**Abstract.** – **Background and Objectives:** *Vernonia (V.) cinerea* Less (Asteraceae) have many therapeutic uses in the practice of traditional medicine. The methanol extract of *V. cinerea*, was screened for antiyeast activity against pathogenic yeast *Candida albicans*.

**Materials and Methods:** The antimicrobial activities were studied by using disc diffusion method and broth dilution method. The effect of the extract on the growth profile of the yeast was also examined via time-kill assay. In addition to the fungicidal effects study, microscopic observations using Scanning (SEM) electron microscopy, Transmission (TEM) electron microscopy and light microscopy (LM) were done to determine the major alterations in the microstructure of *Candida* (*C.*) albicans.

**Results and Discussion:** The extract showed a favorable antimicrobial activity against *C. albicans* with a minimum inhibitory concentration (MIC) value of 1.56 mg/mL. Time-kill assay suggested that *Vernonia cinerea* extract had completely inhibited *Candida albicans* growth and also exhibited prolonged antiyeast activity. The main abnormalities notes from these microscopic observations were the alterations in morphology and complete collapse of the yeast cells after 36 h of exposure to the extract.

**Conclusion:** The extract of *Vernonia cinerea* may be an effective agent to treat the *Candida albicans* infection.

**Key Words:**

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**Materials and Methods**

**Plant Material**

Fresh leaf of *Vernonia cinerea* (authenticated at University Science of Malaysia Herbarium by Dr Shaida Fariza Sulaiman in the School of Biological Sciences) was collected in April 2004 and the specimen was prepared and deposited at the
Herbarium of School of Biological Sciences at University Science of Malaysia with voucher specimen number 11004.

**Preparations of the Crude Extract**

Two hundred grams of dried *V. cinerea* leaf was boiled in a Soxhlet apparatus with 300 ml of 80% methanol (v/v) for 48 h. The entire extract of *V. cinerea* was evaporated to dryness in a rotary evaporator.

**Microorganism**

*Candida albicans* strain 1, *Candida albicans* strain 2, *Candida albicans* strain 3 and *Cryptococcus neoformans* (clinical isolate) were reused as the test organisms and was obtained from a laboratory stock culture. The yeasts were cultured on Sabouraud dextrose agar at 30°C for 24 h. The stock cultures were maintained on Sabouraud dextrose agar slants at 4°C.

**Fungicidal Activity**

The fungicidal activity of the extract was determined following the method described by National Committee for Clinical Laboratory Standards (NCCLS)\(^1\).

**Disk Diffusion Technique**

The test microbes were removed aseptically with an inoculating loop and were transferred to a test tube containing 5 ml sterile distilled water. Sufficient inoculums were added until the turbidity was equivalent to 0.5 McFarland standard (10° colony-forming units ml\(^{-1}\)) (bioMérieux, Marcy-l’Étoile, France). One milliliter of the suspension was added to 20 ml of Sabouraud dextrose agar before setting aside the seeded agar plate (9 cm in diameter) to solidify for 15 min. Nine Whatman’s filter paper no. 1 disks of 6 mm diameter were used to screen the fungicidal activity. Each sterile disk was impregnated with 20 µl of extract (corresponding to 100 mg/mL of crude extract); miconazole nitrate (30 µg/ml, as positive control) and 80% methanol (v/v) (as negative control). The disks were placed on the surface of the seeded plates, incubated at 37°C overnight, and examined for zones of growth inhibition.

**Statistical Analysis**

The data were analyzed by Student \(t\)-test for comparing the *V. cinerea* methanolic extract on the several strains of *C. albicans* and of *C. neoformans* vs control, using SPSS Version 12.0. (SPSS Inc. Chicago, IL, USA). Statistical significance was assumed at the 0.05 levels (\(p<0.05\)).

**Determination of Minimum Inhibitory Concentration (MIC)**

Minimum inhibitory concentration (MIC) of the extract was determined by broth macrodilution assay. A range of concentrations (0.78 to 100.00 mg extract per ml) of plant extract was prepared in yeast extract-peptone-dextrose (YPD) broth medium in flasks. Tween 80 was included at a final concentration of 0.001% (v/v) to enhance extract solubility. 16-h cultures of yeasts were 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 \(5 \times 10^8\) colony forming unit (CFU) ml\(^{-1}\). Subsequently, each flask was inoculated with 20 µl of yeast suspensions. Flasks containing Tween 20 without the plant extract was used as control. The flasks were incubated at 30 ± 2°C, in an orbital shaking incubator (100 rpm) for 48 h. From each flask, 20 µl of cultures were inoculated on YPD agar plates and incubated at 30 ± 2°C for 48 h. The plates were observed and MIC was determined as the lowest concentration of the plant extract which completely inhibits the growth of yeast cells.

