EFFECT OF ETHANOLIC *PSIDIUM GUAJAVA* L. LEAVES EXTRACT ON THE CELL MORPHOLOGY AND RELEASE OF CELL CONSTITUENTS OF MICROORGANISMS

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Abstract. Guava (*Psidium guajava* L.) is a traditional medicinal ingredient widely used in Asia and Africa to treat a variety of diseases, most notably diarrhea. Although several studies have highlighted the antimicrobial properties of guava leaves extracts, research on their effectiveness against foodborne pathogens and spoilage fungi remains limited. This study is to examine the effects of ethanolic *Psidium guajava* L. leaves extracts on the morphology of foodborne pathogens and spoilage fungi. The antimicrobial mode of action was analyzed using crystal violet dye uptake, cell constituent release assay, and scanning electron microscopy (SEM). The highest uptake of crystal violet for *Klebsiella pneumoniae* ATCC13773, *Listeria monocytogenes* ATCC19112, *Candida krusei* ATCC32196, and *Rhizopus oligosporus* ATCC22959 at 4× MIC were 77.00%, 70.25%, 78.00%, and 80.00%, respectively. After 4× MIC treatment, the absorbance of cell constituents released by *K. pneumoniae, L. monocytogenes, C. krusei*, and *R. oligosporus* was 0.937, 0.880, 0.979, and 0.975, respectively. The scanning electron microscopy (SEM) analysis showed that morphological features in treated microorganisms were changed in cell wall shape. The cells were rupturing, and the cytoplasm was leaking out. Meanwhile, untreated cells assume normal oval, rod, or spherical shapes with a smooth surface. The results revealed that ethanolic *P. guajava* L. leaves extracts can be used against *K. pneumoniae* ATCC13773, *L. monocytogenes* ATCC19112, *C. krusei* ATCC32196, and *R. oligosporus* ATCC22959, which are associated with foodborne pathogens and spoilage fungi. The ethanolic *P. guajava* L. leaves extracts showing its potential for further study, especially in food application as natural food sanitizers.

Keywords: Cell constituents release, crystal violet dye, microorganisms, *Psidium guajava* L., SEM

Article Info

Received 7th November 2023
Accepted 29th May 2024
Published 12th June 2024

ISSN: 1823-7010, eISSN: 2600-7444

1. INTRODUCTION

Psidium guajava L. or guava, and also known as *Jambu Batu* in Malaysia, is a medicinal plant. In Asia and Africa*, P. guajava* is used to prevent and treat scurvy [1], and as a remedy for diarrhea, abdominal pain, cough, dysentery, fever, flu, toothache, and headaches [2]. Guava have been documented in Malaysia to be used for the treatment of dermatitis, diarrhea, and stomachache (menstrual disorder) [1-2]. The guava leaves extract has antibacterial and antifungal activity. Some research reports that guava leaves extract with different solvents and concentrations can be effective against several bacteria and fungi [3], but the data for ethanolic guava leaves extract against foodborne pathogens and spoilage fungi remains limited.

K. pneumoniae and *L. monocytogenes* could contaminate foods and cause food poisoning and illness [4]. Meanwhile, *C. krusei* and *R. oligosporus* are spoilage fungi that can make foods unpleasant to eat due to changes in feel, texture, taste, or appearance. They have also been recognized as a possible cause of health problems for people and animals. This is because they produce mycotoxins, which can lead to mycotoxicosis in humans [5].

Food decontamination has been used directly or indirectly during production or processing that helps to increase the self-life of the food. Efficacious decontamination is important for the prevention of the spread of pathogens through the food chain. Customers are currently dissatisfied with decontamination methods such as chemical sanitizers. Many research findings have negative impacts on both people and the environment as a whole. In addition, some decontamination techniques alter the organoleptic and nutritional properties of foods, causing unwanted alterations. It has been reported that chemicals used as decontaminants, such as chlorine sanitizer, have carcinogenic effects [6]. Therefore, preservatives that are derived from plant-based substances, rather than synthetic ones, are gaining popularity. Thereby, this study aimed to determine the effect of ethanolic guava leaves extract on the morphology of *K. pneumoniae* ATCC13773, *L. monocytogenes* ATCC19112, *C. krusei* ATCC32196, and *R. oligosporus* ATCC22959.

