



Tobacco Extract Induces Yeast to Hyphal form Transition in the Human Pathogen, *Candida albicans*

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Abstract

Candida albicans is a commensal microorganism which lives in the oral, digestive and genitourinary tracts. Use of tobacco products such cigarettes and chewing tobacco can cause a number of health issues including oral cancer in humans. The yeast, *C. albicans* which is a normal flora in the mouth can cause oral candidiasis in immunocompromised patients including patients suffering from cancer. In this study, we have examined the effect of the tobacco extract on *C. albicans* morphogenesis. We found that the cold water and cold methanol extracts of chewing tobacco, cigarette and beedi tobacco can induce yeast to hyphal form transition of the human pathogen, *C. albicans*. Gas chromatographic mass spectroscopy analysis of cold methanolic extract of tobacco indicated the presence of various molecules including nicotine. This study indicates that tobacco is a good inducer medium for inducing yeast to hyphal form transition that may enhance the pathogenicity of *C. albicans* in humans.

Keywords: Chewing tobacco; Smoking tobacco; GC-MS analysis; *Candida albicans*; Morphogenesis

Introduction

Candida albicans is an organism commonly found on the mucosal surfaces of the mouth, digestive system and genitourinary tracts of healthy individuals [1]. This organism can be an opportunistic pathogen in immunocompromised patients [2]. Switching from yeast to hyphal form is one of the virulence factors of *C. albicans*. Several factors can induce yeast to hyphal form transition in *C. albicans* such as serum, glucose, *N*-acetylglucosamine, proline, CO₂, temperature, and starvation [3-7]. Tobacco is a product made from curing the leaves of tobacco plant, *Nicotiana glauca*. Tobacco leaves are used in many forms like chewing and smoking. More than 3,000 compounds are found in tobacco [8,9]. About 28 carcinogenic agents are reported in tobacco [10]. The carcinogenic substances can cause alkylation of DNA and lead to cancer [11]. Tobacco consumption as chewing and smoking form is associated with many health problems in humans such as cancers of mouth, oesophagus, lung, and pancreas. Also, it results in digestive problems and increase in stomach acidity, cardiovascular diseases, decaying and falling of teeth, nicotine addiction, increase of heart rate, stroke, nicotine poisoning in children [12-15] and early delivery in pregnant women [13,16,17]. Nicotine can increase blood pressure, catecholamines and free fatty acids [18]. It can inhibit prostacyclin synthesis that can lead to aggregation of platelets which may result in blood clotting. Nicotine content in cured tobacco is between 0.2% and 4.75% by weight depending on many factors like variety of the plant, growing conditions, degree of ripening, fertilizer treatment, and leaf position [19,20]. Non ionized nicotine is freely absorbed by buccal mucosa. Chewing tobacco when buffered to an alkaline pH facilitates absorption of nicotine. Nicotine in chewing tobacco is absorbed more compared to the nicotine in the smoking cigarettes [21]. In this study, we have examined the effect of cold water and cold methanol extracts of chewing and smoking tobacco on *C. albicans* morphogenesis. We found that these extracts can induce yeast to hyphal form transition indicating that tobacco may enhance the pathogenicity of *C. albicans* in humans.

Materials and Methods

Chemicals and media

Chewing tobacco, beedi tobacco (Indigenous smoking tobacco) and cigarette were purchased from local market, Nanded, India. Plates and other media were purchased from HiMEDIA Chemicals Ltd, Mumbai, India.

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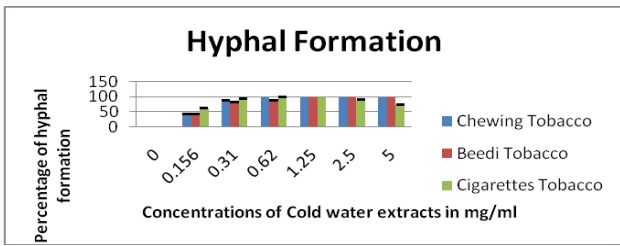


Figure 1: Cold water extracts of chewing tobacco, beedi tobacco, and cigarette inducing yeast to hyphal form transition in *Candida albicans* (ATCC 90028) at 37°C after 2 hours.

