

HOSTED BY



ELSEVIER

Contents lists available at ScienceDirect

## Journal of Traditional and Complementary Medicine

journal homepage: <http://www.elsevier.com/locate/jtcm>

## Original article

Exploration of the anticandidal mechanism of *Cassia spectabilis* in debilitating candidiasis

Angeline Torey<sup>a</sup>, Soundararajan Vijayarathna<sup>a</sup>, Subramanion L. Jothy<sup>a</sup>,  
Sivapragasam Gothai<sup>a</sup>, Yeng Chen<sup>b</sup>, Lachiman Yoga Latha<sup>a</sup>, Jagat R. Kanwar<sup>c</sup>,  
Saravanan Dharmaraj<sup>d</sup>, Sreenivasan Sasidharan<sup>a,\*</sup>

<sup>a</sup> Institute for Research in Molecular Medicine (INFORMM), Universiti Sains Malaysia, USM, Pulau Pinang 11800, Malaysia

<sup>b</sup> Dental Research & Training Unit, and Oral Cancer Research and Coordinating Centre (OCRCC), Faculty of Dentistry, University of Malaya, Kuala Lumpur 50603, Malaysia

<sup>c</sup> Nanomedicine-Laboratory of Immunology and Molecular Biomedical Research (LIMBR), Centre for Biotechnology and Interdisciplinary Biosciences (BioDeakin),

Institute for Frontier Materials (IFM), Deakin University, Waurn Ponds, Victoria 3217, Australia

<sup>d</sup> Faculty of Medical and Health Sciences, Universiti Sultan Zainal Abidin, Kota Campus, Kuala Terengganu, Terengganu 20400, Malaysia

## ARTICLE INFO

## Article history:

Received 12 July 2014

Received in revised form

16 September 2014

Accepted 8 October 2014

Available online 19 January 2015

## Keywords:

Anticandidal mechanism

Cell wall protein

Glucosidase

Morphology

Potassium leakage

Proteinase

## ABSTRACT

*Candida albicans* has become resistant to the commercially available, toxic, and expensive anti-*Candida* agents that are on the market. These factors force the search for new antifungal agents from natural resources. *Cassia spectabilis* had been traditionally employed by healers for many generations. The possible mechanisms of the *C. spectabilis* leaf extract were determined by potassium leakage study and the effect of the extract on the constituents of the cell wall and enzymes as well as the morphological changes on *C. albicans* cells were studied along with cytotoxicity assays. The cytotoxicity result indicated that the extract is nontoxic as was clearly substantiated by a half maximal inhibitory concentration (IC<sub>50</sub>) value of 59.10 µg/mL. The treated cells (*C. spectabilis* extract) demonstrated potassium leakage of 1039 parts per million (ppm) compared to Amphotericin B (AmpB)-treated cells with a released potassium value of 1115 ppm. The effects of the extract on the cell wall proteins illustrated that there were three major types of variations in the expression of treated cell wall proteins: the presence of new proteins, the absence of proteins, and the amount of expressed protein. The activities of two enzymes, α-glucosidase and proteinase, were determined to be significantly high, thereby not fully coinciding with the properties of the antifungal reaction triggered by *C. spectabilis*. The morphology of *C. albicans* cells treated with the *C. spectabilis* extract showed that the cells had abnormalities and were damaged or detached within the microcolonies. Our study verifies *C. spectabilis* leaf extract as an effective anti-*C. albicans* agent.

Copyright © 2014, Center for Food and Biomolecules, National Taiwan University. Production and hosting by Elsevier Taiwan LLC. All rights reserved.

## 1. Introduction

Candidiasis is a threat to public health as an opportunistic infection that affects patients with endocrine disorders, malignant disease, HIV/AIDS,<sup>1</sup> and nosocomial infections.<sup>2</sup> Candidiasis continues to be preponderant, and the yeast *Candida albicans* (C.P. Robin, Saccharomycetaceae family) is the culprit in the majority of cases. *C. albicans* has a staggering morphological feature that

enables it to colonize in a variety of forms ranging from unicellular budding yeast to true hyphae.<sup>3</sup> A frail alteration to the host system physiology transmutes this harmless commensal yeast into a treacherous pathogen, resulting in malaise. Therefore, it is imperative for the host defense system to alleviate the condition by returning the yeast back to its propitious commensal state.

*Candida* strains are presently gaining resistance to antifungal agents due to their contemporaneous usage, which complicates the annihilation of the yeast. Many antifungal agents are insufficient, toxic, and expensive. The revertible nature of *Candida* infestation increases the intricacy of treatment administration, and detracts from the urgency of the development of new antifungal agents.<sup>4</sup> Plants are valuable sources of phytochemicals. Medicinal plants

\* Corresponding author.

E-mail address: [srisasidharan@yahoo.com](mailto:srisasidharan@yahoo.com) (S. Sasidharan).

Peer review under responsibility of The Center for Food and Biomolecules, National Taiwan University.

are employed in the treatment of various diseases. A great number of people are accustomed to the use of these plants as they are affordable and accessible. A quarter of all prescriptions in industrialized countries are predicted to possess one or more components derived from plants.<sup>4</sup>

The focus of this study is the plant *Cassia spectabilis* (syn. *Senna spectabilis*) (DC) Irwin et Barn (Leguminosae family).

*C. spectabilis* numbers among the 600 or so species belonging to the genus *Cassia*; it is widely grown as an ornamental plant in tropical and subtropical areas. The species is extensively recognized for its diverse biological and pharmacological properties, and has been commonly used by traditional healers for many years<sup>5,6</sup>; Brazilian healers, for instance, have long incorporated this plant into treatments for flu and colds, and as a laxative and purgative.<sup>6</sup> Pharmacological reports have also identified antifungal,<sup>7</sup> antibacterial,<sup>8</sup> and antioxidant<sup>9</sup> activities. A recent study by Torey and Sasidharan<sup>10</sup> enunciated the standardization of *C. spectabilis* leaf methanol extract with respect to authenticity, assay methods, and chemical constituent analysis. Torey et al<sup>11</sup> also tested the anti-biofilm activity of *C. spectabilis* leaf methanol extract on a *C. albicans* biofilm and found that it was a good antibiofilm agent. Again, Sangetha et al<sup>7</sup> tested and reported on the toxicity of *C. spectabilis* leaf methanol extract against mice. The researchers affirmed that *C. spectabilis* is nontoxic and recommended it as a safe natural product for commercial utilization. However, the mechanism that vitiates *C. albicans* is yet to be established even though the extract is effective against the pathogenic yeast. This paper reports on the mechanism of action of standardized *C. spectabilis* leaf extract against a candidiasis-causing yeast, with the aim of providing a breakthrough for prediction of the medicinal effects of the extract so that it can be optimized for the eradication of candidiasis.<sup>12</sup> Antimicrobial enhancement for clinical purposes has been focused on targeting five essential components of cellular activity: the cell wall, protein synthesis, RNA synthesis, DNA synthesis, and intermediary metabolism.<sup>13</sup>

## 2. Materials and methods

### 2.1. Plant sampling

Fresh *C. spectabilis* leaves were collected from the University Sains Malaysia (USM) campus at Penang, Malaysia in April 2009. A single specimen was deposited in the Herbarium of the School of Biological Sciences, USM, Pulau Pinang, Malaysia (voucher number 11033) and was authenticated by Mr Shunmugam Vellosamy. The leaves were then separated and washed with water to remove dirt prior to the drying process (40 °C for 3 days).

