably owing to the changes in the composition of fatty acids of the sebaceous glands due to increased androgen concentrations (Rapelanoro et al., 1996). The resulting variations in the tissue milieu are reflected in divergent pathophysiologic manifestations of *Malassezia*-associated skin conditions (Erchiga and Florencio, 2006). The related pathologies reported were dandruff, seborrhoeic dermatitis, pityriasis versicolor, folliculitis, some forms of atopic dermatitis, psoriasis and confluent & reticulate papillomatosis (Marcon and Powell, 1992). For the management of these above mentioned superficial conditions, topical antifungal treatment is beneficial whereas oral treatment may be necessary for chronic infections.

The azoles represent the largest division of antifungals which include ketoconazole, clotrimazole, itraconazole, fluconazole, bifonazole, miconazole, etc. for the management of these infections (Gupta et al., 2004). These drugs are extremely lipophilic, thus accumulating in fatty tissues leading to undesired effects such as toxic hepatitis, acquired cutaneous adherence (Filip et al., 2010; Polsen et al., 1995; Svedhem, 1984), vomiting, loss of appetite, rash, pruritis, menstrual irregularities, gynecomastia and decreased libido. When used in its various topical forms, it was found to have urictant effects (Di Fonzo et al., 2008). Furthermore, these drugs are unable to prevent recurrence, which is the commonest problem. These health hazards necessitated the drive to find alternative antifungal agents with versatile features such as, being non-toxic, eco-friendly, cost effective and also amenable to high throughput and compatibility.

"Natural" means originating from or created by nature. They may be botanicals, animals, minerals, or microorganisms. Throughout the history of mankind, natural products of plant origin have had a very high potential as antimicrobial drugs for treating various skin diseases, and represent a long history of human interactions with the environment since antiquity (Afolayan et al., 2014). The active principles, in different parts of the plant such as root, stem, bark, heartwood, leaf, flower and fruit can be used in various crude dosage forms like pills, powders, essential oils, infusions or poultices.

Cinnamomum verum Presl (CV) (syn: *Cinnamomum zeylanicum* Blume) is a medicinal plant that belongs to the family Lauraceae, The bark is widely used in traditional systems of medicine. The cinnamon bark possesses antimicrobial, anti-diabetic, anti-oxidant, hypoglycemic, anti-inflammatory and anti-ulcer activity (Shan et al., 2007; Chatterjee et al., 2012). Taking this data into account, the present study was aimed to evaluate the effect of aqueous and solvent extracts of *C. verum* bark on the growth of *M. furfur*.

MATERIALS AND METHODS

Test Microorganism

M. furfur (Strain no: 1374 and 1765) was procured from Microbial Type Culture Collection Centre and Gene bank (MTCC), Chandigarh, India. The strains were maintained in modified Emmon's agar medium and Saborauds Dextrose Agar (SDA) supplemented with milk. Leeming-Notman agar (LNA) medium was used for carrying out antimicrobial studies.

Plant materials

Plant material used was chosen according to the Ayurvedic system of medicine considering its therapeutic properties (Jain, 1994). The authentic plant specimen of *Cinnamomum verum* bark was collected from Gandhi Krishi Vignan Kendra (GKVK), Bengaluru, Karnataka and the voucher specimen of the plant was maintained in the Department of Microbiology and Biotechnology, Bangalore University, Bengaluru.

Preparation of the extracts

Aqueous extraction

The aqueous extracts were prepared by maceration method. The dried *C. verum* bark material was pruned with distilled water (1:3 w/v) in a homogenizer. The macerate was sieved through a muslin cloth and centrifuged at 8000 rpm for 10 min. The supernatant was filtered through Whatman No. 1 filter paper, concentrated on water bath and stored at 4˚C for further use (Lakshmeesha et al., 2013).

Solvent extraction

About 65 g of powdered *C. verum* bark was filled in a thimble and sequentially extracted with 200 mL of hexane, chloroform and methanol using a soxhlet extractor for 48 h. The extracts were concentrated using a rotary evaporator and stored at 5℃ in an air tight brown bottle until further use. The prepared extracts were subjected to antifungal studies (Sreelatha et al., 2015).

Antimicrobial activity

Disc diffusion method

Preliminary screening of the antifungal activity of the extracts was done by disc diffusion assay as outlined by NCCLS. The *M. furfur* suspension adjusted to 0.5 McFarland standard was swabbed over the sterile LNA agar medium. Sterile discs were placed on the surface of agar and impregnated with 10 µL of test sample drug at the concentration of 25 mg mL^{-1} and 12.5 mg mL^{-1}. For comparative purposes, ketoconazole was used as reference standard (0.125 mg mL^{-1}) and dimethyl sulphoxide (DMSO) as negative control. The plates were incubated at 32±2˚C for 48 h. Each test was performed in triplicate. At the end of the incubation, the diameter of inhibition zones were measured and recorded in millimeters (Adam et al., 1998).

