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Research Article

Increased expression of GLUT4, catalase and nitric oxide by a crystalised fraction from ethanolic extracts of oak leaf lettuce and okra seed in C2C12 cell line

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ABSTRACT

The fractions obtained from low temperature-induced crystallisation of ethanolic extracts of green- and red-oak leaf and okra seed were accounted for total polyphenol content and the DPPH antioxidant activity, resulting in 5.99, 4.44, and 12.35 mg gallic acid equivalent per g sample, and 0.45, 0.35, and 0.99 mg Trolox equivalent per g sample, respectively. Insulin resistance was the result after incubating C2C12 skeletal muscle cells in high glucose DMEM for 20 h. Oxidative stress and inflammation were triggered by re-incubating these resistant cells with insulin. The expression of p-p38 MAPK and NF-κB proteins and the NF-B p65 transcription factor activity were enhanced. Such inflammatory protein markers were reduced when the crystalised extracts replaced insulin. Increased catalase activity and NO production were also determined for the incubations using the crystalised extracts. It was suitable to include these vegetables in the daily diets of prediabetic individuals for better management of the patient's health status by increasing insulin sensitivity and decreasing inflammation.

Keywords: NF-κB p65 transcription factor, GLUT4, Catalase activity, Nitric oxide, Polyphenols, DPPH antioxidant activity, Oak leaf lettuce, Okra seed, C2C12 cell line

Introduction

The effect on health promotion of vegetables consumed daily has been demonstrated, usually leading to reductions of inflammatory mediators to the levels are not risk for chronic- and degenerative diseases, such as type II diabetes mellitus (T2D), cardiovascular diseases, and neurodegenerative diseases (Stanaway et al., 2022; Godos et al., 2020; Parasoglou et al., 2017). More than 50% of the global deaths have been attributable to these inflammation-related diseases (Furman et al., 2019). Increased consumption of plant-derived flavonoids has improved cognitive function and decreasing risks of age-related neurodegeneration because of intensely suppressing TNF-α and C-reactive protein synthesis (Li et al., 2023). Indeed, there are a variety of secondary metabolites in edible plants that impart health-promoting as well as disease-curing and preventing effects, commonly related to antioxidant, anti-bacterial, anti-cancer, immune-modulating, and/or anti-inflammatory activities (Zheng et al., 2019). However, a plant cultivar has defined sets of secondary metabolites corresponding to plant genetics (Yang et al., 2018). For example, similar phenolic components of green- and red-oak lettuce cultivars have been reported. Apigenin, glucuronide, and dihydroxy-benzoic acid have solely been detected in the green cultivar. In contrast, only luteolin, apigenin conjugates, cyanidin conjugates, and hydroxy-malonyl-hexoside have existed in the red cultivar (Viacava et al., 2017). The pod of *Abelmoschus esculentus* (called okra) is an edible part of the plant, containing ample proteins and fatty acids in the seed (Sami et al., 2013), and mucilage polysaccharides, vitamins, fibres, and minerals in the peel (Zim et al., 2021). In addition, quercetin has been suggested as a major flavonoid in the aqueous extract of okra pod, presenting anti-hyperglycemic activity in the high-fat diet-streptozotocin rat model (Peter et al., 2021). Besides, the mucilage-like substance obtained by boiling okra pods in water has shown anti-diabetic and anti-hyperlipidemic properties in alloxan-induced diabetic mice (Zim et al., 2021). Altogether, the health-beneficial effects of green- and red-oak leaf lettuces and okra seed might be more diverse than expected, leading to an underestimation of their nutritional values. In this research, biological effects concerning anti-oxidation, anti-inflammation, and increased GLUT4 expression of crystallised compounds from ethanolic extracts of green- and red-oak leaf and of okra seed were investigated on C2C12 skeletal muscle cells in normal and high glucose media. The acquired data would be adequately scientific and useful for managing complications related to prediabetes due to increased daily consumption of vegetables (Găman et al., 2020).

Materials and Methods

Preparation of Crystalized Extracts

Okra seeds and green- and red-oak leaves were collected from the Faculty of Natural Resources, Prince of Songkla University, Thailand. The plant materials were dried under the sun to ~95% dryness and ground into fine powder using a blender. Each 50 g was macerated in 150 mL of 95% ethanol (Loba Chemie, India) overnight at room temperature. The supernatant was separated by centrifugation at 6000 x *g* for 15 min. This was 1 cycle, and 3 cycles of the maceration were carried out for a sample. The pooled supernatant of ~400 mL was evaporated using a rotary evaporator at 45^{O}C to obtain viscous extract. This extract was cooled at 6^{O}C overnight for crystallisation induction. The formed crystals were carefully separated, air-dried, and kept in an air-tight container at 6^{O}C until use.