2. MATERIALS AND METHODS

2.1 Preparation of Ethanolic P. guajava Leaves Extract

The samples were obtained from Taman Pertanian, Universiti Putra Malaysia. The maceration and extraction technique were referred to Rukayadi et al. [7]. Dried leaves (100 g) were grinder before soaking in 99.8% ethanol at room temperature for one day with occasional shaking. Furthermore, the filtrate was concentrated for 4 hours using a rotary vacuum evaporator at 40 °C and 99 rpm. The crude extract was subsequently diluted with a dimethyl sulfoxide (DMSO) solution resulting in 1% ethanolic *P. guajava* leaves extract.

2.2 Bacteria and Fungi Strains, Growth Conditions and Inoculum Preparation

The bacterial strains and fungal species employed included *K. pneumoniae* ATCC13773, *L. monocytogenes* ATCC19112, *C. krusei* ATCC32196, and *R. oligosporus* ATCC22959. To create the bacterial and *C. krusei* suspensions, one colony was picked and subsequently transferred to 1 mL of Mueller Hinton Broth (MHB) and Sabouraud Dextrose Agar (SDA), respectively. After a 15-second vortexing step, these suspensions were

incubated at 37 °C overnight for the bacteria and 35 °C for 48 hours for *C. krusei*. The bacterial species and *C. krusei* were then adjusted to meet the 0.5 McFarland standard, with a concentration ranging from 10^6 to 10^8 CFU/mL [8-9]. In the case *of R. oligosporus*, it was cultivated on Potato Dextrose Agar (PDA) at 35 °C for a duration of 7 days. Furthermore, to harvest the fungi, 1-2 mL of 0.85% sterile saline solution was applied to cover the grown colonies, and gentle swabbing with the tip of a pasteur pipette was performed. The resulting suspension contained conidia and hyphal fragments, which were collected and transferred to a sterile tube, allowing the heavier fragments to settle for 5 to 10 minutes. The homogenous mixtures at the top of the tube were collected and vortexed for 15 seconds. Subsequently, an optical density (OD) reading of the conidial suspensions was taken using a spectrophotometer at 530 nm wavelength. The transmittance of *R. oligosporus* fell within the range of 68% to 70% [10].

2.3 Crystal Violet Assay

The bacterial, *C. krusei*, and *R. oligosporus* culture were subjected to centrifugation at 3000 x g for 20 minutes. Subsequently, the pallets formed at the bottom of the centrifuge tubes were subjected to a triple washing process and then resuspended in 0.1% phosphate-buffered saline (PBS, pH 7.0) [11]. Following this, the inoculums were placed in an incubator shaker (200 rpm) at 37 °C for bacteria and 35 °C for fungi, all in the presence of an extract at varying concentrations (0× MIC, 0.5× MIC, 1× MIC, 2× MIC, and 4× MIC). Bacteria and *C. krusei* were incubated for periods of 0.5, 1, 2, and 4 hours, while *R. oligosporus* was incubated for 24, 48, 72, and 96 hours. Afterwards, the samples underwent centrifugation at 9300 x g for 5 minutes, with the removal of the supernatants. The samples were then subjected to shaking in an incubator shaker (200 rpm) for 10 minutes in the presence of 1 mL (10 g/mL) of a prepared crystal violet dye. Following this, the samples were centrifuged at 13400 x g for 15 minutes, and the optical density (OD) of the supernatants was measured at a wavelength of 590 nm. The analysis of crystal violet uptake was conducted using the following formula in Equation 1:

$$\begin{aligned} &Percentage\ uptake\ of\ crystal\ violet \\ &\quad = 100 - (OD\ value\ of\ sample \div OD\ value\ of\ CV\ solution) \times 100\% \end{aligned} \tag{1}$$

2.4 Cell's Constituent Release

The bacterial, *C. krusei*, and *R. oligosporus* suspensions were prepared using the same method as the cell crystal violet assay. Subsequently, these suspensions were placed in an incubator shaker (200 rpm) at 37 °C (for bacteria) and 35 °C (for fungi) with varying extract concentrations of 0× MIC, 0.5× MIC, 1× MIC, 2× MIC, and 4× MIC. The bacteria and *C. krusei* were incubated for durations of 0.5, 1, 2, and 4 hours, while *R. oligosporus* was incubated for 24, 48, 72, and 96 hours. The samples were then loaded into a centrifuge and centrifuged at 13400 x g for 15 minutes. The supernatant was employed to measure the absorbance of released components at 260 nm using a UV-VIS spectrophotometer [12].

