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Protective effect of myricetin derivatives from *Syzygium malaccense* against hydrogen peroxide-induced stress in ARPE-19 cells

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Purpose: Oxidative stress is implicated in the etiology of diabetes and its debilitating complications, such as diabetic retinopathy (DR). Various flavonoids have been reported to be useful in reducing DR progression. Myricetin derivatives (F2) isolated from leaf extract of *Syzygium malaccense* have the potential to serve as functional food as reported previously. The present study was performed with the aim of determining the antioxidant potential and protective effect of myricetin derivatives (F2) isolated from leaf extract of *S. malaccense* against glucose oxidase (GO)-induced hydrogen peroxide (H_2O_2) production that causes oxidative stress in ARPE-19 (RPE) cells.

Methods: Antioxidant properties were assessed through various radical (DPPH, ABTS, and nitric oxide) scavenging assays and determination of total phenolic content and ferric reducing antioxidant power level. ARPE-19 cells were preincubated with samples before the addition of GO (to generate H_2O_2). Cell viability, change in intracellular reactive oxygen species (ROS), H_2O_2 levels in cell culture supernatant, and gene expression were assessed.

Results: F2 showed higher antioxidant levels than the extract when assessed for radical scavenging activities and ferric reducing antioxidant power. F2 protected the ARPE-19 cells against GO-H_2O_2-induced oxidative stress by reducing the production of H_2O_2 and intracellular reactive oxygen species. This was achieved by the activation of nuclear factor erythroid 2-related factor 2 (Nrf2/*NFE2L2*) and superoxide dismutase (*SOD2*), as well as downregulation of nitric oxide producer (*NOS2*) at the transcriptional level.

Conclusions: The results showed that myricetin derivatives from *S. malaccense* have the capacity to exert considerable exogenous antioxidant activities and stimulate endogenous antioxidant activities. Therefore, these derivatives have excellent potential to be developed as therapeutic agents for managing DR.

Diabetic retinopathy (DR) is becoming a leading cause of blindness among one third of patients with diabetes [1]. The combined effects of hyperglycemia and hypertension accelerate the progression of DR among patients with type II diabetes mellitus [2]. The correlation among hyperglycemic condition, oxidative stress, and changes in redox homeostasis is well-known to be among the factors contributing to the pathogenesis of DR. Continuous exposure of retinal microvessels to a high circulating glucose environment causes an increase in oxidative stress through overproduction of reactive oxygen species (ROS), inflammation, activation of protein kinase C (PKC), hexosamine, and polyol pathways, as well as formation of advanced glycosylation end product (AGE) [3-5]. The synergistic effect of oxidative stress and other metabolic changes further accelerates drastic damage of capillary cells in the retinal microvasculature [5,6].

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High levels of superoxide anion have been observed in retinal endothelial cells treated with high glucose [7]. Reduced expression of antioxidant defense enzymes, such as catalase, glutathione peroxidase (GPx) and superoxide dismutase (SOD), has been strongly associated with the progression of DR [5]. Glutathione (GSH), the intracellular antioxidant, has also been reported to be in lower amounts in patients with DR [8]. Nevertheless, studies have confirmed that specific antioxidants and supplements could reduce the rate of DR progression by strengthening the antioxidant defenses [9,10]. Discovery of new drugs, functional foods, or antioxidants for the treatment and prevention of DR either through oral administration or as topical use is ongoing.

The most active fraction isolated from the leaf extract of *Syzygium malaccense* (L.) Merr. & L.M. Perry ("Malay apple") has been reported to contain myricetin derivatives (flavonoid glycosides), i.e., 3-O-L-rhamnoside (myricitrin; 77% v/v), myricetin 3-alpha-L-arabinofuranoside, and myricetin 3′-glucoside [11]. The antioxidant property of the leaf extract was speculated to be mainly attributed to the myricetin derivatives [11]. In addition, the derivatives have been shown to exhibit considerable in vitro antihyperglycemic

potential as evident from their ability to inhibit carbohydrate hydrolyzing enzymes (α-glucosidase and α-amylase) and activate the insulin signaling pathway (similar to insulin) in differentiated 3T3-L1 preadipocytes [12]. The findings support the traditional use of the plant to treat diabetes [13] and reflect the potential use of the derivatives to manage diabetes and its related complications.

Thus, the aim of the present study was to assess the possible protective effect of myricetin derivatives isolated from the ethanolic leaf extract of *S. malaccense* against H_2O_2-induced stress, generated through glucose oxidase (GO) activity in ARPE-19 (RPE) cells. This is the first report to describe the antioxidant and protective potential of the active components and extract of *S. malaccense* against DR using an in vitro model.

METHODS

Materials: ARPE-19 (ATCC® CRL-2302™) RPE cells (Organism: *Homo sapiens*; human) were purchased from American Type Culture Collection (ATCC; Manassas, VA). The species of origin (human) of the cell line (ARPE-19; passage 19; Lot number: 59,270,158) was authenticated by the supplier (ATCC) using the cytochrome C oxidase I gene (COI) assay and short tandem repeat (STR) profiling to verify the human unique DNA profile and rule out intraspecies contamination. According to ATCC, the STR analysis was performed on eight core STR loci (D5S818, D13S317, D7S820, D16S539, vWA, TH01, TPOX, CSF1PO) and gender determining locus (amelogenin). The loci were amplified using the Promega PowerPlex® 18D System (Madison, WI) and the ANSI/ATCC ASN-0002-2011 Standard for Authentication of Human Cell Lines. The amplicons were separated using capillary electrophoresis and data were analyzed using GeneMapper® software (Thermo Fisher Scientific, Waltham, MA). Myricitrin (purity >99.0%), myricetin (purity >96.0%), fetal bovine serum (FBS), o-tolidine, 2′,7′-dichlorofluorescin diacetate (DCFH-DA), and chemicals for antioxidant assays were procured from Sigma Chemical Co., Ltd. (St. Louis, MO). Glucose oxidase from *Aspergillus niger* was purchased from Biochemika Fluka (St. Louis, MO). Gibco® Dulbecco's Modified Eagle Medium/nutrient mixture F12 (DMEM/F12) was purchased from Invitrogen Corporation (Carlsbad, CA). Chemicals and reagents needed for gene expression study were supplied by Qiagen (Frederick, MD). Miscellaneous reagents used were of analytical grade.