Total Phenolic Content

The folin-Ciocalteu method was applied to determine the total phenolic content of samples (Peter et al., 2021). DMSO (RCI Labscan™) was used as the sample solvent. Solution A was prepared by mixing 2% Na_2CO_3, 2% $CuSO_4$, and 4% sodium potassium tartrate at a 100:1:1 volume ratio. A sample of 100 µl and 100 µl of solution A were mixed and incubated for 5 min. Then, 50 µl of 0.5 M sodium hydroxide was added and incubated for 10 min. Next, 200 µl of 1:1 diluted Folin-Ciocalteu reagent (Loba Chemie) in water was added and incubated for 30 min. The OD_{750} was spectrophotometrically measured using a microplate reader (Varioskan LUX, ThermaFisher, USA). Gallic acid (Sigma-Aldrich, Germany) was used as a positive standard, and the concentration range of 0-2 mg/mL was carried out to draw the standard curve. The total phenolic content of a sample was calculated and reported as mg gallic acid equivalent per gram sample.

DPPH Antioxidant Assay

The antioxidant activity of a sample was accounted for by using a DPPH radical scavenging assay (Zim et al., 2021). Briefly, a 100-µl sample solution was mixed with 0.9 mL of 0.1 mM DPPH reagent in methanol (Sigma-Aldrich) using a vortex. A 100-µl DMSO was of a blank control. After incubating for 30 min at 37°C and protected from light, the OD_{517} was measured against the blank. Trolox (Sigma-Aldrich) was used as a positive standard, and the concentration range of 0.05-0.2 mg/mL in methanol was performed to construct the

standard curve. The DPPH antioxidant activity was calculated and reported as mg Trolox equivalent per gram sample.

C2C12 Cell Culture

C2C12 is a myoblast cell line from ATCC. The cells were routinely grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and 1% antibiotic and antimycotic (called normal medium) in a 5% CO_2 incubator at 37°C. The cells were subcultured when their growth approached 70% confluency. When specified, high-glucose DMEM was used to replace the normal medium. Chemicals and reagents used for the cell culture technique were purchased from Gibco™ (ThermoFisher, USA).

Preparation of Test Solutions for Treating Cells

A stock solution of a test extract of 2 mg/mL in DMSO was prepared. This sample stock solution was diluted to a final concentration of 200 µg/mL in a suitable growth medium to treat cells. Treatments were performed on ~80% confluent cells by incubation with a test solution for 20 h at 37°C in a CO_2 incubator. Insulin at a final concentration of 100 nM was handled as the positive control. These treated cells were next used in experiments including quantitative PCR (qPCR), SDS-PAGE and Western blotting, and determinations of p65 transcription factor activity, catalase activity, and nitric oxide (NO) production, as detailed in the following.

qPCR

The total RNA of cells was extracted using Tri Reagent™ and the RNA concentration was fluorometrically measured using Qubit™ RNA Assay Kit (Invitrogen, USA). Procedures related to the kits were in accord with the manufacturers' recommendations. First-strand cDNA was synthesised in a 25-µl reaction containing 12.5 µl of SensiFAST cDNA Synthesis Master Mix (Meridian Bioscience, USA), 200 pmolar of oligo$(dT)_{20}$ primer, and 500 ng of the RNA template. The qPCR of a sample was done in triplicate on the Magnetic Induction Cycler (Mic qPCR) (Bio Molecular Systems, Australia) by using qRT-PCR Brilliant III SYBR Master Mix (Agilent, CA, USA) and a pair of primers regarding inflammatory genes, such as *TNF-α*, *IL-6*, and *NF-κB*, and of *GAPDH* control gene. The primers were of Origene™ with the codes NM_013693, NM_031168, NM_008689, and NM_008084, respectively. The set-up qPCR cycling was 5 min at 95°C, followed by 40 cycles at 95°C for 30 sec and 60°C for 1 min to acquire Ct data. Primer specificity was confirmed by doing a dissociation curve analysis. For a sample, the Ct value of a test gene was normalised by that of GAPDH, and relative expression in fold change was reported by dividing the normalised Ct of a treated sample by the untreated control.