Tobacco extracts preparation

Chewing tobacco, beedi tobacco, and cigarette were extracted by using cold water and cold methanol at room temperature. The prepared extracts were sterilized by using a bacteriological filter.

Culture of *Candida albicans*

Candida albicans (ATCC 90028) was obtained from the Institute of Microbial Technology (IMTECH) Chandigarh, India. The culture was maintained on Yeast extract -Peptone -Dextrose (YPD) agar slant at 4°C and propagated by inoculating a single colony from the YPD agar plates into 50 ml YPD broth in a 250 ml conical flask. Flasks were incubated overnight at 30°C at 100 rpm on an orbital shaking incubator. The cells were harvested by centrifugation at 2000 rpm and washed thrice with sterile 0.1 M Phosphate-Buffered Saline (PBS), pH

7.4 and the cell density was determined by a haemocytometer count. Finally cells were suspended in sterile PBS and were kept as stock cells.

Yeast to hyphal form transition assay

Yeast to hyphal form transition assay was performed in 96-well microtiter plates [22]. *Candida albicans* cells stock was diluted up to 1×10^6 cells/ml in PBS buffer. Various concentrations of cold water and methanol extracts of chewing tobacco, beedi tobacco, and cigarette were prepared and ranged 0.156 mg/ml to 5 mg/ml and added in each well. Wells without tobacco extracts were kept as a control. The final volume was kept at 200 µl in each well. The microtitre plates were incubated at 37°C at 120 rpm on an orbital shaker incubator for 2 hours. After incubation period the cells were observed microscopically by using an inverted light microscope (Metzer, India). Hundred cells were counted and numbers of yeast and hyphal forms were noted. All experiments were carried out in triplicate.

Gas Chromatographic Mass Spectroscopy (GC-MS) analysis

The cold methanol extract of tobacco was analyzed using a Shimadzu QP2010 Gas- Chromatography-Mass. This device employed a fused silica column packed with Elite -5 ms [5% Diphenyl 95% Dimethyl poly siloxane, 30 mm × 0.25 mm × 0.25 µm df] and the components were separated using helium as carrier gas at a constant flow of 1 ml/min. Two microlitre of sample extract was injected into the instrument and it was detected by the turbo gold

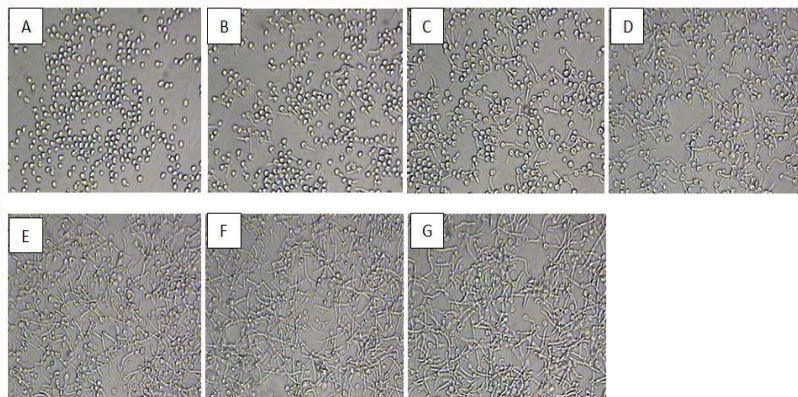


Figure 2: Cold water extract of chewing tobacco inducing yeast to hyphal form transition in *Candida albicans* (ATCC 90028), A) Control; B) 0.15 mg/ml; C) 0.31 mg/ml; D) 0.62 mg/ml; E) 1.2 mg/ml; F) 2.5 mg/ml; G) 5 mg/ml.

