

Evaluation of Antiglycation Effects of Extracts Obtained from *Canarium album* Raeusch Fruit and Beneficial Activity on Advanced Glycation Endproduct-Mediated Oxidative Stress and Inflammation in Monocytes and Vascular Endothelial Cells

Chiung-Tsun Kuo, Tzu-Hao Liu, Fang-Yi Lin, Tai-Hao Hsu, and Hui-Yin Chen

Abstract—Hyperglycemia-mediated accumulation of advanced glycation end-products (AGEs) play a pivotal role in the development of diabetic complications by inducing inflammation. In the present study, we evaluated the possible effects of water/ethanol (1/1, v/v) extracts (WEE) and its fractions from *Canarium album* Raeusch. (Chinese olive) which is a fruit used on AGEs-stimulated oxidative stress and inflammation in monocytes and vascular endothelial cells. Co-incubation of EA.hy926 endothelial cells with WEE and its fractions for 24h resulted in a significant decrease of monocyte-endothelial cell adhesion, the expression of ICAM-1, generation of intracellular ROS and depletion of GSH induced by AGEs. Chinese olive fruit extracts also reduced the expression of pro-inflammatory mediators, such as TNF- α , IL-1 β and IL-6 in THP-1 cells. These findings suggested that Chinese olive fruit was able to protect vascular endothelium from dysfunction induced by AGEs.

Keywords—Advanced glycation end-products (AGEs), *Canarium album* Raeusch, endothelial dysfunction, inflammation.

I. INTRODUCTION

DIABETES mellitus (DM), a group of metabolic diseases characterized by abnormal glucose metabolism, is the most common endocrine disease in nearly all countries. The global figure of the number of adults with DM was estimated at 285 million in 2010 and is expected to rise to 439 million in 2030 [1]. Chronic hyperglycemia may contribute to the pathogenesis of long-term DM complications and have an important role in increasing morbidity and mortality.

C. T. Kuo is with the Department of Bioindustry Technology, Da-Yeh University, Changhua, and Department of Food Science and Technology, Central Taiwan University of Science and Technology, Taichung, Taiwan (e-mail: ctkuo@ctust.edu.tw).

T. H. Liu is with the Department of Food Science and Technology, Central Taiwan University of Science and Technology, Taichung, Taiwan (e-mail: zhow1108@yahoo.com.tw).

F. Y. Lin and T. H. Hsu are with the Department of Bioindustry Technology, Da-Yeh University, Changhua, Taiwan (e-mail: fannylin@mail.dyu.edu.tw th4420@gmail.com).

H. Y. Chen is corresponding author with the Department of Food Science and Technology, Central Taiwan University of Science and Technology, Taichung, Taiwan (phone: 886-4-22391647 ex 3500; fax: 886-4-22396771; e-mail: hychen2@ctust.edu.tw).

Glycation, the nonenzymatic reaction of reducing sugars with amino groups, is increased in hyperglycemic physiological environments, leading to an acceleration of the formation of advanced glycation endproducts (AGEs). Increased accumulation of AGEs can induce multiple cellular changes leading to macro- and micro-vascular complications, such as atherosclerosis, diabetic retinopathy, nephropathy, and neuropathy [2].

The mechanisms of diabetic cardiovascular diseases are multifaceted, involving increased oxidative/nitrosative stress, accumulation of AGEs, enhanced receptor for advanced glycation end product (RAGE), activation of various proinflammatory and cell death signaling pathways, and increased adhesion of circulating monocytes to the vessel wall, etc. [3]-[6]. AGEs mediated inflammation and endothelial dysfunction have been known as a key underlying cause in the development of vascular complications [7]. Studies suggest that AGEs can generate large amounts of pro-inflammatory cytokines through RAGE activation, and these results are related to the modulation of inflammatory molecules through oxidative stress [8], which has been suggested to be the key player in the generation of both micro and macrovascular diabetes complications [9].

For these reasons, investigations on AGEs inhibitors could present a potential preventive and therapeutic method for lowering the development of diabetic complications. Chinese olive (*Canarium album* Raeusch.) is widely cultivated in Taiwan and the southeast area of China. Fresh fruits of Chinese olive are generally processed in the food industry to beverages, candy and confections. From the results of our prior study, the extracts from Chinese olive fruits exhibited well antioxidant activity toward lipid and protein, and scavenging effects on free radicals. The objectives of this study were to investigate the antiglycation and the protection of Chinese olive fruit extracts on the inflammation and dysfunction induced by AGEs in monocytes and endothelial cells.