Isolation of myricetin derivatives (F2) from the S. malaccense ethanolic leaf extract: S. malaccense leaf was subjected to ethanolic extraction, and the myricetin derivative–rich fraction (F2) was isolated from the extract through a standard fractionation protocol established using high-performance liquid chromatography (HPLC) [11]. The samples were stored at − 20 °C. The samples were reconstituted with dimethyl sulfide (DMSO) at an approximate concentration and filter sterilized before use.

Determination of antioxidant properties: Antioxidant assays such as 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), and nitric oxide (NO) free radical scavenging assays for various samples (*S. malaccense* ethanolic leaf extract, myricetin derivative–rich fraction isolated from the extract (F2), and standard compounds such as myricitrin and myricetin) were performed as described in a previous report [11]. Briefly, the DPPH assay was performed by mixing and incubating various samples at different concentrations (5 µl) with 195 µl ethanolic DPPH reagent (100 mM) for 20 min and absorbance was read at 515 nm in a 96-well microtiter plate. The ABTS assay was carried out by incubating 10 µl of samples with ABTS reagent (90 µl) for 4 min. Absorbance of the mixture was measured at 734 nm in a 96-well microtiter plate. The NO assay was conducted by mixing the samples (10 µl) with 90 µl of sodium nitroprusside reagent (final concentration: 10 mM; prepared freshly in phosphate buffered solution, pH 7.3) and incubating the mixture for 2.5 h in the presence of light. This was followed by the addition of 100 µl of Griess-Ilosvay's nitrite reagent and incubation for 30 min in dark before measuring the absorbance at 540 nm using a 96-well microtiter plate. Percentage of radical scavenged (DPPH, ABTS, and NO) was calculated as stipulated below to determine the IC_{50} value for the samples:

$$\%\ \text{of radical scavenged} = \frac{\text{Abs}_{control} - \text{Abs}_{sample}}{\text{Abs}_{control}} x100$$

Abs stands for absorbance. Control is the sample volume replaced with diluents.

The total phenolic content (TPC) was determined by mixing and incubating 50 µl of the samples with 50 µl of Folin-Ciocalteu phenol reagent (10% v/v) for 3 min in dark at room temperature. This was followed by the addition and incubation of the mixture with 100 µl of sodium carbonate solution (10% w/v) for 1 h. Absorbance was read at 750 nm in a 96-well microtiter plate. The average TPC value was calculated and stated as the amount of the phenolic content (mg gallic acid equivalents, GAE) per gram of sample [11]. Additionally, ferric reducing antioxidant power (FRAP) assay was performed based on the protocol described by Benzie and Strain [14] with a slight modification, using 96-well microtiter

plates. Briefly, stock solutions of (a) 0.3 M sodium acetate buffer, (b) 10 mM 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) in 40 mM hydrochloric acid and (c) 20 mM ferric chloride solution were prepared freshly prior to the assay and mixed at a ratio of 10:1:1 (a:b:c). This working solution (FRAP reagent) was protected from exposure to light. The samples (10 µl) at various concentrations were mixed with 300 µl of FRAP reagent, and the respective absorbance was measured after 4 min at 593 nm using a microplate reader. A stock solution of ferrous sulfate, Fe_2SO_4 (final concentration: 0–30 µM), was subjected to the assay to derive a standard curve to calculate FRAP values. The mean FRAP value was expressed as the Fe (II; mM) equivalent in 1 gram of sample.

Cell culture: The ARPE-19 cells were cultured according to the protocol described by the supplier (ATCC). The cells were grown in DMEM/F12 containing 10% (v/v) heat-inactivated FBS and 1% (v/v) penicillin (100 U/ml)-streptomycin (100 U/ml) solutions at 37 °C in a humidified atmosphere of 5% carbon dioxide (CO_2). Cells of passages 21–24 were used for the experiments. The cells were sub-cultured every 3–4 days when they reached 70–80% confluence.

Induction of oxidative stress using glucose oxidase treatment: ARPE-19 cells (10,000 cells/well) were allowed to adhere to the 96-well cell culture plates overnight at 37 °C in 5% CO_2 atmosphere. The cells were pretreated with various concentrations of samples (200 µg/ml or less for the extract and F2 and 40 µg/ml or less for myricitrin and myricetin) for 2 h before incubation with glucose oxidase, GO (final concentration, 12 mUnit/ml; to generate H_2O_2) for 2 h in 96-well cell culture plates. The concentrations described above were not toxic to the cells when determined using the 3,4-5-dimethylthiazol2-yl)-2,5-diphenyl tetrazolium (MTT) assay (Appendix 1). After the incubation period, the culture supernatants were removed, and the wells were rinsed with PBS (136.9 mM sodium chloride, 2.7 mM potassium chloride, 8.1 mM disodium hydrogen phosphate, 1.5 mM potassium dihydrogen phosphate, pH 7.3) and replaced with fresh media. Then, cell viability was determined using the MTT assay. MTT solution (a final concentration of 0.5 mg/ml/well) was added, and the incubation was continued for 3 h at 37 °C. DMSO (100%) solution was added to solubilize the formazon crystals. The culture plate was then placed on a plate shaker for 5 min, and the absorbance was measured at 570 nm using a microplate reader. The percentage of cell viability was determined as stipulated below:

$$\%\ \text{of cell viability} = \frac{\text{Abs}_{\text{treated cells}} - \text{Abs}_{\text{blank}}}{\text{Abs}_{\text{untreated cells}}} x100$$

Untreated cells = cells grown in basal media.

Blank = cells grown in sample diluent (DMSO, final concentration of 0.1%).

The protective effect of the samples was determined through three independent cell stimulation experiments (with three technical replicates in each stimulation).

Measurement of intracellular ROS: The intracellular amount of ROS was determined using the DCFH-DA fluorescent probe as described previously [15] with a slight modification. Cells were pretreated with samples (2 h) followed by GO for 2 h as described in the section above. DCFH-DA (5 µM; final concentration) was introduced 30 min before the end of the incubation period with the oxidant. The adhered cells were rinsed twice with PBS. The fluorescence intensity of the cell monolayers was measured at 495 nm (excitation wavelength) and 525 nm (emission wavelength) using a fluorescence microplate reader (Infinite® M1000 PRO series, Tecan Group Ltd., Mannedorf, Switzerland, coupled with i-control™ data analysis software). The fold change of the ROS was determined as stipulated below:

$$\text{Fold change} = \frac{\text{Fluorescence intensity of cells treated with sample(s)}}{\text{Flourescence intensity of untreated cells}}$$

The fold change was normalized to 10,000 cells for all GO-H_2O_2-treated cells with and without pretreatment of samples by considering the number of viable cells after the treatment based on the cell viability assay results.

