Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2013, 5(3):25-32

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Phytochemical analysis and growth inhibiting effects of *Cinnamomum cassia* bark on some pathogenic fungal isolates

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ABSTRACT

Use of plant based drugs and chemicals for curing various ailments and personal adornment is as old as human civilization. Plants and plant-based medicaments are the basis of many of the modern pharmaceuticals we use today for our various ailments. Cinnamon has a long history both as a spice and as a medicine. In the present study, an attempt was made to determine the phytochemical composition and in vitro effects of methanolic extract of Cinnamomum cassia bark against Candida parapsilosis SN 1980, Candida guilliermondii SN 2006 and Candida glabrata SN 2266. Preliminary phytochemical analysis revealed the presence of phenols, alkaloids, steroids and tannins in varying concentration. In GC-MS analysis, the major compounds were cinnamaldehyde, p-methoxy cinnamaldehyde, cis-2-methoxy cinnamic acid and cinnamic acid. Biological examination was carried out in terms of minimum inhibitory concentration; filter disc assay and growth curve study. SEM examination of treated cells showed severe damage of the membrane. From our data, we conclude that Cinnamomum cassia bark extract can be explored further as an option for efficacious and safe drug for candidiasis.

Keywords: Cinnamon, Candida, phytochemicals.

INTRODUCTION

In nature, plants contain a variety of compounds called phytochemicals and most of them have medicinal properties. Long time ago humanity learned to use plants for disease treatment or control. Today, scientific research reveals that not only the chemical from the plant has effect against a particular disease, but, that the antioxidant property of the plant extract also gives beneficial effect to human health. Down the ages essential oils and other extracts of plants have evoked interest as sources of natural products. Increased fungal infections, toxicity of some antifungal agents and their interaction with other drugs, and development of resistance of some species of fungi have led many studies to search for new antifungal agents [1, 2]. Therefore, antimicrobial efficacies from plant have to be explored. Since, cinnamon is a common food ingredient and has a high human consumption in India. *Candida species* are ubiquitous yeasts and common residents of mucosal surfaces of the human oral cavity, the gastrointestinal and the urogenital tract [3-6]. Essentially all areas of the human gastrointestinal tract can harbour *Candida*. The most commonly isolated species (50 to 70% of yeast isolates) from the human gastrointestinal tract is Candida albicans, followed by *C. tropicalis*, *C. parapsilosis*, and *C. glabrata* [5]. So, it receives direct exposure of cinnamon; it is very interesting to evaluate the anticandidal activity of this plant.

Cinnamon is a native of Southern Asia and South America. Now it is cultivated in many tropical countries such as India, China and the Caribbean. Cinnamon (*Cinnamomum cassia*) of the family *Lauracease* is a favorite spice around the world because of its health benefits, flavors and preserves food [7]. The most favorite chemical constituents of cinnamon are volatile oil (cinnamaldehyde, eugenol, cinnamic acid, and weitherhin), mucilage, diterpenes and proanthocyanidins [8]. Although traditionally known, some recent scientific studies have shown antimicrobial activity of essential oils of *Cinnamomum cassia* presl., *C. osmophloeum* kaneh. and *C. zeylanicum* blume [9-12]. Quattara reported the inhibitory effect of *C. zeylanicum* essential oil on meat deteriorating organisms [13]. Antifungal activity was reported for respiratory tract infecting fungi such as *Aspergillus niger* Tieghem, *A. fumigatus* Fres., *A. nidulans* (Eidam) Winter and *A. flavus* Link [14]. Treating high moisture barley or wheat (*Triticum aestivum* L.) grains with essential oil of *C. zeylanicum* protected them from deteriorating fungi and ochratoxin formation [15, 16]. Similar findings were reported for protection of stored maize (*Zea mays* L.) against *A. flavus* [17].

Although the activity of *Cinnamomum cassia* bark extracts against various yeast species has been demonstrated previously, their mode of action is not clearly understood. This work constitutes the attempt to assess the anticandidal role of *Cinnamomum cassia* bark extract against some recently obtained Candida isolates. The ability of this extract to kill Candida cells was tested and identified by the minimum inhibitory concentration, filter disc assay, growth studies and scanning electron microscopy. Our approach involved the collection, extraction, phytochemical and anticandidal evaluation of bark of this medicinal plant.

