Malaysian Journal of Pharmaceutical Sciences,
Vol. 2, No. 2, 9 — 17 (2004)

PENERBIT USM

MORPHOLOGICAL AND CYTOLOGICAL CHANGES EXHIBITED BY *CANDIDA ALBICANS* CELLS IN RESPONSE TO EXPOSURE TO *GRACILARIA MANILAENSIS* EXTRACT

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Crude extract of Gracilaria manilaensis, a local marine alga which can be found abundantly in Malaysian waters, is a potential antifungal; especially antiyeast agent against C. albicans, a pathogenic yeast that can sometime cause serious human infections. Exposure of the C. albicans cells to 12.5 mg/mL (the MIC value of the extract against C. albicans cells) of the extract resulted in complete suppression and inhibition of the C. albicans growth. Prolonged exposure to the extract (12, 24 and 36 hours) resulted in alteration and deterioration of the C. albicans cells beyond repair. The main abnormalities observed were the altered cells morphology and cytology.

Keywords: *Gracilaria manilaensis, Candida albicans, Antifungal agent, Minimum inhibitory concentration*

INTRODUCTION

In recent years, drug resistance to human pathogenic bacteria has been widely reported all over the world. However, the situation is alarming in developing as well as developed countries due to indiscriminate use of antibiotics. The drug-resistant bacterial and fungal pathogens have further complicated treatment of infectious diseases especially in the immunocompromised, AIDS and cancer patients. In the present scenario of emergence of multiple-drug resistance in human pathogenic microorganisms, this has necessitated a search for new antimicrobial substances from other sources including marine algae (Mayer and Hamann 2002).

The antimicrobial potential of marine algae has been known through the ages. In fact, several marine algae species have been extensively used as therapeutic agents against infections. For example, the red algae *Digenia simplex* and *Chondria* sp. have been exploited in Asia for their content of effective antihelmintic drugs. There are, of course, many active compounds or components that can be extracted from marine algae, and then be exploited for biomedical and pharmaceutical purposes in combating

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pathogenic microorganisms (Caballero and Melian 1990; Faulkner 1993). Marine algae have evolved unique and highly specialized biochemical pathways to adapt to their unique marine environment and pressures. Besides having successfully adapted to the environment that is rich in halogens, sulphates and other concentrated compounds, marine algae have also evolved the ability to produce toxins and deterrents to enhance their survival in the presence of abundant predators (Scheuer 1990). In Malaysia, *Gracilaria manilaensis* from the division of Rhodophyta has been reported to possess some antibacterial activities (Sasidharan *et al.* 2003; Darah and Lim 2004), and was believed to have pharmaceutical and medical values. In order to confirm this, we carried out a preliminary investigation on this alga which can be found abundantly in Malaysian waters. Our preliminary findings showed that the crude extract of *G. manilaensis* exhibited significant activities against various species of pathogenic bacteria, yeasts and fungi which cause infections in human (Darah and Lim 2004). We also found that the antifungal properties in *G. manilaensis* crude extract have a broad spectrum of antiyeast activity.

In this communication, we described the effects of the *G. manilaensis* crude extract on *Candida albicans* cells in order to confirm the previous observations. With the aid of light, scanning and transmission electron microscopy, we described and discussed the cytological alterations that occured in the yeast cells after exposure to the extract.

METHODS

Preparation of crude extract from *Gracilaria manilaensis*

G. manilaensis was collected from Pantai Merdeka, Kedah. The sample was thoroughly washed with sea water to remove epiphytic growths. The sample was then soaked and washed several times in fresh water to reduce the salt content. Finally, the sample was washed in distilled water, rinsed and allowed to dry in the shade for 3—4 days. The dried sample was then kept at dry and cool place until used.

One hundred gram of coarsely powdered dry sample was soaked in 200 mL solution of chloroform-methanol at a ratio of 1:1 for 24 hours. The mixture was then filtered with double-layered cheesecloth before using Whatman No.1 filter paper. The extract was then evaporated to dryness in a rotary evaporator under reduced pressure at a temperature of 40°C.

