ORIGINAL ARTICLE

THE EFFECT OF NON-POLAR SOLVENT EXTRACTION OF *PIPER RIBESIOIDES* (SIREH HUTAN) ON *CANDIDA ALBICANS* MORPHOLOGY.

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Abstract

Introduction: *Piper ribesioides* is a vigorous climber, found in Indonesia and the Peninsular Malaysia, the root of this piper is used to treat an illness caused from asthma, diarrhoea and abdominal pain. Stems of *P. ribesioides* have long been used to flavour food giving its spicy and peppery taste. *Candida albicans* is a commensal life form in the human cutaneous and mucosal verdure, however can turn into a noteworthy human contagious pathogen given the best possible conditions considering hyphal separation, biofilm arrangement, and excess. This fungus is also responsible in causing sexually transmitted infection. The most basic regions for candidiasis are the oral and gastrointestinal tract, the vaginal and the skin. The aim of this study is to identify the potential activities of non-polar extraction of *P. ribesiodes* on *C. albicans* morphology.
Methods: *P. ribesiodes* was extracted using n-hexane solvent and serial dilution method was used to produce concentration from 500, 250, 125, 62.5, 31.3, 15.63, 7.81, 3.91, 1.96 and 0.98 mg/ml. Sensitivity test of *C. albicans* was measured through the diameter of inhibition zone when treated with *P. ribesiodes*. Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentrations (MFC) were determined by using broth micro-diluted method. Morphology study was done by using Scanning Electron Microscope (SEM).
Results: The largest zone of inhibition in 24 hours was at 500 mg/ml concentration. The diameter was nearly 10.333 ± 2.309 mm. The Minimum Inhibitory Concentration and Minimum Fungal Concentration were 15.63 mg/ml and 31.25 mg/ml respectively. Morphology study revealed erosion and damage of cell wall of *C. albicans*.
Conclusion: Morphology of *C. albicans* were altered when treated with n-hexane extraction of *P. ribesiodes*.
Keywords: *Piper ribesiodes*, *Candida albicans*, Antifungal agent, Candidiasis, Natural product

Introduction

Candida species belong to the normal microbiota of an individual's mucosal oral cavity, gastrointestinal tract and vagina [1] which is responsible for various clinical manifestations from mucocutaneous overgrowth to blood stream infections [2]. *Candida albicans* is a normal commensal organism in the human cutaneous and mucosa, however can turn into a noteworthy human contagious pathogen given the best possible conditions considering hyphal separation, biofilm arrangement and excess. The most basic regions for candidiasis are the oral and gastrointestinal tract, the vagina and the skin [3]. In patients encountering vulvo vaginal candidiasis, symptoms and signs are vaginal itching, burning sensation, and excoriations and redness. This condition influences up to 75% of ladies in any event once in their lifetime [4]. The genus Piper is one of the largest and the most important aromatic and medicinal plants of the *Piperaceae* family which comprises four genera and approximately 2000 species distributed in the tropical and subtropical regions [5]. Piper species are used in traditional remedies and folklore medicine all over the world for their antioxidant [6], antimicrobial [7], anti-inflammatory [8] and antifungal activities [9]. *Piper ribesioides* locally known as 'lada rimba', 'akar kalong ular', or 'sireh murai', is a vigorous climber, found in Indonesia and the Peninsular Malaysia. As a traditional medicine the root is used to treat an illness caused from asthma, diarrhea, and abdominal pain. Stems of *P. ribesioides* have long been used to flavour food giving its spicy and peppery taste. The leaves are used to treat abnormalities in body wind element (this is a term used by indigenous) alleviate chest congestion and excrete phlegm while the flowers have been used to treat urticaria [10]. This study was conducted to identify the potential activities of *P. ribesiodes* against *C. albicans*. In this study the *C. albicans* were altered in the morphology when treated with n-hexane extraction of *P. ribesiodes* and the morphological changes was seen under scanning electron microscopic (SEM).