Determination of H_2O_2 levels: The level of H_2O_2 in the culture supernatants of the cells was determined using a protocol described previously [16] with a slight modification. The culture supernatants of incubations with oxidants (with and without pretreatment of samples) were collected. Tolidine (1% w/v) stock solution was prepared by dissolving 10 mg tolidine in 1 ml of absolute ethanol. The working solution comprised 2 units/ml peroxidase and 0.15% of tolidine (from the tolidine stock) in a total volume of 10 ml, 0.2 M sodium acetate buffer, pH 5.0. Fifty microliters of the sample (culture supernatants collected after the incubation) were added to 50 µl of the working solution in a 96-well microtiter plate. The mixture was incubated for 1 h at room temperature, and the absorbance was measured at 625 nm using a microplate reader. A standard curve derived using the H_2O_2 standard

(0–2,500 µM) was used to determine the amount of H_2O_2 present in the culture supernatants of various treated samples.

Gene expression assessment: Samples that showed statistically significant protective effects were subjected to gene expression study. ARPE-19 cells (7.3×10^5 cells/T25 flask) were seeded and allowed to adhere overnight before proceeding with the selected treatments and gene expression analysis. Cells grown in basal media (untreated) served as control. The RNA extraction, determination of the purity and integrity of the RNA samples, synthesis of first-strand cDNA, and quantitative real-time reverse transcriptase-PCR (RT–PCR) were performed according to the method described previously [12]. Briefly, RNA was extracted using Qiazol reagent and miRNeasy Mini Kit. The purity of the RNA samples was determined on Nano-Drop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE) based on optimum absorbance ratio of 230/260 nm and 260/280 nm. Concentration and integrity of the RNA samples were analyzed on Agilent 2000 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA) using Agilent RNA 6000 kit. First strand cDNA was synthesized using RNA samples with high quality of RNA integrity number (RIN) ranging from 9-10. The reverse transcription procedure was performed using RT2 First strand kit (Qiagen) with optimized thermal cycling condition on BioRad C1000™ (Bio-Rad Laboratories Inc., Hercules, CA). Quantitative RT-PCR was conducted using custom RT2 profiler PCR array (CAPH12983) supplied by Qiagen which works based on SYBR Green detection chemistry on Applied Biosystem 7500-Fast Real-Time PCR (Thermo Fisher Scientific Inc., Waltham, MA) system. The program run was performed as stipulated below:

Step1 (1 cycle): 95 ˚C for 10 min.

Step 2 (40 cycles): 95 ˚C for 15 sec followed by 60 ˚C for 1 min. The genes investigated in this study are listed in Table 1. The gene expression data were analyzed using the comparative C_T ($2^{-\Delta\Delta Ct}$) scheme [17]. Final data that were normalized against human 18SrRNA (endogenous control) are expressed as the n-fold of regulation.

Statistical analysis: Results are expressed as the mean ± standard deviation (SD) unless otherwise stated. The values were computed from triplicate measurements using Microsoft Excel 2007 (Redmond, WA). All data were analyzed using the one-way ANOVA Tukey's post hoc test using SPSS software version 17 (Chicago, IL). In statistical analysis, * denotes $p<0.05$, ** denotes $p<0.01$, and *** denotes $p<0.001$. In the paired t test, # denotes $p<0.05$, ## denotes $p<0.01$, and ### denotes $p<0.001$.

Table 1. List of genes assessed in gene expression study.

Genes	Gene symbol	EnsemblGene ID	OMIM number	RefSeq Accession Number	UniGene Number
Human 18S ribosomal RNA	18SrRNA	N/A	180450	X03205	-
BCL2-associated X protein	Bax/BCL2L4	ENSG00000087088	600040	NM_004324	Hs. 624291
C-reactive protein, pentraxin-related	CRP	ENSG00000132693	123260	NM_000567	Hs. 76452
Caspase 3, apoptosis-related cysteine peptidase	CASP3	ENSG00000164305	600636	NM_004346	Hs.141125
Cathepsin D	CTSD/CLN10	ENSG00000117984	116840	NM_001909	Hs. 654447
Glutathione peroxidase 1	GPx1	ENSG00000233276	138320	NM_000581	Hs. 76686
Heat shock 27kDa protein 1	HSPB1/HSP27	ENSG00000106211	602195	NM_001540	Hs. 520973
Interferon, gamma	IFN-γ/IFG	ENSG00000111537	147570	NM_000619	Hs. 856
Lysyl oxidase	LOX	ENSG00000113083	153455	NM_002317	Hs. 102267
Mitogen-activated protein kinase 1	MAPK1/ERK	ENSG00000100030	176948	NM_002745	Hs. 431850
Mitogen-activated protein kinase 14	MAPK14/p38	ENSG00000112062	600289	NM_001315	Hs. 485233
Nitric oxide synthase 2, inducible	NOS2/NOS	ENSG00000007171	163730	NM_000625	Hs. 709191
Nuclear factor (erythroid-derived 2)-like 2	NFE2L2/NRF2	ENSG00000116044	600492	NM_006164	Hs. 744006
Superoxide dismutase 1, soluble	SOD1	ENSG00000142168	147450	NM_000454	Hs. 443914
Superoxide dismutase 2, mitochondrial	SOD2/MnSOD	ENSG00000112096	147460	NM_000636	Hs. 487046
Toll-like receptor 2	TLR2	ENSG00000137462	603028	NM_003264	Hs. 519033

* Gene name and RefSeq Accession number were obtained from the Qiagen product specification sheet. UniGene number with "Hs." prefix indicates "*Homo sapiens*."

Table 2. Antioxidant activities of myricitrin, myricetin, leaf extract and active fraction (F2) of *Syzygium malaccense*.