Protein Electrophoresis and Western Blotting

Cells were lysed on ice using RIPA buffer (Sigma-Aldrich) supplemented with protease inhibitor cocktail (Roche, USA) and centrifuged at 11.000 × g for 15 min at 4°C to remove cell debris. A lysate's protein concentration was quantitated using the Pierce BCA Protein Assay Kit (ThermoFisher). An equal amount of sample proteins was loaded on SDS-polyacrylamide gel for electrophoresis. The separated bands were wet-transferred to the PVDF membrane (Amersham™) at 4°C overnight. The membrane was blocked in Tris-buffered saline containing 0.1% Tween 20 for 2 h at room temperature and probed with 1° antibodies (dilution 1:1000) against NF-κB, p38 MAPK, p-p38 MAPK, GLUT4, or β-Actin protein (Cell Signaling Technology, USA) at 4°C overnight. The binding of the bound 1° antibodies to HRP-conjugated 2° antibody (dilution 1:2000) (Santa Cruz Biotechnology, USA) was carried out at room temperature for 2 h. An ECL reagents kit (ThermoFisher) was used to generate the signal. Band visualisation was accomplished using Luminescent Image Analyzer (GE Healthcare, Sweden), and the bands' density was calculated using ImageQuant™ TL 10.2 analysis software. Variation in the loaded protein concentration was compensated by normalising the band density of a target protein to that of GAPDH.

Preparation of Nuclear Extract

The NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (ThermoFisher) were utilised to separate nuclear extract from cultured cells. In brief, cells in suspension were collected by centrifugation at 500×g for 5 min, washed by resuspending in PBS, transferred of ~5×10^6 cells to a 1.5-mL microcentrifuge tube, and re-centrifuged again at 500×g for 2-3 min. The supernatant was discarded, and the cell pellet was dried as much as possible in the air. Then, the CER I solution was added, mixed vigorously for 15 sec using a vortex and incubated on ice for 10 min. Next, CER II solution was added, mixed for 5 sec, and incubated on ice for 1 min. After centrifugation at 16.000×g for 5 min, the supernatant (the cytoplasmic extract) was separated and stored at -80°C until use. The insoluble fraction in the tube's bottom was resuspended in ice-cold NER solution and mixed for 15 sec for every 10-minute incubation of the total 40 min incubation on ice. The nuclear extract was acquired by centrifugation at 16.000×g for 10 min. The supernatant was immediately aspirated, transferred to a clean pre-chilled tube, and stored at -80°C until use.

Assay of NF-κB p65 Transcription Factor Activity

RayBio® NF-κB p65 Transcription Factor Activity Assay Kit (RayBiotech, USA) was recently used to determine active p65 transcription factors in nuclear extracts. The kit was provided in a 96-well plate format. The wells were pre-coated with double-stranded oligonucleotides containing NF-κB binding sequence (5′-GGGACTTTCC-3′). A sample of nuclear extracts was added to the well and incubated at 4°C overnight before being removed. The primary antibody against p65 was added and incubated for 2 h at room temperature. Unbound antibodies were washed away. Then HRP-conjugated secondary antibody was added, incubated for 1 h at room temperature, and removed. The TMB substrate reagent was added and incubated for colour development at room temperature for 1 h. The stop-solution was added, and the OD_{450} was immediately measured using a microplate reader.

Nitric Oxide (NO) Assay

The principle for assaying NO of Sigma-Aldrich is based on oxidising any present NO to be NO^{2-} (nitrite) and NO^{3-}(nitrate) using a Griess reagent. The reaction products are stable and can be quantitatively measured at OD_{540}. The standard curve can be constructed using accurate concentrations of NO^{2-} for the reaction. NO sample concentration was calculated concerning the standard curve and normalised by the corresponding cells' total protein using Pierce BCA Protein Assay Kit (ThermoFisher). The normalised NO concentration of a test sample was compared with that of the control for reporting the relative NO production.

Catalase Activity Assay

Recently, the determination of catalase activity in samples as cell lysates was carried out by using the Catalase Colorimetric Activity Kit (Invitrogen), according to the manufacturer's protocol. A cell lysate from adhered cells in a well was prepared as follows. The cultured medium was removed, and the cells were gently dislodged with a rubber policeman in 1x assay buffer. After centrifugation of the lysate at 10.000 x g for 15 min at 4°C, the supernatant was collected and used for assaying catalase activity immediately. Catalase standard, as supplied in the kit, was used for constructing the standard curve with a concentration range of 0-5 U/mL, where 1 U of catalase stands for the decomposition of 1.0 μmol H_2O_2 in 1 min at 25°C, pH 7. Samples and catalase standard solutions were separately incubated with mixed hydrogen peroxide reagent at room temperature for 30 min, followed by mixed substrate reagent at room temperature for 15 min before measuring the OD_{560}. Again, the catalase activity determined for a sample was normalised with the corresponding sample protein and compared with the normalised result of the untreated control for reporting the relative catalase activity.

Data Analysis

Each experiment was done in triplicate. Data were reported as Mean ± S.D. Differences between groups were evaluated at $p = 0.05$ using the Student t-test or ANOVA with post hoc test Bonferroni of the IBM SPSS software for multiple comparisons. Results were considered significantly different if p values < 0.05.