Materials and Methods

Plant collection

The *P. ribesioides* leaves were collected from Semenyih, Selangor. Identification of the leaves was confirmed by a certified botanist (UKMB40429). The leaves of *P. ribesioides* were separated from the rest of the plants. The plant materials were then separated in trays and being air dried for 12 hours using an oven at 50˚C in order to increase the surface area of the plants during solvent extraction, the leaves were grounded into fine powder. The ground plant materials were placed in zip lock bag and stored at room temperature.

Figure 1*: P. ribesioides* (Sireh Hutan)

Plant extraction

100 mg of *P. ribesioides* was soaked in 600 ml of n-hexane solvents for 24 hours at ambient temperature. The next day, the mixtures were then filtered using filtered paper into conical flasks leaving a residue on the filter paper. Then, to obtain the extraction, a Rotary Evaporater (Rotavaps) was used. Rotavaps removed the solvents from the reaction mixtures by evaporation process. The filtrate extract need to be concentrated in Rotavaps at 55.4˚C. The resulting filtrates were placed in oven at 50˚C for further evaporation and stored at -18˚C until further analysis.

Concentration preparation

The extract was serially diluted to produce test concentration. Two-fold serial dilution method was used to produce various concentration. The final test concentration decided are 500 mg/ml, 250 mg/ml, 125 mg/ml, 62.5 mg/ml, 31.25 mg/ml, 15.63 mg/ml, 7.82 mg/ml, 3.91 mg/ml, 1.96 mg/ml and 0.98 mg/ml. 1g of pure *P. ribesioides* extract was dissolved in 1ml dimethyl sulfoxide (DMSO) and used as stock solution.

Sensitivity Test

The standard culture of *C. albicans* (ATCC 10231) was purchased from the supplier. The sensitivity test of *C. albicans* was determined through agar disc diffusion assay. *C. albicans* were cultured on Sabouraud dextrose agar (SDA). Sterile swab stick was used to dip into the broth solution containing *C. albicans* and cultured transfer to SDA plate using streaking technique. The discs were slowly infused with various defined concentration of n-hexane extract of *P. ribesioides* and placed onto the inoculated surface of agar. Dimethyl sulfoxide (DMSO) and Fluconazole was used as negative and positive control. After incubation for 24 hours at 37˚C, zone of inhibition was determined by measuring the diameter of inhibited zone in mm. All tests were repeated three times to reduce the test error.

Determination of Minimum Inhibitory Concentration (MIC)

MIC was determined by broth micro dilution through two-fold serial dilution. Test was performed using 96-well microdilution plates [9]. 100 µL of *P. ribesioides* extract of each concentration from 500, 250, 125, 62.5, 31.3, 15.63, 7.81, 3.91, 1.96 and 0.98 mg/ml) were pipetted into each well and mix well. All tests are performed in triplets. The 96- well microdilution plates were incubated for 24 hours at 37˚C. Next, a drop of Methylcyclopentadienyl manganese tricarbonyl (MMT) was added to observe the colour change. The 96-well microdilution plate was again incubated for 2 hours at 37˚C. This reagent was used to differentiate between live and dead microorganism. The live microorganism can convert the dye into purple colour. The MIC value was recorded by observing the lowest concentration at which there was no colour change at all.

Determination of Minimum Fungicidal Concentrations (MFC)

MFCs were determined by culturing 10uL of the culture from wells showing no visible growth and culture them on to Sabouraud dextrose agar (SDA) plates. The plates were then incubated for 24 hours and MFCs were determined as the lowest concentration of extract showing no visible growth.

Morphological study using Scanning Electron Microscope (SEM)

The sample for SEM was treated with 15.63mg/ml (MIC value) *P. ribesioides* extract. Then, it was fixed with 4% (v/v) glutaraldehyde at 4˚C for more than 4 hours. The fixed yeast cells were washed twice with 0.1 M sodium sacodylate buffer (10 minute for each step) and post fixed with 1% (v/v) osmium tetroxide at 4˚C for 1 hour. Then, the sample was washed with double distilled water for two times with 10 minutes for each step. After the washing, the sample were dehydrated in series of ethanol (30%, 50%, 70%, 80%, 90%, 95%) each had done for 15 minutes. Next, the samples were dehydrated with ethanol and acetone with ratio of 3:1, 1:1 and 1:3 for 15 minutes each. After that, the samples were dehydrated three times in pure acetone for 20 minutes. Dehydrated samples were mounted on stubs with double stick tape and coated with gold in sputter coater. Lastly the samples were observed using scanning electron microscopic.