### 2.2. Extraction

The leaf extract was prepared by macerating the dry powdered plant material in methanol (Sigma -Aldrich, Germany) for 3 days. Powdered leaves with an approximation of 200 g were macerated with methanol under agitation conditions for 72 hours. The extracted materials were then filtered with No. 1 Whatman filter paper (Whatman, Maidstone, UK) and further vaporized to dryness using the rotary evaporator (BUCHI Rotary Evaporator R110, Buchi, Switzerland).

### 2.3. Cytotoxicity assay

The cytotoxicity of the extract on Vero cells (ATCC: Manassas, VA, USA) was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT (Sigma, USA)) assay. The Vero cell lines were supplied by the INFORMM tissue culture laboratory.

The cell lines were grown as a monolayer culture in Roswell Park Memorial Institute (RPMI-1640) medium (Gibco, Invitrogen, UK), supplemented with 10% fetal bovine serum (FBS, Gibco, Invitrogen, UK), 100 units/mL penicillin (Serva Co, Germany), 100 µg/mL streptomycin (Merck Co, Germany), and 1 g NaHCO<sub>3</sub>. The cultures were maintained at 37 °C in humidified 5% CO<sub>2</sub> conditions. The leaf extract of *C. spectabilis* was tested for *in vitro* cytotoxicity using Vero cells by the MTT assay as described by Mosmann<sup>14</sup> with some modifications.<sup>15</sup> Briefly, 100 µL of media (RPMI-1640) was added to the control wells of the 96-well microtiter plate. Then, 100 µL of the *C. spectabilis* methanol extract (200, 100, 50, 25, 12.5, 6.25, 3.13, 1.56, and 0.78 µg/mL) was added into the respective treatment wells. The final volume for each well was 100 µL. The cultured Vero cells were harvested by trypsinization and pooled in a 50 mL vial. Then, 100 µL of the cell extract was plated into each well (1 × 10<sup>5</sup> cells per well) and the microtiter plate was incubated at 37 °C in a humidified 5% CO<sub>2</sub> incubator for 24 hours. After the incubation period, MTT (20 µL of 5 mg/mL) was added into each well and the cells were incubated for 4 hours at 37 °C until a purple precipitate was clearly visible under a microscope. Then, the medium together with the MTT (190 µL) was aspirated from the wells, dimethyl sulfoxide (DMSO; 100 µL) was added, and the plates were shaken for 5 minutes. (DMSO was added to dissolve the formazan crystals that had formed.) The absorbance for each well was measured at 540 nm using a microtiter plate reader (Sunrise Tecan). The percentage cell viability (CV) was calculated using the following formula:

$$CV (\%) = \frac{\text{Average absorbance of drug wells}}{\text{Average absorbance of control wells}} \times 100$$

A dose–response curve was plotted and the half maximal inhibitory concentration (IC<sub>50</sub>) value was obtained with Probit Analysis using SPSS version 16.0 software (SPSS Inc., Chicago, IL, USA).

### 2.4. Determination of the minimal inhibitory concentration

An 18-hour culture of *C. albicans* was diluted with a sterile physiologic saline solution [PS; 0.85% (w/v) sodium chloride] with reference to the 0.5 McFarland standard to achieve inoculums of approximately 10<sup>8</sup> colony forming units (CFU)/mL. A serial dilution was carried out to give final concentrations between 1.563 mg/mL and 200.00 mg/mL of crude extract of *C. spectabilis*. The tubes were inoculated with 500 µL yeast suspension per mL Sabouraud dextrose broth (SDB) (Oxoid, Basingstoke, UK), homogenized, and incubated at 37 °C. After incubation, 20 µL was withdrawn from each tube, inoculated on SDA agar plates, and incubated at 37 °C for 24 hours. The minimal inhibitory concentration (MIC) value was determined as the lowest concentration of the crude extract in the broth medium that inhibited a visible growth of the test microorganism.<sup>10</sup>

### 2.5. Potassium leakage

Ion leakage kinetics was performed according to a previously reported method.<sup>16</sup> Briefly, *C. albicans* was suspended in yeast nitrogen base (YNB) to achieve a final concentration of 5 × 10<sup>6</sup> cells/mL. The MIC concentration (6.25 mg/mL) of the *C. spectabilis* leaf extract was added to the test tubes. Amphotericin B (AmpB; 10 µg/mL) was the positive control, and methanol was used as the negative control. Samples of 10 mL were retrieved for ion concentration determination immediately after adding the extract and AmpB at 15-minute intervals for a period of 60 minutes. Equivalent samples were taken from the negative control at the same time of incubation. After centrifugation, the supernatant was used for ion determination with a Perkin Elmer 403 atomic absorbance

spectrophotometer. The concentration of potassium ions was determined after calibration with KCl at 383 nm. The evaluation was performed in triplicate.

## 2.6. Isolation of cell wall proteins

Fresh *C. albicans* isolates were first cultured in yeast peptone dextrose (YPD) broth for 14 hours at 37 °C. The culture was divided into two upon which one sample was treated with 6.25 mg/mL of the *C. spectabilis* leaf extract for 10 hours at 37 °C while methanol was added to the other sample and used as an untreated negative control. The isolation of cell walls from *C. albicans* was performed following the procedures by Pitarch et al.<sup>17</sup> The cells were harvested by centrifugation at 4500 g for 5 minutes. The cell pellet was washed in ice-cold sterile distilled water and resuspended in ice-cold lysis buffer (10 mM Tris–HCl, pH 7.4, 1 mM phenylmethylsulfonyl fluoride, PMSF). Then, 0.5 mm of chilled, acid-washed glass beads (Sigma, St. Louis, MO, USA) were added and the cells were mechanically disrupted. After centrifugation, the pellet was successively washed with cold water and gradual concentrations of NaCl (5%, 2%, and 1%) in 1 mM PMSF. Sodium dodecyl sulfate/dithiothreitol (SDS/DTT)-extractable proteins were extracted by boiling the purified cell walls in SDS extraction buffer (50 mM Tris–HCl, pH 8.0, 0.1 M EDTA, 2% SDS, 10 mM DTT) at 100 °C for 10 minutes. The sample was centrifuged for 10 minutes at 1000 g and the supernatant was precipitated using trichloroacetic acid (TCA). The pellet obtained from the above was used for the subsequent extraction using mild alkali conditions. The cell walls were resuspended in ice-cold wash buffer (0.1 M sodium acetate, pH 5.5, 1 mM PMSF), centrifuged for 10 minutes at 1000 g, and the supernatant was discarded. This step was repeated six more times. The cell walls were then resuspended in ice-cold extraction solution (30 mM NaOH, 1 mM PMSF) and the cell wall suspension was incubated at 4 °C for 17 hours. The suspension was centrifuged for 10 minutes at 1000 g and the supernatant was collected and precipitated using TCA. TCA (20% final concentration) was added to the supernatant obtained from the procedures above and the samples were incubated for 30 minutes on ice. The suspension was then centrifuged at 10,000 g for 15 minutes and the supernatant was discarded. The proteins were visible as a white, stringy mass. The protein pellet was washed twice with cold acetone to remove residual TCA and the pellet was air-dried and stored at 80 °C until further use.