EXPERIMENTAL SECTION

Collection and preparation of plant extract

The fresh bark of *Cinnamomum cassia* was purchased from the stock market, New Delhi, India. A voucher of specimen (D 11) was stored in laboratory for further reference. The bark was ground to coarse powder and extracted with methanol in Soxhlet apparatus for period of 72 h. It was filtered with whattman filter paper and filtrate was evaporated to dryness. The extract was used for the phytochemical and antifungal investigation after clearance of biosafety and ethical committee of the Institute.

Phytochemical analysis

The methanolic extract was subjected to various phytochemical tests to find out the major constituents. The tests were performed for alkaloids (Mayer's/ Wagner's test), phenols/ flavanoids (NaOH, $NaOH/H_2SO_4$), glycoside (Keller-Killiani test), tannins (Braemer's test), steroids and terpenoids (Salkowski test) and saponins [18-20].

GC-MS analysis

GC-MS analysis of the extract was carried out using a Shimadzu 2010 gas chromatograph fitted with an AB-Wax column. Helium was used as carrier gas. Sample (0.1 ml) was injected in the splitless mode. The chemical component from the extract was identified by comparing the retention time of the chromatographic peaks with those of authentic compound using the WILEY8.LIB and NIST05s.LIB.

Strains and growth media

The clinical isolates of *Candida parapsilosis* SN 1980, *Candida guilliermondii* SN 2006 and *Candida glabrata* SN 2266 used in this study were obtained from Department of Microbiology, Vardhaman Mahavir Medical College, New Delhi, India. For confirmation of the isolates germ tube test, microscopic morphology on Corn Meal Agar, Hi Chrome Agar, carbon and nitrogen assimilation test and ascospore production on malt-extract agar was done. The working strains were maintained on YEPD slants containing 2% (w/v) glucose, 2% peptone, and 1% yeast extract at -20^0C and were subcultured twice prior to testing to ensure viability and purity. All media constituents were obtained from Hi-Media (India). All chemicals and solvents were of analytical grade and obtained from Merck (India). For all experimental studies the yeast cells were maintained on yeast extract-peptone-dextrose (YEPD) medium at 30^0C.

Determination of Minimum Inhibitory Concentration

MICs of the strains were determined using a broth microdilution method. Cells were resuspended in a 0.9% normal saline solution to give an optical density at 600 nm (OD_{600}) of 0.1. The diluted cell suspensions were added to the wells of round-bottomed 96-well microtiter plates containing equal volumes of medium and different concentrations

of test extract [21]. A drug-free control was also included. The MIC test end point was evaluated visually and is defined as the lowest extract concentration that showed significant inhibition of growth compared to the controls.

Filter disc assays

Assays were performed according to the standard guidelines (M2-A7) of the National Committee for Clinical Laboratory Standards (NCCLS), using a modified Kirby–Bauer disk diffusion method. All the organisms were stored at -20^{0}C until use. Cells were grown at 30^{0}C in YEPD broth (approximately 10^5 cells/ml) and were passaged at least twice on solid agar. Broth cultures were swabbed onto agar to achieve a lawn of confluent yeast growth. Stock solutions of the test extract were prepared in 1% DMSO. Paper disks impregnated with different extract concentrations were placed on each plate. One disc impregnated with 1% DMSO was placed in the centre of the plate that served as solvent control. The plates were incubated at 30^{0}C for 48 h. The diameter of zone of inhibition was recorded in millimeters, after 48 hours.

Growth curve studies

In this experiment 10^6 cells (optical density A595 = 0.1) of strains were grown aerobically in automated shaker set at 30^{0}C until stationary phase. Growth was followed turbidometrically at 595 nm using spectrophotometer. Required concentrations of test extract were added to culture. The growth rates of cells alone and with inhibitor were performed. Optical density was recorded for each concentration against time (hrs). The growth rate is equivalent to the slope of log (optical density) versus time during the exponential phase [22].

