*applied microbiology*

*Communication*

# Pterostilbene, a Natural Methoxylated Analog of Resveratrol, Exhibits Antifungal Activity Induced by Reactive Oxygen Species Production and Plasma Membrane Injury

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**Abstract:** Pterostilbene has been shown to exhibit antifungal activities. However, the detailed action mechanism is unknown. Here, we analyzed the antifungal mechanism of pterostilbene against *Saccharomyces cerevisiae*. The minimum growth inhibitory and fungicidal concentrations of pterostilbene were 120 and 240 μM in a YPD medium, respectively. Although pterostilbene produced reactive oxygen species (ROS), resveratrol did not. This effect was maximized at 120 μM. Furthermore, α-tocopherol acetate inhibited ROS production and reversed pterostilbene-induced growth inhibition. At 240 μM, pterostilbene showed fungicidal effects accompanied by the leakage of intracellular potassium ions, suggesting the involvement of membrane injury in addition to oxidative stress in fungicidal action. Nevertheless, the antioxidants gradually reversed the decrease in cell viability caused by pterostilbene, suggesting that ROS production mainly contributed to the lethal effect. As *Pterocarpus marsupium* extract, mainly containing pterostilbene, is used as a traditional medicine, pterostilbene has great potential for development as a preservative with fewer adverse effects.

**Keywords:** antifungal; pterostilbene; reactive oxygen species; plasma membrane injury; *Saccharomyces cerevisiae*

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## 1. Introduction

Controlling fungal growth is essential to prevent frequent food spoilage, which leads to significant economic losses [1]. Despite advancements in food preservatives, food spoilage caused by yeasts such as *Saccharomyces cerevisiae* and *Wickerhamomyces anomalus* cannot be entirely prevented. These yeasts are major sources of spoilage in preserved foods and beverages due to their high tolerance to weak acid preservatives and ethanol [2,3]. The brewing yeast *S. cerevisiae* is the primary microorganism involved in alcoholic fermentation. Among film-forming yeasts, *W. anomalus* frequently causes spoilage in beer [4,5] and bakery-related products [6,7]. Furthermore, non-*Saccharomyces* yeasts, including *W. anomalus*, contribute to the complex aroma of wine [8]. Traditional preservatives such as sorbic acid and its potassium salt are added to wine with residual sugars to prevent re-fermentation by yeasts such as *S. cerevisiae* and *W. anomalus*, but their use is limited due to their slight odor and fatty acid-like taste. Therefore, safe and effective antifungal preservatives are required to control these yeasts. Although several theories have been proposed to inhibit yeast growth using this class of compounds, the weak antifungal activities of such organic acid-based preservatives have not yet been overcome [9]. Thus, there is a pressing need to develop effective fungus-targeting preservatives to reduce food losses.

Pterostilbene is a type of trans-stilbene, which is a natural methoxylated analog of resveratrol (Figure 1). It can be derived from several plants, including *Pterocarpus marsupium* (Indian kino tree), *Pterocarpus santalinus* (red sandalwood), *Vitis vinifera* (common grape vine), and

*Vaccinium ashei* (rabbit-eye blueberry) [10–13]. Pterostilbene, in addition to resveratrol, exhibits various biological activities that are beneficial to human health, including anticancer, anti-inflammatory, antioxidant, and analgesic properties [14–17]. It also has anti-biofilm activity against the human pathogenic fungus *Candida albicans* [18]. In addition to its human health benefits, this stilbene exhibits antifungal effects against several plant pathogens, including *Phomopsis viticola*, *Phomopsis obscurans*, and *Botrytis cinerea* [19–21]. However, the antifungal mechanism of pterostilbene against fungi, including food spoilage yeasts, has not yet been thoroughly investigated. Therefore, we analyzed its action mechanism using the fungal model microorganism *S. cerevisiae* at the molecular level.