## 2.7. SDS-polyacrylamide gel electrophoresis

Samples were taken up in SDS loading solution and 20 µL was loaded onto 10–20% BioRad Criterion gel. Precision Plus protein molecular mass markers were used as standards. The gel was run for 160 minutes at a voltage of 140 V. The gel was stained using the colloidal Coomassie Brilliant Blue G-250. All SDS-polyacrylamide gel electrophoresis (SDS-PAGE) dyes, reagents, and equipment were from Bio-Rad (Hercules, CA, USA).

## 2.8. $\alpha$ -Glucosidase activity

The  $\alpha$ -glucosidase activity was measured using *p*-nitrophenyl- $\alpha$ -D-glucopyranoside (Sigma, Germany) as a substrate based on a previously described method with some modifications.<sup>18</sup> Intact organisms of  $4 \times 10^6$  cells in 1.1 mL PBS were incubated with 6.25 mg/mL of the *C. spectabilis* extract and 5 mM substrate in 1.0 mL of 0.1M phosphate-citrate buffer (pH 6.0) for 2 hours at 37 °C. A negative control containing methanol was used for comparison. The reaction was terminated by the addition of 2 mL of ice-cold 0.1M Na<sub>2</sub>CO<sub>3</sub>. The *p*-nitrophenol that was released in the supernatant was then read at an optical density (OD) of 405 nm. The  $\alpha$ -glucosidase activity was

calculated by using a standard curve of *p*-nitrophenol. The testing was done in triplicate. The preparation of the standard curve was done by preparing *p*-nitrophenol with concentrations of 140, 70, 35, 17.5, 8.75, and 0 µM in test tubes. The tubes were vortexed and the absorbance was read at OD 405 nm. A standard curve was plotted using the *p*-nitrophenol standard and the amount of  $\alpha$ -glucosidase released was calculated using the following formula:

$$\alpha - \text{glucosidase activity in the sample (mU/mL)} \\ = \frac{\frac{1}{\text{Slope}} \times \text{OD}_{405} \times \text{Total volume}}{\text{Time} \times \text{Sample volume}}$$

## 2.9. Proteinase activity

The culture conditions and treatment with plant extract were carried out according to a previously reported method.<sup>19</sup> YYG medium (15 mL) (1.17% yeast carbon base, 0.01% yeast extract, 0.27% glucose) containing 6.25 mg/mL of the *C. spectabilis* leaf extract was inoculated with 106 CFU of *C. albicans* and incubated at 37 °C for 48 hours while shaking at 60 rpm. Methanol was used as a negative control. The proteinase assay was undertaken using a modification of the previous method<sup>20</sup> by adding 0.2 mL of the supernatant to 2 mL of 0.1 M citrate buffer (pH 3.2) containing 0.2% bovine serum albumin (BSA) and incubating at 37 °C for 2 hours. The reaction was stopped using 5% ice-cold trichloroacetic acid. The mixture was centrifuged at 5000 rpm for 30 minutes and the supernatant was read at an absorbance of 750 nm on a spectrophotometer.

## 2.10. Effect on the morphology of *C. albicans*

The *C. albicans* cells were treated with 6.25 mg/mL<sup>10</sup> (the MIC<sup>21</sup> obtained from our previous study) of the *C. spectabilis* leaf extract for 36 hours. After treatment with *C. spectabilis* extract, scanning electron microscopy (SEM) observation was carried out on *C. albicans* biofilm. The plate containing 25 mL potato dextrose agar medium was seeded with 1 mL of the *C. albicans* suspension containing 10 cells per mL from a 24-hour-old culture. The extract (1 mL), at the concentration of 6.25 mg/mL, was then dropped onto the inoculated agar and was further incubated for another 36 hours at 37 °C. A 1.0% DMSO-treated culture was used as a control. Segments (5–10 mm) were cut from cultures growing on potato dextrose plates at 36 hours for SEM examination. The specimen was placed on the double-stick adhesive tabs on a planchette. Subsequently, the planchette was placed on a Petri plate. In a fume hood, a vial cap containing 2% osmium tetroxide in water was placed in an unoccupied quadrant of the plate. After being covered, the plate was sealed with parafilm, and vapor fixation of the sample proceeded for 1 hour. Once the sample was vapor-fixed, the planchette was plunged into slush nitrogen (210 °C) and transferred on to the “peltier-cooled” stage of the freeze dryer (Emitech K750), and freeze drying of the specimen proceeded for 10 hours. Finally, the freeze-dried specimen was sputter-coated with 5–10 nm of gold before viewing in the SEM (FESEM LEO Supra 50 VP; Carl Zeiss, Germany) operating at 5 kV at 5.00 k $\times$  of magnification.

## 2.11. Statistical analysis

All data were analyzed and compared using a Student *t* test in SPSS software. A *p* value <0.05 was considered statistically significant.

### 3. Results

#### 3.1. Cytotoxicity assay

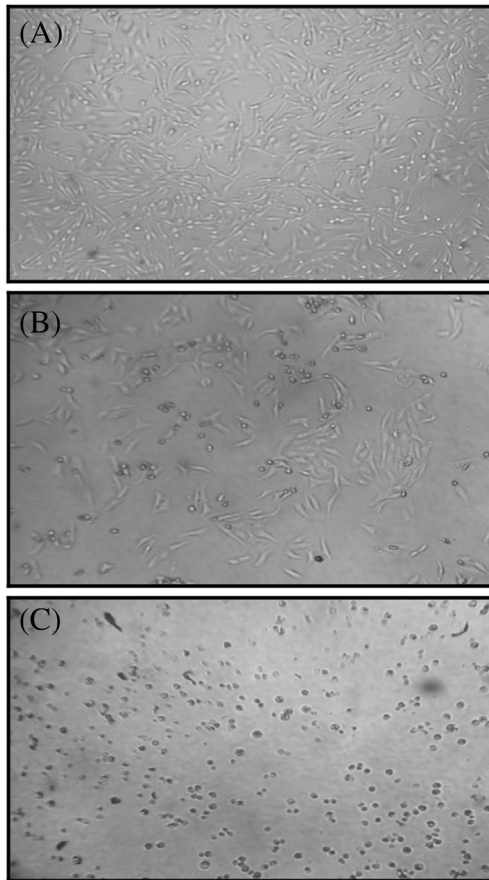
The IC<sub>50</sub> value obtained from the MTT assay was 59.10 µg/mL. Microscopic images of Vero cells at different concentrations are displayed in Fig. 1A–C. Normal, untreated Vero cells are presented in Fig. 1A—the cells appear as a monolayer of elongated cells with nuclei positioned in the middle and attached to the surface of the microtiter plate. Fig. 1B shows *C. spectabilis*-treated (50 µg/mL) Vero cells—this concentration correlates with the IC<sub>50</sub> concentration acquired earlier, hence elucidating the damage in 50% of the Vero cells, which is depicted with shriveled and detached characteristics on the microtiter plate surface. By contrast, Fig. 1C shows Vero cells treated with 200 µg/mL of the *C. spectabilis* leaf extract; no viable cells are observed here.