Scanning electron microscopy (SEM) analysis

In this microscopy experiment tested cell suspensions from overnight cultures were prepared in YEPD medium. Test extract at equivalent to minimum inhibitory concentration was added to the cell (~1×10^6) and incubated for 16 hours at 30^{0}C and then prepared for an electron microscopy. All cells were fixed with 2% glutaraldehyde in 0.1% phosphate buffer for 1 h at room temperature (20^{0}C) [23, 24]. Washed with 0.1M phosphate buffer (pH 7.2) and post fixed 1% O_SO_4 in 0.1M phosphate buffer for 1h at 4^{0}C. For SEM, samples were dehydrated in acetone and dropped on round glass cover slip with HMDS and dried at room temperature then sputter coating with gold and observed under the SEM (Zeiss EV040).

RESULTS AND DISCUSSION

Phytochemical analysis

Table 1 shows the result of the preliminary phytochemical analysis. The results showed the phenols, alkaloids, steroids and tannins showed the positive result. Saponins and glycosides were absent in the test extract (Table 1). In the GC-MS analysis 35 phytochemical compounds were identified in the methanolic extract of *Cinnamomum cassia* bark. As indicated in (Table 2) the identified compounds were thirty five, out of which six were found in abundance and rest twenty nine compounds were in traces. The identification of phytochemicals is based on the molecular weight, molecular formulae and peak area. The major compounds of the extract were cinnamaldehyde (24.58%), p-methoxy cinnamaldehyde (9.87%), cis-2-methoxy cinnamic acid (9.22%), cinnamic acid (7.39%) and coumarin (5.31%).

Table 1: Phytochemical analysis of *Cinnamomum cassia* bark extract. The test was based on the colour intensity.

Phytochemicals	Name of Test	Colour observed	Colour intensity
Alkaloids	Wagner Mayer	Pale yellow Brown ppt.	+ +
Phenols/ Flavonoids	NaOH $NaOH/H_2SO_4$ $AlCl_3$	Deep yellow Colourless Yellow	++ ++ +
Steroids/Terpenoids	Salkowaski	Red colour	++
Glycosides	H_2SO_4 Keller Killiani	Dark brown Brown ring	- -
Tannins	Braemer Iodine	Dark blue faint bluish	+ +
Saponins	Frothing		-

- = Negative (absent), + = Positive (slightly present), ++ = Positive (moderately present)

Table 2: Chemical compositions of *Cinnamomum cassia* bark extract.

