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Antifungal activity of ethanolic *Psidium guajava* L. leaf extract against food spoilage fungi

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ABSTRACT

Aims: This study was aimed to determine the antifungal activity of ethanolic *Psidium guajava* L. leaf extract against food spoilage fungi.
Methodology and results: The ethanolic *P. guajava* L. leaf extract was tested for antifungal activity against *Candida albicans* ATCC10231, *C. glabrata* ATCC2001, *C. krusei* ATCC32196, *C. parapsilosis* ATCC22019, *Aspergillus fumigatus* ATCC26430, *A. niger* ATCC9029, *Rhizopus oligosporus* ATCC22959, and *R. oryzae* ATCC22580 in terms of well diffusion assay, minimum inhibitory concentration (MIC), minimum fungicidal concentration (MFC), and timekill curve assay, using Clinical and Laboratory Standard Institute (CLSI) methods. The inhibition conidia germination assay was determined qualitatively and quantitatively. The well diffusion agar results showed the inhibition zones of extract at a concentration 1% against *C. albicans* ATCC10231, *C. glabrata* ATCC2001, *C. krusei* ATCC32196, *C. parapsilosis* ATCC22019, *A. fumigatus* ATCC26430, *A. niger* ATCC9029, *R. oligosporus* ATCC22959, and *R. oryzae* ATCC22580 in the range from 7.00 ± 0.00 to 8.50 ± 0.00 mm. The MICs and MFCs values ranged from 0.31 to 2.50 mg/mL and 5.00 to >5.00 mg/mL, respectively. The time-kill analysis was done for *C. albicans* ATCC10231, *C. glabrata* ATCC2001, *C. krusei* ATCC32196, and *C. parapsilosis* ATCC22019 with 0× MIC, 0.5× MIC, 1× MIC, 2× MIC and 4× MIC. The results revealed that the extract was only able to reduce 3 $\log_{10}$ CFU/mL at 4× MIC for all *Candida* strains. The inhibition of conidial germination assay was done with 0× MIC, 1× MIC, 2× MIC, 4× MIC, and 8× MIC. The results showed that *A. fumigatus* ATCC26430, *R. oligosporus* ATCC22959, and *R. oryzae* ATCC22580 were completely killed at 8× MIC, whereas *A. niger* ATCC9029 reduced conidia germination by 20%. In the qualitative assay, *R. oligosporus* ATCC22959 and *R. oryzae* ATCC22580 can be inhibited with the extract up to day 14. On the other hand, 8× MIC of extract only succeeds in inhibiting conidial germination of A. *fumigatus* ATCC26430 up to day 10, while *A. niger* ATCC9029 was inhibited up to day 5.
Conclusion, significance and impact of study: The current study shows that ethanolic *P. guajava* L. leaf extract has antifungal activity and can inhibit germination of the spoilage fungi. Thus, it can be considered one of the candidates for natural food preservatives.

Keywords: Antifungal, *Aspergillus*, *Candida*, *Psidium guajava* L., *Rhizopus*

INTRODUCTION

Food spoilage can also be caused by fungi such as *Rhizopus, Aspergillus*, and *Candida*. They can produce various metabolic by-products, making foods distasteful and unsuitable for consumption due to changes in feel, texture, flavour, or appearance (Akinmusire, 2011; Rawat, 2015). The presence of spoilage fungi in food is also considered a contributing factor to the global problem of food waste and loss. This was later identified as a potential source of human and animal health hazards due to its ability to produce mycotoxins. Cancer, cytotoxicity, teratogenicity, estrogenicity, immune suppression, and neurotoxicity are all possible outcomes of mycotoxins

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(Akinmusire, 2011; Adeyeye, 2016; Zahra *et al*., 2019; Tolosa *et al*., 2021).

Food preservatives like sodium benzoate, acetic acid, propionic acid, and calcium propionate can prevent the growth of spoilage fungi. Regardless of their benefits, chemical preservatives and fungicides are harmful to human health. Recently, natural preservative agents, especially from edible medicinal plants have become widespread interest since they are relatively safe for human health as compared to synthetic or chemicals preservatives (Hintz *et al*., 2015; Campêlo *et al*., 2019). Plants have the ability to against spoilage fungi due to active compounds such as phenols, aldehydes, ketones, ethers, and hydrocarbons (Cowan, 1999; Aneja *et al*., 2014; Barberis *et al*., 2018; Putnik *et al*., 2020; Shwaiki *et al*., 2021). Guava *(Psidium guajava* L.) leaf is one of the medicinal plants that have antifungal properties (Wang *et al*., 2018; Saleh and Al-Mariri, 2020).

Psidium guajava L. is a member of the Myrtaceae family and the genus *Psidium* (Singh, 2011; Mishra and Bahadur, 2019). Investigations of ethnobotany or ethnopharmacology of *P. guajava* L. leaf have been used in traditional treatment in both tropical and subtropical regions to treat diarrhoea, dysentery, cholera, digestive problems, skin problems, sore throats, ulcers, and scabies (Chai, 2000; Kamath *et al*., 2008; Maroyi, 2011; Naseer *et al*., 2018; Nguanchoo *et al*., 2019). According to some research, guava leaf extract can inhibit the growth of *C. albicans* and *C. tropicalis* (Morais-Braga *et al*., 2015); *C. krusei* and *C. glabrata* (Ferreira *et al*., 2013); *A. niger* (Pandey and Shweta, 2011); *A. niger* and *R. oryzae* (Wang *et al*., 2018). Regardless of this, there have been few antifungal studies of ethanolic *P. guajava* L. leaf extract, especially against food spoilage fungi. Thus, the goal of this study was to measure the antifungal activity of *P. guajava* L. leaf extract, which include *C. albicans* ATCC10231, *C. glabrata* ATCC2001, *C. krusei* ATCC32196, *C. parapsilosis* ATCC22019, *A. niger* ATCC9029, *A. fumigatus* ATCC26430, *R. oligosporus* ATCC22959, and *R. oryzae* ATCC22580.