Results

There are the results of preliminary study to identify the potential activities of *P. ribesioides* against *C. albicans* tested with n-hexane extracts of dried *P. ribesioides.* Table 1 shows the result of Agar disc diffusion sensitivity test of n-hexane

extraction of *P. ribesioides* on *C. albicans*. The result shows that largest zone of inhibition was at 500 mg/ml concentration and the diameter was nearly 10.333 ± 2.309 mm. The minimum zone of inhibition was at 62.5, 31.25, 3.91, 1.96 mg/ml concentration and the diameter was nearly 7.000 ± 0.000mm. In the table 2 it shows result of MIC and MFC n-hexane extraction of *P. ribesioides* on *C. albicans*. Whereas the morphological alteration of *C. albicans* treated with fluconazole, n-hexane extraction of *P. ribesioides* and DMSO is presented in figure 1,2 and 3.

Discussion

Results of this study revealed that n-hexane extraction of *P. ribesioides* was able to induce change to the morphology against *C. albicans*. The antifungal susceptibility tests which were being applied here were the sensitivity test, broth microdilution and for the morphological changes viewed under Scanning Electron Microscopic (SEM)[18] has proven the n-hexane extraction of *P. ribesioides* was able to alter the morphology.

In sensitivity test, the largest zone of inhibition in 24 hours was at 500 mg/ml concentration. The diameter was nearly 10.333 ± 2.309 mm. The lowest zone of inhibition was at 62.5, 31.25, 3.91, 1.96 mg/ml concentration. The diameter was nearly 7.000 ± 0.000mm. Moreover, fluconazole was used as positive control and the diameter of zone of inhibition was 19 mm. DMSO was used as negative control and the diameter of zone of inhibition was 7mm.

Determination of MIC was done by using broth dilution method. The minimum inhibitory concentration was 15.63 mg/ml concentration. MIC reading was taken as the lowest concentration which inhibit the *C. albicans* where there was no colour change at all. Minimum Fungal Concentration was 31.25 mg/ml concentration where the reading was taken when there is no visible growth.

The morphology change was identified by using Scanning Electron Microscope. In positive control with fluconazole, the cell wall was swollen, the surface was rough and shape was totally altered. At the end of both right and left (vertically) cell wall, there was enlargement of the cell wall where this might lead to rupture. At the bottom of the surface of *C. albicans*, there was erosion which indicated that *C. albicans* was killed by fluconazole (refer to figure 2). In negative control which was DMSO the cell wall was not ruptured or swollen. The surface was smooth and solid. There were no major changes in the morphology (refer to figure 3). However, in n-hexane extraction of *P. ribesioides* the cell wall was swollen and there is enlargement of both right and left side (vertically). At the right side of cell wall there was a rough surface and a deep hole which was due to erosion (refer to figure 4). Significantly, n-hexane extraction of *P. ribesioides* can be treated to killed *C. albicans*.

Conclusion

In conclusion, this study was conducted to identify the potential activities of *P. ribesiodes* against *C. albicans*. In this study the *C. albicans* were altered in the morphology when treated with n-hexane extraction of *P. ribesiodes*. This promising result indicated that *P. ribesiodes* has the potential to be developed into an antifungal agent. Further studies are recommended to allow different extraction method of *P. ribesiodes* and test them against other types of fungi to expand the antifungal activities of this promising plant.

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Table 1: The result of Agar Disc Diffusion Sensitivity Test of n-hexane extraction of *P. ribesioides* on *C. albicans*.