Samples	Scavenging assays (IC_{50}; µg/ml)			FRAP (mM Fe (II) /gram sample)	TPC (mg gallic acid /gram sample)
	DPPH	ABTS	NO		
Myricitrin	3.61±1.04	9.84±1.11	>60	3813.67±54.09	644.13±46.87
Myricetin	2.45±1.07	5.20±1.08	>60	1937.5±76.03	894.63±31.77
Syzygium malaccense	16.65±1.17[a]	47.27±1.05[a]	333.00±1.19 [a]	847.24±21.59	128.32±20
F2	7.62±1.09***	17.21±1.14***	42.73±1.15***	2160.30±89.12***	353.70±35.10***

[a]Adapted from [11] for comparison. Data are shown as mean ± standard error (n=3). Paired *t* test between *S. malaccense* extract and F2 showed the significance value of ***p<0.001.

RESULTS

Antioxidant property and TPC of S. malaccense leaf extract and its active fraction (F2): Table 2 depicts the TPC, the concentration of the sample needed to scavenge 50% of the radicals (IC_{50}), i.e., the DPPH, ABTS, and NO and FRAP levels of myricetin, myricitrin, leaf extract of *S. malaccense*, and its active fraction, F2. Standard myricitrin and myricetin were also included in this study as a comparison to the *S. malaccense* leaf extract and F2. Myricetin and myricitrin are classified as flavonols, a subgroup of flavonoids, and are known for their good radical scavenging property [18,19]. In addition, the aglycone myricetin is a major metabolic product of the flavonoid glycoside, myricitrin [20,21].

The IC_{50} values (DPPH, ABTS, and NO) of the *S. malaccense* extract were adapted from our previous study [11]. F2 showed statistically significant improvement in its antioxidant property (based on its reduced IC_{50} and increased FRAP values) when compared to the ethanolic leaf extract (Table 2). F2 was able to scavenge DPPH and NO radicals efficiently and showed good ferric reducing antioxidant power that was comparable to the effect exerted by myricetin. Myricitrin and myricetin showed better DPPH and ABTS scavenging activities than the leaf extract of *S. malaccense* and F2. Nevertheless, F2 was a better nitric oxide scavenger compared to myricitrin and myricetin.

GO-H_2O_2-induced stress model: The ARPE-19 cells were preincubated for 2 h with the samples before GO treatment to allow H_2O_2 production. The protective effect of the test samples was monitored using the cell viability (MTT) assay as well as changes in intracellular ROS and H_2O_2 levels in culture supernatants. Figure 1 shows the cytoprotective effects of the test samples to improve and restore the viability of the ARPE-19 cells that were exposed to GO-H_2O_2-induced oxidative stress. Two hours of 12 mUnit of GO treatment resulted in 45% reduction in cell viability (Figure 1). Interestingly, myricetin and F2 that were preincubated with the cells before the GO treatment showed statistically significant protection in a dose-dependent manner against GO-H_2O_2-induced cell death (Figure 1). Myricetin (40 µg/ml) showed protection by restoring the viability of the cells by 88% (p<0.001) followed by F2 (200 µg/ml) by 91% (p<0.001). Myricitrin and *S. malaccense* leaf extract exhibited statistically significant protection (p<0.001) of the retinal cells against GO-H_2O_2-induced cell death. However, the effect was not dose-dependent. F2 at a concentration of 50 µg/ml contained 39 µg of myricitrin (calculated based on liquid chromatography mass spectrometry, LCMS data of 77% volume [11]). F2 improved the viability of the retinal cells but not as effectively as myricitrin at 40 µg/ml. However, after the concentration of F2 was increased to 100 µg/ml (myricitrin content, 77 µg), the fraction was able to improve the cell viability up to 75%, which was similar to the effect of myricitrin (40 µg/ml). At 200 µg/ml, F2 (myricitrin content, 154 µg) enhanced the cell viability up to 91%, which was higher than the effect exerted by the *S. malaccense* leaf extract (Figure 1).

The GO-H_2O_2-treated cells showed an approximate 12-fold increase in the intracellular ROS level (Figure 2) with the presence of approximately 1,950 µM H_2O_2 in the culture supernatant (Figure 3). These values were statistically significantly reduced when the cells were preincubated with the samples for 2 h before the introduction of GO-H_2O_2-induced stress (Figure 2 and Figure 3). Myricetin, F2, and myricitrin attenuated intracellular ROS (Figure 2) and H_2O_2 generation (Figure 3) statistically significantly (p<0.001) in a dose-dependent manner. The *S. malaccense* leaf extract scavenged ROS at concentrations ≤25 µg/ml (p<0.001) and reduced the H_2O_2 levels statistically significantly at all concentrations tested.

Gene expression study: The preincubation of myricetin (40 µg/ml) and F2 (200 µg/ml) for 2 h showed statistically significant protection of the ARPE-19 cells against GO-H_2O_2-induced stress (Figure 1). Thus, the molecular mechanism of these compounds was assessed using the

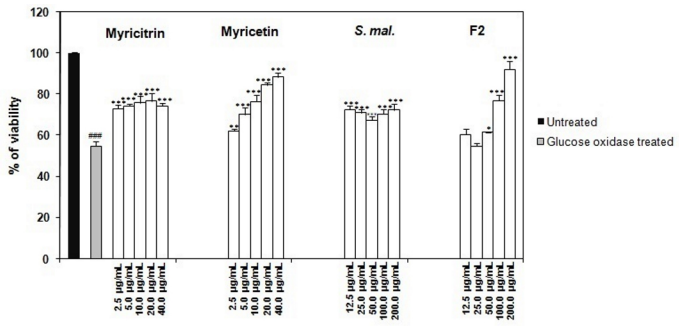

Figure 1. Cytoprotective effects of myricitrin, myricetin, leaf extract of *Syzygium malaccense*, and F2 (myricetin derivatives) when pretreated for 2 h against glucose oxidase-H_2O_2 (GO-H_2O_2)-induced oxidative stress in ARPE-19 cells. The concentration of GO used is 12 mUnit/ml. Control (100%) is the untreated cells grown in basal medium. Data are expressed in mean ± standard deviation (SD); n=3. The paired *t* test between the GO-treated cells and control showed statistical significance at ###p<0.001. The one-way ANOVA Tukey's post hoc test showed statistical significance at *p<0.05, ** p<0.01, and *** p<0.001 when the test samples were compared to the GO-treated cells. *S. mal* = *Syzygium malaccense*.

