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PHYTOCHEMICAL AND BIOLOGICAL INVESTIGATION OF GALLOTANNINS FROM RED MAPLE (*ACER RUBRUM*) SPECIES

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PHYTOCHEMICAL AND BIOLOGICAL INVESTIGATION OF GALLOTANNINS FROM RED MAPLE (*ACER RUBRUM*) SPECIES

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ABSTRACT

This study investigated the phytochemical constituents, primarily gallotannins, present in a proprietary extract, namely Maplifa^{TM}, from leaves of the red maple (*Acer rubrum* L.) species as well as their biological activities and mechanisms of action. Although the red maple species has been traditionally used as folk medicine by Native American Indians for numerous health benefits, the bioactive chemical constituents of the leaves of the red maple still remain unknown. This study carried out the identification of phytochemicals targeting gallotannins, a class of polyphenols, from red maple leaves by using various chromatographic separation techniques and spectroscopic approaches. Furthermore, compounds from the red maple species were evaluated for bioactivities including anti-oxidant, anti-diabetic (α-glucosidase enzyme inhibition and inhibition of advanced glycation end products, AGEs) and skin lightening effects (anti-tyrosinase and anti-melanogenesis) by using a panel of in-house bioassays. In addition, the mechanisms of the inhibitory effects of red maple phytochemicals on α-glucosidase were characterized by using kinetic assay, fluorescence assay, FTIR and CD (circular dichroism) spectra. Moreover, the mechanisms of inhibition of the gallotannins on the formation of AGEs were elucidated at each individual stage of AGEs formation by applying MALDI-TOF mass spectroscopy, HPLC-FL analysis, G.K. peptide assays, and assessing the conformational changes induced by protein glycation by using CD.

Plant derived natural products have served as an important resource for medicinal compounds over decades. The red maple is an indigenous plant species of eastern North America and was extensively used as an herbal remedy by the indigenous peoples of

eastern North American region for many ailments including abdominal pain, diarrhea and eyes diseases.

Recent studies have shown that extracts of red maple plant parts exhibit free radical scavenging capacities and this antioxidant property was associated with its polyphenolic content. Red maple extracts have also been investigated for their antimicrobial and anticancer effects. In addition, our group has recently demonstrated that a red maple bark extract was able to decrease blood glucose levels in mice after sucrose loading indicating the presence of bioactive compounds that could be relevant to diabetes management. Furthermore, our laboratory has recently isolated a number of new gallotannins, named maplexins A-I, from the stem and bark of the red maple species and has shown that these compounds display potent α-glucosidase inhibitory activity *in vitro*. As the total polyphenolic content level in red maple leaves is higher than in bark, it is logical to investigate the chemical constituents of red maple leaves for gallotannins which could also serve as α-glucosidase inhibitors. Therefore, we propose a phytochemical study to develop a proprietary extract from red maple leaves and to isolate and identify phenolic compounds therein and evaluate these compounds for their *in vitro* antioxidant and anti-diabetic activities. This study also proposes to elucidate the mechanisms of inhibitory effects of maple gallotannins on the α-glucosidase enzyme by using various biophysical tools. Understanding the mechanisms of inhibitory action of these compounds will be critical for the further development of α-glucosidase inhibitors from maple for diabetes management.

Beside the inhibitory effects on the α-glucosidase enzyme, the red maple gallotannins were also evaluated for their inhibitory effects on the formation of advanced

glycation end products (AGEs), another therapeutic target of diabetes. AGEs are a heterogeneous group of irreversible adducts from non-enzymatic glucose-protein condensation reactions. It has been reported that AGEs as oxidative derivatives are increasingly being implicated as a potential risk for diabetes. Numerous studies have demonstrated that phenolics with anti-oxidative capacity from fruits or vegetables could effectively interrupt the condensation reaction of glucose and proteins and further decrease the formation of AGEs. Therefore, we hypothesize that gallotannins from red maple species might act as antioxidants and inhibit the formation of AGEs.

In addition to anti-diabetic activities, the skin lightening effects of red maple gallotannins, as a treatment strategy for hyperpigmentary disorders, were also evaluated in this study. Hyperpigmentary disorders, such as melasma, age spot, freckles and lentigies, are caused by the accumulation of abnormal melanin. In the melanin biosynthetic pathway, tyrosinase is an exclusive enzyme responsible for the formation of melanins. Emerging data suggests that several plant-derived constituents may act as natural tyrosinase inhibitors which are of interest to the food (as anti-browning agents) and cosmetic (as skin lightening agents) industries. Therefore, we also evaluated the anti-tyrosinase and anti-melanogenic properties of gallotannins isolated from red maple leaves.