Name of compound	Molecular formula	Molecular weight	Retention time	% of prescence
Erythritol	$C_4H_{10}O_4$	122	9.017	0.98
Pyrocatechol	$C_6H_6O_2$	110	14.350	0.76
2-Furaldehyde, 5-(hydroxymethyl)-	$C_6H_6O_3$	126	15.058	1.38
Cinnamaldehyde	C_9H_8O	132	16.342	24.58
o-Methoxy cinnamaldehyde	$C_{10}H_{10}O_2$	162	18.717	2.63
cis-2-Methoxy cinnamic acid	$C_{10}H_{10}O_3$	178	19.075	9.22
Cinnamic acid	$C_9H_8O_2$	148	19.825	7.39
Coumarin	$C_9H_6O_2$	146	20.217	5.31
Copaene	$C_{15}H_{24}$	204	20.858	1.68
alpha.-Amorphene	$C_{15}H_{24}$	204	21.350	2.79
p-Methoxy cinnamaldehyde	$C_{10}H_{10}O_2$	162	21.967	9.87
5,8-Dimethylquinoline	$C_{11}H_{11}N$	157	22.292	0.85
Dimethyl 4-methylheptanedioate	$C_{10}H_{18}O_4$	202	22.392	1.70
4a-Methyl-trans-2-decalinone	$C_{11}H_{18}O$	166	22.992	1.18
o-(Trimethylsilyl) phenol	$C_9H_{14}OSi$	166	23.292	1.18
Cubenol	$C_{15}H_{26}O$	222	23.658	1.35
alpha.-Cadino	$C_{15}H_{26}O$	222	24.325	2.35
3-Hydroxycarbofuran phenol	$C_{10}H_{12}O_3$	180	26.390	1.85
1,2,3,4-Tetrahydroisoquinoline	$C_9H_{11}N$	133	27.725	1.51
beta.-Phenethyl cinnamate	$C_{17}H_{16}O_2$	252	28.258	1.25
n-Tridecanoic acid	$C_{13}H_{26}O_2$	214	28.892	1.35
Benzeneacetaldehyde, .alpha.-(3-methylbutylidene)-	$C_{13}H_{16}O$	188	29.233	0.99
Palmitaldehyde	$C_{16}H_{32}O$	240	30.225	1.03
n-Tetradecanoic acid	$C_{14}H_{28}O_2$	228	30.917	3.91
Homosyringic acid	$C_{10}H_{12}O_5$	212	31.292	0.26
3-Methyl-2,3-dihydro-benzofuran	$C_9H_{10}O$	134	32.092	0.51
8,11-Octadecadienoic acid, methyl ester	$C_{19}H_{34}O_2$	294	33.058	4.27
Tetraline	$C_{10}H_{12}$	132	33.325	0.89
Olealdehyde	$C_{18}H_{34}O$	266	33.658	2.89
Tricosenoic acid	$C_{23}H_{44}O_2$	352	33.958	0.75
2-Pentadecanone	$C_{15}H_{30}O$	226	34.625	0.57
2-O-Benzyl-d-arabinose	$C_{12}H_{16}O_5$	240	35.292	0.53
9-Methyl-10,12-hexadecadien-1-ol acetate	$C_{19}H_{34}O_2$	294	37.192	0.88
Octadecanoic acid, 2,3-dihydroxypropyl ester	$C_{21}H_{42}O_4$	358	37.833	1.20
cis-9-Hexadecena	$C_{16}H_{30}O$	238	40.042	1.05

Biological activity Investigation

Minimum inhibitory concentration

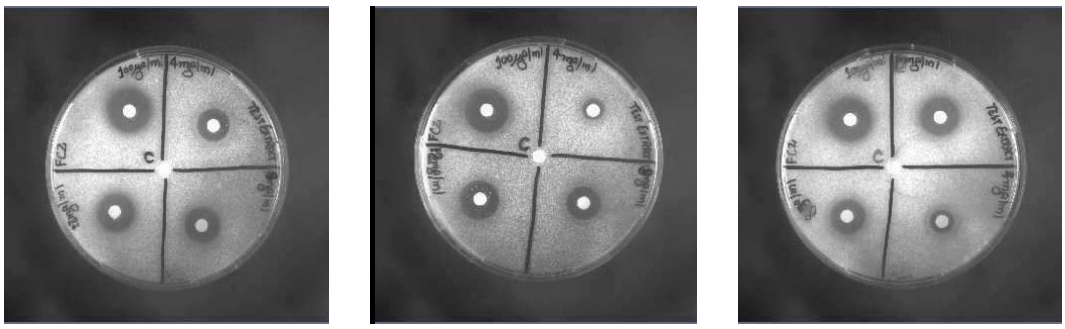
A more generally accurate method of assessment is the broth dilution technique. In this study, therefore, the broth dilution method was used in determining the activities measured as MIC. In using this method, higher degrees of differences in susceptibility among Candida species were not observed. The crude methanol extract was found to have minimum inhibitory concentration of 800 µg/ml, 800 µg/ml and 1600µg/ml aganist *Candida parapsilosis* SN 1980, *Candida guilliermondii* SN 2006 and *Candida glabrata* SN 2266 respectively. The results revealed that the test extract showed more killing in case of *Candida parapsilosis* SN 1980 and *Candida guilliermondii* SN 2006 respectively It would appear that *Candida glabrata* SN 2266 is the less sensitive yeast to the test extract. The results obtained provide us obvious evidence that the test extract used in the study has a substantial level of antifungal activity.