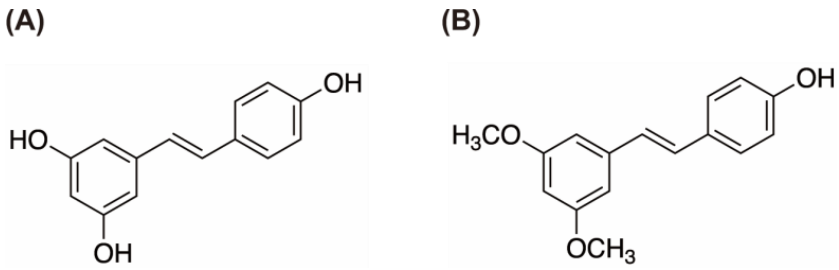


**Figure 1.** Chemical structures of resveratrol (**A**) and pterostilbene (**B**).

## 2. Materials and Methods

### 2.1. Chemicals

Pterostilbene and resveratrol were purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). α-Tocopherol acetate and 2′,7′-dichloro-fluorescein diacetate (DCFH-DA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). *N*,*N*-dimethylformamide (DMF) was obtained from Wako Pure Chemicals (Osaka, Japan). Pterostilbene and resveratrol were dissolved in DMF before use. α-Tocopherol acetate and DCFH-DA were diluted in ethanol before use. The $K^+$ ion assay kit was purchased from HACH (Loveland, CO, USA). All the other chemicals were of analytical grade.

### 2.2. Strain and Culture Conditions

The strain *S. cerevisiae* BY4741 was purchased from Thermo Fisher Scientific (Waltham, MA, USA). The yeast cells were grown in YPD broth, consisting of 1% Bacto yeast extract (Difco Laboratories, Detroit, MI, USA), 2% Bactopeptone (Difco Laboratories), and 2% D-glucose at 30 °C for 16 h without shaking prior to experiments, unless otherwise stated. All experiments, including an assay for cellular reactive oxygen species (ROS) levels, were performed in the dark.

### 2.3. Measurement of Cell Growth and Viability

To obtain an initial cell density of approximately $10^6$ cells/mL, the precultured cells were inoculated into freshly prepared YPD medium in a test tube (diameter, 10 mm) and incubated at 30 °C without shaking in the presence or absence of the test compound at various concentrations. After 24 h of culturing, we measured cell turbidity at 595 nm to estimate the minimum growth inhibitory concentration (MIC). MIC was defined as the lowest concentration of the test compound that causes a decrease in cell turbidity of less than 80% in the absence of drugs. After measuring cell turbidity, 100 µL portions of the cell culture were plated on a YPD medium containing 1.8% (*w*/*v*) agar. After 48 h of incubation at 30 °C, colony formation was assessed to confirm cell viability and to determine the minimum fungicidal concentration. The minimum fungicidal concentration (MFC) was determined as the lowest concentration of the test compound to prevent colony formation on agar plates.

### *2.4. Leakage of $K^+$ Ions*

Yeast cells cultured overnight in YPD medium were harvested by centrifugation, washed twice, and resuspended in 50 mM succinate buffer (pH 6.0) at a cell density of $5 \times 10^7$ cells/mL. The cell suspensions were supplemented with or without each compound and incubated with vigorous shaking at 30 °C for 3 h. The supernatant obtained after removing the cells by centrifugation was used for the quantification of $K^+$ ions released from the cells. The $K^+$ ions from the cells react with sodium tetraphenylborate to form potassium tetraphenylborate, an insoluble white solid. The amount of turbidity produced is proportional to the $K^+$ concentration. The measurement wavelength of the spectrophotometer was 650 nm. Quantification was performed using a $K^+$-ion assay kit based on the tetraphenylborate method [22].

### *2.5. Determination of Cellular Reactive Oxygen Species (ROS) Levels*

Cellular ROS production was measured using the DCFH-DA method, which is dependent on intracellular deacetylation and oxidation of 2′,7′-dichlorodihydrofluorescein (DCFH) to the fluorescent compound 2′,7′-dichlorofluorescein (DCF) [23,24]. This probe is highly reactive with hydrogen peroxide and has been used to evaluate ROS generation in yeast. Following preincubation ($1 \times 10^7$ CFU/mL) in YPD medium with 40 µM of DCFH-DA at 30 °C for 60 min, 3 mL of cell suspension was withdrawn and further treated with the test compounds against the yeast cells for 60 min. After treatment, the cells were washed and resuspended in 330 µL of phosphate-buffered saline buffer. The fluorescence intensity of the cell suspension was measured using a GENios (TECAN, Grödig, AT, USA) (excitation at 485 nm and emission at 535 nm). Arbitrary units were based on fluorescence intensity per $10^7$ cells.