MATERIALS AND METHODS

Plant sampling and extraction

Psidium guajava L. leaf was collected from Taman Pertanian, Universiti Putra Malaysia (UPM). The extraction method was performed using the maceration technique as described by Rukayadi *et al*. (2008), with slight modifications. The leaves were washed under running tap water, oven-dried at 40 °C for 24 h, and stored at room temperature. One hundred gram of dried samples were ground using a dry blender Panasonic MK-5087M (Panasonic Corporation, Osaka, Japan) and extracted with 400 mL of absolute ethanol (99.8%) (SYSTERM®, Selangor, Malaysia). After that, ground leaves were soaked in 99.8% ethanol (SYSTERM®, Selangor, Malaysia) for 24 h at room temperature in a water bath (SintifikMaju, Selangor, Malaysia) with occasional shaking. Twenty-four hours later, the soaked leaves were vacuum-filtered through Whatman filter paper No. 2 (Whatman International Ltd, Middlesex, England) by EYELA A-1000S aspirator pump (Tokyo Rikakikai Co., Tokyo, Japan). The filtrate was concentrated by using a rotary vacuum evaporator (BUCHI Rotavapor R-200, Switzerland) at 40 °C with the speed of 99 rpm for 3 to 4 h. Ethanolic *P. guajava* L. leaf extract was made by diluting 100 mg of the crude extract into 1.00 mL of dimethyl sulfoxide (DMSO) (R & M chemicals, Selangor, Malaysia) to obtain 100 mg/mL (10%) and then further diluted in 1:10 (v/v) distilled water to get a final concentration of 10 mg/mL (1.00%) stock solution.

Preparation of fungi tested

Different bacterial strains including *C. albicans* ATCC10231, *C. glabrata* ATCC2001, *C. krusei* ATCC32196, *C. parapsilosis* ATCC22019, *A. niger* ATCC9029, *A. fumigatus* ATCC26430, *R. oligosporus* ATCC22959, and *R. oryzae* ATCC22580 were obtained from the American Type Culture Collection (ATCC), Maryland, United States and commonly cause food spoilage. The *Candida* spp. were grown on Sabouraud Dextrose Agar (SDA) (Difco, USA) at 37 °C for 24-48 h and the moulds were cultured on Potato Dextrose Agar (PDA) (Difco, USA) at 35 °C for 3-7 days (Rukayadi and Hwang, 2007). Sterile cotton swabs were used to transfer three to five colonies of yeast into 1 mL of Sabouraud Dextrose Broth (SDB) (Difco, USA), then vortexed for 15 sec. After the vortex, the yeast suspension was incubated for 24-48 h, and then 10 µL of yeast suspension were taken and transferred into 10 mL of SDB. The turbidity of inoculums was standardized to 10^6-10^8 CFU/mL prior to the test by using the standard broth microdilution method (Rukayadi *et al*., 2013; CLSI, 2017) and inoculum quantification (Indira, 2014). Inoculum quantification was performed by plating 25 µL of yeast suspension on SDA and counts the visible colonies after incubation at 37 °C for 24-48 h. The inoculums of filamentous fungi were prepared as described in CLSI standard: M38 (CLSI, 2017). Fungi strains were grown on PDA for 3-7 days at 35 °C. After 7 days, approximately 1-2 mL of 0.85% sterile saline was placed to cover the grown fungi and the colonies were gently probed with the tip of the Pasteur pipette. The mixture containing conidia and hyphal fragments was collected and transferred to a sterile tube, and let the heavy particles settle for 5 to 10 min. The homogenous suspensions of the mixture at the top of the tube were collected and vortexed for 15 sec. A spectrophotometer was used to read the optical density (OD) of the conidial suspensions and adjusted at a wavelength of 530 nm. The transmittance of the *A. niger* ATCC9029 and *A. fumigatus* ATCC26430 were ranged from 80% to 82%, while it was 68% to 70%, for *R. oryzae* ATCC22580 *and R. oligosporus* ATCC22959, respectively (Santos *et al*., 2006). The conidial suspensions were diluted 1:50 in sterile distilled water. Then the inoculum dilutions corresponded to 2× density (almost 0.4×10^4-5×10^4 CFU/mL which is equivalent to 0.5 McFarland standard) (Espinel-Ingroff *et al*., 2012).

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Well diffusion assay

According to the Clinical and Laboratory Standard Institute (CLSI, 2017) the well diffusion assay (WDA) was used to test the antifungal activity of ethanolic *P. guajava* L. leaf extract. The *Candida* sp. and filamentous fungi grew in SDA and PDA media, respectively. A sterile cotton swab was used to apply 30 µL of fungal suspension to each medium. After that, a cork borer was dipped into the media to make four holes or wells in the media. Each well was labelled and accordingly filled with 30 µL of 1% ethanol *P. guajava* L. leaf extract, 0.1% commercial Amp B was used as a positive control, and 10% DMSO was used as a negative control. The plates were placed in a 37 °C incubator for 24 h. There is a clear zone around the well. The clear zone around the wells is evidence and an indicator for fungal growth inhibition, and the diameter was measured in mm. Data analysis was conducted three times in triplicate (n = 3 × 3).

Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) was determined using a sterile and disposable 96-well U-shaped microtiter plate and a two-fold standard broth microdilution method, as suggested by the Clinical and Laboratory Standard Institute (CLSI, 2017), with an inoculum previously prepared at nearly 10^6-10^8 CFU/mL (*Candida*) and 0.4 × 10^4-5 × 10^4 CFU/mL (*Aspergillus* and *Rhizopus*). Each well of the microtiter plate from column No. 12 until column No. 3 received 100 µL of the inoculum fungal strains, then two-fold diluted with 100 µL of the sample extract starting from column No. 12 to column No. 3. Column No. 1 of the microtiter plate was filled with 200 µL of plain SDB/PDB as a negative control, whereas column No. 2 was filled with 100 µL of broth plus 100 µL of inoculum suspension where acts as a positive control. The microdilution was carried out at a concentration of the extract ranging from 5.00 mg/mL in column No. 12 to 0.009 mg/mL in column No. 3 for *Candida* spp. and filamentous fungi strains. The microtiter plates were incubated for 24-48 h at 35 °C (*Candida*) and for 3-7 days at 30 °C (filamentous fungi). The lowest concentration of antifungal agent that was able completely to inhibit fungal visible growth was considered the MIC (Zainin *et al*., 2013).