#### 3.2. Determination of the MIC

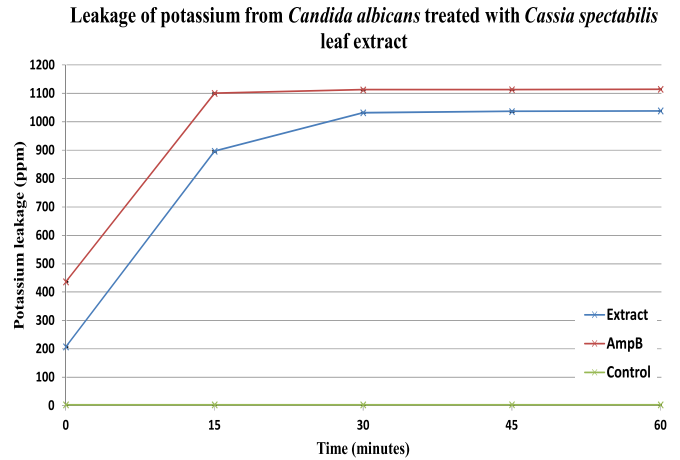
The broth dilution method recorded the MIC value of 6.25 mg/mL against the *C. albicans* test strain. Therefore, this concentration was used for further testing.

#### 3.3. Potassium leakage

Potassium (K<sup>+</sup>) ion leakage was estimated using atomic absorption spectroscopy (AAS) and the resulting patterns are presented in Fig. 2. It was found that the methanol extract of



**Fig. 1.** Microscopic images of Vero cells. (A) Untreated Vero cells. (B) Cells treated with *Cassia spectabilis* leaf extract at the concentration 50 µg/mL. (C) Cells treated with *C. spectabilis* leaf extract at the concentration 200 µg/mL.



**Fig. 2.** Leakage of potassium found in *Candida albicans* cells treated with *Cassia spectabilis* leaf extract,  $p < 0.05$ .

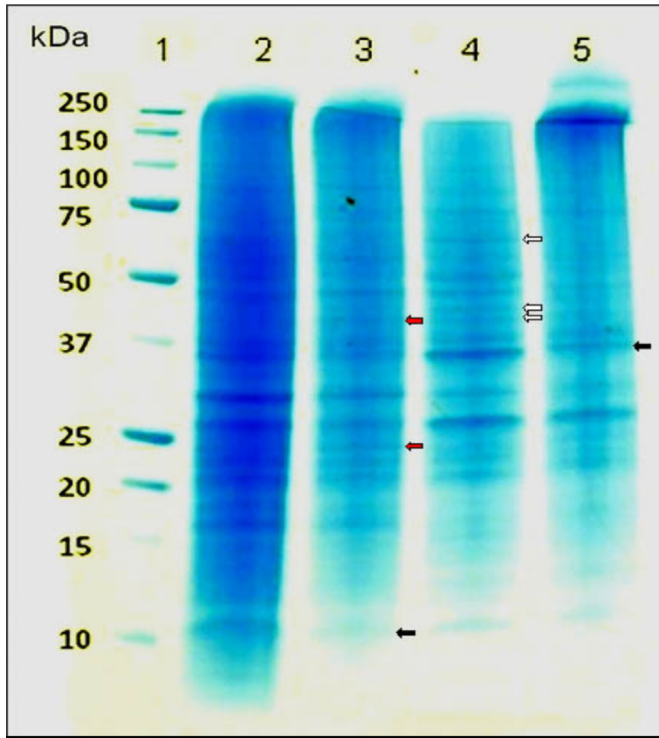
*C. spectabilis* was effective in leakage of K<sup>+</sup> ions by releasing 1039 ppm whereas AmpB (positive control) released comparatively higher ions than the extract (1115 ppm). The K<sup>+</sup> ions were instantly detected within the culture supernatant upon extract treatment initiation, to which progressive increments were observed peaking at 30 minutes of exposure, after which they declined. By contrast, *C. albicans* cells treated with AmpB managed to achieve their peak after 15 minutes. Untreated *C. albicans* cells served as a negative control whereby the cells had small traces of K<sup>+</sup> ions in the culture supernatant. This result illustrates that K<sup>+</sup> ions are leaked from the cells within 60 minutes of interaction between the organism and the extract.

#### 3.4. Effect on *C. albicans* cell wall proteins

The result obtained from the SDS-PAGE is shown in Fig. 3. There were two extraction methods: SDS/DTT and NaOH extractions. On extraction with SDS/DTT, the *C. albicans* cell walls expressed two new proteins (red arrows). One of these proteins was measured to be in the range of 37–50 kDa in molecular weight whereas the other protein was between 20 kDa and 25 kDa in molecular weight. As for the extraction using NaOH, three major proteins were discovered to be absent in the cell walls of treated *C. albicans* compared to the untreated ones (white arrows). When compared to the untreated sample, two of these absent proteins were identified to be within the range 37–50 kDa in molecular weight while the final one was estimated to be in the range 50–75 kDa in molecular weight. A thinner and fainter protein band in the treated sample (black arrow) indicated lower protein expression—this was observed with both SDS/DTT and NaOH extraction methods. The low-expressed proteins were noted to be about 10 kDa in the SDS/DTT extraction sample and around 37 kDa in the NaOH extraction sample.

#### 3.5. $\alpha$ -Glucosidase activity

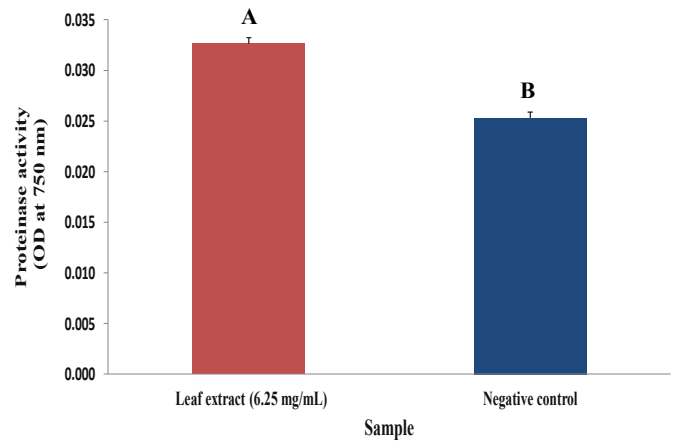
Fig. 4 depicts the  $\alpha$ -glucosidase activity of the *C. albicans* cells treated with 6.25 mg/mL of the leaf extract. The activity of the treated cells was compared to those of an untreated control. It was found that the cells treated with the extract possessed a significantly higher  $\alpha$ -glucosidase activity compared to the untreated control. The activity of the treated cells was  $0.432 \pm 0.003$  mU/mL, while the activity of the untreated cells was  $0.048 \pm 0.001$  mU/mL ( $p < 0.05$ ).