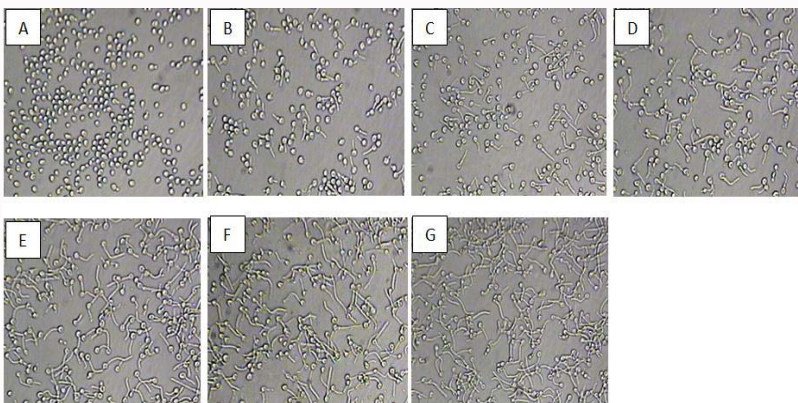


Figure 3: Cold water extract of beedi tobacco inducing yeast to hyphal form transition in *Candida albicans* (ATCC 90028), A) Control; B) 0.15 mg/ml; C) 0.31 mg/ml; D) 0.62 mg/ml; E) 1.2 mg/ml; F) 2.5 mg/ml; G) 5 mg/ml.

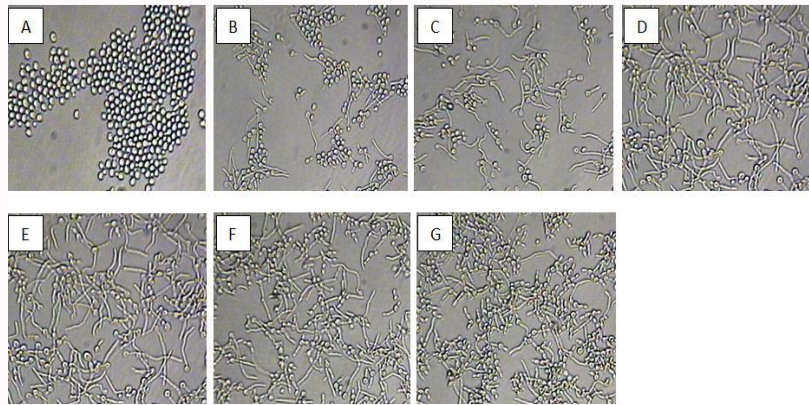


Figure 4: Cold water extract of cigarette tobacco inducing yeast to hyphal form transition in *Candida albicans* (ATCC 90028), A) Control; B) 0.15 mg/ml; C) 0.31 mg/ml; D) 0.62 mg/ml; E) 1.2 mg/ml; F) 2.5 mg/ml; G) 5 mg/ml.

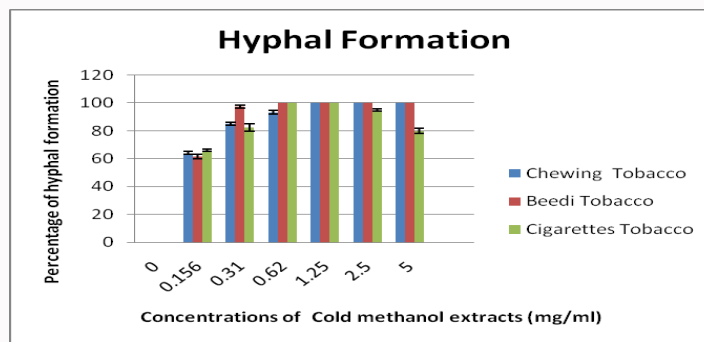


Figure 5: Cold methanol extracts of chewing tobacco, beedi tobacco, and cigarette inducing yeast to hyphal form transition in *Candida albicans* (ATCC 90028) at 37°C after 2 hours.