Test microorganism

A clinical isolate of *Candida albicans,* obtained from the School of Medical Sciences, Health Campus, Universiti Sains Malaysia, was used throughout the study. The yeast culture was grown and maintained on Sabouraud glucose agar slant at 30°C.

Minimal inhibitory concentration (MIC)

The MIC of the extract against *C. albicans* was determined by liquid dilution method. A series of dilution was prepared to obtain concentrations of 3.13—100.00 mg/mL of the extract in Sabouraud glucose broth (Darah and Halim 1995; Darah and Jain 2001). To each tube, containing 10 ml of the test medium, 0.1 mL of *C. albicans* suspension containing $1x10^9$ cells per mL was added and incubated at 30°C for 24 hours. The lowest concentration which did not show any growth of the tested microorganism after macroscopic evaluation was determined as the MIC.

Morphological and cytological study of the *C. albicans* cells after exposure to the extract

One loopfull of *C. albicans* cells was added into 5 ml Sabouraud glucose broth which contained the extract at a final concentration of 12.50 mg/mL. The mixture was incubated at 30°C. Sterile distilled water-treated culture was included as a control. The culture was not replenished with fresh medium during the whole assay period. Samples (0.1 ml) of the mixture were taken at various time intervals (0, 12, 24 and 36 hours); fixed and examined by light, scanning and transmission electron microscopies. The experiment was done in triplicate.

RESULTS AND DISCUSSION

The MIC value of the extract against *C. albicans* cells was found to be about 12.50 mg/mL. Therefore, this value was used to study the effects of the extract on the yeast cells. Figure 1 shows the light microscopy micrographs of the control (sterile water treated cells) and extract treated cells of *C. albicans*. Figure 1A shows control cells, that are oval in appearance and distinct cells with buddings. After 12 hours of exposure (Figure 1B), the *C.*

albicans cells seems to be closer to each other. The cell walls became thicker and the cells was not oval in appearance anymore, but more seems to be rounded cells. After 24 hours of exposure (Figure 1C), the cells became attached to each other with thicker cell wall formation and less budding. A closer examination revealed that the inner part of the cells became coarse and irregular shape of the cells were seen. After 36 hours of exposure (Figure 1D), the cells became rounder with even thicker cell walls formation. However, some of the cells seems to be broken, and some showed shrunken phenomenon.