Minimum fungicidal concentration

The results from minimum fungicidal concentration (MIC) were sub-cultured on SDA and PDA to determine the MFC. Ten µL of each of the suspension in the MIC wells from the microtiter plate dropped onto the above-mentioned agar plates (from columns 1 to 12). The plates were incubated for 24-48 h at 35 °C for *Candid*a and for 3-7 days at 30 °C for filamentous fungi. The lowest concentration that showed no visible growth was considered as the MFC value (Andrews, 2002).

Time-kill curve analysis for *Candida* spp.

Determination of the timekill curve for *Candida* sp. was referred to CLSI, (2017). Time kill curve assay was conducted on the representative *Candida* according to the MIC values obtained previously in the microdilution assay by using ethanolic *P. guajava* L. leaf extracts. Five different concentrations of the extract were used to perform this assay. The final concentrations of (0× MIC, 0.5× MIC, 1.0× MIC, 2.0× MIC and 4.0× MIC) for each *Candida* were obtained by diluting the extract with the SDB containing approximately 10^6-10^8 CFU/mL of the inoculums. The assay was carried out at different interval times of incubation of the mixture (0, 0.5, 1, 2, and 4 h). A 100 µL of the mixture was serially diluted into 1% PBS and streaked in SDA and incubated at 35 °C for 24-48 h. After incubation, the total plate count (TPC) was expressed in Log_{10} CFU/mL and plotted it versus time. All the tests were carried out three times with three replications (n = 3 × 3).

Inhibition conidial germination assay for filamentous fungi

Qualitative analysis

The inhibition conidial germination assay was carried out for both qualitative and quantitative assay according to the method described by Rukayadi and Hwang (2007). PDB medium was used to dilute the adjusted inoculum suspension of 5 × 10^4 CFU/mL in 1:100 ratios to give a final inoculum concentration of 5 × 10^3 CFU/mL. *Psidium guajava* L. leaf extract at different concentrations of 0× MIC, 1× MIC, 2× MIC, 4× MIC and 8× MIC were obtained previously, and mixed with the cultures. The mixtures were incubated for 14 days at 35 °C. This assay was performed based on daily visual observation.

Quantitative analysis

The standardized inoculum suspension, where it was prepared in the same method as the qualitative analysis, was mixed with the final concentrations of *P. guajava* L. leaf extract of 0× MIC, 1× MIC, 2× MIC, 4× MIC and 8× MIC. Then, 1 mL of the final mixtures was incubated at 35 °C for 48 h for *A. niger* ATCC9029, *A. fumigatus* ATCC26430, *R. oligosporus* ATCC22959 and *R. oryzae* ATCC22580. Next, 25 µL of the incubated mixtures were spread on PDA and incubated at 35 °C for 48 h. The number of conidia was then determined, and the percentage of the conidial germination inhibition was calculated by the following formula (Jin *et al*., 2004):

$$\text{Germinataion inhibition \%} = \frac{\text{Average conidial germination \% of control} - \text{Average conidial germination \% of treatment}}{\text{Average conidial germination \% of control}} \times 100$$

Data analysis

The data was analyzed using Minitab Statistical Software, Version 16. A one-way ANOVA was carried out, followed

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by a *post-hoc* Tukey's test to analyse the significance among different treatments at a 95% confidence level (p< 0.05).

RESULTS

Well diffusion assay

The diameter of inhibition zone of ethanolic *P. guajava* L. leaf extract presented in Table 1. The inhibition zone ranged was from 12.75 ± 0.25 to 7.00 ± 0.00 mm. Results showed that *C. glabrata* and *C. krusei* were susceptible compared to other fungi with a value of 8.50 ± 0.00 mm. Meanwhile, other food spoilage microorganisms have an inhibition zone of as much as 7.00 ± 0.00 mm.

Table 1: Inhibition zone of ethanolic *Psidium guajava* L. leaf extract against spoilage fungi.

	Inhibition zone (mm)		
Fungi Strains	*P. guajava* L. leaf extract	Amp B	DMSO
C. albicans ATCC10231	7.00±0.00^{Bb}	12.50±0.71^{Ca}	n.a
C. glabrata ATCC2001	8.50±0.00^{Ab}	18.00±0.00^{Aa}	n.a
C. krusei ATCC32196	8.50±0.00^{Ab}	14.00±0.00^{Ba}	n.a
C. parapsilosis ATCC22019	7.00±0.00^{Bb}	18.00±0.00^{Aa}	n.a
A. fumigatus ATCC26430	7.00±0.00^{Bb}	7.00±0.00^{Bb}	n.a
A. niger ATCC9029	7.00±0.00^{Bb}	7.00±0.00^{Bb}	n.a
R. oligosporus ATCC22959	7.00±0.00^{Bb}	7.00±0.00^{Bb}	n.a
R. oryzae ATCC22580	7.00±0.00^{Bb}	7.00±0.00^{Bb}	n.a

n.a: no activity; diameter of inhibition zones in mm (including disc); Positive control (Amp B; 0.1%); Negative control (DMSO; 10%); Results were expressed as means ± standard deviation (SD); n = 3 × 3; Mean values ± standard deviation with different lowercase or uppercase letters in the same column and row are significantly different (p<0.05).