**Fig. 3.** SDS-PAGE of *Candida albicans* cell wall proteins under the effect of the *Cassia spectabilis* leaf extract. Lanes: 1, Precision Plus molecular mass markers; 2, SDS/DTT-extracted proteins (untreated control); 3, SDS/DTT-extracted proteins (treated with 6.25 mg/mL *C. spectabilis* leaf extract); 4, NaOH-extracted proteins (untreated control); 5, NaOH-extracted proteins (treated with 6.25 mg/mL *C. spectabilis* leaf extract). Molecular mass standards are expressed on the left of the gel (in kilodaltons, kDa). White arrows indicate the absence of that protein in the treated counterpart; black arrows indicate less protein expression compared to the untreated counterpart; red arrows indicate a new protein compared to the untreated counterpart. DTT = dithiothreitol; PAGE = polyacrylamide gel electrophoresis; SDS = sodium dodecyl sulfate.

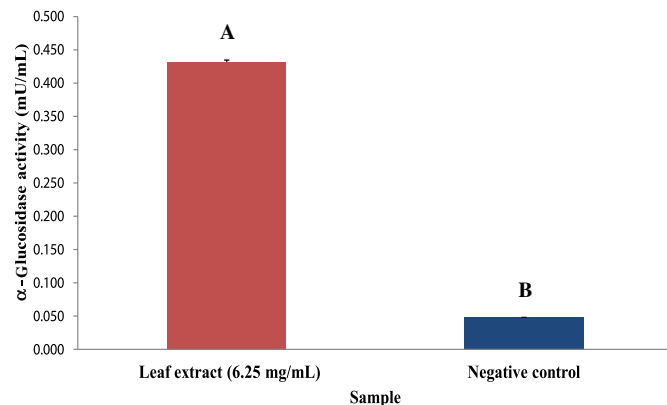
3.6. Proteinase activity

**Fig. 5** shows the results of the proteinase activity of the *C. albicans* cells. The activity of the treated cells was compared to those of an untreated control. It was observed that the cells treated with the *C. spectabilis* leaf extract (6.25 mg/mL) exhibited a significantly higher proteinase activity compared to the control. The absorbance value (at 750 nm) of the supernatant for the treated

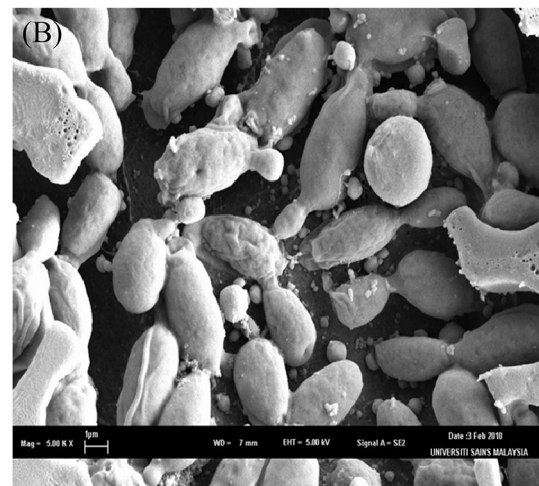
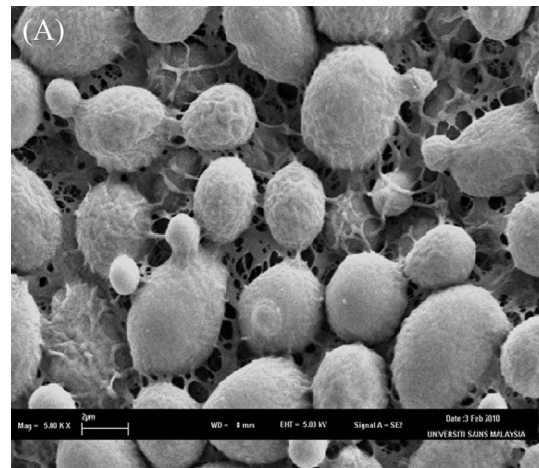


**Fig. 5.** Proteinase activity of *Candida albicans* treated with *Cassia spectabilis* leaf extract.

cells was  $0.033 \pm 0.001$ , while the untreated cells gave an absorbance value of  $0.025 \pm 0.001$  ( $p < 0.05$ ).



**Fig. 4.**  $\alpha$ -Glucosidase activity evaluated from *Candida albicans* treated with *Cassia spectabilis* leaf extract.



**Fig. 6.** Morphological changes observed on (A) untreated control *Candida albicans* cells and (B) *C. albicans* cells treated with 6.25 mg/mL *C. spectabilis* leaf extract.

### 3.7. Effect on the morphology of *C. albicans*

The SEM photomicrographs of the untreated and *C. spectabilis*-treated cells of *C. albicans* at 36 hours are displayed in Fig. 6. Fig. 6A depicts the untreated *C. albicans* cells that were used as a negative control; the presence of biofilm that connects the cells together can be seen along with budding cells. By contrast, the treated cells appear to be loosened from each other indicating a deteriorated biofilm (Fig. 6B); the cells seemed to develop small ruptures on the cell surface actuating in cell convulsion; the treated cells were more elongated compared to the untreated cells.

## 4. Discussion

In our study, a few characteristics were disclosed ascertaining the possible patterns by which *C. spectabilis* leaf extract may act upon *C. albicans*. Fig. 7 depicts the anticandidal mechanistic pattern of *C. spectabilis* leaf extract. The activity of the extract suggests a promising mechanism for altering cell wall and cell membrane components, inhibiting DNA, and affecting protein synthesis and metabolism of *C. albicans*. An extract possessing distinctive active compounds is associated with multiple mechanisms of action, each ascribable to a specialized compound or group of compounds. The rigid cell wall of *C. albicans* is composed mainly of glucans and chitins<sup>22</sup> offering the notion that some compounds might be responsible for the initial step of cell wall penetration, before allowing the rest of the crude extract compounds to access further into the cell causing more damage.

The complete procedure for discovering and developing a new therapeutic agent is highly expensive and tedious, often stretching over a 12-year period. Identifying the potential toxic compound is an imperative step for early drug development. The IC<sub>50</sub> value obtained when Vero cell lines were treated with *C. spectabilis* extract was 59.10 µg/mL; this value was considered nontoxic because it was more than 20 µg/mL.<sup>23</sup> A previous study conducted by Sangetha et al.<sup>7</sup> on the same leaf extract using mice oral acute toxicity tests further verifies the nature of *C. spectabilis* leaf extract as nontoxic. Toxic effects of an antimicrobial agent should be pondered deliberately especially when concerning host cells. Any single substance from a crude extract is capable of inevitably causing perturbation.<sup>24</sup> Nevertheless, it was surprising that the toxic effect of *C. spectabilis* leaf extract was exhibited on *C. albicans* rather than on Vero cells, suggesting that the extract displayed selective toxicity towards yeast cells. Although there are many commercial drugs readily

available to treat candidiasis, *C. spectabilis* fortuitously reveals its competence in inhibiting *C. albicans* growth (Fig. 1B) as well as being less toxic to Vero cells (Fig. 1A). It can be corroborated that *C. spectabilis* leaf extract is a potent *in vitro* anticandidal agent. Cell culture can be used to screen for toxicity both by estimation of the basal functions of the cell (i.e., those processes common to all types of cells) or by tests on specialized cell functions.<sup>25</sup> General toxicity tests, aimed mainly at detection of the biological activity of test substances, can be carried out on many cell types (e.g., fibroblasts, HeLa cells, and hepatoma cells). A number of parameters including vital staining, cytosolic enzyme release, cell growth, and cloning efficiency are used as end-points to measure toxicity.<sup>26</sup> Hence, in this study Vero cells from a monkey were used for the cytotoxicity assay.