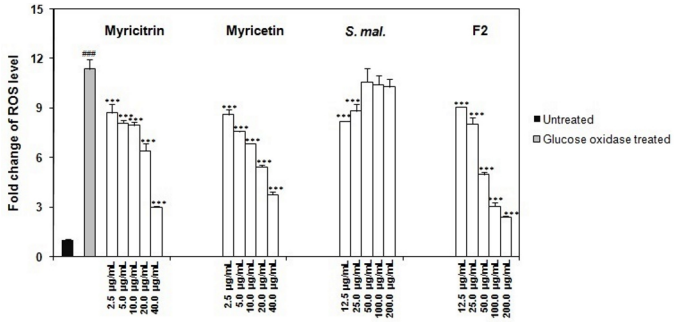

Figure 2. Intracellular reactive oxygen species (ROS) levels in ARPE-19 cells when pretreated with myricitrin, myricetin, leaf extract of *Syzygium malaccense*, and F2 (myricetin derivatives) for 2 h before exposure to GO-H_2O_2 induced stress. The concentration of glucose oxidase (GO) used is 12 mUnit/ml. Intracellular ROS that was generated during the incubation period was monitored using the 2′,7′-dichlorofluorescin diacetate (DCFH-DA) fluorescent probe. Control (100%) is the untreated cells grown in basal medium. The fold change was normalized to 10,000 cells for all GO-treated cells with and without pretreatment of samples by considering the number of viable cells after the treatment. Data are expressed in mean ± standard deviation (SD), n=3. The paired *t* test between the GO-treated cells and control showed statistical significance at ###p<0.001. The one-way ANOVA Tukey's post hoc test showed statistical significance at *p<0.05, ** p<0.01, and *** p<0.001 when the test samples were compared to the GO-treated cells. *S. mal* = *Syzygium malaccense*.

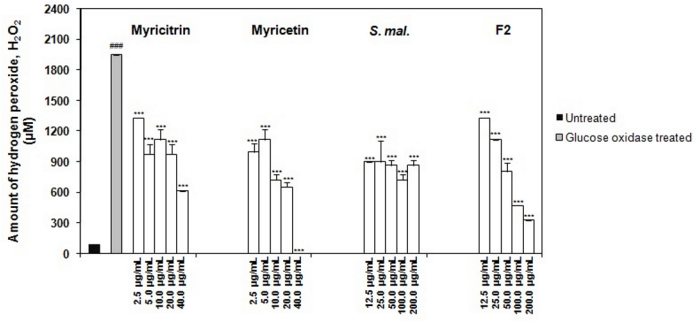

Figure 3. Hydrogen peroxide (H_2O_2) levels in ARPE-19 cell culture supernatants when pretreated with myricitrin, myricetin, leaf extract of *Syzygium malaccense*, and F2 (myricetin derivatives) for 2 h before exposure to GO-H_2O_2 induced stress. The concentration of GO used is 12 mUnit/ml. Control (100%) is the untreated cells grown in basal medium. Data are expressed in mean ± standard deviation (SD); n=3. The paired *t* test between the GO-treated cells and control showed statistical significance of ###p<0.001. The one-way ANOVA Tukey's post hoc test showed statistical significance at *** p<0.001 for all the test samples and the GO-treated cells when compared to the GO-treated cells only. *S. mal* = *Syzygium malaccense*.

quantitative real-time RT–PCR technique. Table 1 depicts the list of genes investigated in this study. Figure 4 shows the gene expression results for the GO-H_2O_2-treated cells in the presence or absence of preincubation with F2 and myricetin.

GO-H_2O_2-induced stress caused statistically significant upregulation of the *MAPK1*, *NFE2L2*, *SOD2*, *GPx1*, *CASP3*, *Bax*, *HSPB1*, *IFN-γ*, *NOS2*, and *LOX* genes (Gene ID, OMIM, RefSeq Accession and UniGene numbers are listed in Table 1) and downregulation of the *MAPK14* gene when compared to the control (untreated retinal cells). The introduction of oxidative stress did not result in changes in the expression of the *SOD1*, *CTSD*, *CRP*, and *TLR2* (Gene ID, OMIM, RefSeq Accession and UniGene numbers are listed in Table 1) genes. Treatment of F2 along with GO caused enhanced upregulation of the *NFE2L2* ($p<0.001$) and *SOD2* ($p<0.001$) genes. F2 reversed the expression of the *NOS2* (downregulation; $p<0.01$) gene compared to the GO-H_2O_2-treated cells. F2 treatment did not affect the pattern of gene expression influenced by GO-H_2O_2-induced stress for the *MAPK1*, *MAPK14*, *GPx1*, *CASP3*, *Bax*, *HSPB1*, *IFN-γ*, and *LOX* genes.

Myricetin downregulated the expression of the *MAPK1* ($p<0.001$), *GPx1* ($p<0.001$), *CASP3* ($p<0.01$), *Bax* ($p<0.01$), *HSPB1* ($p<0.05$), *IFN-γ* ($p<0.01$), and *LOX* ($p<0.001$) genes when compared to the GO-H_2O_2-treated cells. Treatment of myricetin did not show statistically significant changes in the expression of the *MAPK14* and *NOS2* genes that were affected by GO-H_2O_2-induced stress. It did not upregulate the *NFE2L2* and *SOD2* genes further as observed with the F2 treatment.

DISCUSSION

Antioxidant capacity of myricetin, myricitrin, leaf extract of S. malaccense, and its active fraction: The pure standard compounds (myricitrin and myricetin) showed higher TPC compared to F2 and the crude extract as the values were determined per gram of the sample and reflected the purity of the respective samples. Free radical scavenging activity is mainly attributed to the presence of phenolic compounds, such as phenolic acids, phenolic diterpenes, and flavonoids, in plants [22]. In the present study, myricetin exhibited a better radical (DPPH and ABTS) scavenging property than myricitrin (Table 2). This result concurs with another report that showed the aglycone myricetin was more active than its glycosidic form (myricitrin or myricetin-3-rhamnoside) when assessed for antioxidant property in methyl linoleate, a non-polar lipid model widely used in antioxidant testing [23]. Myricetin is generally more potent than its glycosides due to the presence of more hydroxyl groups in the benzene ring structures, namely, the presence of a 3-hydroxyl group (C-ring) and a 5-hydroxyl group (A-ring), a 3′,4'-dihydroxy structure (B-ring), and a 2,3-double bond in combination with a 4-oxo group (C-ring) [24,25]. The weaker antioxidant property of myricitrin has been attributed to steric hindrance contributed by the rhamnoside attached to the flavonol at the C3 position [23]. Nevertheless, myricitrin showed higher ferric reducing power compared to myricetin.