### *2.6. Statistical Analysis*

Statistical evaluation was performed using Student's *t*-test; $p < 0.05$ was considered to represent statistical significance.

## 3. Results and Discussion

Pterostilbene constitutes approximately 90% of the extract from the dried heartwood of *P. marsupium* [11]. The *Pterocarpus* extract (Sabinsa Japan Corp., Tokyo, Japan) was preliminarily found to restrict the colony formation of *W. anomalus* NBRC (Biological Resource Center, NITE, Tokyo, Japan) 10213 and *S. cerevisiae* NBRC 100929 on potato dextrose agar plates after 48 h incubation at concentrations more than 100 µg/mL. Therefore, pterostilbene is thought to be the active constituent of the extract and is expected to be a candidate preservative with antifungal properties. To analyze the antifungal mechanism of pterostilbene, we evaluated the antifungal activities of resveratrol and pterostilbene after 24 h of incubation with the drugs based on cell turbidity in liquid YPD media. Although resveratrol did not show growth-inhibitory effects on *S. cerevisiae* at concentrations up to 480 µM (data not shown), the MIC of pterostilbene was 120 µM, as shown in Figure 2. After determining the MIC, the MFC was determined using cell suspensions. The MFC of pterostilbene was 240 µM. These results indicate that the two methoxy residues contribute to the antifungal activity of pterostilbene compared with the chemical structure of resveratrol. The two methoxy residues of pterostilbene are demethylated in resveratrol (Figure 1). In resveratrol, the hydroxyl residues are distributed at the ends of the molecule. Additionally, due to the presence of two methoxy residues at one end of the molecule and one hydroxy residue at the other end, pterostilbene possibly exhibits nonionic surfactant-like characteristics, with both hydrophobic and hydrophilic properties within the molecule. As an example of nonionic surfactants, hydroxyl groups in *n*-alkanols would interact with hydrophilic groups thrust on the membrane to form hydrogen bonds [25]. Nonpolar carbon chains in the alkanols might be folded into membrane bilayers [26–28]. Therefore, nonionic surfactants such as *n*-alkanols and alkyl gallates physically disrupt the orderly structure of the lipid bilayer in the plasma membrane, thereby affecting membrane permeability [26–28]. Therefore, we examined the effect of pterostilbene on membrane permeability in *S. cerevisiae* cells.

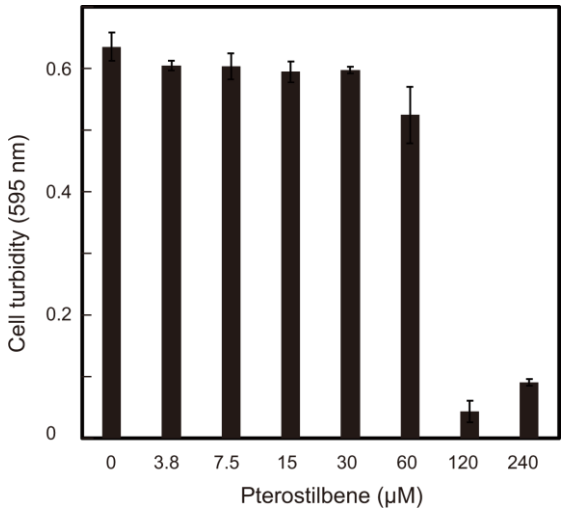


**Figure 2.** Effect of pterostilbene on the growth of *S. cerevisiae*. The exponentially growing yeast cells were incubated in YPD broth with 0–240 μM of pterostilbene at 30 °C for 24 h. After incubation, the cell turbidity was measured at 595 nm to evaluate growth inhibitory effect of pterostilbene. Each data point represents the mean ± SD of triplicate assays.

Leakage of potassium ions from cells was confirmed to evaluate the effect of pterostilbene on membrane permeability. A traditional polyene antifungal, amphotericin B (AmB), interacts with fungal ergosterols, thereby forming pores to leak potassium ions selectively [29]. It was used as a positive control in this study, causing an increase in membrane permeability. AmB at MFC significantly induced leakage after 120 min of incubation with the cells (Figure 3). Similarly, pterostilbene at MFC also significantly induced leakage, but the leakage was gradual and at lower levels than that in the AmB treatment. Therefore, the fungicidal effect of pterostilbene cannot be explained solely by its effect on membrane permeability, and other factors are possibly involved.