Results and Discussion

Extraction Yields, Total Phenolic Content, and DPPH Antioxidant Activity

Amorphous crystals of 3 g, 6 g, and 4.5 g were obtained by extracting 50 g dried powder from okra seed, green-oak leaf, and red-oak leaf with three times repeated 150-mL 95% ethanol. This resulted in a 6%, 12%, and 9% yield. All the extracts were sparingly soluble in ethanol and methanol but freely dissolved in DMSO. Stock solutions of 2 mg/mL in DMSO of the extracts were prepared and diluted in methanol to examine total phenolic content (TPC) and DPPH antioxidant activity using gallic acid and Trolox as the corresponding standards, respectively. The extract from okra seed showed TPC and antioxidant activity of about 2 times and 2.8 times greater than those of green and red leaf, respectively. Moreover, the measured antioxidant activities corresponded with the TPC values (Table 1).

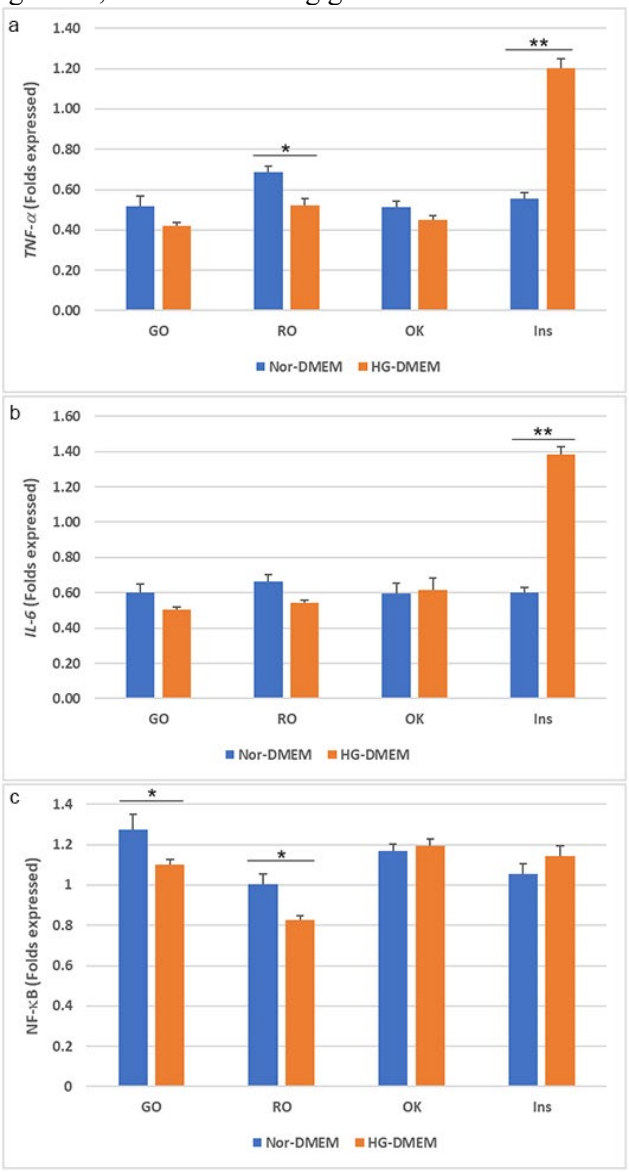
Table 1 The values of TPC and DPPH antioxidant activity for the extracts of okra seed, green-oak leaf and red-oak leaf

The extract origin	TPC (mg gallic acid per g sample)	DPPH antioxidant activity (mg Trolox per g sample)
Green-oak leaf	5.99 ±0.22 [a,b]	0.45 ±0.02 [c,d]
Red-oak leaf	4.44 ±0.06 [a,b]	0.35 ±0.05 [c,d]
Okra seed	12.35 ±0.15 [b]	0.99 ±0.08 [d]

a and c, significant difference with $p < 0.05$; b and d, significant difference with $p < 0.01$

Transcriptions of TNF-α, IL-6, and NF-κB Genes

The transcription of *TNF-α* and *IL-6* genes seemed to be silenced in the normal glucose medium because the folds expressed were less than 1 (Figure 1). Transcriptional silencing of these genes was observed for all incubations whether using insulin or the extracts of green- and red-oak leaf and of okra seed. However, significant elevation of such gene transcriptions was evident after treating the cells with insulin in the high glucose medium (Figure 1a and b). For the *NF-κB* gene transcription in normal glucose, incubations using green-oak leaf and okra seed extracts resulted in transcriptional activation of the gene, leading to the folds increased of > 1 (Figure 1c). This gene transcription was not affected when challenged with red-oak leaf extract and insulin (folds changed ~1). In the high glucose medium, it was noted that the NF-κB transcription was suppressed by green- and red-oak leaf extracts but slightly activated by insulin. Likely, the effect of okra seed extract on the NF-κB transcription in both the normal- and the high-glucose media was equivalent and stimulatory, indicating a 1.2-fold change.