Concentration (mg/ml)		**Mean ± Standard Deviation**
n-hexane	500	10.333 ± 2.309
	250	7.333 ± 0.577
	125	7.667 ± 1.155
	62.5	7.000 ± 0.000
	31.25	7.000 ± 0.000
	15.63	7.333 ± 0.577
	7.82	8.000 ± 0.000
	3.91	7.000 ± 0.000
	1.96	7.000 ± 0.000
	0.98	8.000 ± 0.000

MIC AND MFC

Table 2: The result of MIC and MFC n-hexane extraction of *Piper ribesioides* on *Candida albicans*

Antifungal agent	**MIC (mg/ml)**	**MFC (mg/ml)**
P. ribesioides extract in n-hexane	**15.63**	**31.25**

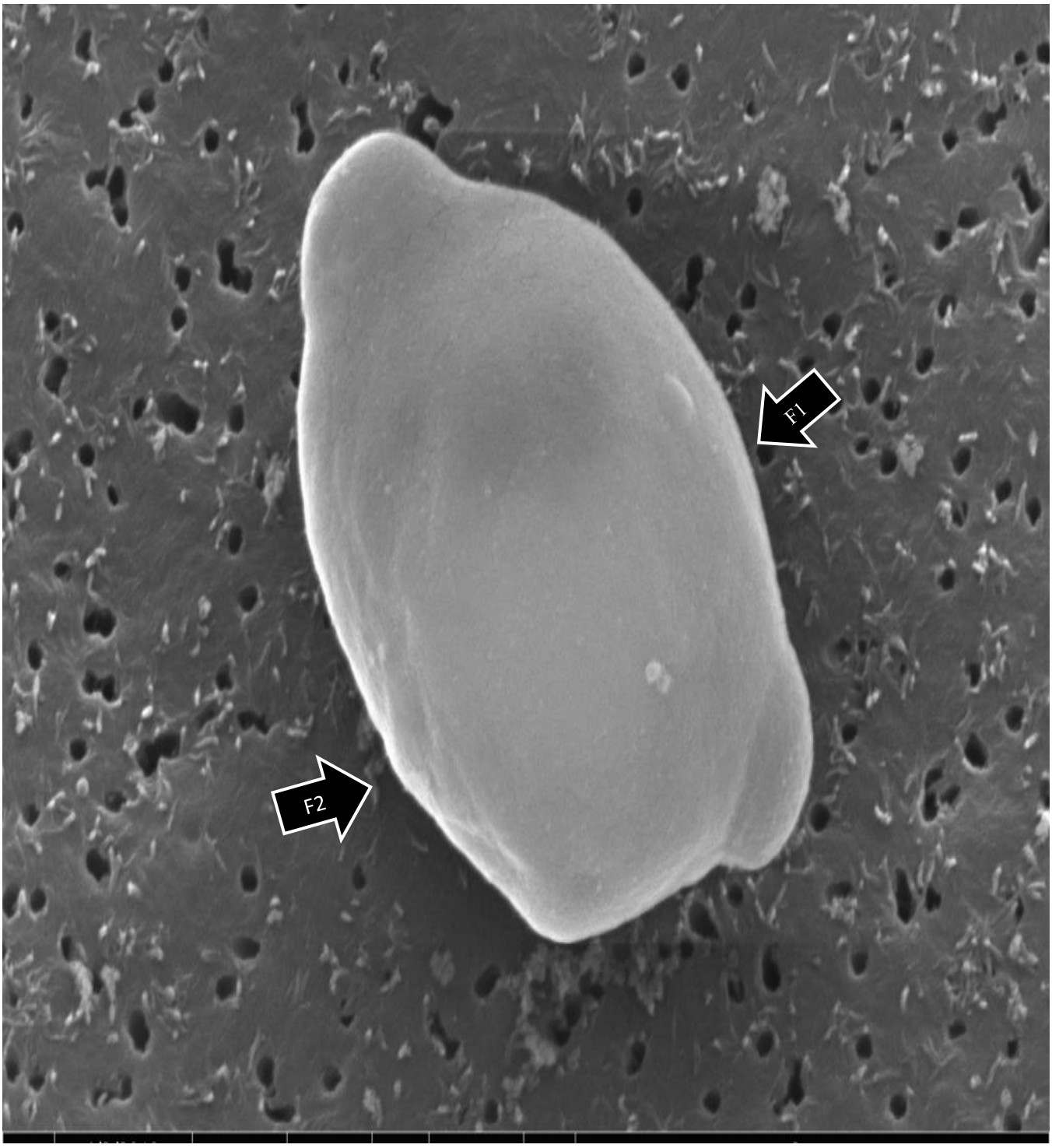

Figure 2: SEM image under 50x magnification of *C. albicans* treated with fluconazole (positive control). (F1- swollen of cell wall, F2- erosion of cell wall)