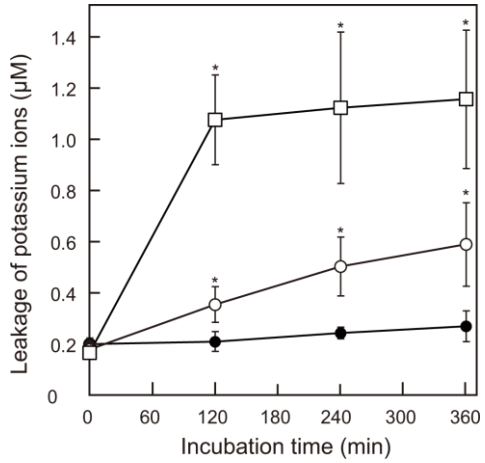


**Figure 3.** Effects of pterostilbene on $K^+$ ion leakage from cells. Exponentially growing yeast cells were incubated at 30 °C in S-buffer alone (closed circle) and the buffer containing 10 μM of AmB (open square) or 240 μM of pterostilbene (open circle). Each data point represents the mean ± SD of triplicate assays. Asterisks indicate significant differences compared to the cells incubated in S-buffer without drugs ($p < 0.05$).

Dodecanol is a medium-chain normal aliphatic alcohol that also shows nonionic surfactant-like characteristics, attributed to a hydrophobic tail alkyl chain and a hydrophilic head hydroxyl moiety. This alcohol induces oxidative stress by inducing the production of cellular reactive oxygen species (ROS) against *S. cerevisiae* [30]. In addition, AmB

also induces cellular ROS production against human pathogenic yeast species, including *Candida albicans* [31]. Thus, we evaluated the effects of resveratrol and pterostilbene on cellular ROS production using a ROS detection fluorescence probe, DCFH-DA. Prior to determining cellular ROS levels, yeast cells were incubated at 30 °C for 1 h in the YPD broth with or without the drugs. Resveratrol did not induce ROS production at concentrations up to 240 μM (Figure 4). In contrast, pterostilbene significantly induced ROS production at 120 and 240 μM, indicating that the drug caused oxidative stress in yeast cells. Maximum ROS production was observed at a concentration of 120 μM. However, it is unclear why ROS production was reduced in the 240 μM pterostilbene treatment. The nonionic surfactant nature of pterostilbene may have leaked DCFH-DA molecules, thereby reducing the intracellular concentration of the fluorescent probe. As one of the other possibilities, the reaction rate may reach a plateau point in the ROS production, where all of the oxygen source was consumed by pterostilbene.