II. MATERIALS AND METHODS

A. Plant Material and Extraction

Fresh Chinese olive (*Canarium album* Raeusch.) fruits were purchased in Hsinchu, Taiwan. Lyophilised sample powder (10g) was repeatedly extracted twice with 250ml water, water / ethanol (1/1, v/v), ethanol, methanol, acetone or ethyl acetate at room temperature for 15min, respectively. The water / ethanol (1/1, v/v) extracts (WEE) was further fractionated sequentially with ethyl acetate, acetone, ethanol, methanol, and water based on their polarity in order to obtain antioxidant and antiglycation rich fractions.

B. In vitro Glycation of Bovine Serum Albumin

Glycation of bovine serum albumin (BSA) was performed by incubating BSA (20mg/ml) with 0.5M glucose in PBS (pH 7.4) containing 0.02% sodium azide at 37°C for 6 weeks under sterile conditions. The degree of BSA glycation was detected by AGEs-specific fluorescence (Ex 370 nm/ Em 440 nm).

C. Cell Cultures

The human endothelial cell line EA.hy926 was obtained from American-type culture collection (ATCC) and cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 0.37% NaHCO₃, 10% fetal bovine serum (FBS, Invitrogen), 100U/ml penicillin and 100 µg/ml streptomycin. The human monocytic cell line THP-1 (Bioresources Collection and Research Center, Hsinchu, Taiwan) was maintained in RPMI-1640 (Invitrogen) supplemented with 10% FBS, 100 U/ml of Penicillin and 100 lg/ml of Streptomycin. The cells were maintained at 37°C in a humidified atmosphere of air and 5% CO₂. The cytotoxicity of AGEs and samples was determined by MTT assay.

D. Co-Cultures and in vitro Monocyte Adhesion Assay

EA.hy926 (4×10⁴ cells/well) in 96-well plates were treated with AGEs and/or samples for 24h. Cells of the human monocytic leukemic cell line THP-1 (1×10⁶ cells/ml) were labeled with 1mM calcein AM (Molecular Probes Inc.) in RPMI 1640 medium containing 10% FBS. In the co-culture system, the labeled THP-1 cells were seeded at a density of 1×10⁵ cells onto EA.hy926 cells and incubated for 45min. The non-adherent THP-1 cells were removed by careful washing twice with PBS. The quantitative results were obtained using a FLUOstar Optima microplate reader (BMG Labtechnologies, Jena, Germany) at 485nm excitation and 538nm emission wavelengths.

E. Determination of Intracellular Reactive Oxygen Species (ROS)

EA.hy926 cells grown in 96-well plates were pretreated with AGEs and samples for 24h. Then, the cells were washed and then incubated with 100µl 1mM H₂DCFDA in the dark at 37°C for 30min. After incubation, the cells were washed thrice with PBS to remove unbound H₂DCFDA and the fluorescence image was measured at 485nm excitation and 538nm emission wavelengths.

F. ELISA Assays

EA.hy926 cells were grown in Petri dishes to reach confluence. Cells were starved in serum free DMEM for 24h and then treated with AGEs and/or samples in DMEM containing 10% FBS for a further 24h. The soluble adhesion molecules levels of ICAM-1 in cell supernatant were measured with a commercial ELISA kit (Invitrogen) and glutathione (GSH) and superoxide dismutase (SOD) levels in cells were determined using a commercially available kit according to the manufacturer's instructions (Enzo Life Sciences).

THP-1 cells were stimulated with AGEs for 24h in presence or absence of samples. At the end of 24h, the cell supernatant was used for cytokine measurements including IL-1β, IL-6 and TNFα with ELISA kit (Invitrogen).

G. Statistical Analysis

All data are expressed as means ± SD. Data were analyzed using the Statistical Analysis System software package. Analyses of variance were performed using ANOVA procedures. Significant differences between means were determined using Duncan's multiple range tests.