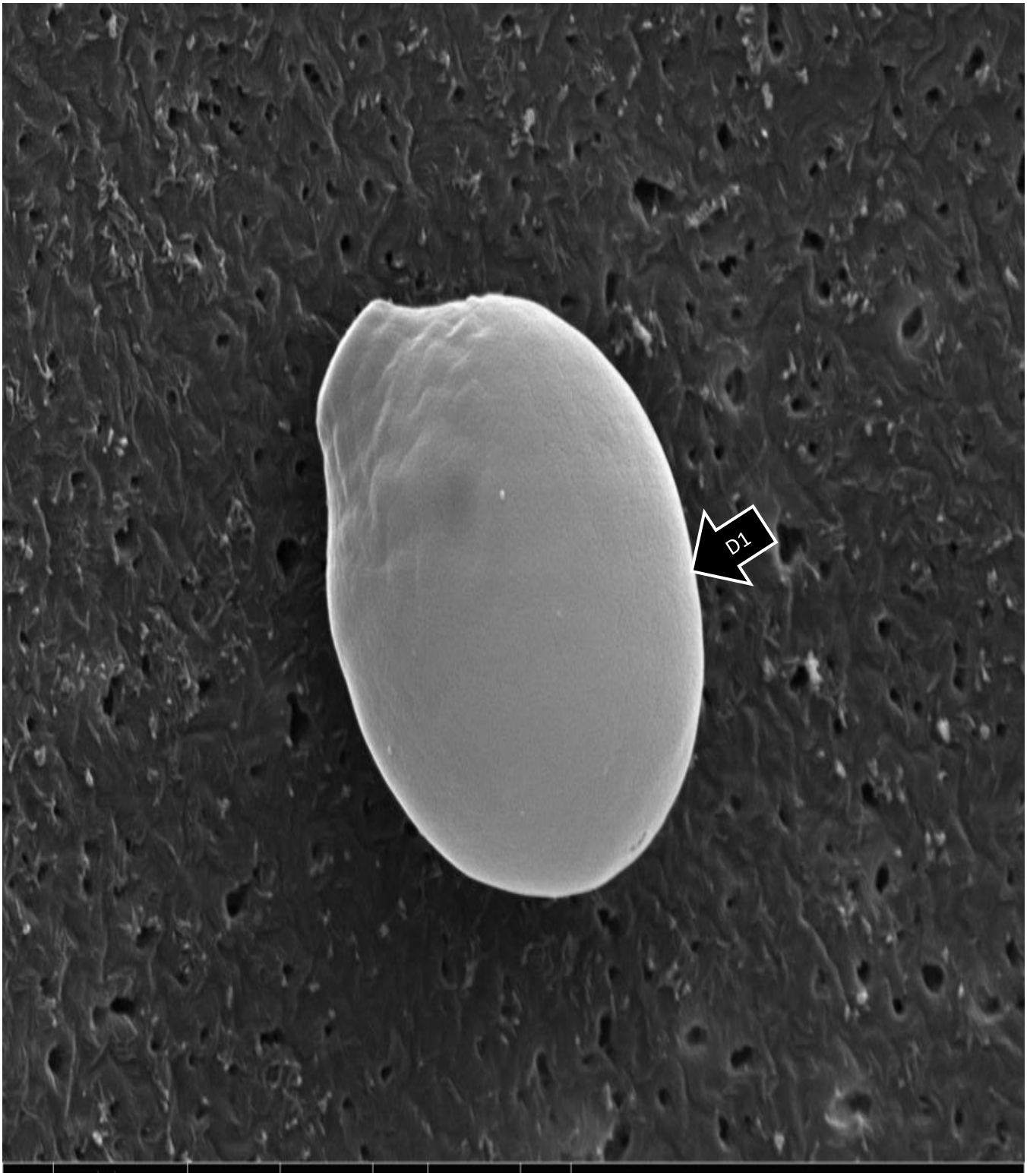

Figure 3: SEM image under 40x magnification of *C. albicans* treated with DMSO (negative control). (D1- smooth and solid cell wall)