Determination of minimum inhibitory concentration and minimum fungicidal concentration

Determination of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values of ethanolic *P. guajava* L. leaf extract against spoilage fungi listed in Table 2. The MIC values for all tested fungal were in the range of 0.31 to 2.50 mg/mL. *Rhizopus oligosporus* ATCC22959 and *R. oryzae* ATCC22580 were found to have more susceptibility to the extract compared to other strains, with an MIC value of 0.31 mg/mL. In fact, *A. fumigatus* was the most resistant fungus, with a MIC value of 2.50 mg/mL. The MFC values were in the range of 5 mg/mL to >5 mg/mL, where *A. fumigatus* gave a value as low as 5 mg/mL. *Candida albicans*, *C. glabrata*, *C. krusei*, *C. parapsilosis*, *A. niger*, *R. oligosporus*, and *R. oryzae* that had values greater than 5 mg/mL.

Table 2: Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of ethanolic *Psidium guajava* L. leaf extract against food spoilage microorganisms.

Fungi strains	MIC (mg/mL)	MFC (mg/mL)
C.albicans ATCC10231	0.62	>5.00
C. glabrata ATCC2001	0.62	>5.00
C. krusei ATCC32196	0.62	>5.00
C.parapsilosis ATCC22019	0.62	>5.00
A.fumiatus ATCC26430	2.50	5.00
A. niger ATCC9029	0.62	>5.00
R.oligosporus ATCC22959	0.31	>5.00
R. oryzae ATCC22580	0.31	>5.00

Timekill curve for *Candida* species

The result of timekill assay for *C. albicans* (Figure 1), *C. glabrata* (Figure 2), *C. krusei* (Figure 3), and *C. parapsilosis* (Figure 4) only reduced to less than 3 Log_{10} CFU/mL at 4× MIC within 4 h of incubation.

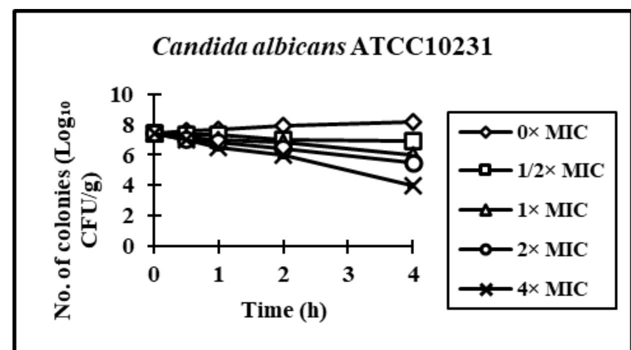

Figure 1: Timekill curve plots for *C. albicans* ATCC10231 following exposure to ethanolic *Psidium guajava* L. leaf extract at 0× MIC, 0.5× MIC, 1× MIC, 2× MIC and 4× MIC (0, 0.315, 0.625, 1.250, 2.520, mg/mL respectively).

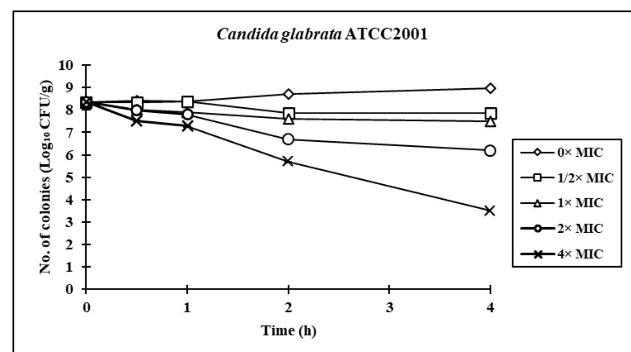

Figure 2: Timekill curve plots for *C. glabrata* ATCC2001 following exposure to ethanolic *Psidium guajava* L. leaf extract at 0× MIC, 0.5× MIC, 1× MIC, 2× MIC and 4× MIC (0, 0.315, 0.625, 1.250, 2.520, mg/mL respectively).

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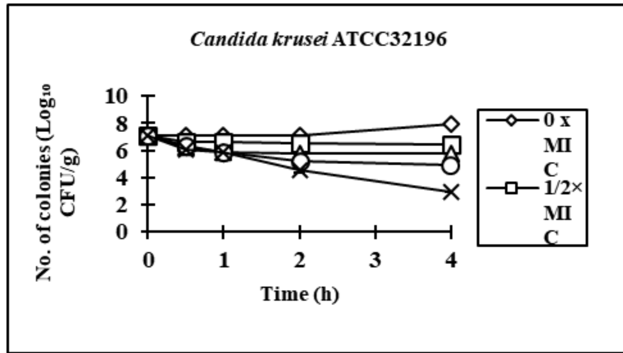

Figure 3: Timekill curve plots for *C. krusei* ATCC32196 following exposure to ethanolic *Psidium guajava* L. leaf extract at 0× MIC, 0.5× MIC, 1× MIC, 2× MIC and 4× MIC (0, 0.315, 0.625, 1.250, 2.520, mg/mL respectively).

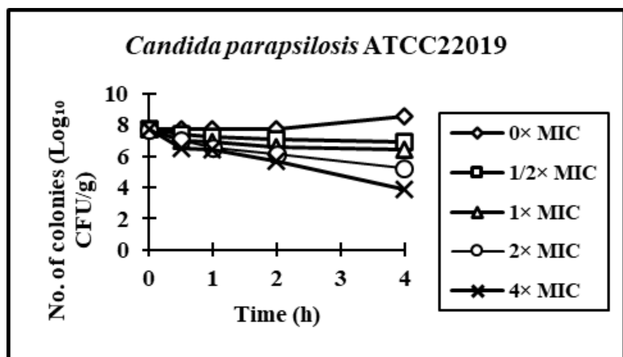

Figure 4: Timekill curve plots for *C. parapsilosis* ATCC22019 following exposure to ethanolic *Psidium guajava* L. leaf extract at 0× MIC, 0.5× MIC, 1× MIC, 2× MIC and 4× MIC (0, 0.315, 0.625, 1.250, 2.520, mg/mL respectively.