In **Manuscript I:** the objective was to develop a proprietary standardized red maple leaf extract, named Maplifa^{TM}, for potential nutraceutical and/or cosmeceutical applications. In addition, the total phenolic content of Maplifa^{TM}, was determined as gallic acid equivalents (GAEs) and the major gallotannins present in Maplifa^{TM} were identified as

maplexin B, ginnalin B, ginnalin C, ginnalin A, maplexin F and a pair of isomers, 6-*O*-digalloyl-2-*O*-galloyl-1,5-anhydro-D-glucitol and 2-*O*-digalloyl-6-*O*-galloyl-1,5-anhydro-D-glucitol. The quantitative HPLC-UV analysis revealed that ginnalin A was the predominant gallotannin in Maplifa™ (56.3% by dried weight). Furthermore, Maplifa™ was evaluated for various bioactivities including anti-oxidant, anti-tyrosinase, anti-α-glucosidase and anti-AGEs. Maplifa™ showed potent anti-oxidant activity in the DPPH assay with an IC_{50} of 78.2 ppm. Also, Maplifa™ showed inhibitory effects on the tyrosinase and α-glucosidase enzymes (IC_{50} = 154.5 ppm and 37.8 ppm, respectively). Lastly, Maplifa™ displayed inhibitory activity against the formation of AGEs (IC_{50} = 8.6 ppm), which was 10-fold more potent than aminoguanidine as the positive control. Findings from this study suggest that Maplifa™ could have potential cosmetic (for e.g. skin-whitening/lightening) and/or nutraceutical (for e.g. anti-diabetic) applications but further *in vivo* studies would be required to confirm this.

In **Manuscript II,** as our previous phytochemical studies on red maple (*Acer rubrum*) species lead to the identification of a series of maple gallotannins as potent α-glucosidase inhibitors, we aimed to provide insights into the ligand-enzyme interactions and the binding mechanisms of the maple gallotannins and the α-glucosidase enzyme. The inhibitory effects of four gallotannins from red maple species, namely ginnalin A, ginnalin B, ginnalin C and maplexin F, as well as a synthetic derived gallotannin, namely maplexin J, were evaluated against α-glucosidase. The inhibitory effects of the gallotannins against the α-glucosidase enzyme were determined as a noncompetitive mode. The interactions between the gallotannins and α-glucosidase were further

elucidated by using spectroscopic means. The results revealed that the gallotannins inhibited α-glucosidase by forming stable ligand-enzyme complexes which consequently lead to the quenching of their intrinsic fluorescence statically. In addition, a fluorescent probe 1,1′-bis(4-anilino-5-napththalenesulfonic acid (bis-ANS), was used to explore the binding regions of the gallotannins. Lastly, FTIR and CD data showed that the gallotannins bound to α-glucosidase and induced conformational changes to the enzyme. These findings were further discussed in the context of structure activity relationship (SAR) for the potential of developing structural gallotannin analogs as potent α-glucosidase inhibitors.

In **Manuscript III**, the objective of this study was to evaluate the inhibitory effects of a series of maple gallotannins, namely ginnalins A-C and maplexins F and J, at individual stage of protein glycation using a combination of analytical methods including fluorescence spectroscopy, HPLC-FL, CD, and MALDI-TOF. Both early and middle stages of protein glycation inhibition by the maple gallotannins was determined by using the BSA-fructose assay. In this assay, maplexin F showed the most potent inhibitory effect against the formation of AGEs with an IC_{50} value of 15.8 μM. In addition, MALDI-TOF analysis revealed that the maple gallotannins were able to reduce the number of fructose that adducted to BSA protein indicating that they were able to inhibit the production of Amadori products. Moreover, the maple gallotannins were effective in the G.K. peptide-ribose assay suggested that the protein cross-linking formation at the late stage of glycation could be prevented by maple gallotannins. Lastly, the

spectroscopy analyses revealed that the maple gallotannins were able to reduce the conformational changes of BSA protein that were induced by glycation.