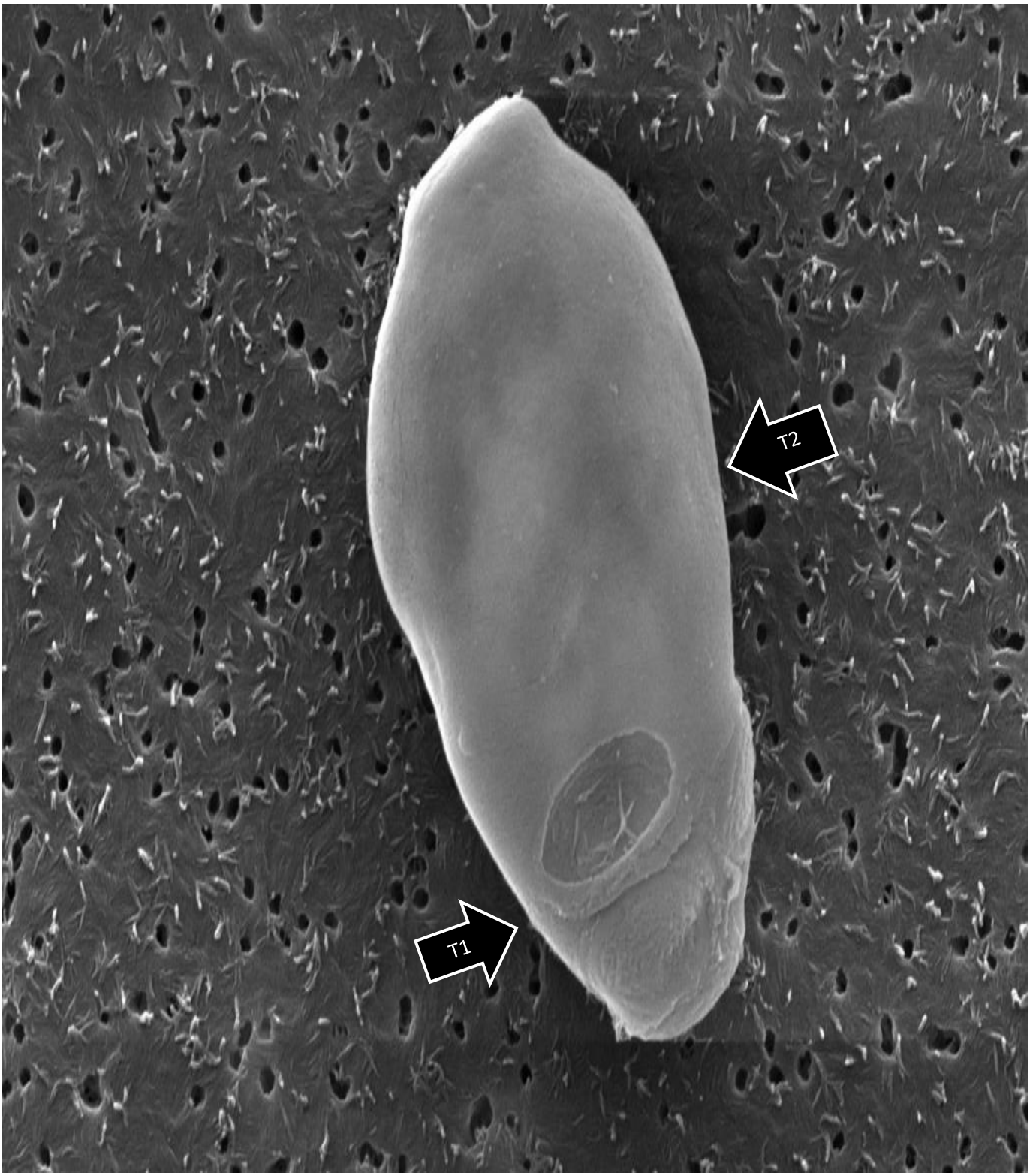

Figure 4: SEM image under 40x magnification of *C. albicans* treated with n-hexane extraction of *Piper ribesioides*. (T1-deep erosion of cell wall, T2- swollen of cell wall)

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