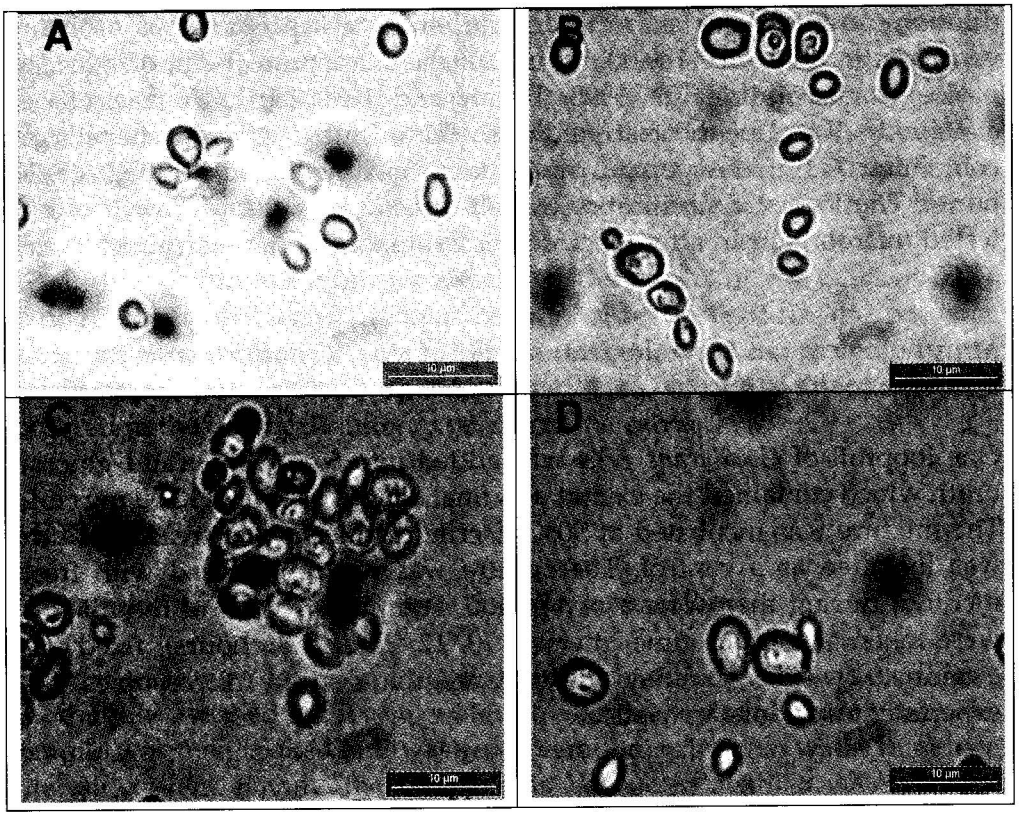

Fig. 1: Light microscopy micrographs of control and extract treated cells of *C. albicans* (12.5 mg/mL). (A) Control cells, (B) 12 hours of exposure, (C) 24 hours of exposure and (D) 36 hours of exposure to the extract.

A clearer view of the effects can be seen through scanning electron micrsocopy (SEM) micrographs (Figure 2). Control cells (Figure 2A) shows distinct smooth surface oval shaped cells with buddings. After 12 hours of

exposure (Figure 2B), the growth of the cells seems to be inhibited with retarded budding. The surface of the cells became slightly irregular and rough. The shape of the cells became rounder and the sizes were not uniform. After 24 hours of exposure to the extract (Figure 2C), the cells showed severe altered stages. There were no uniformity in the sizes and no distinct cells showed. The cells were altered and the growth inhibited. The cells became irregular and collapsed, and sticked to each other. Some of the cells were not in oval or round shape, but seems to loose the whole shape. Figure 2D shows the effect on *C. albicans* cells after 36 hours of exposure. The growth of the cells were altered and some cells showed the formation of cavitation or perhaps collapsed cells. In fact, some completely shrunken cells can be seen. Furthermore, no new budding cells were seen. This figure shows that the *C. albicans* cells were altered beyond repair. The shrunken cells could be due to the leakage of the cell walls and cell membranes.