Inhibition conidial germination for filamentous fungi

Qualitative assay

In the qualitative test, the growth of *A. fumigatus* ATCC26430 (Figure 5) was shown at a concentration of 1× MIC and 2× MIC on day 7 while at 4× MIC started on day 10 after being treated with extract, whereas the growth of *A. niger* ATCC9029 started at 1× MIC on day 4 while other concentrations started on day 5 (Figure 6). The same observation has been performed on *R. oligosporus* ATCC22959 and *R. oryzae* ATCC22580 as shown in (Figures 7 and 8). The *R. oligosporus* and *R. oryzae* showed no growth after 14 days incubation after being treated with the extract.

Quantitative assay

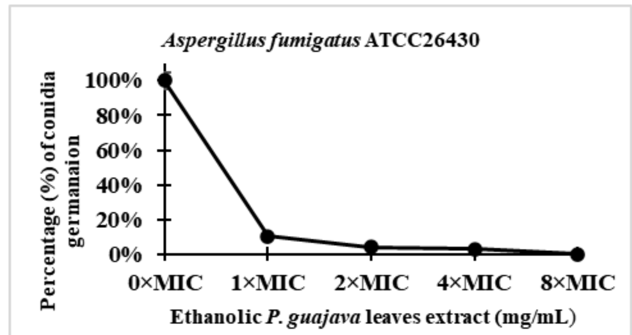

Figure 9: Effect of *Psidium guajava* L. leaf extract against conidia germination of *A. fumigatus* at concentrations of 0× MIC, 1× MIC, 2× MIC, 4× MIC and 8× MIC (0, 2.50, 5.00, 10.00 and 20.00 mg/mL respectively).

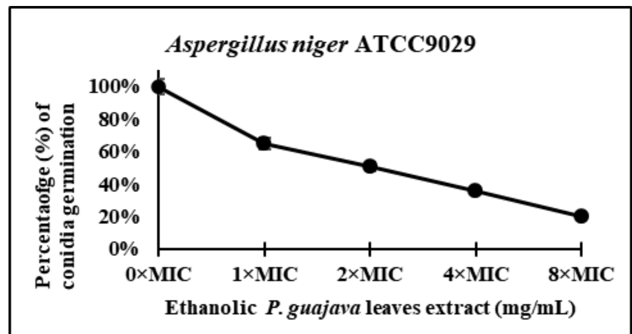

Figure 10: Effect of *Psidium guajava* L. leaf extract against conidia germination of *A. niger* at concentration of 0× MIC, 1× MIC, 2× MIC, 4× MIC and 8× MIC (0, 0.625, 1.25, 2.50 and 5.00 mg/mL respectively).

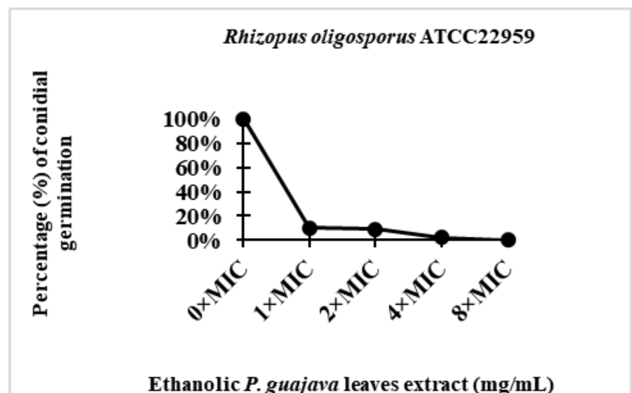

Figure 11: Effect of *Psidium guajava* L. leaf extract against conidia germination of *R. oligosporus* at concentration of 0× MIC, 1× MIC, 2× MIC, 4× MIC and 8× MIC (0, 0.315, 0.625, 1.25 and 2.50 mg/mL respectively).

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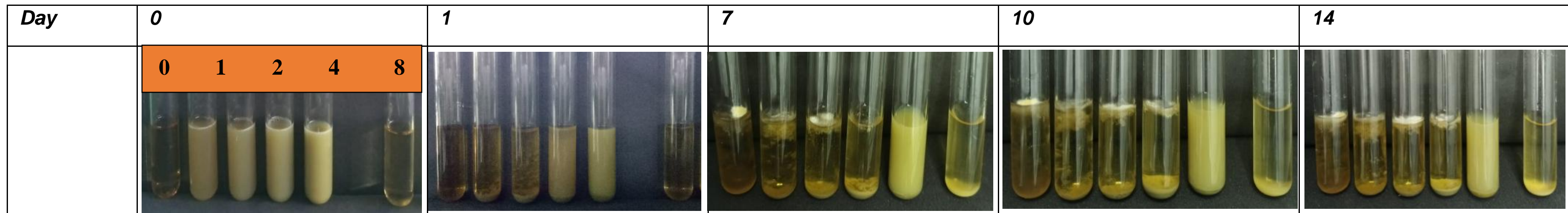

Figure 5: Inhibition germination conidia assay (qualitative) of *A. fumigatus* ATCC26430 incubated up to 14 days treated with *Psidium guajava* L. leaf extract at 0× MIC, 1× MIC, 2× MIC, 4× MIC, and 8× MIC.