Filter disc assay

Antifungal activity (*in vitro*) of the test extract was studied against three Candida isolates at three different concentrations (4mg/ml, 8mg/ml and 12mg/ml) respectively. The results, summarized in Table 3, and shown in (Figs. 1a, 1b & 1c) suggest that the test extract shows significant antimicrobial activity against various tested pathogens. At 4mg/ml the activity shown was very less. The degree of inhibition varied with the concentration of the test extract. The highest zone of inhibition i.e. 11 mm was measured in *Candida guilliermondii* SN 2006 when treated against 12mg/ml of the test extract, followed by 10 mm measured in *Candida parapsilosis* SN 1980 when treated with the same concentration of the test extract, respectively. In case of *Candida glabrata* SN 2266 at highest concentration of the test extract the zone of inhibition was only 8mm. In comparison fluconazole at 100µg/ml showed 17mm, 15mm and 15mm, zone of inhibition in *Candida parapsilosis* SN 1980, *Candida guilliermondii* SN 2006 and *Candida glabrata* SN 2266. Comparison between antifungal activities of the different extract

concentrations and standard drug is shown in Fig 2. The results show that, in case of control disc no zone of inhibition was observed so as far as our study is concerned 1% DMSO, as a solvent is having no effect on the tested organisms. Hence we can effectively conclude here that whole of the antifungal effect is due to the different concentration of the test extract used in this study.

Fig. 1: Typical photographs obtained in filter disc assay assay of test extract against some fungal isolates (a) *C. guilliermondii* SN 2006 (b) *C. parapsilosis* SN 1980 (c) *Candida glabrata* SN 2266.



(a) **(b)** **(c)**

Fig. 2: Bar diagram showing comparison between antifungal activities of different test extract concentrations and standard antifungal drug against *C. parapsilosis* SN 1980, *C. guilliermondii* SN 2006 and *Candida glabrata* SN 2266.

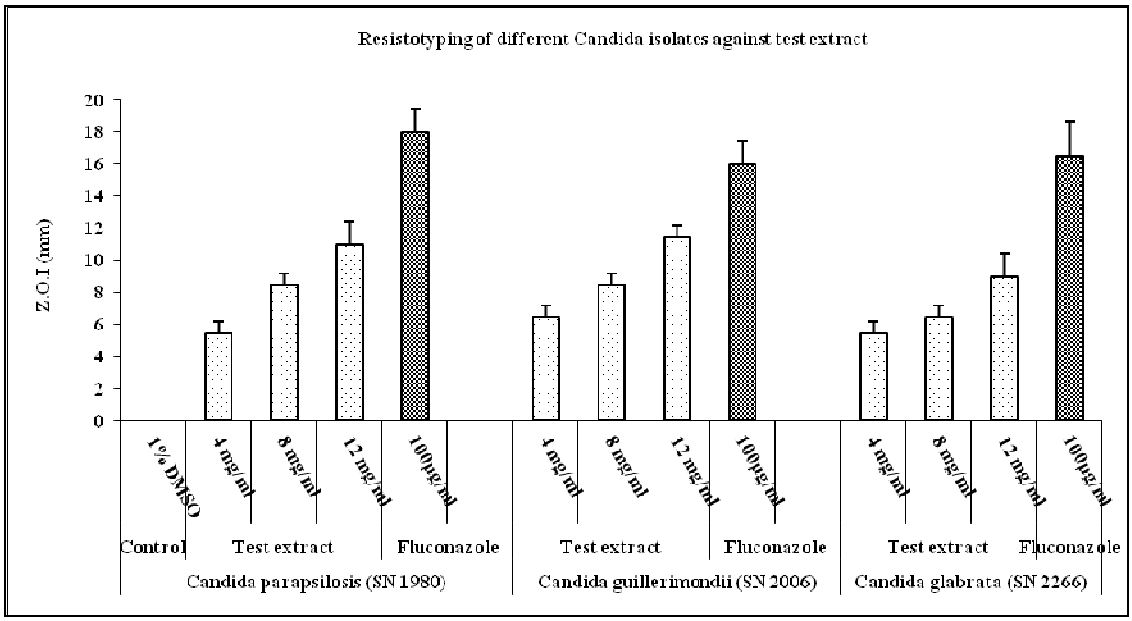


Table 3: Antifungal activity screening data for different test extract concentrations and fluconazole.