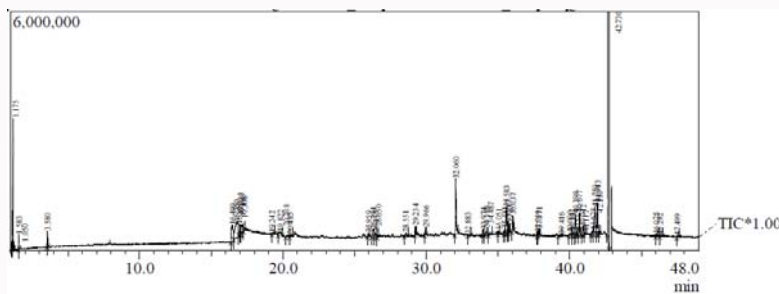


Figure 6: GC-MS Chromatogram of the cold methanol extract of tobacco.

mass detector with aid of turbo mass 5.2 software. The oven in meantime of GC Process was maintained at the temperature of 110°C with 2 min holding and the injector temperature was set at 250°C. The temperature of inlet line was 200°C and source temperature was 200°C. Mass spectra were taken at 70 eV, a scan period of 0.5 S and fragment from 45 to 450 Da. The MS detection was completed within 48 min. The mass spectrum GC-MS interpretation was done using the database of National Institute Standard and Technology (NIST and WILEY) spectroscopy at Central analytical facility University College of Technology, Osmania University, Hyderabad, India which contain more than 62,000 patterns. The spectrum of unknown components was stored in the NIST and WILEY library [23].

Statistical analysis

Values of samples were compared using student's t-test. A value of $p < 0.05$ was considered statistically significant.

Results

Cold water and cold methanol extracts of chewing tobacco and smoking tobacco induces yeast to hyphal form transition in *C. albicans*

The cold water extract of chewing tobacco induced yeast to hyphal form transition in a concentration dependent manner. Hundred percent of hyphal formation was seen at 5 mg/ml, 2.5 mg/ml, and 1.25 mg/ml (Figures 1 and 2). The cold water extract of beedi tobacco also induced yeast to hyphae in *C. albicans*. At the concentration of 5 mg/ml, 2.5 mg/ml and 1.25 mg/ml, a 100% of hyphal formation was observed (Figures 1 and 3). The cold water extract of cigarette also induced yeast to hyphal form transition where 100 % of hyphal formation was observed at 1.25 mg/ml and 0.62 mg/ml (Figures 1 and 4). Cold methanol extracts of chewing tobacco, beedi tobacco and cigarette induced yeast to hyphal formation. Hundred percent