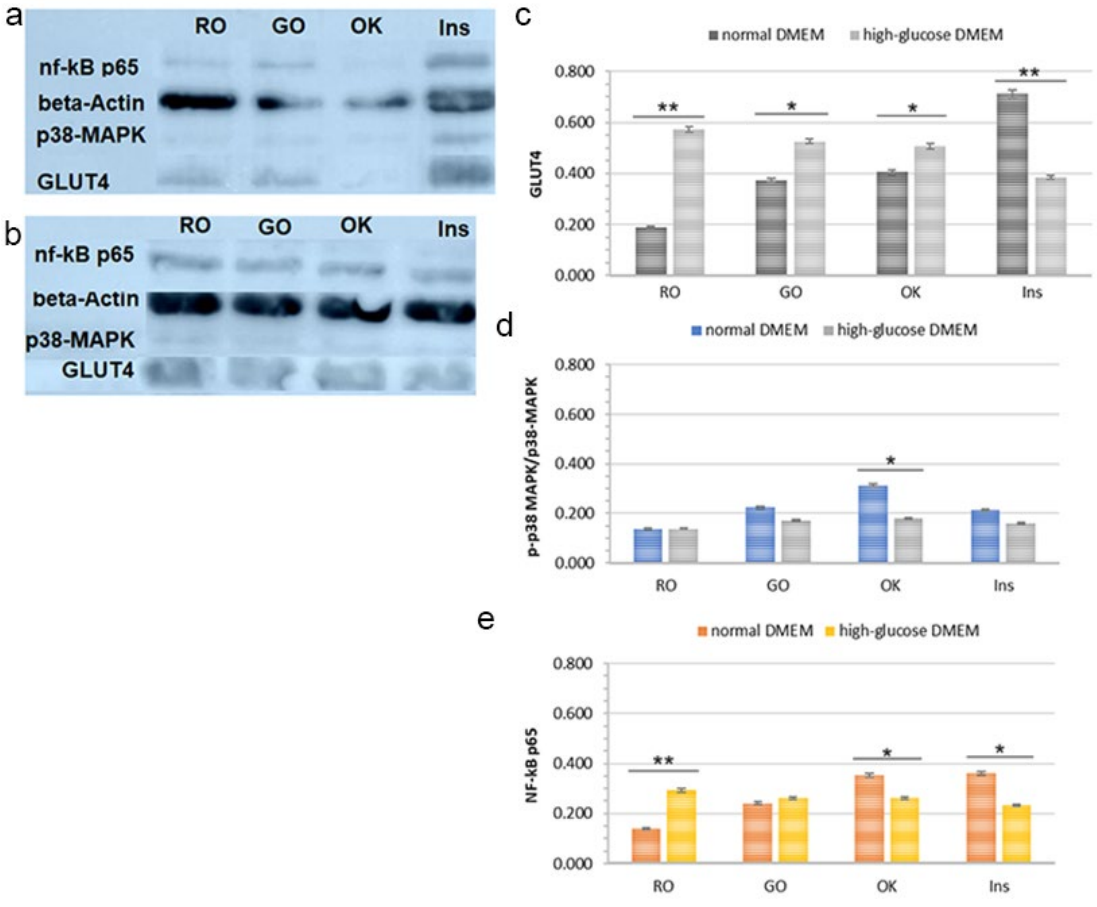


Symbols: $p < 0.05$; **, $p < 0.01$

Figure 1. Transcriptional expression in folds changed of genes *TNF-α*, *IL-6*, and *NF-κB* of C2C12 cells grown in normal- and high-glucose DMEM determined by qPCR method; Insulin of 100 nM (Ins) or 200 µg/mL extracts from green-oak leaf (GO), red-oak leaf (RO), or okra seed (OK) was independently incubated with the cells for 20 h before running the qPCR.