2.5 Scanning Electron Microscope (SEM)

K. pneumoniae, *L. monocytogenes*, *C. krusei*, and *R. oligosporus* were subjected to treatment with an ethanolic extract derived from *P. guajava* leaves at a concentration of 4× MIC value for a duration of 2 hours. Subsequently, the pellets were centrifuged at 5000 × g for 10 minutes and then fixed in 2.5% (v/v) glutaraldehyde for a period of 4 to 6 hours at 4

°C. The pellets underwent three times of washing with 0.1 M sodium cacodylate buffer, each time for ten minutes. The pellets were then post-fixed for 2 hours at 4 °C using a 1% osmium tetroxide buffer and washed three times for 10 minutes in each round. Dehydration was carried out for 15 minutes at various acetone concentrations (35%, 50%, 75%, and 95%). Finally, the pellets were subjected to three rounds of dehydration in 100% acetone, each lasting 15 minutes. The cell suspension was subsequently transferred to a specimen basket and dried for 30 minutes in a critical dryer. The specimen was mounted on a stub and coated with a layer of gold. The scanning electron microscope was employed to examine alterations in the morphology of *K. pneumoniae*, *L. monocytogenes*, *C. krusei*, and *R. oligosporus* after their exposure to an ethanolic extract from *P. guajava* leaves.

2.6 Statistical Analysis

The data were analyzed for analysis of variance (ANOVA) using Minitab 19, with Tukey's multiple range test determining significant difference ($p<0.05$). The results of cell constituents' releases, and crystal violet assay that presented the antimicrobial mode of action of ethanolic *P. guajava* leaves extract was presented as mean ± standard deviation of triplicate experiments (n = 3) and analyzed by using Microsoft Excel version 16.0.

3. RESULTS AND DISCUSSION

3.1 Crystal Violet Assay

Figure 1(a) to (d) showed the uptake of crystal violet after exposed the ethanolic guava leaves extract at concentration 4 x MIC. The highest uptake of crystal violet for *K. pneumoniae*, *L. monocytogenes, C. krusei*, and *R. oligosporus* were 77.00%, 70.25%, 78.00%, and 80.00%, respectively. The ethanolic extract caused a damage of the cell surface from all tested microorganims. It is indicated by the increased intracellular material leakage. The result was measured by the absorbance which showed the higher uptake of crystal violet.According to Nilles et al. [13], crystal violet can dye proteins and nucleic acids (DNA and RNA). Huang et al. [14] state that essential oils, flavonoids, alkaloids, and terpenoids have antimicrobial activity. Any phytochemical compound may act on various sites of action in a variety of ways.

The chemical association of antimicrobial compounds with the outer layer of microbial cells, according to Zhang et al. [15], may involve a number of processes that disrupt cell integrity. Aggregation of the cell plasma membrane is one example in this case. Farid et al. [16] had reported, this situation may eventually result in cell death. Analysis of the crystal violet assay data revealed significant differences among different concentrations at various time exposure for all treated microorganisms as the p-value was smaller than the significance level ($p<0.05$).