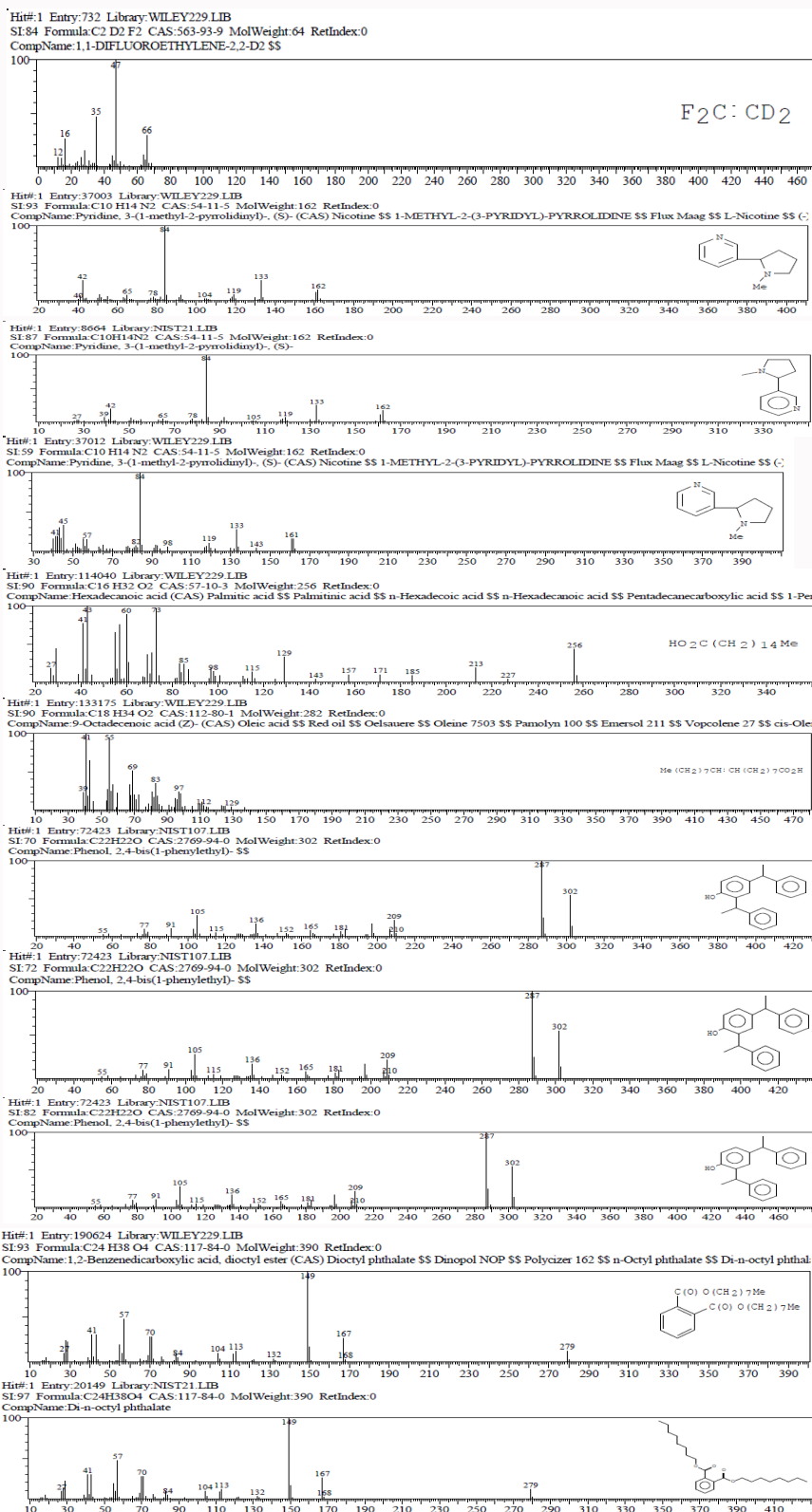


Figure 7: Structures of eleven major compounds in the cold methanol extract of tobacco.

of hyphal formation was seen at 5 mg/ml, 2.5 mg/ml, and 1.25 mg/ml of both chewing and beedi tobacco extracts (Table 1 and Figure 5). Hundred percent of hyphal formation also was observed at 0.62 mg/ml of cold methanol extract of beedi tobacco (Table 1 and Figure 5). The cold methanol extract of cigarette tobacco induced hyphal

formation where at the concentration of 1.25 mg/ml and 0.62 mg/ml, 100% of hyphal formation was observed (Table 1 and Figure 5).

GC-MS analysis of tobacco extract

Eleven major chemical compounds were identified in the

Table 1: Concentrations of cold methanol extracts of chewing tobacco, beedi tobacco and cigarette can induce yeast to hyphal form transition in *Candida albicans* (ATCC 90028).

S. No	Concentrations (mg/ml)	% of hyphal formation in <i>C. albicans</i> induced by cold methanol extracts		
		Chewing tobacco	Beedi tobacco	Cigarette
1	5	100	100	80
2	2.5	100	100	95
3	1.25	100	100	100
4	0.62	95	100	100
5	0.31	85	96	85
6	0.156	65	62	65
7	Control	0	0	0

Table 2: Major components in the cold methanol extract of tobacco as analyzed by GC-MS.