The higher TPC in F2 compared to the extract is attributed to the predominant presence of myricitrin (77% based on LCMS analysis [11]). Myricetin derivatives isolated from the ethanolic leaf extract of *S. malaccense* (F2) clearly showed an improved and considerable antioxidant property when

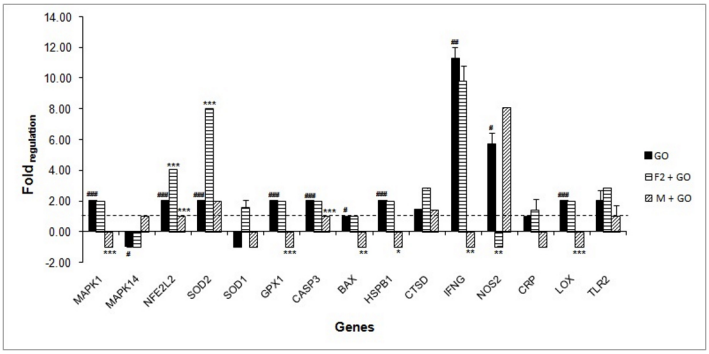

Figure 4. Gene expression profile of selected markers in glucose oxidase-hydrogen peroxide (GO-H_2O_2) –induced stress treated ARPE-19 cells that were subjected to a 2 h pretreatment with samples (M and F2). M (40 µg/ml) and F2 (200 µg/ml) represent myricetin or myricetin derivative–rich fraction, respectively. The concentration of glucose oxidase (GO) used is 12 mUnit/ml. Quantitative real-time reverse transcriptase-PCR was performed using a custom RT^2 profiler PCR array. y-axis represents the n-fold of regulation (mean ± standard deviation (SD) of two separate experiments) against control (untreated cells grown in basal media) after normalization with human 18SrRNA, the endogenous control. The dotted line represents the fold regulation of the untreated control (1.00). The paired *t* test between the GO-treated cells and control showed statistical significance at $^{\#}p<0.05$, $^{\#\#}p<0.01$, and $^{\#\#\#}p<0.001$, respectively. The one-way ANOVA Tukey's post hoc test showed statistical significance at *p<0.05, ** p<0.01, and *** p<0.001 when the test samples (M and F2) were compared to the GO-treated cells only.

compared to the extract. F2 reflected lower TPC, DPPH, and ABTS scavenging activities in contrast to the standard compounds (myricetin and myricitrin). The presence of other myricetin derivatives that contain arabinofuranoside and glucoside in addition to rhamnoside (myricitrin) could possibly caused a concerted effect in attenuating the scavenging property. The FRAP potential of F2 was higher than that of myricetin but lower than that of myricitrin. However, the NO scavenging property of F2 was stronger than myricetin and myricitrin (Table 2). This clearly reflected the presence of the synergic antioxidant potential of the isolated compounds. The varied antioxidant property of F2 indicates that antioxidant activity is not necessarily always correlated to the phenolic content of the compounds [26,27]. The antioxidant property of phenolic compounds is dependent on their modes of action (ability to scavenge, inhibit, or chelate), as well as their stereochemical structures [28].

Protective effect of S. malaccense leaf extract and myricetin derivative–rich fraction against oxidative stress in ARPE-19 cells: Retinal cells are often subject to high risk of oxidative stress due to ROS, particularly H_2O_2 as they are exposed to oxygen tensed-environment [29,30]. H_2O_2 is constantly generated through oxygen metabolism in mitochondria, light irradiation of melanin, and outer segment phagocytosis of shed photoreceptors [31-33]. H_2O_2 easily crosses cell membranes, confines into various parts of cells, and breaks down to hydroxyl radicals, which contribute to the significant cell cytotoxic condition [31,34,35]. The oxidant can be added directly in pulse or through enzymatic (GO) production in experimental models. However, the latter has been proposed to be useful as it generates a sustained level of H_2O_2 using glucose in the medium as substrate. This would be favorable for establishing a steady chronic or mild stress condition which can be followed by downstream measurement of the injury in cells in addition to evaluating the protective effects of antioxidants [36]. ARPE-19 is a common retina model used to assess oxidative stress particularly H_2O_2-induced injury to cells [37,38].

In the present study, oxidative stress was induced in ARPE-19 cells using glucose oxidase (to generate H_2O_2) to mimic the pathogenesis of DR. A pretreatment period of the samples for 2 h before the introduction of GO-H_2O_2-induced stress was used in view of the relatively short half-life of myricetin at a higher pH range. Myricetin, an aglycone, is a major metabolic product of the flavonoid glycoside, myricitrin [20,21] and therefore, was used as a standard for comparison in the present study. The stability of bioflavanoids has been shown to be predominantly affected by the pH of the environment [39]. Myricetin and myricitrin have been shown to be unstable at neutral and alkaline pH compared to acidic pH when incubated for 3 h at 37 °C. Myricetin has a shorter half-life (0.51 h) than myricitrin (13.08 h) at pH 6.8 [39] and is more rapidly degraded. A separate study reported a half-life and a degradation rate of 2.11 ± 0.34 h and 0.3343 ± 0.0547 h^{-1}, respectively, for myricetin in phosphate buffer at pH 6.8 [40]. An incubation period of 2 or 4 h was used for myricetin in a previous study to assess the independent potential of the compound to induce transcription factor Nrf2 (phase 2 detoxifying enzyme) in ARPE-19 cells [41]. Thus, in the present study the preincubation period of myricetin, myricitrin, F2 containing myricitrin was optimized to 2 h, followed by an additional 2 h incubation with GO-H_2O_2 to induce oxidative stress. Although the flavonoid compounds used in this study have shown antioxidant properties, one should not rule out the possibility that after a longer period of incubation, they may behave as prooxidants. This can be attributed to the stress level contributed by the compound's structural characteristics and the concentration used [42,43]. The focus of the present study was to assess the direct protective effects of the bioflavonoids against oxidative stress in retinal cells. However, future investigations should include a longer incubation period to confirm the possible prooxidant effect.