In **MANUSCRIPT IV**, we initiated a project to investigate the cosmetic skin lightening/whitening applications of Maplifa^{TM}, a proprietary extract purified from the leaves of the red maple species, in enzyme and cell based assays. Maplifa^{TM} is standardized to ca. 45-50% of ginnalin A along with other gallotannins including ginnalin B and C and maplexins. SAR studies showed that increasing the number of galloyl groups attached to the 1,5-anhydro-D-glucitol moiety resulted in greater inhibitory effects on the tyrosinase enzyme. Consequently we synthesized maplexin J, a tetragalloyl-glucitol (contains the maximum number of 4 galloyl groups on the1,5-anhydro-D-glucitol core) and confirmed our SAR observations. Purified ginnalins A-C, as representative gallotannins in Maplifa^{TM}, were assayed for their inhibitory effects on melanin production in murine B16F10 cells. Ginnalin A (contains 2 galloyl groups) clearly reduced the melanin content at 50 μM whereas ginnalin B and C (contain 1 galloyl group of each) showed only minor anti-melanogenic effects. Lastly, the mechanisms of the inhibitory effects of ginnalins A-C on melanogenesis in B16F10 cells were elucidated by using real-time PCR and Western blot experiments. The results indicated that ginnalins were able to down-regulate the expression of MITF, TYR, TRP-1 and TRP-2 gene levels in a time and dose-dependent manner and significantly reduce the protein expression of TRP-2 gene. The findings in our study indicate that phytochemicals in red maple leaves possess anti-melanogenic effects and thus may have potential cosmetic skin-whitening applications.

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PREFACE

This dissertation was prepared according to the University of Rhode Island "Guidelines for the Format of Theses and Dissertations" standards for Manuscript format. This dissertation comprises of four manuscripts that have been combined to satisfy the requirements of the department of Biomedical and Pharmaceutical Sciences, College of Pharmacy, University of Rhode Island.

MANUSCRIPT-I: Phytochemical and biological evaluation of Maplifa™, a standardized extract of red maple (*Acer rubrum*) leaves

This manuscript has been prepared for submission to the 'Journal of Agriculture and Food Chemistry'

MANUSCRIPT-II: Comparison of inhibitory activities and mechanisms of red maple gallotannins against *α*-glucosidase

This manuscript has been prepared for submission to the 'Journal of Agricultural and Food Chemistry'

MANUSCRIPT-III: Inhibitory effects and mechanistic studies of red maple gallotannins on the formation of advanced glycation endproducts

This manuscript has been prepared for submission to the 'Journal of Agricultural and Food Chemistry'

MANUSCRIPT-IV: Cosmetic applications of a red maple (*Acer rubrum*) leaf extract (Maplifa™) and its purified gallotannin constituents: Inhibition of melanogenesis via down-regulation of tyrosinase and melanogenic gene expression in B16F10 melanoma cells.

This manuscript has been prepared for submission to 'Experimental Dermatology'

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LIST OF ABBREVIATIONS

HPLC, High Performance Liquid Chromatography; UV, Ultraviolet; AUC, area under curve; NMR, Nuclear magnetic resonance; TM, trademark; GAEs, gallic acid equivalents; DPPH, diphenylpicrylhydrazyl; BHT, butylated hydroxytoluene; MPLC, Medium Pressure Liquid Chromatography; pNPG, p-Nitrophenyl-α-D-glucopyranoside; AGEs, Advanced Glycation Endproducts; RMLE, red maple leaves extract; DMSO, Dimethyl sulfoxide; GA, ginnalin A; GB, ginnalin B; GC, ginnalin C; MF, maplexin F; MJ, maplexin J; bis-ANS, 1,1′-bis(4-anilino-5-napththalenesulfonic acid; SAR, structure activity relationship; NIDDM, non-insulin-dependent diabetes mellitus; AGI, *α*-glucosidase inhibitors; PGG, pentagalloyl glucose; MGO, Methylglyoxal; AG, Aminoguanidine; ROS, Reactive Oxygen Species; RCS, Reactive Carbonyl Species; Gly, Glyoxal; 3-DG, 3-Deoxyglucosone; BSA, Bovine Serum Albumin; PD, 1,2-Phenylenediamine; DQ, 2,3-Dimethylquinoxaline; TFA, Trifluoroacetic Acid; ATR-IR, Attenuated total reflection - infrared spectroscopy; MALDI, Matrix-assisted laser desorption/ionization; CD, Circular Dichroism; 2-MQ, 2-Methylquinoxaline; B16F10, murine metastatic melanoma cells; TYR, tyrosinase; TRP-1, tryosinase-related protein-1;TRP-2, tryosinase-related protein-2; DOPA, 3,4-dehydroxyphenylalanine; DCT, DOPA-chrome tautomerase; DHICA, 5,6-dihydroxyindol-2-carboxylic acid; MITF, Microphthalmia-associated transcription factor; WB, western blotting; RT-PCR, Real-time polymerase chain reaction; RNA, Ribonucleic acid; cDNA, complementary deoxyribonucleic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; DMEM, Dulbecco's Modified Eagle's medium; FDA, Food and Drug Administration.