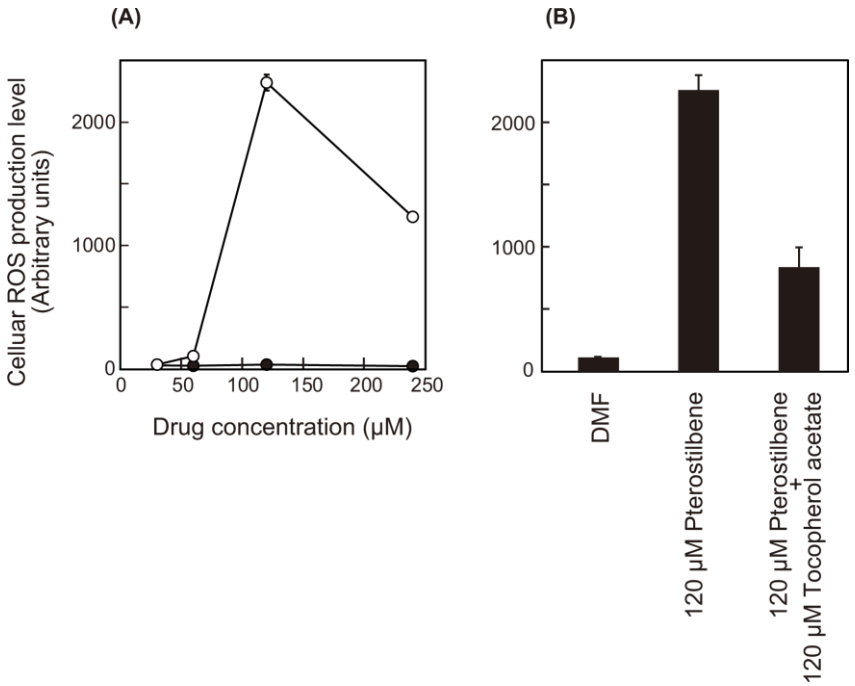


**Figure 4.** Effects of pterostilbene on cellular ROS production. (**A**) Effects of resveratrol (closed) and pterostilbene (open) concentrations on cellular ROS production. The DCFH-DA pre-stained cells were incubated in YPD broth with each drug at 30 °C for 1 h prior to the measurement of DCF fluorescence. Each data point represents the mean ± SD of triplicate assays; (**B**) effect of tocopherol acetate on pterostilbene-induced ROS production. The DCFH-DA pre-stained cells were incubated in YPD broth with 120 μM of pterostilbene and 120 μM of tocopherol acetate at 30 °C for 1 h prior to the measurement of DCF fluorescence. Each data point represents the mean ± SD of triplicate assays.

Cellular ROS production originates mainly from the mitochondrial respiratory chain [32], in addition to lipid peroxidation of the plasma membrane [33]. Moreover, in previous studies, cellular ROS production was effectively inhibited by the addition of antioxidants to the yeast culture [34,35]. Therefore, we investigated the effect of hydrophilic and lipophilic antioxidants on pterostilbene-induced ROS production. To measure ROS levels, pterostilbene was used at a concentration of 120 μM, because the maximum level of ROS production was observed at this concentration. Yeast cells were co-treated with each antioxidant and the indicated drugs at 30 °C for 1 h. The hydrophilic antioxidant ascorbic acid (1.0 mM) reduced ROS production by 23% (data not shown). In contrast, the lipophilic antioxidant, 120 μM of α-tocopherol acetate, reduced ROS production by 63% (Figure 4B). Next, we examined the effects of the two antioxidants on the decrease in cell turbidity induced by 120 μM of pterostilbene. Pterostilbene at 120 μM reduced cell turbidity by 90% and 97%, respectively, compared to drug-

free treatment after 24 and 48 h of incubation, respectively. Although ascorbic acid (1.0 mM) did not restore the decrease in cell turbidity (data not shown), 120 μM of α-tocopherol acetate restored cell turbidity by up to 30% and 90% after 24 and 48 h of incubation, respectively (Figure 5). These results indicated that the hydrophilic antioxidant ascorbic acid did not affect pterostilbene-induced ROS production or growth inhibition, whereas lipophilic α-tocopherol acetate reduced ROS production and restored growth inhibition. As ascorbic acid probably behaves as a dissociated ion in media such as a YPD broth, it is difficult for dissociation to penetrate the cell membrane and be incorporated into the cytoplasm. Therefore, higher concentrations of ascorbic acid may be required to induce its antioxidant effects. In contrast, α-tocopherol acetate readily penetrates the cell membrane, gets deacetylated, and integrates into the membranes. Therefore, α-tocopherol probably inhibits ROS production originating from membranes of cell organelles, such as the mitochondrial inner membrane, where ROS production is caused by the blockade of electron transfer in complexes I and III of the electron transport chain [36,37]. On the other hand, with regard to treatment with a fungicidal concentration of pterostilbene, α-tocopherol acetate at 240 μM did not restore yeast growth after 24 h of incubation, but after 72 h incubation, the growth was restored to 85% of the control condition (data not shown), indicating that it takes a long time to recover growth after treatment with the lethal concentration.