The viability of retinal cells was affected statistically significantly by GO-H_2O_2 induced stress (Figure 1). This was attributed to the cytotoxic effect of H_2O_2 (oxidant) released through the GO enzymatic reaction [36]. The protection against stress-induced cytotoxicity conferred by the test samples (concentration range of 40 or 50 µg/ml) in descending order is as follows: myricetin (40 µg/ml) > myricitrin (40 µg/ml) > *S. malaccense* leaf extract (50 µg/ml) > F2 (50 µg/ml; Figure 1). F2 exerted better protection of retinal cell viability than the extract at higher concentrations due to the presence of pure myricetin derivatives. The pattern of protection corresponded with the antioxidant properties of the samples (Table 2) as discussed previously in which myricetin was shown to be the most potent antioxidant followed by F2, myricitrin, and the leaf extract. This concurred with the ability of the samples to attenuate intracellular ROS in ARPE-19 cells (Figure 2) and H_2O_2 level in the culture supernatants (Figure 3), effectively. The consistent lower protective effect exerted by myricitrin when compared to myricetin could be attributed to the presence of an additional bulky glycoside group on the compound that could hinder its reaction with ROS.

The findings of the in vitro investigation may not be literally extrapolated to the in vivo condition. Although F2 showed effective protection at a higher concentration, 200 µg/ml (myricitrin content, 154 µg [11]), upon metabolic

digestion, it will be degraded to myricetin and other minor components [20,21]. Therefore, the effective dose of the metabolites is likely to be considerably lower. Administration of myricitrin to rats (100 mg/rat; n=5) has been shown to result in urinary excretion of myricetin (1.46 mg) and 3,5-dihydroxy-phenylacetic acid (0.48 mg) as the metabolites in 24 h [21]. It is feasible to speculate that myricetin will be bioavailable at a far lower dose approximately 1.5% (3 µg/ml) from the consumption of F2 at a concentration of 200 µg/ml containing 154 µg of myricitrin. Myricetin was shown to be statistically significantly effective in protecting retinal cells against oxidative stress even at a very low concentration (2.5 µg/ml) as shown in the present study. Thus, even after metabolic digestion to yield myricetin, the compound could greatly attenuate stress-induced complications through direct inhibition or activation of the endogenous protective mechanism. Myricetin is also well-known for its numerous health benefits which include antioxidant, antihyperglycemic, anti-inflammatory, antihypertensive, and anticancer properties [12,44,45]. Therefore, F2 has potential as an alternative therapeutic agent to manage DR in addition to offering other medicinal values.

Molecular mechanism of F2 and myricetin in protecting ARPE-19 cells against oxidative stress: The mixture of myricetin derivatives present in F2 was found to exert a more prominent and consistent protective effect compared to myricitrin only (Figure 1, Figure 2, Figure 3). Myricitrin was expected to reflect similar effects in the gene expression study. However, myricetin, a major metabolic product of myricitrin, showed considerably better protection against oxidative stress in the retinal cells compared to all the tested compounds and extract in the present study. Therefore, the molecular mechanism of F2 and myricetin (instead of myricitrin) in preventing GO-H_2O_2-induced stress in retinal cells was assessed. An F2 concentration of 200 µg/ml (myricitrin content, 154 µg) was selected for the purpose of determining the molecular mechanism as this concentration showed higher restoration of cell viability (91%) which was comparable to the protective effect (88%) exerted by myricetin (40 µg/ml).

The antioxidant adaptive mechanism in retinal cells is crucial and thus, serves as a good cellular model to be explored [46]. SOD is one of the major antioxidant enzymes responsible for protecting RPE cells [47]. The cytosolic (CuZnSOD; *SOD1*) and mitochondrial (MnSOD; *SOD2*) enzymes dismutate superoxides into oxygen and H_2O_2 [48,49]. H_2O_2 is degraded to water (H_2O) by catalase (CAT) or glutathione peroxidase (GPx) [48]. GO-H_2O_2-induced oxidative stress caused upregulation of *SOD2* and *GPx1* when compared to the untreated ARPE-19 cells (Figure 4). Presumably, the cells activated the internal antioxidant system immediately to combat stress factors. The introduction of stress along with F2 or myricetin pretreatments showed different response patterns in the expression of antioxidant enzyme genes. F2 upregulated *SOD2* ($p<0.001$) to exert its protective effect, but myricetin did not affect the expression of *SOD2* induced by GO-H_2O_2-induced stress. However, myricetin downregulated *GPx1* gene expression. The compound possibly scavenged or inactivated the radicals directly, thus attenuating the activation of the internal antioxidant defense mechanism. Expression of the *SOD1* gene was not statistically significantly affected in the presence of stress with or without the samples (myricetin or F2). However, F2 activated *SOD2* to manage the oxidative stress in ARPE-19 cells.

The nuclear factor erythroid 2-related factor 2-antioxidant response element (Nrf2/ARE) pathway plays an important role in protecting RPE cells through activation of the endogenous protection mechanism against oxidative stress [15,50]. Nrf2 also targets and interacts with apoptosis-related pathways [50,51]. F2 showed statistically significant upregulation of *NFE2L2* indicating activation of the Nrf2 pathway to exert its protective effect (Figure 4). This finding corresponds to previous reports that showed the protective effects of bioactive compounds, such as simvastatin, 6-shogaol, and astaxanthin, against H_2O_2-induced stress through the activation of the Nrf2 pathway [15,52,53]. Upregulation of *NFE2L2* which indicates activation or expression of Nrf2 was not observed with myricetin treatment against GO-H_2O_2-induced stress (Figure 4) in the present study. This appears to contradict a previous report that showed myricetin induces the expression of Nrf2 independently when incubated for 2 or 4 h in ARPE-19 cells [41]. In the present study, the effect of myricetin alone was not assessed; instead, the effects of GO-H_2O_2 in the presence of myricetin were assessed. Pretreatment of myricetin (40 µg/ml) showed a negligible presence of H_2O_2 in culture supernatant (Figure 3). It is possible that myricetin exerted a direct inhibitory effect on GO-H_2O_2 and thus, conferred exogenous protection before the compound could activate the endogenous phase 2 enzyme protective mechanism (Nrf expression) in the cells during the incubation period.