Broth microdilution assay

Minimum inhibitory concentrations (MICs) were determined in liquid culture by using 96-well plate assay as recommended by the NCCLS, with minor modifications. Stock solution of the extract was prepared in 10 % DMSO by two-fold serial dilution ranging from 12.5 mg mL^{-1} to 0.097 mg mL^{-1}. The cells in 1X phosphate buffer saline with turbidity equivalent to 0.5 McFarland standard was inoculated in each well of the microdilution plate. The plates were incubated at 34 ± 2˚C for 48 h. After 48 h of incubation, 15 µL of *p*-iodonitotetrozolium violet dye (INT) was added in each well and further incubated for 30 min for a colour change from colourless to purple. The lowest concentration of the drug that prevented the colour change from colourless to purple was determined as MIC. The standard reference used in these studies was ketoconazole at the concentration of 0.125 mg mL^{-1} and 10% DMSO served as negative control. The procedure was performed in octuplicate under aseptic conditions. Further, Minimal Yeast-cidal Concentration (MYC) has been determined with all treated samples to find out the concentration that inhibits the complete yeast growth after incubation (Kavyashree et al., 2015).

Qualitative Phytochemical screening

The preliminary phytochemical screening was carried out to detect the chemical groups of the extracts using standard procedures (Harborne, 1984; Kaur and Arora, 2009; Raaman, 2006; Rajeshkumar and Sundararaman, 2012). The extracts were evaluated for the presence of phytochemicals *viz*., alkaloids, carbohydrates, flavonoids, glycosides, naphthoquinones, phenols, proteins, steroids, tannins, and terpenoids.

High-performance Thin Layer Chromatographic (HPTLC) analysis of hexane extract of *Cinnamomum verum*

Test samples were applied as 10 mm wide bands, positioned 12 mm from the bottom of the pre-coated thin layer silica plate60 F_{254} 10 × 10 cm, E-Merck. HPTLC plates (10 X 10 cm; 0.25 mm layer thickness; Merck), using a Camag (Mutten, Switzerland) Linomat IV automated TLC applicator. The chromatographic separation was performed in a Camag twin trough glass chamber pre -saturated with 20 mL mobile phase of Toluene : Ethyl acetate (9.5 : 0.5) (v/v) for 20 min. Mobile phase was allowed to run to a height of 8.5 cm under the laboratory conditions of 25 ± 5℃ and relative humidity of 40-50 %. After development, the chromatograms were dried and were visualised under UV at 254 and 366 nm. They were also derivatised in Vanillin-sulphuric acid reagent [1 % Vanillin solution (w/v) in methanol and 10 % sulphuric acid (v/v) in methanol; mixed in the ratio of 1:1] and R_f values were recorded (Kumar et al., 2012).

Statistical Analysis

Statistical analysis was performed using SPSS software: Version 16.0. The results were expressed as mean ± SD (n=3). Multivariate analysis followed by post hoc analysis - Tukey HSD was applied for statistical analysis with the level of significance set at $P<0.05$. Results with $P<0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSION

Extraction and preliminary phytochemical analysis of plant extracts

The extract yield of *C. verum* was not constant with any particular solvent. The hexane extract of *C. verum* bark yielded 0.95 % based on dry weight of the bark whereas aqueous extract, methanol extract and chloroform extract yielded 1.15%, 3.72% and 0.23% respectively. The phytochemical analysis of hexane extraction of bark revealed the presence of alkaloids, tannins, phenols and terpenoids. The chloroform extraction revealed the presence of alkaloids, carbohydrates, flavonoids, glycosides, naphthoquinones, phytosteroids, terpenoids whereas proteins, tannins and phenols were absent. In methanolic and aqueous extract, carbohydrates, flavonoids, glycosides, naphthoquinones, proteins, tannins, phenols and terpenoids were present. The results of the phytochemical analysis were tabulated in Table 1.

Table 1. Qualitative phytochemical screening of different extracts of *C. verum*

Sl. No.	Phytochemical tests	Water extract	Methanol extract	Chloroform extract	Hexane extract
1	**Alkaloids**				
	a) Mayer's test	-	-	+	+
	b) Dragendorff's test	-	-	+	+
2	**Carbohydrates**				
	a) Fehling's test	+	+	+	-
3	**Flavonoids**				
	a) Shinoda test	+	+	+	-
4	**Glycosides**				
	a) Legal's test	+	+	+	-
5	**Naphthoquinones**				
	a) Juglone test	+	+	+	-
6	**Phytosteroids**				
	a) Libermann- Buchard test	-	-	-	-
7	**Proteins**				
	a) Ninhydrin test	+	+	-	-
	b) Xanthoproteic test	+	+	-	-
8	**Tannins and phenols**				
	a) Ferric chloride test	+	+	-	+
	b) Lead acetate test	+	+	-	+
9	**Terpenoids**				
	a) Salkowski test	+	+	+	+