Expression of GLUT4, p38 MAPK, p-p38 MAPK, and NF-κB Proteins

Cells of C2C12 treated with 100 nM insulin or 200 µg/mL extracts of green- and red-oak leaf and of okra seed were subjected to SDS-PAGE and immunoblotting against NF-κB, p38 MAPK, p-p38 MAPK, and GLUT4 proteins. Results are shown in Figure 2. The expressed GLUT4 protein after insulin challenge in the normal medium was higher than that affected by other used extracts. However, such expression was intensely suppressed by insulin in the high-glucose medium. Lower levels of GLUT4 protein in normal glucose were determined for the incubations using green- and red-oak leaf and okra seed extracts compared to that using insulin. Of note, the expression levels were increased while replacing the normal medium with another the containing high glucose (Figure 2c). The expressed ratio of p-p38 MAPK and p-38 MAPK proteins after incubations with green- and red-oak leaf and insulin was not altered whether normal glucose or high glucose medium was supplied (Figure 2d). Instead, such the ratio was decreased after incubated with okra seed extract using the high glucose medium compared to that of the normal one. The expressed levels of NF-κB in the normal medium were arranged according to the treatments as red-oak leaf < green-oak leaf < okra seed = insulin (Figure 2e). When changed to the high glucose medium, decreased expression of NF-κB was examined for treatments using okra seed extract and insulin. However, a similar amount of this expressed protein was indicated after incubation using green oak leaf extract in either the normal or the high glucose medium. Notably, increased expression of NF-κB protein was apparent for red-oak extract treatment in the high glucose culture.



Symbols: *, $p < 0.05$; **, $p < 0.01$

Figure 2. Expressions of GLUT4, p-38 MAPK, p-p38 MAPK, and NF-κB proteins determined by Western blotting method; C2C12 cells grown in normal- or high- glucose DMEM were incubated with 100 nM insulin (Ins) or 200 µg/mL extracts from green-oak leaf (GO), red-oak leaf (RO), or okra seed (OK) for 20 h followed by SDS-PAGE of the cells grown in normal DMEM (a) and those in high glucose DMEM (b); The density of protein bands was calculated by using ImageQuant™ TL 10.2 analysis software and graphed for GLUT4 (c), the p-p38 MAPK/p-38 MAPK ratio (d), and NF-κB (e).

NF-κB p65 Transcription Factor Activity

p65 is one of the five components that form the NF-κB transcription factor family, which is present in most cells and mediates inflammatory responses through the NF-κB signalling pathway (Zhang et al., 2020). In this research, the transcriptional activity of p65 in C2C12 cells was investigated using the kit as specified. Results are shown in Fig. 3. Increased p65 transcriptional activity was found in normal glucose after incubation with green-oak leaf extract compared to that of okra seed and red-oak leaf. Suppression of the activity was suggested for insulin, whether using the normal or the high glucose medium. Instead, reduction of the p65 activity was significant after being treated with the extracts of okra seed and green oak leaf in the high-glucose medium. However, slight decreasing the activity was seen for the incubation using the red oak extract.

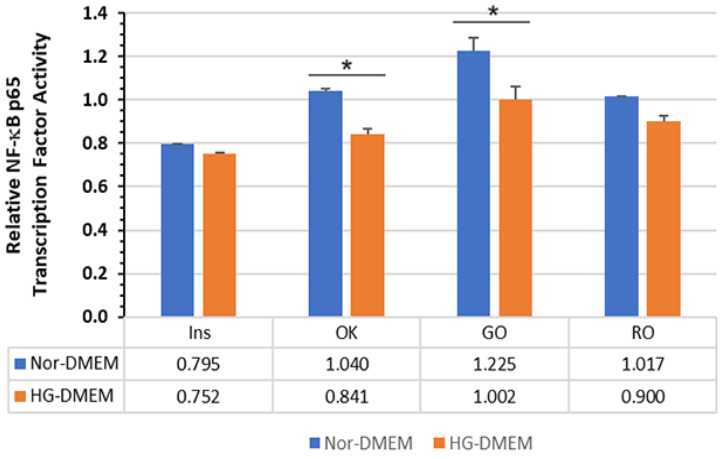


Figure 3. Relative p65 transcription factor activity: C2C12 cells grown in normal- or high- glucose DMEM were treated with 100 nM insulin (Ins) or 200 µg/mL extracts from green-oak leaf (GO), red-oak leaf (RO) or okra seed (OK) for 20 h before the assay using nuclear extracts. The untreated cells were of the control. Symbols: *, $p < 0.05$

NO Production

The production of NO by C2C12 cells grown in the normal or the high glucose medium and treated with insulin or the extract of either green- and red-oak leaf or okra seed was measured using cell supernatants. Results are shown in Fig. 4. NO was actively produced by the cells after incubation with okra seed extract, and the lower producing levels were observed after incubation with green- and red-oak leaf extracts. Meanwhile, the effect of insulin on inducing NO production was insignificant, whether in the normal or the high glucose medium.