The cytotoxic effect of steady H_2O_2 triggers apoptosis in cells due to DNA damage [54,55]. Generally, the MAPK1 (ERK) or MAPK14 (p38MAPK) signal transduction pathway is activated instantly in the presence of oxidative stress. However, activation of the pathways reported previously showed contradictory results of either inhibition or further enhancement of cell death in the retinal cell model [56-58]. In the present study, GO-H_2O_2-induced ARPE-19 cell death was accompanied by the upregulation of *MAPK1* and the

downregulation of *MAPK14* (Figure 4). Myricetin showed downregulation of the *MAPK1* gene, and this inverse expression of the gene suggested a counter-protective mechanism of myricetin in the stress model. However, F2 and myricetin did not exhibit statistically significant changes in the expression of the *MAPK14* gene. Caspase 3 (*CASP3*) was activated twofold with the GO-H_2O_2 cytotoxicity treatment (Figure 4), and this concurred with a previous report that confirmed *CASP3* activation and its association with apoptosis in ARPE-19 cells caused by H_2O_2 [59]. Myricetin normalized the upregulation of *CASP3* caused by the stress. *HSPB1* was activated upon GO-H_2O_2 treatment in this study, which concurred with a previous report [58], but it was downregulated by myricetin. Myricetin also showed an expression pattern of the *Bax* gene (a proapoptotic protein) that implied elimination of the basal apoptosis of the cells during the preincubation for 2 h. *CTSD* is a lysosomal enzyme which is involved in the phagocytosis of photoreceptor outer segment in RPE. The protein has been recognized as an important marker in the pathogenesis of several vitreoretinal diseases which includes DR [60]. However, GO-H_2O_2 treatment with or without the presence of the samples (F2 or myricetin) did not statistically significantly affect the expression of the *CTSD* gene when compared to the untreated control.

The expression of proinflammatory cytokine *IFN-γ* was upregulated in ARPE-19 cells approximately by 11-fold upon exposure to GO-H_2O_2-induced oxidative stress (Figure 4), and this expression was reduced statistically significantly in the myricetin-treated cells. Introduction of IFN-γ is known to be able to stimulate nitric oxide synthase and thus, NO production in RPE cells [61]. Inducible nitric oxide synthase (iNOS) is believed to be involved in the breakdown of the blood–retinal barrier in DR [62]. In addition, in vivo iNOS expression has been shown to induce apoptosis in the retina and thus, retinal degeneration in ischemic proliferative retinopathy [63]. In this study, upregulation of *NOS2* led to activation of *IFN-γ* in GO-H_2O_2-induced oxidative stress (Figure 4). Interestingly, F2 downregulated *NOS2* which supported its NO scavenging property as described previously. The findings indicate that F2 attenuated the cell death caused by GO-H_2O_2-induced oxidative stress partly through the downregulation of the *NOS2* gene though it did not affect the expression pattern of the other apoptosis-related factors (the *MAPK1*, *CASP3*, *HSPB1*, and *Bax* genes; Figure 4). The focus of the gene expression analysis in the present study was mainly on investigating the effect of H_2O_2-induced cytotoxicity followed by apoptosis in DR. However, the present data are not sufficient to clearly rule out the possible occurrence of necrosis in the cells due to the injury induced by GO-H_2O_2 and thus, warrants further investigations.

Lysyl oxidase (LOX) is an enzyme responsible for maintaining the stability and integrity of the extracellular matrix (ECM) of retinal cells. The activity and expression of LOX were shown to be increased upon exposure to vascular endothelial growth factor (VEGF), a proangiogenic agent closely related to retinopathy [64]. In this study, GO-H_2O_2-induced oxidative stress also upregulated *LOX* indicating disorganization of the ECM (Figure 4). Myricetin downregulated the expression of the *LOX* gene, which is indicative of myricetin's protective activity against GO-H_2O_2-induced oxidative stress compared to F2 treatment.

CRP is another inflammatory marker believed to be associated with the pathogenesis of DR [65]. Whereas TLR2 has been recently recognized as a DR marker as its mRNA and protein expression was significantly elevated in the hyperglycemic condition. TLR2 plays a vital role in inflammation and innate immune responses. The rise in the expression of TLR2 was linked to inflammation and ROS [66]. However, the expression of the *CRP* and *TLR2* genes for the samples did not show any statistically significant differences compared to the untreated or GO-H_2O_2 treated controls.

In conclusion, myricetin derivatives isolated from leaf extract of *S. malaccense* (F2) showed considerable potential as potent antioxidants and exerted good protective effect against GO-H_2O_2-induced oxidative stress in ARPE-19 cells. The derivatives deactivated the nitric oxide producer (*NOS2*) and activated the expression of Nrf2, as well as the antioxidant enzyme (*SOD2*) to manage the oxidative stress and exert a protective effect at the transcriptional level. In addition, myricetin, the potential metabolic product of F2, prevented apoptosis of the retinal cells by controlling various proapoptotic factors and inflammatory markers at the molecular level. However, the possible molecular mechanisms of F2 and myricetin against GO-H_2O_2-induced oxidative stress using an in vitro model in the present study must be further validated through protein expression, as well as in vivo studies. Nevertheless, the present study has clearly reflected that myricetin derivatives of *S. malaccense* leaf extract have the ability to protect retinal cells against oxidative stress and therefore, may be used as a potential agent for managing DR.

APPENDIX 1. PERCENTAGE OF ARPE-19 CELL VIABILITY WHEN TREATED WITH MYRICITRIN, MYRICETIN, LEAF EXTRACT OF *SYZYGIUM MALACCENSE* AND F2 (MYRICETN DERIVATIVES) FOR 24 H.

Control is the untreated cells grown in basal medium. Data are expressed in mean ± standard deviation (SD); n = 3. The one way ANOVA Tukey's post hoc showed statistical

significance at *p < 0.05, ** p < 0.01 and *** p < 0.001 when compared to the control. *S. mal* = *Syzygium malaccense*. To access the data, click or select the words "Appendix 1."

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