**Growth Profile of *C. Albicans* in the Presence of the *V. Cinerea* Extract and Light Microscopy Study**

In order to assess the antifyeast activity with MIC, 1/2 MIC, and 2 MIC concentration over time, growth profile curves were plotted\(^12\). A 16-h culture was harvested by centrifugation, washed twice with phosphate saline, and resuspended in phosphate saline. The suspension was adjusted using the McFarland standard and was then further diluted in phosphate saline to achieve approximately \(10^7\) CFU ml\(^{-1}\). *V. cinerea* extract was added to aliquots of 25 ml Mueller-Hinton broth (MHB) in a 50-ml Erlenmeyer flask (37°C) to achieve a concentration of 0 (control), 1.56 mg/ml (MIC), 0.78 mg/mL (1/2 MIC), and 3.13 mg/ml (2 MIC) after addition of the inocula. One ml inoculums were added to all Erlenmeyer flasks. Finally, 1 ml portion was removed and the growth of *C. albicans* was monitored using this portion by measuring optical density at 540 nm (UV-9100; Ruili Co., Beijing, China).
Antiyeast activity of *Vernonia Cinerea*

**Results**

**Antifungal Activity**

The result of antifungal activity of the extract against *C. albicans* is given in Table 1. The extract inhibited all the yeast tested in this study. The zone of clearance produced by the commercial antibiotic disk was larger than that produced by the extract disk ($p<0.05$). The agar dilution method recorded the MIC value in the range of 1.56 to 6.25 mg/ml for the yeast tested. Further study was concentrated on *C. albicans* strain 1 since it’s showed the lowest MIC value.

**Time-Kill Study**

Time-kill studies were performed over a period of 48 h with yeast being exposed to 1, 1/2 or 2 × MIC of the *V. cinerea* extract. The result of the time-kill curves for *C. albicans* was shown in Figure 1. At 1/2 × MIC, *V. cinerea* extract demonstrated a large drop in OD after 16 h, which leads to the stationary phase of yeast growth compared with the control. At MIC and 2 × MIC, *V. cinerea* extract produced absolute yeast eradication after only 4 h. The time-kill curves described above show the potency of *V. cinerea* extract as an antifungal agent against *C. albicans*.

Furthermore, microscopic observation on the effect of the *V. cinerea* extract on yeast cells was performed by light microscopy study revealed that *V. cinerea* extract produced considerable morphological changes in the test yeast. The cells were destroyed and collapsed as the result of exposure to *V. cinerea* extract. Figure 2 able to visualize the damaged yeast cells such as hole formation and distorted cell membrane compare with control.

**Table 1.** Antiyeast activity (zone of inhibition and MIC) of *Vernonia cinerea* extract compared with commercial antibiotic miconazole nitrate.

<table>
<thead>
<tr>
<th>Yeast</th>
<th>Zone of inhibition [mm]</th>
<th>MIC [mg/ml] of the extract</th>
<th>Methanol extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crude extract</td>
<td>Miconazole nitrate</td>
<td></td>
</tr>
<tr>
<td><em>Candida albicans</em> strain 1</td>
<td>20.00</td>
<td>24.00</td>
<td>1.56</td>
</tr>
<tr>
<td><em>Candida albicans</em> strain 2</td>
<td>19.00</td>
<td>22.00</td>
<td>3.13</td>
</tr>
<tr>
<td><em>Candida albicans</em> strain 3</td>
<td>19.0</td>
<td>23.00</td>
<td>3.13</td>
</tr>
<tr>
<td><em>Cryptococcus</em> neoformans</td>
<td>13.0</td>
<td>21.00</td>
<td>6.25</td>
</tr>
</tbody>
</table>

*Agar dilution method, mean value n = 3. The values (average of triplicate) are diameter of zone of inhibition at 100 mg/ml crude extract and 30 µg/ml miconazole nitrate.
Scanning (SEM) and Transmission (TEM) Electron Microscope Observations

The SEM photomicrographs of the untreated and extract treated cells of *C. albicans* at various time of exposure to the crude extract of *V. cinerea* were shown in Figure 3. Untreated cells (Figure 3a) showed many oval and smooth cells in appearance and some at a budding stage. After 12 hours of exposure (Figure 3b), a mild effect of the extract was observed compared to the untreated control cells. The 24 h treated cells (Figure 3c) shows hole formation and distorted cell membrane due to cell leakage as compared to the normal *C. albicans* cell and followed by the collapsed cells. After 36 hours of exposure (Figure 3d), completely collapsed and cavitated cells were seen. It was believed that at this stage, the cells had lost its metabolic functions completely.