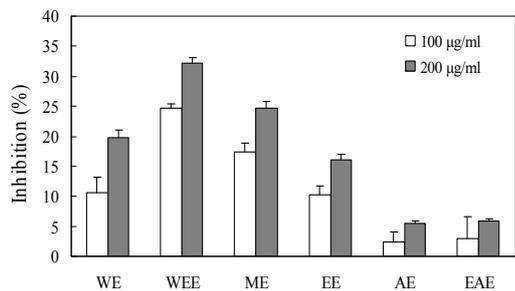
III. RESULTS AND DISCUSSION

Fig. 1 (a) shows the inhibitory effects of various extracts from Chinese olive fruit compared to Trolox and aminoguanidine (AG). AGEs-related fluorescence assay revealed significant inhibition by various Chinese olive extracts. The antiglycation effects of six extracts decreased in the order of WEE>methanol extracts (ME)>water extracts (WE) ≥ ethanol extracts (EE)>acetone extracts (AE) ≅ ethyl acetate (EAE). Our previous study reported that WEE and WE from Chinese olive fruit exhibited well free radical-scavenging activity and antioxidant activity against LDL oxidation. Thus, we next attempted to investigate the effects of fractions obtained from WEE on AGEs formation and the protective activity against AGEs-induced monocytic inflammation and vascular endothelium dysfunction. All fractions (200µg/ml) obtained from WEE significantly suppressed AGEs-related fluorescence and the inhibitory effects between 23% and 57% (p<0.05). Among the five fractions examined, strongest efficiency for water fractions (WF) was found and was similar to that of Trolox (Fig. 1 (b)).

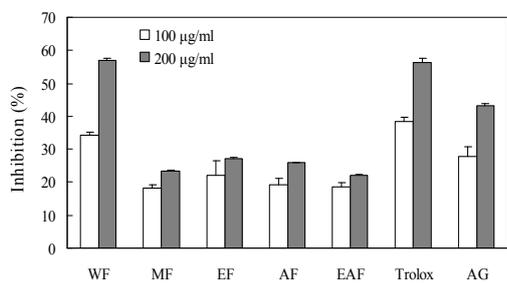
As shown in Fig. 2 (a), EA.hy926 cells treated with AGEs significantly increased intracellular ROS generation. Whereas WEE and its fractions from Chinese olive fruit inhibited 59%~85% AGEs-induced ROS formation. As a positive control, Trolox and AG at the concentration of 100µg/ml showed 29% and 13% reduction, respectively.

Co-incubation of EA.hy926 cells with AGEs for 24h resulted in a significant decrease of SOD activity (Fig. 3 (a)) and GSH level (Fig. 3 (b)). After incubation of endothelial cell in the co-presence of Chinese olive fruit samples with AGEs for 24h, WEE and its fractions significantly increased SOD activity and GSH level. AG and fractions obtained from WEE exhibited better inhibitory effects than that of Trolox in increasing SOD activity. However, Trolox showed the strongest protection on

GSH depletion induced by AGEs. These data verify that Chinese olive fruit possessed significant prevention against AGEs-mediated oxidative stress in endothelial cells.



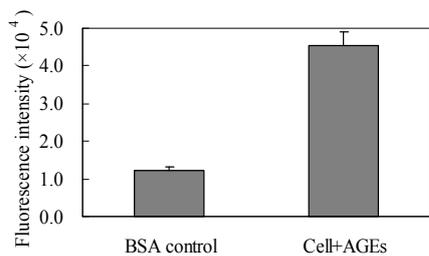
(a)



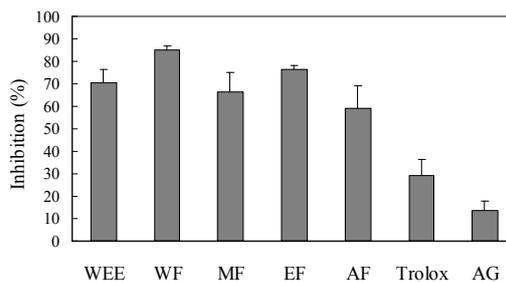
(b)

Fig. 1 The inhibitory effect of (a) various extracts from Chinese olive fruit and (b) various fractions from WEE of Chinese olive fruit on the formation of AGEs by incubating BSA(20mg/ml) with glucose (500mM) in 0.2M phosphate buffer (pH 7.4) at 37°C for 35 days

The incubation of EA.hy926 cell with 100µg/mL AGEs resulted in a considerable increase in THP-1 cell adhesion compared to non-stimulated cells (Fig. 4 (a)). This increased cell adhesion was reduced by fractions from WEE of Chinese olive fruit treatments (Fig. 4 (b)). The WEE and fractions had significantly higher inhibitory effects than that of Trolox and AG.