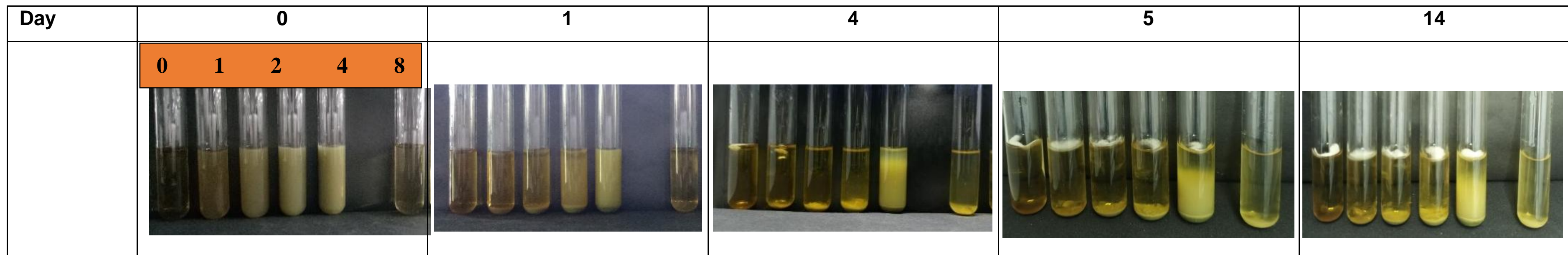

Figure 6: Inhibition germination conidia assay (qualitative) of *A. niger* ATCC9029 incubated up to 14 days treated with *Psidium guajava* L. leaf extract at 0x MIC, 1× MIC, 2× MIC, 4× MIC, and 8× MIC.

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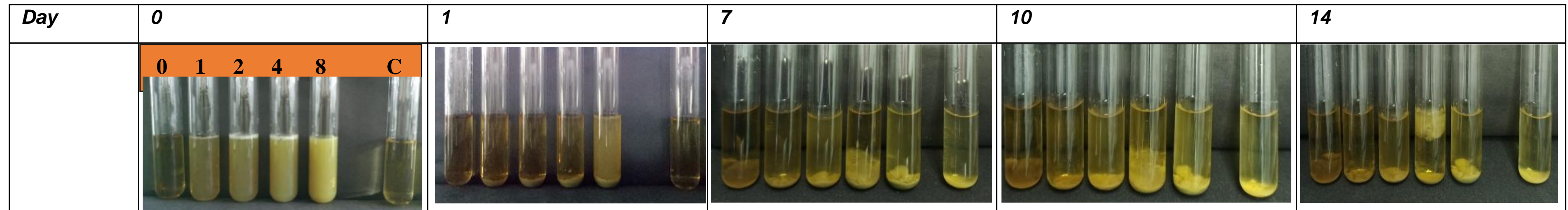

Figure 7: Inhibition germination conidia assay (qualitative) of *R. oligosporus* ATCC22959 incubated up to 14 days treated with *Psidium guajava* L. leaves extract at 0× MIC, 1× MIC, 2× MIC, 4× MIC, and 8× MIC.

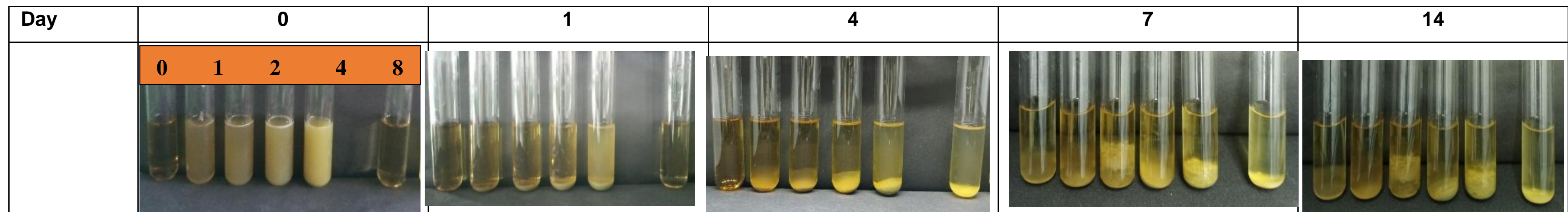

Figure 8: Inhibition germination conidia assay (qualitative) of *R. oryzae* ATCC22580 incubated up to 14 days treated with *Psidium guajava* L. leaf extract at 0× MIC, 1× MIC, 2× MIC, 4× MIC, and 8× MIC.

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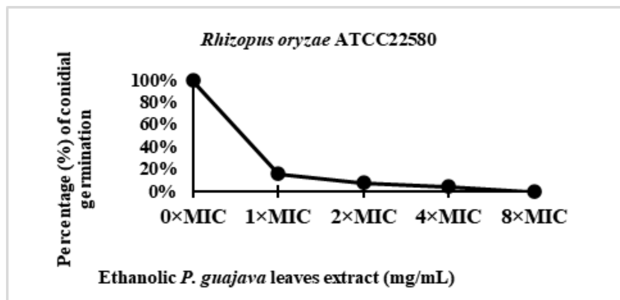

Figure 12: Effect of *Psidium guajava* L. leaf extract against conidia germination of *R. oryzae* at concentration of 0× MIC, 1× MIC, 2× MIC, 4× MIC and 8× MIC (0, 0.315, 0.625, 1.25 and 2.50 mg/mL respectively).

The quantitative inhibition conidia germination assay in this study used different MIC concentrations (0× MIC, 1× MIC, 2× MIC, 4× MIC, and 8× MIC) of the ethanolic *P. guajava* L. leaves extract and used 4 filamentous fungal strains (*A. fumigatus*, *A. niger*, *R. oligosporus*, and *R. oryzae*). As shown in Figure 9, 100% of the *A. fumigatus* conidia were inhabited at 8× MIC. For *A. niger*, as can be seen in Figure 10, only 20% of the conidia were inhibited at 8× MIC. Meanwhile, the *R. oligosporus* (Figure 11) and *R. oryzae* (Figure 12) conidia were completely killed at 8× MIC.