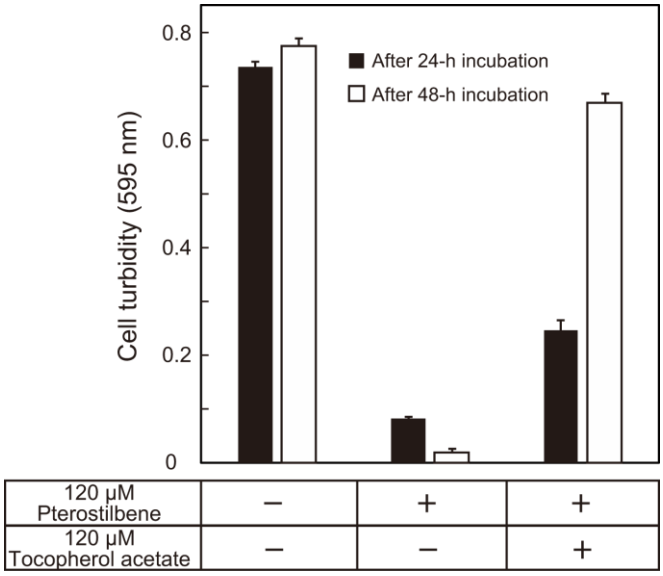


**Figure 5.** Effect of tocopherol acetate on pterostilbene-induced growth inhibition. The cells were incubated in YPD broth with or without 120 μM of pterostilbene supplemented with or without 12 μM of tocopherol acetate at 30 °C. After incubation, the cell turbidity was measured at 595 nm. Each data point represents the mean ± SD of triplicate assays.

Recently, the involvement of oxidative stress in lethal effects has been reported in antimicrobial drugs [38,39]. The drugs have been believed to disrupt cellular metabolism and the membrane as primary targets. In contrast, bacteriostatic drugs do not induce oxidative stress, such as the production of hydroxyl radicals [39]. Although antifungal AmB induces plasma membrane injury, such as the formation of pores and leakage of potassium ions against fungi, including human pathogenic *Candida albicans*, the fungicidal action of AmB is related to membrane depolarization, decreasing metabolic activity, and increasing ROS production in addition to membrane injury [40]. A sesquiterpene dialdehyde, polygodial, also acts primarily by damaging the permeability barrier of *S. cerevisiae* cells [41]. In addition, this dialdehyde also exerts fungicidal action via ROS production caused by the depletion of intracellular glutathione [42]. In this case, α-tocopherol can lower the cellular levels of ROS produced, thereby partly reducing the fungicidal effect. Following treatment with fungistatic concentrations of pterostilbene, cellular ROS were produced at maximum

levels, but membrane injury was not observed (data not shown). Pterostilbene induced fungicidal action via membrane injury and ROS production, the levels of which were lower than those induced by the fungistatic concentration. The involvement of cellular damage induced by two or more pathways in fungicidal action was consistent with previously reported AmB and polygodial fungicidal mechanisms, although slightly different. Farnesol showed growth-inhibitory effects against *S. cerevisiae*, and the mechanism involved ROS production without membrane injury [43]. This is consistent with our results showing that pterostilbene-induced fungistatic effects are dependent on ROS production.

Although pterostilbene has been reported to show antioxidative activities [14–16] also, including the blockade of ROS's surge in mammalian cell lines [44], its antioxidative effects seem to be inconsistent with its ability to induce ROS production in fungi. This discrepancy was also reported for other compounds. When phenolic compounds with potent antioxidant activity react with ROS and scavenge radicals, they produce highly active phenoxy and hydroxyl radicals, which in turn, produce new ROS [45–47]. Whether pterostilbene exhibits an antioxidant effect or exerts oxidative stress may depend on the species of drug applied. Further investigations are needed to clarify the possibility of pterostilbene-induced fungus-specific oxidative stress.

## 4. Conclusions

We showed that pterostilbene exerts fungistatic effects via cellular ROS production and fungicidal effects via plasma membrane injury in addition to ROS production. ROS contributes to both fungistatic and fungicidal activities. Phenolic compounds derived from plants, such as flavonoids and dihydroxycinnamic acids, also induce ROS production depending on the concentrations used [47]. Therefore, pterostilbene and *Pterocarpus* extract may serve as potential preservatives when used to restrict fungal growth at concentrations that do not cause side effects in humans. The replacement of certain preservatives with pterostilbene might contribute to a reduction in food losses.

**Author Contributions:** Conceptualization, K.M. and K.-I.F.; methodology, N.M., M.I., H.K., K.M., A.O. and K.-I.F.; validation, K.M., A.O. and K.-I.F.; formal Analysis, N.M. and A.O.; investigation, N.M., M.I., H.K., K.M., A.O. and K.-I.F.; data curation, A.O.; writing—original draft preparation, A.O. and K.-I.F.; writing—Review and editing, N.M., M.I., H.K., K.M., A.O. and K.-I.F.; supervision, K.M. and K.-I.F.; project administration, K.M. and K.-I.F.; funding acquisition, K.M., A.O. and K.-I.F. All authors have read and agreed to the published version of the manuscript.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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