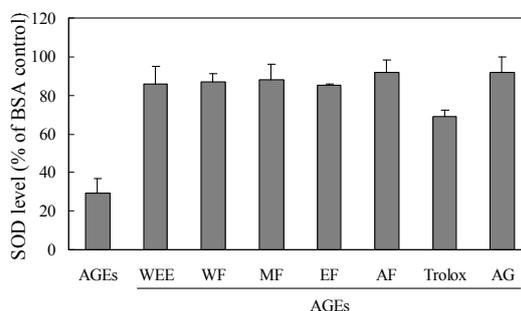


(a)

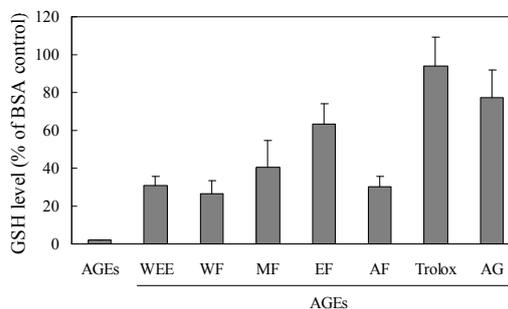


(b)

Fig. 2 Effect of WEE and its fractions from Chinese olive fruit on ROS generation induced by AGEs(100µg/ml) in EA.hy926cell.EA.hy926 cells were incubated with AGEs (100µg/ml)in the (a) absence and (b) presence of Chinese olive fruit samples (100µg/ml) for 24h. ROS levels were measured using fluorescent probe, H₂DCFDA

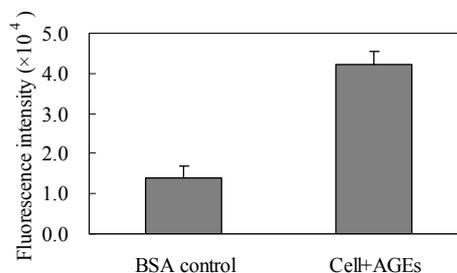


(a)

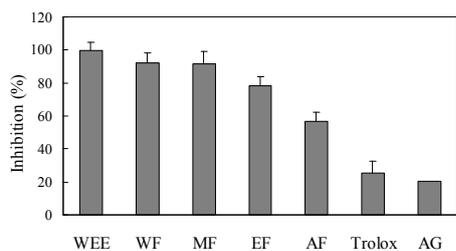


(b)

Fig. 3 The protection of WEE and its fractions (300µg/ml) from Chinese olive fruit on depletion of intracellular (a) SOD and (b) GSH induced by AGEs (200µg/ml)in EA.hy926 cell



(a)

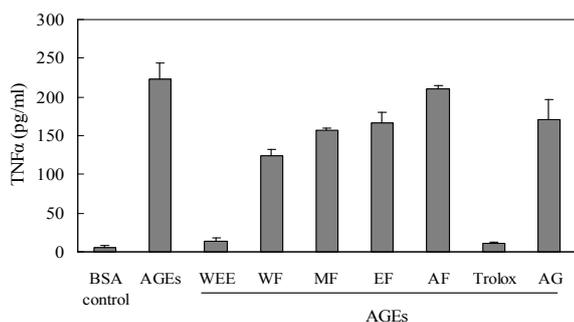


(b)