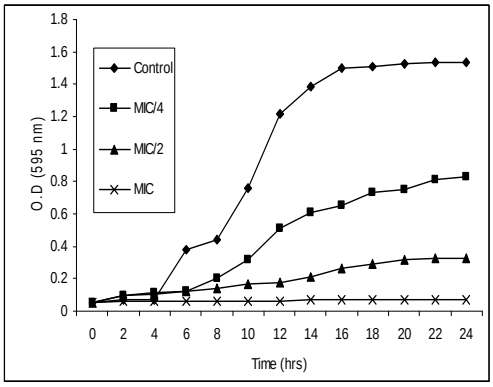
Zone of Inhibition (mm)			
Test extract	*Candida parapsilosis* (SN 1980)	*Candida guillerimondii* (SN 20006)	*Candida glabrata* (SN 2266)
4mg/ml	6	6	5
8mg/ml	8	9	6
12mg/ml	10	11	8
Fluconazole [a] (100µg/ml)	17	15	15
Control [b] (1% DMSO)	-	-	-

Positive Control[a]
Solvent Control[b]

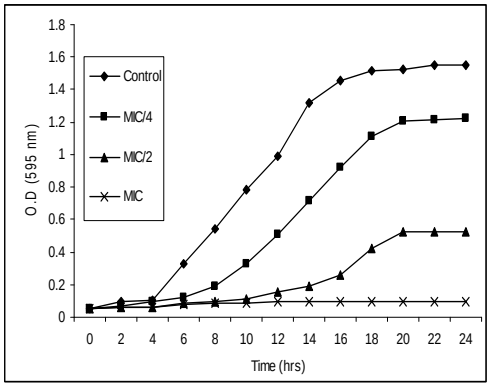
Growth studies (Turbidometric measurement)

Growth pattern of Candida species was investigated at different concentrations of methanolic extract of *Cinnamomum cassia* bark. (Figs. 3a, 3b & 3c) shows the effect of different concentration of test extract on growth pattern of *Candida parapsilosis* SN 1980, *Candida glabrata* SN 2266 and *Candida guilliermondii* SN 2006 respectively. Control cells showed a normal pattern of growth with lag phase of 4 hrs, active exponential phase of 8-10 hrs before attaining stationary phase. The absorbance obtained for the growth control (only organism) showed that the culture reached the stationary growth phase after 16 h showing a normal growth pattern. The curve depicts a lag phase in the initial phase of growth, active log phase and stationary phase. Increase in concentration of test extract leads to significant decrease in growth with suppressed and delayed exponential phases with respect to control. At minimum inhibitory concentration values almost complete cessation of growth was observed which is indicated by a flat line.

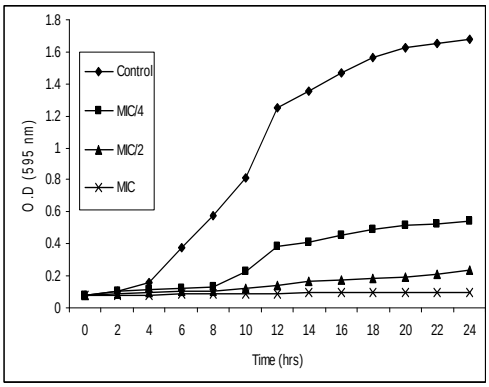
Fig. 3: Effect of different concentrations of test extract on growth of Candida species. Growth curve plotted against absorbance at 595 nm and time (hrs) shows complete inhibition of growth at MIC values. (a) Against *C. parapsilosis* SN 1980 (b) Against *C. glabrata* SN 2266 (c) Against *C. guilliermondii* SN 2006.