DISCUSSION

The difference in the results of the well diffusion assay may be due to the more complex structure of the fungal cell wall. Free (2013) reported that chitin is a major cell wall component of fungi, making up almost 1-15% of the cell wall mass, with yeast species generally having 1-2% chitin and filamentous fungi having up to 15% chitin. In addition, Garcia-Rubio *et al.* (2020) stated that chitin makes up between 10% to 20% of the dry weight of cell walls in filamentous fungus, a substantially higher percentage of the cell wall than in yeast. For cellular integrity and vitality, the fungal cell wall is a crucial structure with considerable flexibility. The regulation of cellular permeability and defense against osmotic and mechanical stress are just two biological processes that the cell wall is crucial for. There is diversity in the cell wall composition of the fungus, and certain species have distinctive qualities that set them apart from other fungi. However, the explanation above provides an understanding that between *Candida* species, it is possible that there are differences in the results of the well diffusion assay. According to Wong and Ramli, (2021) the 1% of ethanolic *Centella asiatica* extract had no antifungal activity against *C. albicans* ATCC 10231 and *Aspergillus niger* ATCC 9029 when they used the agar disc diffusion method. It means 1% ethanolic *P. guajava* L. leaf extract had antifungal activity slightly better compared to 1% ethanolic *C. asiatica*. Well diffusion assay (WDA) method is known for its inexpensiveness and simplicity. Nonetheless, the data obtained from this assay appears insufficient because the assay focuses on qualitative data and functions as a preliminary screening for antibacterial activity (Gonelimali *et al.*, 2018). Therefore, MIC and MFC assays were required to determine accurate quantitative data for the antifungal activity of the ethanolic *P. guajava* L. leaf extract. The MIC of an antimicrobial or plant extract is the lowest concentration that inhibits visible bacteria or fungi growth after overnight incubation. Meanwhile, the MFC is the lowest antimicrobial or plant extract concentration required to kill at least 99% of the fungi (Pankey and Sabath, 2003). The results of MIC and MFC compared to Wong and Ramli, (2021) and Wong *et al.* (2022) have different values. The MIC and MFC values from 1% ethanolic *P. guajava* L. leaf extract compared with Wong *et al.* (2022) was significantly lower. Furthermore, Wong and Ramli, (2021) showed that 50% ethanolic *Centella asiatica* leaves extract has weak activity against *C. albicans* and *A. niger*. If the result of 50% ethanolic *C. asiatica* extract was compared to 1% ethanolic *P. guajava* L. leaf extract, it showed that 1% ethanolic *P. guajava* L. was better against *C. albicans* and *A. niger*. It means the ethanolic *P. guajava* L. leaf extract only has fungistatic activity against the *Candida* species. Morais-Braga *et al.* (2015), reported that MIC of hydroethanolic (70%) and aqueous *P. guajava* leaf extracts at a concentration of 150 mL of H_2O/10 g of leaves can inhibit both *C. albicans* and *C. tropicalis* by up to 8192 µg/mL. Meanwhile, Ferreira *et al.* (2013) discovered that MIC value of ethanolic *P. guajava* leaf extracts inhibited *C. albicans* and *C. glabrata* up to 62.50 µg/mL, but only 15.62 µg/mL for *C. krusei*. The 1% ethanolic *P. guajava* leaf extract performed better than Ferreira *et al.* (2013) and Morais-Braga *et al.* (2015).

Concentration-dependent fungal killing occurs when the rate and extent of killing increase with progressively higher antifungal concentrations, and time-dependent killing occurs when increasing antifungal concentrations to more than the MIC does not result in proportional increases in killing (Pankey and Sabath, 2003). Figure 1 shows that *C. albicans* was reduced by about 3 Log_{10} after 4 h of incubation. Tangarife-Castaño *et al.* (2011) showed that the extract of *Morinda royoc* showed fungicidal activity on *C. albicans* at 2× MIC after 8 h. Wong *et al.* (2022) reported *C. albicans* was killed by ethanol leaves extract of *Syzygium polyanthum* 4× MIC after 4 h of incubation time. At the same time, *Cympobogon citratus* had a timekill effect on *C. albicans* at a concentration of 0.63 mg/mL after 4 h of incubation (Zulfa *et al.*, 2016). This means ethanolic *P. guajava* L. leaf extract had weaker antifungal activity compared to *C. citratus*. As shown in Figure 2, the population of *C. glabrata* was observed to reduce by about 3 Log_{10} after 4 h of incubation. According to De Toledo *et al.* (2016), the *Cymbopogon nardus* extract at a concentration of 1 mg/mL can completely kill *C. glabrata* within 24 h of incubation time. Wong *et al.* (2022) reported that *C. glabrata* could be completely killed after it had been

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exposed to ethanolic *Syzygium polyanthum* extract at 2.52 mg/mL for 2 h. It means ethanolic *P. guajava* L. leaf extract had weaker antifungal activity compared to *C. nardus* and *S. polyanthum*. As shown in Figure 3 the population of *C. krusei* was observed to reduce by about 3 Log_{10} after 4 h of incubation. However, Wong *et al.* (2022) stated the population of *C. krusei was* reduced to below 3 Log_{10} CFU/mL at 4× MIC of ethanolic *S. polyanthum* leaf extract concentration for 4 h. At the same time, Correa-Royero *et al.* (2010) stated oil extraction from *Chenopodium ambrosioides* on *C. krusei was* found to have a significant fungicidal effect (CFU/mL >3 Log_{10} units) at 4× MIC (31.128 g/mL) after 4 h incubation time. This means *C. krusei* could not be killed by the ethanolic *P. guajava* L. leaf extract, ethanolic *S. polyanthum* leaves extract, or oil of *C. ambrosioides.* The timekill curve of *C. parapsilosis* after treatment with 1% ethanolic *P. guajava* L. leaf extract is shown in Figure 4. The population of *C. parapsilosis* could be reduced by about 3 Log_{10} after 4 h of incubation. Wong *et al.* (2022) reported the population of *C. parapsilosis* reached the endpoint of 4 h of incubation after being exposed to ethanolic *S. polyanthum* leaf extract at concentrations of 2 and 4× MIC. This means ethanolic *P. guajava* L. leaf extract had weaker antifungal activity compared to *S. polyanthum.* The qualitative assay for filamentous fungi showed different results for each strain (Figures 5 - 8). There was also no growth on both *Rhizopus* strains after being treated with the antifungal commercial Amp B. This ethanolic *P. guajava* L. leaf extract, if compared to antifungal Amp B, had the same condition. This means the extract of *P. guajava* L. leaf had better antifungal activity against *Rhizopus* sp. According to De Lira Mota *et al*. (2012), the essential oil of *Thymus vulgaris* L. was able to reduce the conidia germination of *R. oryzae* after exposure at concentrations of 256 µg/mL (1× MIC) and 512 µg/mL (2× MIC) for 24 h of incubation. The percentages of conidia germination inhibition were 55 ± 6% (1× MIC) and 66 ± 2% (2× MIC). This observation shows that 1% ethanolic *P. guajava* L. leaf extract had better antifungal activity against *R. oryzae* than *T. vulgaris* extract. These results suggest that an ethanolic extract of *P. guajava* L. leaf extract can inhibit conidia germination.