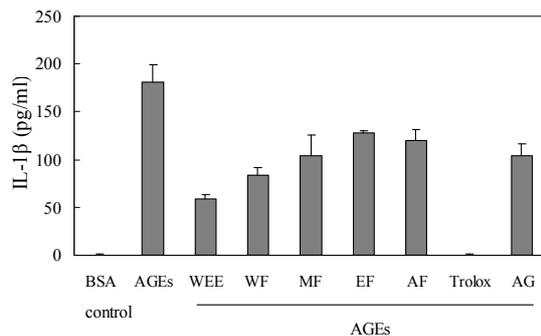
Fig. 4 Effects of WEE and its fractions from Chinese olive fruit on AGEs-induced monocyte (THP-1 cell) adhesion to EA.hy926 cell. EA.hy926 cells were incubated with AGEs (100µg/ml) in the (a) absence and (b) presence of Chinese olive fruit samples (100µg/ml) for 24h, and washed extensively before the addition of fluorescence-labeled monocytes and co-incubation continued for 45min

As shown in Fig. 5, the expressions of various pro-inflammatory intermediates, including TNF-α, IL-1β and IL-6 were significantly increased by AGEs treatments. The WEE and fractions of Chinese olive fruit potently inhibited the production of TNF-α, IL-1β and IL-6. The inhibitory effects of WEE on the expression of TNF-α and IL-6 were similar to that of Trolox, and better than AG. Among five fractions studied, WF showed the highest inhibitory effect on the expression of pro-inflammatory molecules induced by AGEs.

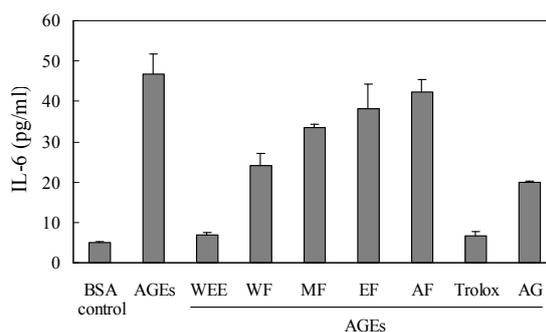
ICAM-1, a biomarker of inflammation, was shown to be induced by pro-inflammatory cytokine TNF-α. ICAM-1 in endothelial cells incubated with AGEs for 24h was significantly increased (Fig. 6). The anti-inflammatory effect of Chinese olive fruits was evaluated by measuring its inhibition of AGEs-induced ICAM-1 expression. Compared with Trolox and AG, the expression of ICAM-1 in endothelial cells incubated with WEE and its fractions were significantly suppressed.



(a)



(b)



(c)

Fig. 5 The inhibition of WEE and its fractions from Chinese olive fruit on the expression of pro-inflammatory mediators in AGE-stimulated THP-1 cells. Monocytic THP-1 cells were incubated with AGEs (600 µg/ml) in the absence and presence of Chinese olive fruit samples (200 µg/ml) for 24 h. The cell supernatants were assayed for (a) TNF-α, (b) IL-1β and (c) IL-6 production by ELISA as described in Materials and Methods

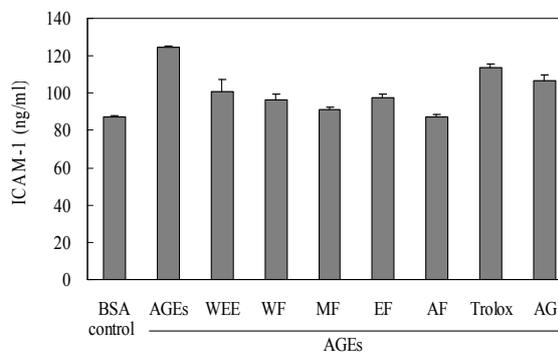


Fig. 6 Effects of WEE and its fractions from Chinese olive fruit on ICAM-1 expression in AGE-stimulated EA.hy926 cells. EA.hy926 cells were incubated with AGEs (600µg/ml) in the absence and presence of Chinese olive fruit samples (200µg/ml) for 24h. The cell supernatants were assayed for ICAM-1 production by ELISA as described in Materials and Methods

Our results showed that WEE and its fraction from Chinese olive fruit markedly suppressed intracellular ROS production by scavenging ROS and increasing SOD activity and GSH level, and inhibited inflammatory signaling cascades in THP-1 cells and the expression of ICAM-1 and monocyte-endothelial

adhesion induced by AGEs in EA.hy926 endothelial cells. Considering its protection on AGEs-mediated oxidative stress and inflammation in monocytes and vascular endothelial cells, Chinese olive fruit could be applied to prevent the development of diabetic complications.

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