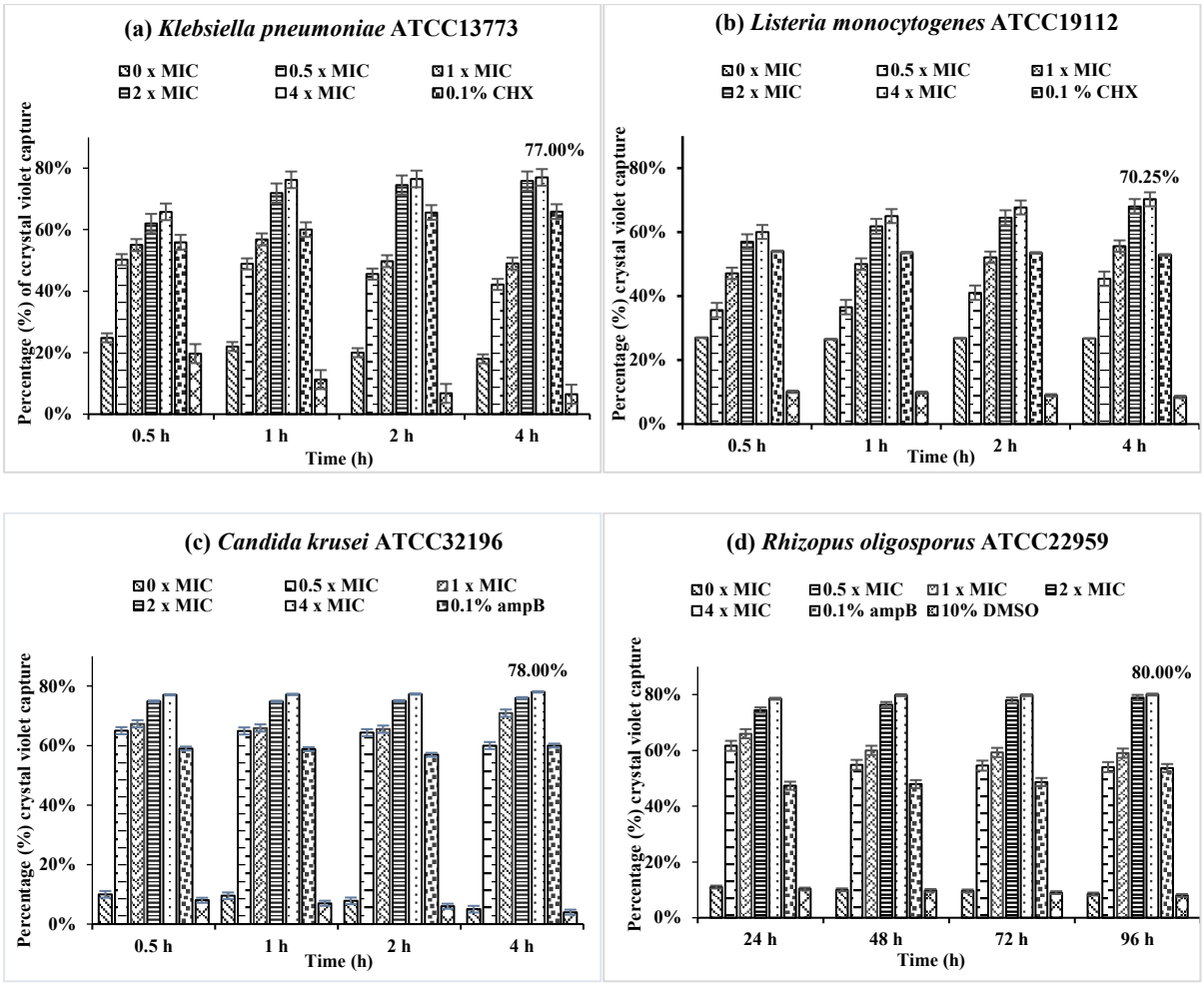

Figure 1: Crystal violet analysis of (a) *K. pneumoniae*, (b) *L. monocytogenes*, (c) *C. krusei*, and (d) *R. oligosporus* after being exposed with ethanolic *P. guajava* leaves extract at concentration from 0× MIC to 4× MIC

3.2 Cell's Constituent Release

The result of the constituent release assay is shown in Figure 2(a) to (d) below. The highest absorbance indicated the greatest cell release with 0.937, 0.880, 0.979, and 0.975, respectively, for *K. pneumoniae, L. monocytogenes, C. krusei,* and *R. oligosporus* after being exposed to 4× MIC. The results show that as the concentration of leaf extract used increased and the incubation time was extended, the release of cell constituents also increased. This result also indicated that the cytoplasmic membranes were permanently damaged.

According to Tavares et al. [17], the leakage of intracellular components suggests that the formation of pores in the plasma membrane may be a critical effect of antimicrobial agents on tested microorganisms. Patel et al. [18] states, the amount of UV-absorbing materials released was used to quantify cell lysis and non-selective pore formation. The p-value was smaller than the significance level ($p<0.05$) for all treated microorganisms, indicating significant differences in cell constituent release between extract concentrations at each exposure time. These findings suggest a dose-dependent effect of the extract on cell constituent release, with higher concentrations leading to greater membrane leakage.