The osmotic balance of a cell can be easily affected by the changes in potassium ions (K<sup>+</sup>), and ion leakage from a cell may result in cell lysis. These ions play crucial roles in activating essential enzymes that function as organic catalysts mediating prominent biochemical reactions.<sup>27</sup> *C. albicans* cells treated with *C. spectabilis* were evaluated for K<sup>+</sup> leakage and the efficacy was compared to AmpB (antifungal agent, positive control). AmpB binds to ergosterol causing the formation of aqueous channels that increase membrane permeability to univalent cations resulting in cell death.<sup>28</sup> A gradual leakage of K<sup>+</sup> ions by the treated *C. albicans* cells was shown here (Fig. 2) corresponding to the time of treatment exposure; this result was as good as the effect of the commercial antifungal drug AmpB on K<sup>+</sup> leakage in *C. albicans*. The leaf extract pattern coined a perception that the extract had altered the membrane of the *C. albicans* intensifying its permeability to release K<sup>+</sup> ions. The fungal plasma membrane is constituted predominantly by phospholipids, sphingolipids, and sterols that together serve as a permeability barrier concurrent to the transport of small molecules. Among other things, these components also transmit signals and serve as a matrix for proteins with various functions.<sup>29,30</sup> Therefore, when this membrane is compromised, it will cause an efflux of intracellular potassium. Cox et al.<sup>31</sup> studied the influence of tea tree oil on *C. albicans* and drew the conclusion that leakage of K<sup>+</sup> ions was an indication of cell membrane structure damage. Similarly, Wei et al.<sup>32</sup> performed an analysis of K<sup>+</sup> leakage to investigate plasma membrane injury in *C. albicans* and asserted that the outflow of K<sup>+</sup> ions was associated with yeast cell membrane permeability. This result can also be related to other antifungal mechanisms such as binding of the drug to sterols to increase permeability, inhibition of ergosterol synthesis, or even dysfunctional membranal enzymes.<sup>33</sup>

Other methods can also be used to assess the membrane damage caused by antifungal agents, for example, assessment of ATP leakage by fungal cells was employed by Kulakovskaya et al.<sup>34</sup> According to Nagarsekar et al.,<sup>35</sup> cytoplasmic membrane damage can be evidenced by leakage of cellular K<sup>+</sup> ions. Hence, it is concluded that the *C. spectabilis* leaf extract caused membrane damage in the *C. albicans* cells.

Proteomic analysis of *C. albicans* cell wall proteins was performed to substantiate the association of K<sup>+</sup> ion leakage with cell membrane damage. The *C. albicans* cell wall is very significant in terms of biology and pathogenicity.<sup>36</sup> The mechanical and osmotic balance of the yeast is achieved by its cell wall, which is constructed mainly of carbohydrates with a basic network complex of β-glucans, chitin, and mannan.<sup>37–40</sup> The yeast cell wall forms a definite boundary in providing mechanical stability and cell turgor,<sup>40</sup> in addition to its role in the construction of the outer fungal structural surface. The cell wall, being the first point of contact when subjected to the *C. spectabilis* leaf extract, pointedly provokes interruption in the organization or functions of the cell wall components. These alterations within the cell wall organization are

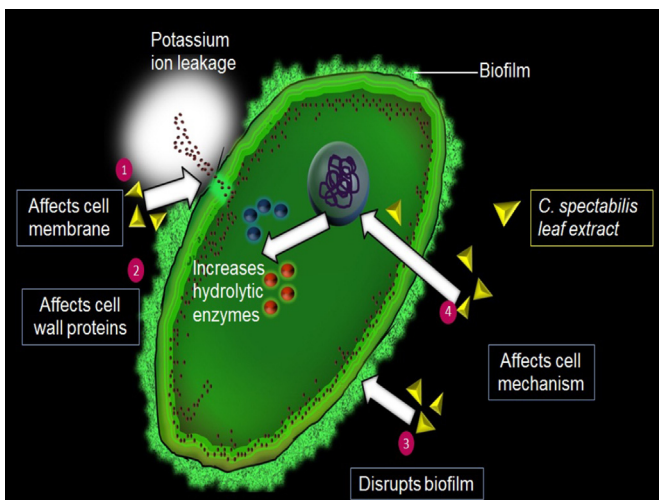


Fig. 7. Mechanistic patterns displayed by *Cassia spectabilis* on *Candida albicans*.

inspected at the molecular level through its cell wall protein expression. Considering the fact that proteins are the ultimate gene products and representatives of various physiological and pathological processes of the living cell, the field of proteomics has made allowances for the influence of different conditions on the expression of proteins.<sup>41,42</sup> For this reason, the cell wall protein expression was investigated to ratify the alteration involving the cell wall constituents caused by the *C. spectabilis* leaf extract. Different biochemical approaches have been used to identify proteins that are randomly scattered along the cell wall. The non-covalently attached proteins are extracted using SDS/DDT while covalently bound proteins are extracted by NaOH treatment (mild alkali treatment).<sup>40</sup> The presence of new proteins can be proposed as the action of *C. spectabilis* leaf extract in disintegrating cell wall moieties into smaller peptides. Low protein expression levels may result from the possible disintegration of proteins into smaller peptides making them imperceptible between the bounds of the standard molecular mass weights. Mahmoud and Aly<sup>43</sup> reported the differences in cell wall protein expression using SDS-PAGE; they uncovered an increase in phosphopeptidomannan of molecular weights 45 kDa, 25 kDa, and 15 kDa when *C. albicans* was treated with a polymer. Other conditions are also capable of modifying the composition of the cell wall. For example, an elevated level of *Candida* Pir2-related protein was found to be a reason for vulnerability of *C. albicans*.<sup>39</sup> Then again, the featured changes in cell wall proteins solidly attest the changes in protein expression.

*C. albicans* is well advanced in secreting an assortment of hydrolytic enzymes such as  $\alpha$ -glucosidase and proteinase. The yeast secretes these enzymes as part of its mechanism to invade and proliferate. It causes destruction to the host cells when it degrades healthy tissues to supply its nutrient needs.<sup>44</sup> The enzymes  $\alpha$ -glucosidase and proteinase are culpable in the virulence of *C. albicans* and were found to be significantly higher in treated *C. albicans* cells in contrast with untreated control cells. Fekete Forgács et al<sup>45</sup> examined the possibility of  $\alpha$ -glucosidase being a virulence factor. The enzyme activity was compared between a fluconazole-resistant *C. albicans* strain and a fluconazole-sensitive strain and they found that  $\alpha$ -glucosidase plays an insignificant role in the virulence of *C. albicans*. There was no correlation between  $\alpha$ -glucosidase and proteinase.