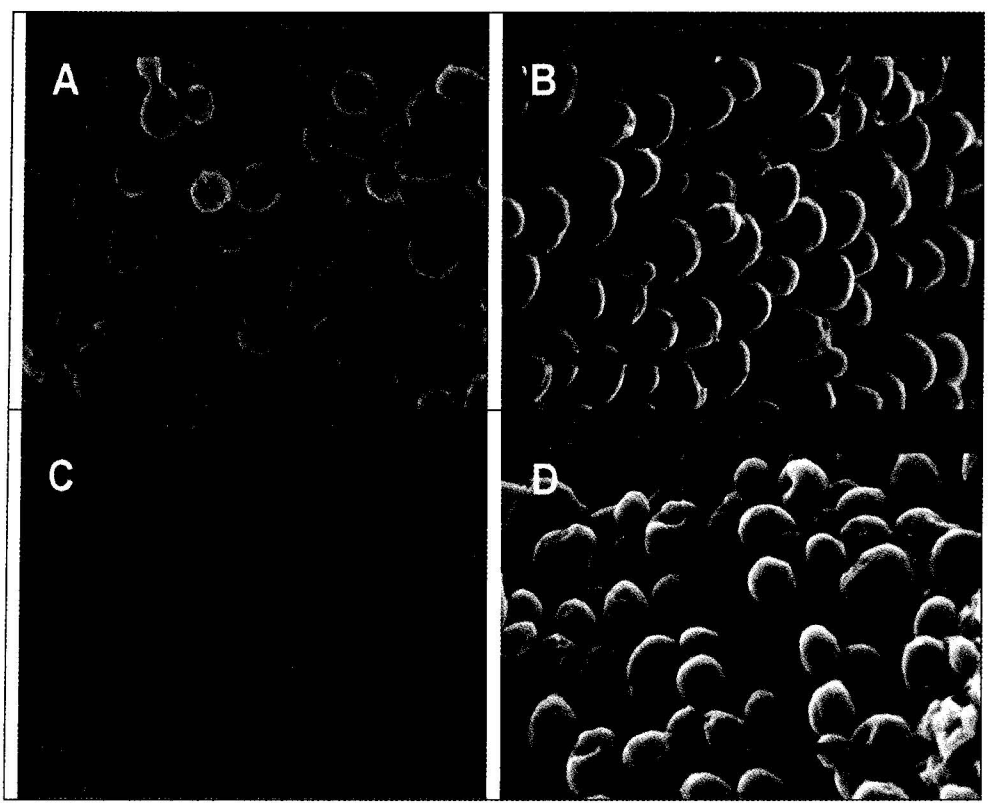

Fig. 2: SEM micrographs of *C. albicans* cells after exposure to *G. manilaensis* crude extract (12.5 mg/mL) at different time intervals. (A) Control cells, (B) 12 hours of exposure, (C) 24 hours of exposure and (D) 36 hours of exposure to the extract.

Further evidences of these phenomena can be seen in the transmission electron microscopy (TEM) micrographs (Figure 3). Figure 3A shows a TEM micrograph of a control cells. It shows a typical *C. Albicans* cells with no budding formation yet. The cell wall, cell membrane and the cell protoplasm were uniform. After 12 hours of exposure to the extract (Figure 3B), some alteration occured to the cell protoplasm. There was an empty space occured and the cytoplasm became densed. The abnormality occured in the cell protoplasm and it was clearly seen when compared to the control cells. After 24 hours of exposure (Figure 3C), the cell membrane altered slightly and the cytoplasm became densed and more empty spaces occured. Finally, after 36 hours of exposure (Figure 3D), the whole *C. albicans* cells was altered beyond repair. The cell membrane and cell wall were affected with uneven thickness of the cell wall. The cell membrane seemed to leak and the cell protoplasm became abnormal. The shape of the cells became irregular and severed. At this stage, the yeast cell was believed to be in a very severe and worst condition, which eventually led to cell lysis and cell death.

The sequence of morphological alterations in *C. albicans* cells after exposure to the extract of *G. manilaensis* can be observed in three different forms. The first form was when the mother cell became very dense with its vesicles or perhaps organelles and membraneous bodies dispositioned within the cell; which in TEM micrographs, it can be observed as dense cytoplasmic aggregation (Figure 3B). Another form of alteration is shown in Figure 3C. The mother cell was joined to the cell wall of the growing bud, however, the cytoplasmic materials failed to be transfered to the daughter cells. Therefore, the small cell protrusion was actually an extension of the profused cell wall of the daughter cell. When the cells were exposed to the extract for a longer period of time, both mother and daughter cells undergo structural degeneration. This final form can be clearly shown in the deformation of the shape and formation of large vacuoles within the cytoplasm. Cytoplasmic shrinkage, irregular cell membrane, invaginations and thick cell wall were obvious. Ultimately, these cells lysed and disintegrated.

It is difficult to explain the nature and the significance of the numerous vesicles and membraneous bodies which represent a typical cytological change in spatially related to cell membrane. The formation of vesicles, as well as the involvement of the cell membrane and cell wall, may be the consequences of the interaction of the extract with the cell metabolism on the assumption that treated cells tried to eliminate unphysiologically

synthesized materials by exocytosis. These kind of changes were also seen in dermatophytes (Darah and Jain 2001), but they differ in that their vesicles penetrated the cell wall of dermatophytes but not in the case of *C. albicans*. This difference could be due to the difference in the cell wall structure of both species.