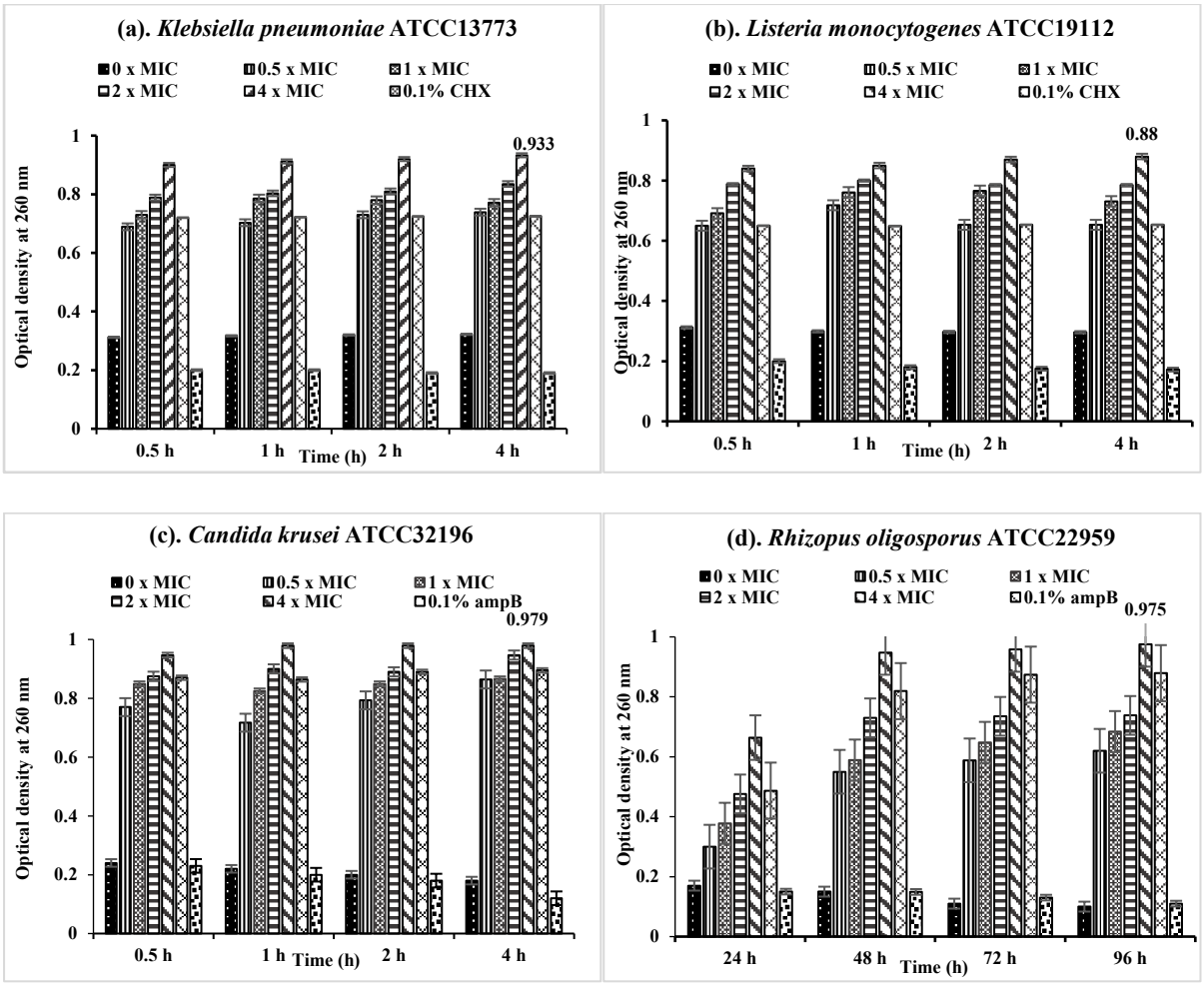

Figure 2: Cell constituent release analysis of (a) *K. pneumoniae*, (b) *L. monocytogenes*, (c) *C. krusei*, and (d) *R. oligosporus* after exposed with ethanolic *P. guajava* leaves extract at concentration from 0× MIC to 4× MIC

3.3 Scanning Electron Microscope (SEM)

Observations of changes in the microbial cell morphology were conducted after 2 hours of incubation. At this time, the effects of the extract treatment were visible. Untreated *K. pneumoniae* and *L. monocytogenes* exhibited normal cell characteristics, including a rod shape and an intact cell surface (Figures 3(a) and 4(a)). Furthermore, the treated bacteria cells appeared to be damaged or ruptured after being exposed to ethanolic *P. guajava* leaves extract, with some irregularities in surfaces and shape (Figure 3(b) and 4(b)). This is consistent with the discovery by Solo'rzano-Man et al. [19] that plant essential oils are hydrophobic. The compounds could disrupt cell structure and increase bacterial cell permeability by binding to lipids in cell wall membranes and mitochondria. The cell will then extinguish due to massive ion and molecule leakage.