The proteinase activity of *C. albicans* cells is divulged generally as a repercussion of virulence. Some time ago, it was reported that a *C. albicans* culture filtrate manifested many types of hydrolytic enzymes including proteinases, phospholipases, acid phosphatases, chitinases, esterases, and glucoamylases.<sup>38</sup> Cutler<sup>46</sup> showed that the release of proteinase was associated with cell death. Given that *C. spectabilis* had earlier demonstrated antibiofilm activities towards *C. albicans*, the feeble yeast cells of Cutler's study can be used to rationalize the high proteinase activities. Cassone et al<sup>47</sup> discovered the presence of proteinase in pathogenic strains isolated from cases of candidal vaginitis and those of carriers. Schreiber et al<sup>48</sup> did not find a correlation between the volume of proteinase generated by various clinical and normal *C. albicans* strains with the invasion level of the strains. More to the point, another study found similar production of proteinase in a low-virulence mutant of *C. albicans* and virulent parent strains.<sup>49</sup> The increase in proteinase activity alone is not sufficient to be considered a virulence factor as Dubois et al<sup>50</sup> revealed that a systemic infection-causing mutant *C. albicans* in mice transformed from being virulent to avirulent when proteinase was overexpressed. The authors believe that the overexpressed proteinase might have resulted from compromised yeast cell walls, otherwise the mice host immune system would not be able to eliminate these *C. albicans* unless they were susceptible. In general terms, the high  $\alpha$ -glucosidase and proteolytic activity of *C. albicans* is an

indication of altered metabolism caused by the *C. spectabilis* leaf extract.

*C. albicans* cells were exposed to the *C. spectabilis* leaf extract for 36 hours, and the morphological alterations were viewed through SEM along with other untreated cells. Few structural differences were noted in the treated cells. The exterior outline of the cells seemed to shrivel and elongate. The cells also appeared to be detached from each other elucidating certain disturbance in the biofilm formation. One of the major contributors to candidiasis occurring at an alarming rate is the propensity of *C. albicans* for biofilm formation. The biofilm forms when single cells attach to a surface and grow into microcolonies, followed by blended production of a complex three-dimensional (3D) structure held by hyphae and exopolymer matrix.<sup>51</sup> The biofilm acts as a barrier, preventing penetration by immune system components or even antifungal agents.<sup>51–53</sup> Critically, SEM revealed the after-effect of the *C. spectabilis* leaf extract on *C. albicans* biofilm construction. The destruction of the biofilm that occurs in treated cells facilitates the host immune system to eliminate the pathogen, thereby eradicating candidiasis.

## 5. Conclusion

Taken together, our findings demonstrated that *C. spectabilis* leaf extract is nontoxic to Vero cells and is effective against *C. albicans* biofilms. Future studies will be necessary to identify the protein changes that occur within the cell wall during treatment, as well as the isolation and the characterization of active compound(s) with antibiofilm activity. In conclusion, *C. spectabilis* leaf extract has strong anticandidal activity with various mechanisms of action.

## Conflicts of interest

All authors declare no conflicts of interest.

## Acknowledgments

This project was funded by USM Short Term Grants (304/CIPPM/639040). Angeline Torey was supported by the USM Fellowship Scheme of the Institute for Postgraduate Studies.

## References

- Martinez JP, Gil ML, Lopez-Ribot JL, Chaffin WL. Serologic responses to cell wall mannoproteins and proteins from *Candida albicans*. *Clin Microbiol Rev.* 1998;11:121–141.
- Nucci M. Prevention and treatment of nosocomial candidiasis. *Int J Infect Dis.* 2010;12. Article ID e118 <http://dx.doi.org/10.1016/j.ijid.2010.02.1525>.
- Sudbery P, Gow N, Berman J. The distinct morphogenic states of *C. albicans*. *Trends Microbiol.* 2004;11:317–324.
- Runyoro DKB, Ngassapa OD, Matee MIN, Joseph CC, Moshi MJ. Medicinal plants used by Tanzanian traditional healers in the management of *Candida* infections. *J Ethnopharmacol.* 2006;106:158–165.
- Viegas JRC, Bolzani VS, Furlan M, et al. Further bioactive piperidine alkaloids from the flowers and green fruits of *Cassia spectabilis*. *J Nat Prod.* 2004;67:908–910.
- Lorenzi H, Matos FJA. *Plantas Medicinais do Brasil Nativas e Exóticas*. Nova Odessa, Brazil: Instituto Plantarum; 2002:291.
- Sangetha S, Zuraini Z, Sasidharan S, Suryani S. Fungicidal effect and oral acute toxicity of *Cassia spectabilis* leaf extract. *Nippon Ishinkin Gakkai Zasshi.* 2008;49:299–304.
- Sangetha S, Zuraini Z, Sasidharan S, Suryani S. Antibacterial, antifungal and cytotoxic activities of *Cassia spectabilis*. *Asian J Pharm Clin Res.* 2008;1:17–20.
- Sangetha S, Zuraini Z, Sasidharan S, Suryani S. Free radical scavenging activity of *Cassia spectabilis* and *Cassia fistula*. *Int J Nat Eng Sci.* 2008;2:111–114.
- Torey A, Sasidharan S. Anti-*Candida albicans* biofilm activity by *Cassia spectabilis* standardized methanol extract: an ultrastructural study. *Eur Rev Med Pharmacol Sci.* 2011;15:875–882.
- Torey A, Sasidharan S, Yeng C, Latha LY. Standardization of *Cassia spectabilis* with respect to authenticity, assay and chemical constituent analysis. *Molecules.* 2010;15:3411–3420.