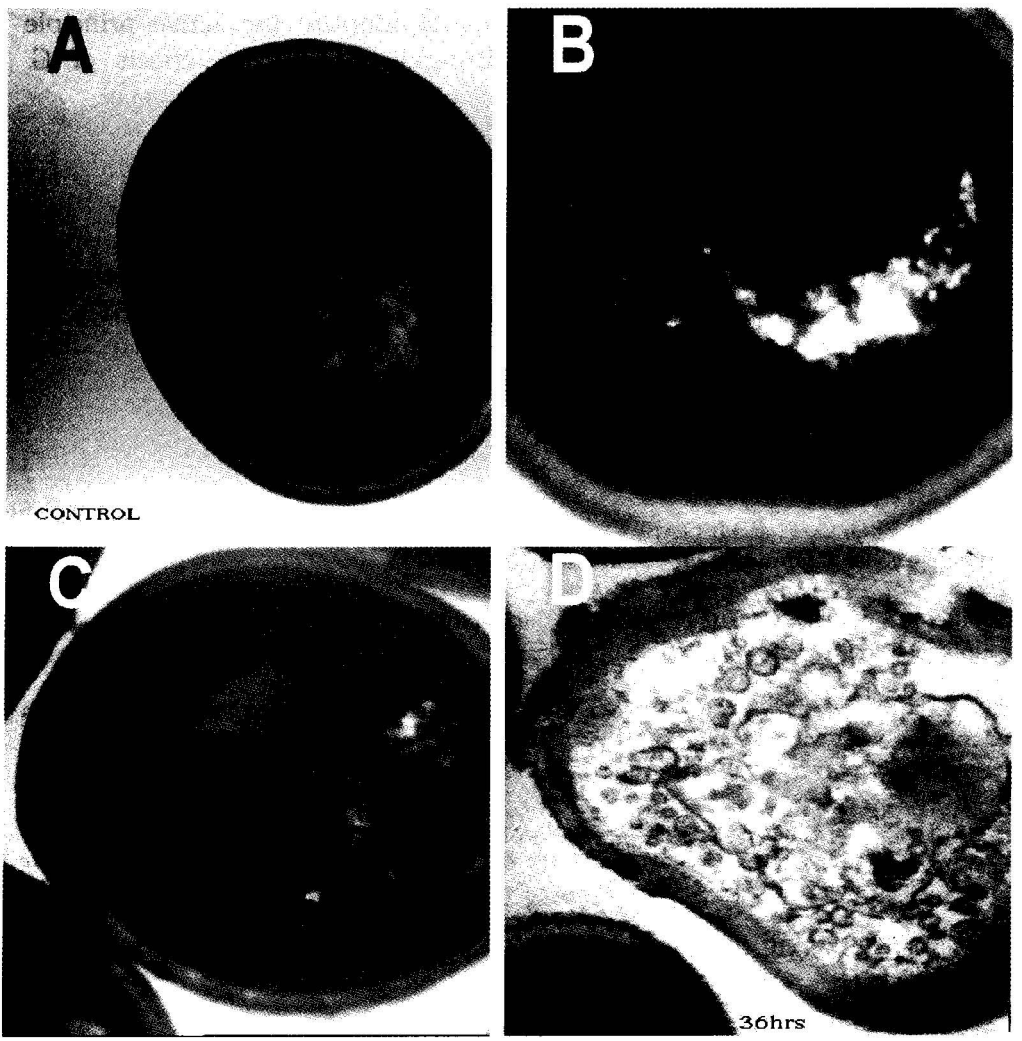

Fig. 3: TEM micrographs of *C. albicans* cells after exposure to *G. manilaencis* crude extract (12.5 mg/mL) at different time intervals. (A) Control cells, (B) 12 hours of exposure, (C) 24 hours of exposure and (D) 36 hours of exposure to the extract.

CONCLUSION

The results of the present work confirmed that the marine algae, *Gracilaria manilaensis* possesses antifungal or antiyeast properties. It further underlines the importance of the ethnobotanical approach for the selection of marine algae in the discovery of new bioactive compounds. Further phytochemical research is still ongoing to identify the active principle compounds responsible for the antifungal or antiyeast effects of *G. manilaensis*.

ACKNOWLEDGEMENTS

The authors would like to thank the National Biotechnology Directorate, Minister of Science, Technology and Innovation of Malaysia (MOSTI) for the grant provided to support this investigation .

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