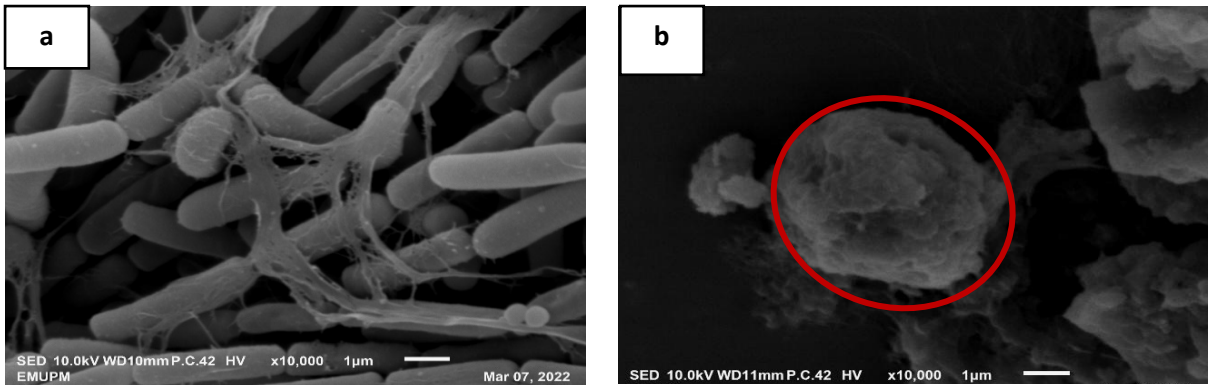

Figure 3: Morphology observation of *Listeria monocytogenes* ATCC19112 (a) untreated cell and (b) after treating by ethanolic *P. guajava* leaves extract at 4× MIC for 2 hours of incubation

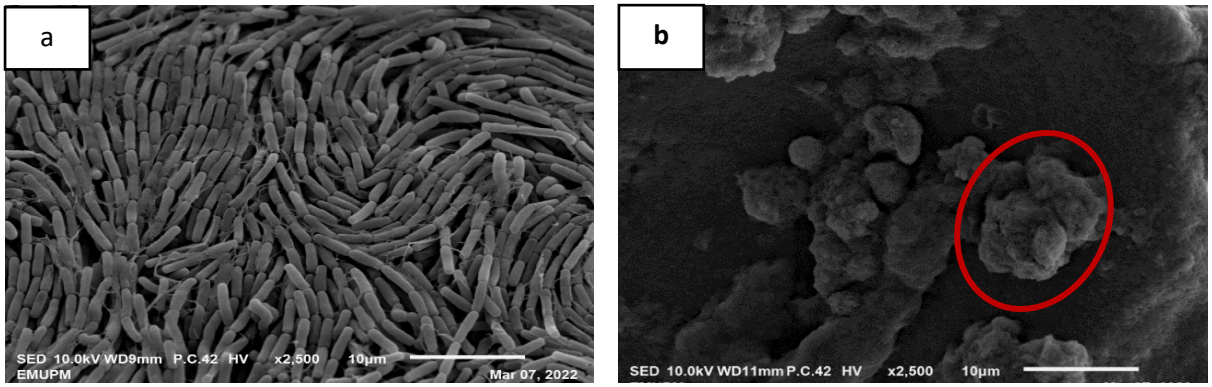

Figure 4: Morphology observation of *Klebsiella pneumoniae* ATCC1377 (a) untreated cell and (b) after treating by ethanolic *P. guajava* leaves extract at 4× MIC for 2 hours of incubation

Scanning electron microscopy for *C. krusei* and *R. oligosporus* showed morphological changes after exposure to the ethanolic *P. guajava* leaves extract. Figures 5(a) and 6(a) showed untreated *C. krusei* and *R. oligosporus* with intact cells, respectively. Meanwhile, treated cells showed the rupture of cells and leakage of their cytoplasm on *C. krusei* (Figure 5(b)) and damage of conidial heads on *R. oligosporus* (Figure 6(b)). Wong et al. [20] observed the same result on fungi after treating them with ethanolic *Syzygium polyanthum* leaves extract. The treated *C. albicans* membrane cells showed some dissolution of cytoplasmic content due to electron density loss. The cell wall had ruptured and was leaking. The treatment extract altered the morphology of *Aspergillus niger*, resulting in spores with distorted and perforated structures.