12. Hart CP. Finding the target after screening the phenotype. *Drug Discov Today*. 2005;10:513–519.
13. Stratton CW. Mechanisms of activity and resistance for fluoroquinolones. *Antimicrob Infect Dis News*. 1995;14:69–72.
14. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Meth*. 1983;65:55–63.
15. Kavitha N, Noordin R, Chan KL, Sasidharan S. Cytotoxicity activity of root extract/fractions of *Eurycoma longifolia* Jack root against vero and Hs27cells. *J Med Plants Res*. 2010;4:2383–2387.
16. Polacheck I, Levy M, Guizie M, Zehavi U, Naim M, Evron R. Mode of action of the antimycotic agent G2 isolated from alfalfa roots. *Zentralbl Bakteriol*. 1991;275:504–512.
17. Pitarch A, Nombela C, Gil C. Cell wall fractionation for yeast and fungal proteomics. *Methods Mol Biol*. 2008;425:217–239.
18. Bramono K, Tsuboi R, Ogawa H. A carbohydrate-degrading enzyme from *Candida albicans*: correlation between alpha-glucosidase activity and fungal growth. *Mycoses*. 1995;38:349–353.
19. Patel M, Gulube Z, Dutton M. The effect of *Dodonaea viscosa* var. *angustifolia* on *Candida albicans* proteinase and phospholipase production and adherence to oral epithelial cells. *J Ethnopharmacol*. 2009;124:562–565.
20. Sugita T, Kurosaka S, Yajitate M, Sato H, Nishikawa A. Extracellular proteinase and phospholipase activity of three genotypic strains of a human pathogenic yeast, *Candida albicans*. *Oral Microbiol Immun*. 2002;46:881–883.
21. Vijayarathna S, Zakaria Z, Chen Y, Latha LY, Kanwar JR, Sasidharan S. The antimicrobial efficacy of *Elaeis guineensis*: characterization, *in vitro* and *in vivo* studies. *Molecules*. 2012;17:4860–4877.
22. Shepherd MG. Cell envelope of *Candida albicans*. *Crit Rev Microbiol*. 1987;15:7–25.
23. Zirihni GN, Mambu L, Guede-Guina F, Bodo B, Grellier P. *In vitro* antiplasmodial activity and cytotoxicity of 33 West African plants used for treatment of malaria. *J Ethnopharmacol*. 2005;98:281–285.
24. Sasidharan S, Darah I, Jain K. *In vivo* and *In vitro* toxicity study of *Gracilaria changii*. *Pharm Bio*. 2008;46:413–417.
25. Ekwall B. Screening of toxic compounds in mammalian cell cultures. *Ann N.Y. Acad Sci*. 1983;407:64–77.
26. Ekwall B, Silano V, Paganuzzi-Stammati A, Zucco F. Toxicity tests with mammalian cell cultures. In: Bourdeau P, Somers E, Richardson GM, Hickman JR, eds. *Short-Term Toxicity Tests for Non-Genotoxic Effects*. New York, NY: John Wiley & Sons; 1990:75–97.
27. Arya V, Yadav S, Kumar S, Yadav JP. Antimicrobial activity of *Cassia occidentalis* L (leaf) against various human pathogenic microbes. *Life Sci Med Res*. 2010;9:1–12.
28. Groll AH, Shah PM, Mentzel C, Schneider M, Just-Nuebling G, Huebner K. Trends in the postmortem epidemiology of invasive fungal infections at a university hospital. *J Infect*. 1996;33:23–32.
29. Oehlschlager AC, Czyzewska E. Rationally designed inhibitors of sterol biosynthesis. In: Sutcliffe J, Georgopapadakou NH, eds. *Emerging Targets in Antibacterial and Antifungal Chemotherapy*. New York, NY: Chapman & Hall; 1992:437–475.
30. Georgopapadakou NH, Tkacz JS. The fungal cell wall as a drug target. *Trends Microbiol*. 1999;3:98–104.
31. Cox SD, Mann CM, Markham JL, et al. The mode of antimicrobial action of the essential oil of *Melaleuca alternifolia* (tea tree oil). *J Appl Microbiol*. 2000;88:170–175.
32. Wei MK, Wu QP, Huang Q, Wu JL, Zhang JM. Plasma membrane damage to *Candida albicans* caused by chlorine dioxide (ClO<sub>2</sub>). *Lett Appl Microbiol*. 2008;47:67–73.
33. Sionov E, Roth D, Sandovsky-Losica H, et al. Antifungal effect and possible mode of activity of a compound from the marine sponge *Dysidea herbacea*. *J Infect*. 2005;50:453–460.
34. Kulakovskaya TV, Kulakovskaya EV, Golubev WI. ATP leakage from yeast cells treated by extracellular glycolipids of *Pseudozyma fusiformata*. *FEMS Yeast Res*. 2003;3:401–404.
35. Nagarsekar KS, Nagarsenker MS, Kulkarni SR. Evaluation of composition and antimicrobial activity of supercritical fluid extract of leaves of *Vitex negundo*. *Indian J Pharm Sci*. 2010;72:641–643.
36. Cassone A. Cell wall of *Candida albicans*: its functions and its impact on the host. *Curr Top Med Mycol*. 1989;3:248–314.
37. Chauhan N, Li D, Singh P, Calderone R, Kruppa M. The cell wall of *Candida* spp. In: Calderone RA, ed. *Candida and Candidiasis*. Washington, DC: ASM Press; 2002:159–175.
38. Chaffin WL, Lopez-Ribot JL, Casanova M, Gozalbo D, Martinez JP. Cell wall and secreted proteins of *Candida albicans*: identification, function, and expression. *Microbiol Mol Biol R*. 1998;62:130–180.
39. Kapteyn JC, Van Den Ende H, Kli FM. The contribution of cell wall proteins to the organization of the yeast cell wall. *Biochim Biophys*. 1999;1426:373–383.
40. Teparic R, Stuparevic I, Mrsa V. Increased mortality of *Saccharomyces cerevisiae* cell wall protein mutants. *Microbiology*. 2004;150:3145–3150.
41. Ellepola A, Morriss CJ. Laboratory diagnosis of invasive candidiasis. *J Microbiol*. 2005;43:65–84.
42. Pfaller MA. Laboratory aids in the diagnosis of invasive candidiasis. *Mycopathology*. 1992;120:65–72.
43. Mahmoud YA, Aly MM. Anti-*Candida* and mode of action of two newly synthesized polymers: a modified poly (methyl methacrylate-co-vinylbenzoylchloride) and a modified linear poly (chloroethylvinylether-co-vinylbenzoylchloride) with special reference to *Candida albicans* and *Candida tropicalis*. *Mycopathology*. 2004;157:145–153.
44. Bramono K, Yamazaki M, Tsuboi R, Ogawa H. Comparison of proteinase, lipase and alpha- glucosidase activities from the clinical isolates of *Candida* species. *Jpn J Infect Dis*. 2006;59:73–76.
45. Fekete Forgacs K, Jeney A, Varga G, Lenkey B. Investigation of glucosidase as a potential virulence factor of *Candida albicans*. *J Basic Microbiol*. 2000;40:105–110.
46. Cutler JE. Putative virulence factors of *Candida albicans*. *Annu Rev Microbiol*. 1991;45:187–218.
47. Cassone A, Debernardis F, Mondello F, Ceddia T, Agatensi L. Evidence for a correlation between proteinase secretion and vulvo-vaginal candidiasis. *J Infect Dis*. 1987;156:777–783.
48. Schreiber B, Lyman CA, Gurevich J, Needham CA. Proteolytic activity of *Candida albicans* and other yeasts. *Diagn Microbiol Infect Dis*. 1985;3:1–5.
49. Edison A, Manning-Zweerink M. Comparison of the extracellular proteinase activity produced by a low-virulence mutant of *Candida albicans* and its wild-type parent. *Infect Immun*. 1988;56:1388–1390.
50. Dubois N, Colina AR, Aumont F, Belhumeur P, de Repentigny L. Overexpression of *Candida albicans* secretory aspartyl proteinase 2 and its expression in *Saccharomyces cerevisiae* do not augment virulence in mice. *Microbiology*. 1998;144:2299–2310.
51. LaFleur MD, Kumamoto CA, Lewis K. *Candida albicans* biofilms produce antifungal-tolerant persister cells. *Antimicrob Agents Chemother*. 2006;50:3839–3846.
52. Jain N, Kohli R, Cook E, Gialanella P, Chang T, Fries BC. Biofilm formation by and antifungal susceptibility of *Candida* isolates from urine. *Appl Environ Microbiol*. 2007;73:1697–1703.
53. Meyer B. Approaches to prevention, removal and killing of biofilms. *Int Biodeter Biodegr*. 2003;51:249–253.