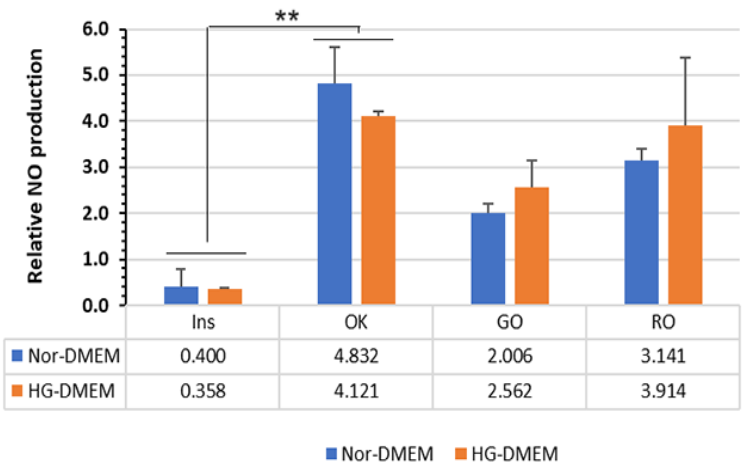


Figure 4. Relative NO production: C2C12 cells grown in normal- or high- glucose DMEM were treated with 100 nM insulin (Ins) or 200 µg/mL extracts from green-oak leaf (GO), red-oak leaf (RO), or okra seed (OK) for 20 h before the assay using cell supernatants. The untreated cells were of the control. Symbols: **, $p < 0.01$

Catalase Activity

Catalase is an enzyme found in nearly all living cells exposed to oxygen and is important by protecting cells from oxidative stress caused by reactive oxygen species (ROS). This enzyme, among others, has the highest turnover rate in converting millions of hydrogen peroxide molecules to water and oxygen in a second. In Fig. 5, increased catalase activity was found for the cells grown in the normal glucose medium and after treatments using okra seed and green- and red-oak leaf extracts. In comparison, less catalase activity was investigated for the cells of insulin challenge. In high glucose cultures, the enzyme activity decreased significantly when incubated with insulin as well as red oak leaf extracts and okra seed extracts. However, the activity disappeared after incubation using green oak leaf extract.

Fasting blood glucose is one of the criteria for diagnosing prediabetes (also called insulin resistance), about which plasma glucose reached in 2 h of an oral 75-g glucose challenge ranges between 101 and 125 mg/dL (5.6–6.9 mM). Importantly, about 50% of the diagnosed prediabetic individuals have been noted to develop T2D within five years and are also at a higher risk of developing other metabolic disorders and cancers (Merz & Thurmond, 2020; Owei et al., 2019). Lifestyle interventions through exercise and diet are the current strategies that are potential to cure prediabetes, including the fact that the co-administration of metformin has improved insulin sensitivity conditions. However, using this drug for prediabetes has not been ratified by the US FDA because it

sounds ineffective compared to lifestyle interventions in addition to the drug's side effects (Centers for Disease Control and Prevention, 2017). The skeletal muscle is the largest organ system of the body, essential for movements in daily life. Over 80% of oral glucose uptake is absorbed and metabolised to ATP by the muscle cells, corresponding to insulin's function. Desensitisation to insulin of these cells impacts health by increasing blood glucose concentrations, the preceding diabetes condition (Khan et al., 2019).

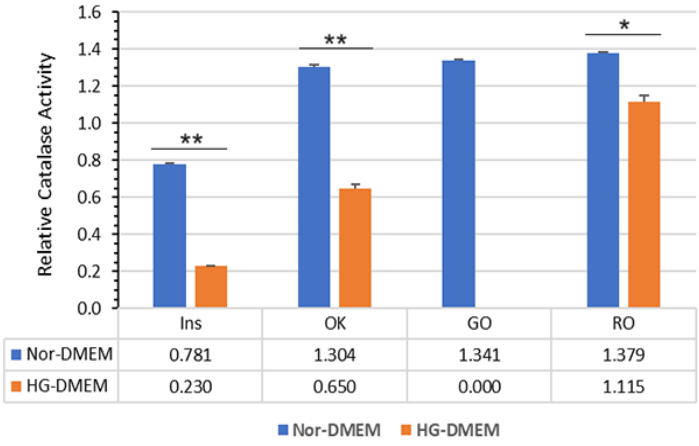


Figure 5. Relative catalase activity: C2C12 cells grown in normal- or high- glucose DMEM were treated with 100 nM insulin (Ins) or 200 µg/mL extracts from green-oak leaf (GO), red-oak leaf (RO) or okra seed (OK) for 20 h before the assay using cell lysates. The untreated cells were of the control. Symbols: *, $p < 0.05$; **, $p < 0.01$