From the SEM findings, it can be suggested that the cells had undergone some distinct morphological and cytological alterations. Further evidence of these changes was obtained from TEM studies. TEM observation also re-affirms some form of disorganization of *C. albicans* and destruction of its organelles. The TEM photomicrograph of the untreated cells of *C. albicans* was shown in Figure 4a. It showed a typically structured nucleus and vacuoles. The cytoplasm contains several element of endomembrane system and enveloped by a typical structure of cell wall. After 12 hours of exposure to the extract (Figure 4b), cells were densed with the vesicles and membranous bodies dispositioned within the cells. After 24 hours of exposure (Figure 4c), the cells exhibited notable alterations in the cell membrane and the cell wall. The cytoplasmic volume decreased, leaving a state of structural disorganization within the cell cytoplasm. The effect of the extract on the yeast cells after 36 h of exposure is shown in Figure 4d. It shows that the yeast cells collapsed and lysed.

Discussion

The inhibitory effect of *V. cinerea* methanolic extract on pathogenic yeasts *C. albicans* has been investigated. In this study, we particularly concentrated on *C. albicans* for the reason that the management of *Candida* infections faces a number of problems including limited number of effective antifungal agents, toxicity of the available antifungal agents, resistance of *Candida* to commonly used antifungals, relapse of *Candida* infections, and the high cost of antifungal agents.
From this study, it appears that the extract exhibits a favorable antiyeast activity against *C. albicans* with the MIC value to be only 1.56 mg/mL. The MIC value defined as the lowest amount of extract necessary to completely inhibit the growth of *C. albicans*. Effectiveness of antiyeast activity inversely correlated with their MIC values. Fabry et al.\textsuperscript{16} reported that the extract having activities where the MIC values are below 8 mg/mL are considered to possess some antimicrobial activity. Time-kill assay was utilized in this study to verify MIC findings and to evaluate the ability of *V. cinerea* extract to eliminate *C. albicans* growth in vitro. In the case of 1 and 2 times MIC concentrations, the extract inhibited the yeast growth within 4 h and subsequent regrowth was not seen. The time-kill assay suggested that *V. cinerea* extract completely inhibited *C. albicans* growth and it also exhibited prolonged antiyeast activity against the *C. albicans* as determined by time-kill curves. Furthermore, the Light Microscope, SEM and TEM studies showed that the extract could completely collapse the yeast cell and inhibit the growth of *C.
Figure 3. SEM micrograph of the untreated and extract treated cells of *Candida albicans*. (×5,000 Magnifications). 
A, Control cells of *Candida albicans*. B, 12 h *C. albicans* cells treated with 1.56 mg/ml of crude extract of *Vernonia cinerea*. C, 24 h *C. albicans* cells treated with 1.56 mg/ml of crude extract of *Vernonia cinerea*. D, 36 h *C. albicans* cells treated with 1.56 mg/ml of crude extract of *Vernonia cinerea*.

Figure 4. TEM micrograph of a cross section of the untreated and extract treated cells of *Candida albicans*. (×20,000 Magnifications). A, Control cells of *Candida albicans*. B, 12 h *C. albicans* cells treated with 1.56 mg/ml of crude extract of *Vernonia cinerea*. C, 24 h *C. albicans* cells treated with 1.56 mg/ml of crude extract of *Vernonia cinerea*. D, 36 h *C. albicans* cells treated with 1.56 mg/ml of crude extract of *Vernonia cinerea*. 
The anti-yeast activity of Vernonia Cinerea albicans. The commercial antibiotics Miconazole nitrate showed significantly ($p<0.05$) larger zone of clearance compared to the extract because not all the compounds presence in the extract reacted as antimicrobial agent.

To the best of our knowledge this is the first report wherein the inhibition of $C.\ albicans$ by $V.\ cinerea$ is shown in detail. However, antimicrobial activity of $V.\ cinerea$ has been reported by Gupta et al$^{17}$ against pathogenic bacteria. They used $V.\ cinerea$ extract extracted with benzene before testing their antimicrobial activity against 9 microbial species, including Bacillus subtilis, Staphylococcus aureus, Staphylococcus epidermidis, Micrococcus luteus, Escherichia coli, Klebsiella pneumoniae, Salmonella typhi, Shigella dysenteriae and Psudomonas aeruginosa. The benzene extract of $V.\ cinerea$ exhibited a good antibacterial activity at 250 µg/ml tested concentrations against all the tested microorganisms. The presence of flavonoids and tannins in the methanol extract of $V.\ cinerea$ was previously reported by Harbone$^{18}$. It is possible that this compound was mainly responsible for the observed antifungal effects in this study.

In summary, the research presented in this article conclusively demonstrates the anti-Candida potential of $V.\ cinerea$ extract. $V.\ cinerea$ extract is proven to be fungistatic, fungicidal and antifungal agent for $C.\ albicans$. Furthermore, it may have potential for clinical treatment of candidiasis although this use will require additional investigation. Further purification of active compound(s) and its individual antiyeast activity study can be suggested on the basis of the present study.

References