+ positive; - negative

Antifungal activity of the extracts

Antifungal activity evaluated in terms of zone of inhibition in millimetres of the four extracts of *C. verum* in different solvents *viz*., water, methanol, chloroform and hexane (sequentially extracted with non-polar to polar solvents) tested on *M. furfur* was recorded in Table 2. It was observed that hexane extract significantly inhibited both the strains of *M. furfur* (1374 and 1765) with the zone of inhibition of 14.16±0.76 mm and 15.5±0.4 mm at 25 mg mL^{-1} and 12.5±0.5 mm and 13.11±0.51 mm at 12.5 mg mL^{-1}. The observed MIC and MYC of *M. furfur* strain1374 was found to be 0.391 mg mL^{-1} and 0.781 mg mL^{-1} respectively whereas those of strain 1765 was found to be 0.195 mg mL^{-1} and 0.39 mg mL^{-1}, respectively. The results obtained concur with the previously published results of Mariappan et al. (2013) which concluded that methanolic extract of *C. verum* has potential antifungal activity against *Malassezia* species. But in contrary, our study reported the antimalassezial activity of hexane extract to methanol extract.

Table 2. Antifungal activity of *Cinnamomum verum* extracts on *Malassezia furfur*

Tested strain	Extraction solvent	Inhibition zone in diameter (mm)		MIC	MYC
		Phytoextracts (mg mL^{-1})			
		25	12.5		
M. furfur MTCC-1374	Water	0.0±0.0^{a}	0.0±0.0^{a}	nd	nd
	Methanol	0.0±0.0^{a}	0.0±0.0^{a}	nd	nd
	Chloroform	0.0±0.0^{a}	0.0±0.0^{a}	nd	nd
	Hexane	14.16±0.76^{b}	12.5±0.5^{b}	0.391	0.781
M. furfur MTCC-1765	Water	0.0±0.0^{a}	0.0±0.0^{a}	nd	nd
	Methanol	0.0±0.0^{a}	0.0±0.0^{a}	nd	nd
	Chloroform	0.0±0.0^{a}	0.0±0.0^{a}	nd	nd
	Hexane	15.55±0.40^{c}	13.11±0.5^{b}	0.195	0.39
Positive control: Ketoconazole (125 µg mL^{-1})	Ethanol	16.06±0.15		0.004	0.007
Negative control (DMSO)		0.0±0.0			

Data are the means of three independent experiments ± Standard deviation (n=3) analysed in triplicate. Mean values within the column with different superscript letters are significantly different when subjected to Tukey HSD ($P < 0.05$). nd- not determined.

HPTLC analysis of hexane extract of *Cinnamomum verum*

The fine separation of the extracts was achieved by using mobile phase of Toluene: Ethyl acetate (9.5: 0.5 v/v) after standardizing different compositions of mobile phase (Fig. 1). The HPTLC profile of hexane extract of *C. verum* was scanned under UV (254 nm and 366 nm) and derivatised using Vanillin-H_2SO_4 acid reagent and R_f values obtained were in the range of 0.07 to 0.58.

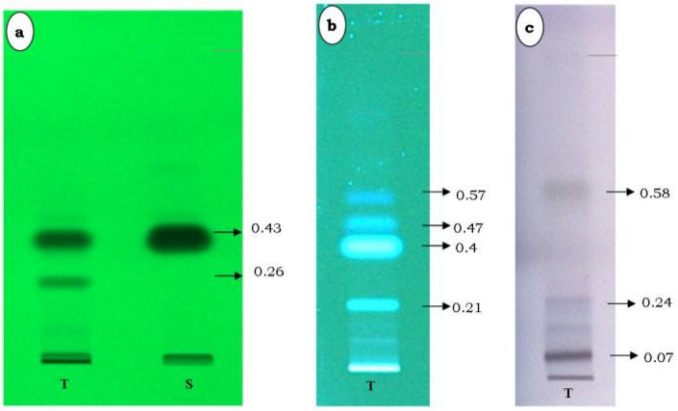

Figure 1. HPTLC profile of *n*– hexane extract of *Cinnamomum verum* bark

a. Under short wave UV (254 nm); **b.** Under long wave UV (366 nm) and **c.** After derivatization in Vanillin– Sulphuric acid reagent.
T-Test solution: **S**-Standard

DISCUSSION

Cinnamon, a widely used spice has active components *viz.*, eugenol and cinnamaldehyde, which are reported to possess broad spectrum antimicrobial activity (Gill and Holley, 2004). The possible mechanism of inhibition hypothesised by Wendakoon and Sakaguchi (1993) was that carbonyl group on cinnamaldehyde might hold to proteins to avoid the action of amino acid decarboxylases and hydroxyl group of eugenol combine with the protein inhibiting enzyme action. Another hypothesised fact by Wendakoon and Sakaguchi was that synergistic effects of

eugenol and cinnamaldehyde acted on diverse proteins or enzymes. The mechanism of fungicidal activity associated with cinnamaldehyde, a electronegative compound are cytoplasm granulation, cytoplasmic membrane rupture, and inactivation and/or inhibition of intracellular enzymes. These interrupting physiological processes may take place separately or concomitantly, concluding the growth inhibition. To conclude, the result signifies the potential of using those extracts in combating superficial infections caused by *Malassezia* infections. Additionally, detailed toxicological studies are needed to understand the nature of these extracts.

Conflict of interest statement
We declare that we have no conflict of interest.

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