The glucose concentration of the recently used high glucose DMEM has been mimicked to prediabetes's fasting blood glucose level. This was used to desensitise the cultured C2C12 cells to insulin to cause insulin resistance. Accordingly, the responses of these insulin-resistant cells to all test extracts in high glucose mediums would be different from those of the insulin effects. Regarding some inflammatory genes' markers, depletion of *TNF-α*, *IL-6*, and *NF-κB* transcriptions after incubation with green- and red-oak leaf extracts was greater than that of okra seed incubation (Figure 1). However, suppression of p-p38 MAPK, p-38 MAPK, and NF-κB proteins was potential for okra seed extract compared to those of green- and red-oak leaf samples (Figure 2d and e). For insulin effects, the promotion of NF-B transcription was suggested with decreased NF-B translation (Figure 2e). For reliability, the NF-κB signal transduction was validated by measuring NF-κB p65 transcription factor activity using a specific reagent kit. The extracts of okra seed and green-oak leaf, or even of red-oak leaf, were indicated to suppress the p65 transcription factor activity, and these results were different from the impacts of insulin (Figure 3). As such, all of the test extracts would mitigate inflammations as induced by high glucose condition but not by insulin. The promotion of GLUT4 expression was also determined after incubation with the extracts, and the highest protein expression was suggested for the red-oak leaf sample. Again, reduction of the expressed GLUT4 protein was significantly observed for insulin supplement in the high glucose medium (Figure 2c). It was certain that the extracts of green- and red-oak leaf and of okra seed had the potential for improvement of prediabetic conditions due to increasing insulin sensitivity and glucose uptake (Bird and Hawley, 2017).

Hyperosmotic stress of C2C12 cells possibly happened while grown in the high glucose medium, leading to water efflux and cell shrinking. Although oxidative and osmotic stress differ significantly, responses to these stresses by the affected cells have overlapped (Gayathri et al., 2023; Mager et al., 2000). There was a rationale to monitor transcriptional and translational extents associated with inflammations of C2C12 cells after being cultured in iso-osmotic or hyper-osmotic medium recently. In general, cells have defence mechanisms to mitigate stresses and inflammations. NO production and dissipation are parts of biological processes identified to date for stabilising normal cellular oxidative stress. However, depending on its location and concentration, NO has been suggested to elicit pro-inflammatory or anti-inflammatory effects. For instance, localised suppression of NO has been inflammatory by increasing pleural exudate volumes, leukocyte counts, and activities of enzymes related to oxidative stress of rats with carrageenan-induced acute inflammation. However, these signs have been dissipated by supplementing NO locally (Iwata et al., 2020). This project suggested increased NO production by C2C12 cells after incubation with green-oak leaf, red-oak leaf, and okra seed extracts by two, three, and four times, respectively. For insulin-challenged cells, however, very little NO was produced. It was noted that trends of NO production for the cells in the normal- and the high- glucose media were not very varied (Figure 4). Consequently, all test extracts were supposed to be great in resolving oxidative stress and inflammation in the high glucose medium compared to insulin, which might be associated with the existing antioxidant activities (Table 1) and the ability to induce NO production (Figure 4).

Catalase deficiency and malfunctioning have been etiological factors of age-associated degenerative diseases, including T2D (Nandi et al., 2019). A strategy has been proposed to mitigate these diseases using catalase enzymes in food supplement forms (Chandrasekaran et al., 2017). In the present

project, C2C12 cells grown in the normal medium were stimulated to produce catalase after incubations using the extracts from green-oak leaf, red-oak leaf, and okra seed. However, less induction of the enzyme by these extracts was found for the cells in the high glucose medium. The potential of insulin to induce catalase production was much inferior to the extracts (Fig. 5). Although increased catalase expression was liable for C2C12 cells treated with the extracts, mechanisms at molecular levels are further explored.

Conclusion

In summary, the phenolic fractions obtained by cool-induced crystallisation of ethanolic extracts from green- and red-oak leaf and okra seed were determined to present DPPH-radical scavenging activity in a dose-dependent response. Cells of the skeletal muscle cell line, C2C12, were insulin-resistant after being cultured in high glucose DMEM for 20 h. Inflammations were indicated to increase by repeat incubation of these resistant cells with insulin, leading to increased expression of p-p38 MAPK and NF-κB proteins and NF-κB p65 transcription factor activity. Instead, using all of the extracts for the incubation seemed beneficial, as the activations of such inflammatory proteins and the transcription factor were diminished. Besides, increased catalase activity and NO production were apparent by incubations using the extracts. Consequently, these three vegetables might be helpful when included in the daily diets of prediabetic individuals for increasing insulin sensitivity and reducing oxidative stress and inflammation.

Compliance with Ethical Standards

Conflict of interest: The author(s) declares that they have no actual, potential, or perceived conflict of interest for this article.

Ethics committee approval: The authors declare that this study does not include experiments with human or animal subjects, so ethics committee approval is not required.

Data availability: Data will be made available on request.

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Disclosure: -

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