S. No.	RT	Compound Name	Molecular Formula	Molecular Weight	Peak Area %
1	1.175	1,1-Difluoroethylene	C ₂ D ₂ F ₂	64	4.93
2	16.49	Pyridine, 3-(1-methyl-2-pyrrolidinyl)	C ₁₀ H ₁₄ N ₂	162	2.39
3	16.567	Pyridine, 3-(1-methyl-2-pyrrolidinyl)	C ₁₀ H ₁₄ N ₂	162	4.4
4	17.183	Pyridine, 3-(1-methyl-2-pyrrolidinyl)	C ₁₀ H ₁₄ N ₂	162	1.17
5	32.06	Hexadecanoic acid	C ₁₆ H ₃₂ O ₂	256	3.71
6	35.583	9-Octadecenoic acid (Z)	C ₁₈ H ₃₄ O ₂	282	1.86
7	40.399	Phenol, 2,4-bis(1-phenylethyl)-	C ₂₂ H ₂₂ O	302	1.48
8	40.677	Phenol, 2,4-bis(1-phenylethyl)	C ₂₂ H ₂₂ O	302	1.41
9	41.75	Phenol, 2,4-bis(1-phenylethyl)	C ₂₂ H ₂₂ O	302	2.47
10	41.943	Di-n-octylphthalate	C ₂₄ H ₃₈ O ₄	390	2.03
11	42.73	Di-n-octyl phthalate	C ₂₄ H ₃₈ O ₄	390	58.59

cold methanol extract of tobacco by using Gas Chromatographic Mass Spectroscopy (GC-MS). The chemical compounds with their Retention Time (RT), Molecular Formula (MF), Molecular Weight (MW) and concentration (Peak area %) are tabulated in Table 2. The major prevailing compounds were Di-n-octyl phthalate (58.59%), 1,1 Difluoroethylene (4.93%), Pyridine, 3-(1-methyl-2-pyrrolidinyl) (4.40%), Hexadecanoic acid (3.71%), Phenol, 2,4-bis(1-phenylethyl) (2.47%), Pyridine, 3-(1-methyl-2-pyrrolidinyl) (2.39 %), Di-n-octylphthalate (2.03), 9-Octadecenoic acid (Z) (1.86%), Phenol, 2,4-bis(1-phenylethyl) (1.48%), Phenol, 2,4-bis(1-phenylethyl) (1.41%), Pyridine, 3-(1-methyl-2-pyrrolidinyl) (1.17%). The GC-MS Chromatogram of tobacco extract is presented in Figure 6. Structures of eleven major compounds are represented in Figure 7.

Discussion

Yeast to hyphal form transition in *Candida albicans*, can be induced by serum, neutral pH, high temperature, contact, glucose, proline, N-acetylglucosamine, CO₂ and starvation. In this study, we have shown that extract of chewing and smoking tobacco induced yeast to hyphal form transition (Table 1 and Figures 1 to 5). This makes us suggest that *C. albicans* may play an auxiliary role in oral cancer. GC-MS analysis of cold methanolic extract of tobacco indicated presence of various molecules. Out of these components eleven major components including nicotine are revealed (Table 2). These compounds are known to have bioactive properties. Di-n-octyl phthalate is a chemical compound used in the manufacture of plasticizers and has anti venom [24], antimicrobial [25-28] and antifouling properties [29]. 1,1difluoroethylene is a flammable gas known as vinylidene fluoride and is used in the manufacture of polyvinylidene fluoride. Pyridine, 3-(1-methyl-2-pyrrolidinyl) is

a nicotine derivative found in tobacco and is used as an addictive agent [30,31]. Hexadecanoic acid, known as palmitic acid, is a saturated fatty acid that has pesticide and antimicrobial properties [32]. Phenol, 2, 4-bis (1-phenylethyl) is a phenolic compound used as antioxidant agent [33] and it has antimicrobial properties [34]. 9-Octadecenoic acid (Z) is a saturated fatty acid known as oleic acid that has fungistatic properties [35]. Arendorf et al. reported that *Candida* species are more prevalent in tobacco smokers than in non-smokers [36]. The antifungal activity of nicotine is reported. Zaidi et al. found that nicotine has antifungal activity against all fungi except *C. albicans*, *C. tropicalis*, *Microsporium canis*, *Epidermophyton floccosum*, and *Alternaria infectoria* [37]. Gunasegar et al. showed that nicotine can enhance the growth of planktonic and biofilm of oral fungal pathogens like *C. albicans* and *C. parapsilosis* [38]. This is the first report on tobacco extract inducing a virulence factor in *C. albicans*. Our study indicates that tobacco is a good inducer of yeast to hyphal form transition and may enhance the pathogenicity of *C. albicans* in humans.

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