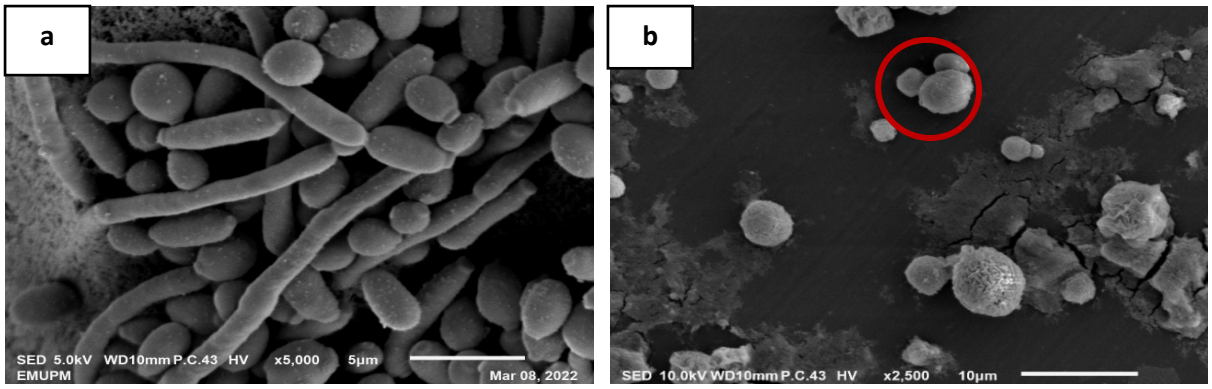

Figure 5: Morphology observation of *Candida krusei* ATCC32196 (a) untreated cell and (b) after treating by ethanolic *P. guajava* at 4xMIC for 2 hours of incubation

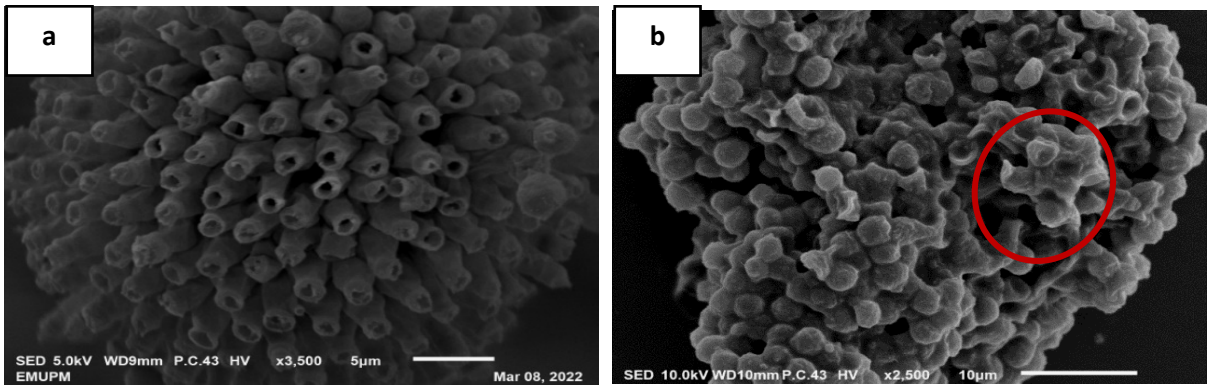

Figure 6: Morphology observation of *Rhizopus oligosporus* ATCC22959 by using SEM (a) untreated cell and (b) after treating by ethanolic *P. guajava* at 4xMIC for 2 hours of incubation

4. CONCLUSIONS

This study discovered that ethanolic *P. guajava* leave extract may contain essential phytochemical compounds capable of permanently damaging the cell membranes of *K. pneumoniae, L. monocytogenes, C. krusei,* and *Rh. oligosporus,* resulting in intracellular leakage. Therefore, the ethanolic *P. guajava* leaf extract could have been promoted for further study as a natural antimicrobial to be applied in the food industry.

Acknowledgements

The authors acknowledge the Southeast Asian Regional Center for Graduate Study and Research in Agriculture (SEARCA) PhD Research Scholarship

Author Contributions

All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure of Conflict of Interest

The authors have no disclosures to declare

Compliance with Ethical Standards

The work is compliant with ethical standards

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