Meanwhile, the quantitative assay for filamentous fungi showed almost the same results as the qualitative results. Figure 9 shows the inhibition of conidia germination for *A. fumigatus*. Based on the results obtained, *A. fumigatus* was completely inhibited after being treated with the highest concentrations (8× MIC) of ethanolic *P guajava* L. leaf extract. After 24 h of incubation, conidia germination was reduced to 0%. At the lowest concentration (1× MIC), inhibition of conidia germination was reduced to 10%, and a sharp reduction was observed at 1× MIC. Islam *et al.* (2003) reported the germination of *A. fumigatus* has been affected by *Vinca rosea* and *Lowsernia inermis* extract with concentrations of 200 g/L after 5 min of incubation. They were reduced by almost 95% and 56%. This showed that inhibition of germination conidia was depending on the concentration of extract and time of exposure.

Figure 10 shows the inhibition of germination in conidia of *A. niger* after treatment with ethanolic *P. guajava* L. leaf extract. The results showed that *A. niger* was not fully inhibited after being treated with the highest concentration (8× MIC) of extract. However, conidia germination had been reduced to 20%. The result also showed a sharp reduction of 65% at 1x MIC. According to Islam *et al.* (2003), extract of *Curcuma longa* had inhibitory effects on *A. niger* when the conidia germination was reduced to 83% after 5 min of exposure and 67% within 30 min of exposure. However, after 5 min and 30 min treatments with *Azadiracta indica* extract, it reduced germination conidia of *A. niger* by 34% and 23%, respectively. Figure 11 shows the inhibition of germination in conidia of *R. oligosporus* after treatment with ethanolic *P. guajava* L. leaf extract. Based on the results obtained, the inhibition of conidia germination endpoint was determined at a concentration of 2.50 mg/mL (8× MIC) after 48 h of incubation time. Begum *et al.* 2010 have reported that the *Azadirachta indica* leaf extract was able to reduce the conidia germination of *Rhizopus* spp. by up to 72% after being treated at a concentration of 5%. Figure 12 shows the inhibition of germination in conidia of *R. oryzae* after treatment with ethanolic *P. guajava* L. leaf extract. Based on the results obtained, the inhibition of conidia germination endpoint was determined at a concentration of 2.50 mg/mL (8× MIC) after 48 h of incubation time. According to De Lira Mota *et al.* (2012), the essential oil (EO) of *Thymus vulgaris* L. was able to reduce the conidia germination of *R. oryzae* after exposure at concentrations of 256 µg/mL (1× MIC) and 512 µg/mL (2× MIC) for 24 h of incubation. The percentages of conidia germination inhibition were 55 ± 6% (1× MIC) and 66 ± 2% (2× MIC). This observation shows that 1% ethanolic *P. guajava* L. leaf extract had better antifungal activity against *R. oryzae* than *T. vulgaris* extract. The use of ethanolic *P. guajava* L. leaf in this study of antifungal activity against *Candida* species and filamentous fungi could be as a result of the synergistic effect of these phytocompounds found in the extract as revealed by GC-MS and LC-MS method. Nursanty *et al*. (2023) report the presence of 15 major volatile compounds in the ethanolic *P. guajava* L. leaf analysed by GC-MS, i.e., pyrogallol, α-copaene, caryophyllene, aromadendrene, α-humulene**,** alloaromadendrene, γ-muurolene, β-selinene, α-selinene, α-muurolene, β-bisabolene, β-bisabolene, (-) globulol, caryophyllene oxide, α-muurolol, and epiglobulol. Furthermore, the non-volatile compounds identified by LC-MS were quinic acid, catechin, apigenin-7-O-β-D-glucuronopyranoside, quercetin, luteolin-7-O-glucuronide, epigallocatechin (4β,8)-gallocatechin, naringenin4′-O-glucopyranoside, and myrecitin. Lima *et al.* (2011) discovered that pyrogallol from *P. cattleianum* (strawberry guava) has antimicrobial activity against *Candida* species. Caryophyllene, α-Copaene, and α- Humulene have antimicrobial properties, as documented by Soliman *et al.* (2016) and Thenmozhi and Rajah (2015). Then, Liu *et al.* (2020) report that quinic acid compound has antimicrobial activity. Meanwhile, Yang *et al.* (2020) found that

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quercetin can against *Aspergillus flavus.* Based on the results of this research, it is suggested that ethanolic *P. guajava* L. leaf extract has potential as a food preservative. However, more detailed and comprehensive research is required before applying ethanolic *P. guajava* L. leaf to the food industry.

CONCLUSION

Research findings suggest that 1% ethanolic *P. guajava* L. leaf extract can be used effectively as an antifungal agent against a broad range of food spoilage microorganisms. The extract also has fungistatic activity against *Candida* strains after 4 h incubation with the highest concentration (4× MIC). Thus, the ethanolic *P. guajava* L. leaf extract can inhibit conidia germination of filamentous fungi and would be helpful for controlling and decreasing pathogenic fungi-caused food spoilage. Further research is required.

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DECLARATION OF INTEREST

The authors have no conflict of interest to declare.

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