(a)



(b)

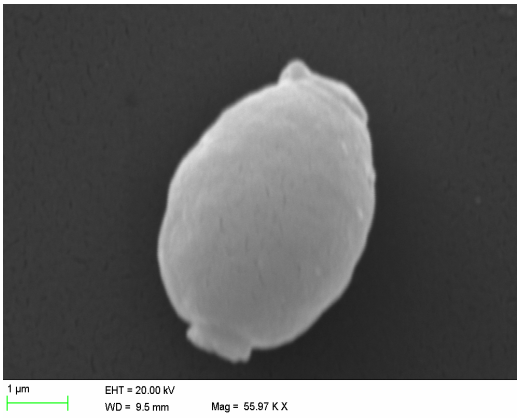


(c)

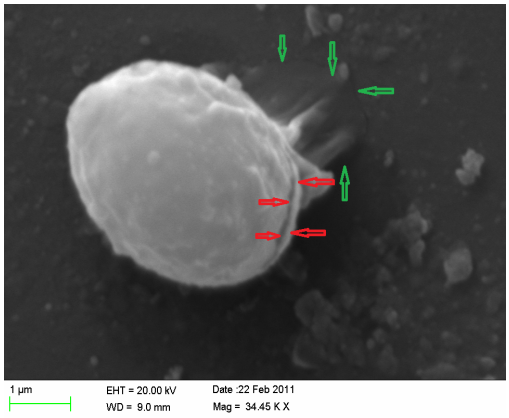
Scanning electron microscopy

Towards the end we focused on attempts to determine the possible mode of action of this test extract on the morphology of the treated cells by scanning electron microscopy. Present microscopic analyses of the *Candida guilliermondii* SN 2006 cells, clearly showed differences in morphology between untreated and treated cells**.** The SEM micrograph for untreated *C. guilliermondii* SN 2006 shows well defined shape with normal and smooth surface in (Fig. 4 a). Comparatively test extract treated cells showed membrane breakage. In addition to this leakage of cellular content was also noticed (Fig. 4 b).

Fig. 4: Scanning electron micrographs of *C. guilliermondii* SN 2006 (a) Untreated control and (b) Treated with test extract. Arrows indicate cell breakage and leakage of the intracellular content.



(a)



(b)

Natural products provide an unparalleled source of chemical scaffolds with diverse biological activities and have profoundly impacted antimicrobial drug discovery. The potential for developing antimicrobials from plants appears rewarding, as it will lead to the development of phytomedicine to act against microbes. This is significant finding as Candida is normal resident of oral cavity and genitourinary tract; it receives direct exposure of cinnamon. Our findings suggest options for expanding the utility of plant active principles as antifungal agents. We have demonstrated that methanolic bark extract of *Cinnamomum cassia* exhibits fungicidal activity by filter disc assay and growth curve study against three different Candida isolates. Recently obtained clinical isolates were found evidently sensitive to the test extract at varying extents. *In vitro* studies have shown that this test extract significantly inhibited the growth of candida isolates. The test extract displayed significant MIC,s against different Candida isolates ranging from 800 to 1600µg/ml. The extract was found to be highly active against *Candida parapsilosis* SN 1980, *Candida guilliermondii* SN 2006 followed by *Candida glabrata* SN 2266. Growth kinetic studies as a function of varied concentration also follow the same pattern. MIC/4 treated cells showed depressed growth curve with clearly differentiated phases. While as MIC/2 treated cells showed suppressed and delayed exponential phases. At MIC value S shaped curve declined to flat line showing almost complete arrest of cell growth (Fig. 3). On solid media (filter disc assay) effective inhibition of growth of Candida sp. by test extract was found to increase in concentration dependent manner (Fig. 1). Our findings by scanning electron microscopy suggest that this potential bioactive extract has distinct influence on Candida cell by causing breakage in the cell membrane and leakage of cellular content (Fig. 4b).

CONCLUSION

To conclude, the test extract shows significant anticandidal activity both in liquid and solid medium. Tentative mechanism of actions appears to originate from membrane breakage. This work is an additional effort to the development of new therapeutic agent which is fungicidal, less toxic and prevents drug resistance. Further investigation need to be done, which is fundamentally important and may help facilitate its application as future anticandidal agent.

Acknowledgements
The authors are highly thankful to Sheikh Shreaz, SRF (ICMR) for assisting in carrying out various biological experiments in this study.

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