Manuscript-I

To be submitted for publication in ***Journal of Agriculture and Chemistry***

Phytochemical and Biological Evaluation of Maplifa™, a Standardized Extract of Red Maple (*Acer rubrum*) Leaves

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Running Head: Phytochemical and biological evaluation of Maplifa™

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ABSTRACT

Our previous studies revealed that extracts of maple (*Acer*) species possess numerous biological activities attributed to their polyphenolic contents. In order to utilize maple extracts for potential nutraceutical and cosmeceutical applications, we developed a proprietary maple extract, namely Maplifa™, from red maple (*Acer rubrum* L.) leaves. In addition, the total phenolic contents of Maplifa™ were determined in tgallic acid equivalents (GAEs) and the major polyphenols present in Maplifa™ were identified as maplexin B (**1**), ginnalin B (**2**), ginnalin C (**3**), ginnalin A (**4**), maplexin F (**5**) and two isomers (**6a** and **6b**), 6-*O*-digalloyl-2-O-galloyl-1,5-anhydro-D-glucitol (**6a**) and 2-*O*-digalloyl-6-*O*-galloyl-1,5-anhydro-D-glucitol (**6b**). Quantitative HPLC-UV analysis revealed that ginnalin A was the predominant phenolic in Maplifa™ (56.3% of dried weight). Furthermore, Maplifa™ was evaluated for various bioactivities including anti-oxidant, anti-tyrosinase, anti-α-glucosidase and anti-AGEs. Maplifa™ showed potent anti-oxidant activity in the DPPH assay with an IC_{50} of 78.2 ppm. Also Maplifa™ showed inhibitory effects on tyrosinase and α-glucosidase (IC_{50} = 154.5 ppm and 37.8 ppm, respectively) enzymes. Lastly, Maplifa™ displayed the inhibitory activity against the formation of advanced glycation end products, AGEs (IC_{50} = 8.6 ppm), which was 10-fold more potent than aminoguanidine, the positive control. Findings from this study suggest that Maplifa™ could have potential cosmetic (for e.g. skin-whitening/lightening) and/or nutraceutical (for e.g. anti-diabetic) applications but further *in vivo* studies would be required to confirm this.

Keywords: Acer rubrum, red maple, proprietary extract, total phenolic contents, gallotannins, anti-oxidant, anti-tyrosinase, anti-diabetes

INTRODUCTION

Plants based natural product extracts have been intensively studied for numerous biological and pharmacological properties (*1, 2*). Apart from playing an important role as resources of leading compounds for drug discovery, plants are also considered as preferable substances for the nutraceutical and/or cosmeceutical uses since they tend to be safer than synthetic agents (*3*). It has been demonstrated that many bioactive phytochemicals in plant extracts such as phenolic compounds, alkaloids and sterols are of great value in human health and beauty applications.

Maple (*Acer*) species, especially the sugar maple (*Acer saccharum* L.) and red maple (*Acer rubrum* L.), are widely regarded for their sap which is used for the production of maple syrup, a natural sweetener. These maple species have been traditionally used by the indigenous peoples of eastern North America as a folk medicine for skin and digestive disorders (*4*). Recently, maple extracts have attracted research interests for their biological activities including anti-oxidant, antibacterial, anti-cancer and anti-depressant effects (*5-7*). In our own phytochemical and biological investigations of various plant parts of the sugar maple and red maple species, our group has isolated a series of gallotannins with anti-oxidant, anti-cancer and anti-α-glucosidase activities (*8-11*). It was notable that the gallotannin, ginnalin A, which is the most abundant phenolic compound present in the red maple species, displayed potent free radical scavenging capacity and anti-proliferative effects *in vitro*. Meanwhile, a new gallotannin isolated from red maple bark, namely maplexin F, was 20 times more active than acarbose (a clinical α-glucosidase inhibitor) in the anti-α-glucosidase assay. Furthermore, our group has shown that a red maple bark extract was able to reduce blood glucose level after

carbohydrate challenge in an animal model (*12*). Therefore, we hypothesized that red maple extracts could have potential nutraceutical application for diabetes management.

The formation of advanced glycation endproducts (AGEs) is often associated with diabetes patients in the hyperglycemic condition and it triggers the generation of oxidative stress which further leads to numerous diabetic complications including kidney failure, cardiovascular diseases and atherosclerosis (*13, 14*). Therefore, plants extracts with inhibitory effects against AGEs formation could be a therapeutic strategy for related diabetic complications.

Plants extracts with free radical trapping capacity have been desired as alternatives of skin whitening agents (*15*). Tyrosinase is a rate-limiting oxidase that converts a monophenol to *o*-quinone and then catalyzes a set of reactions that eventually produce melanin as skin pigments (*16*). Thus, tyrosinase inhibitors, in particular from natural sources, are highly sought as skin whitening agents for the cosmeceutical industry.

Due to the aforementioned biological bioactivities of maple extracts and their potential applications for the treatments of diabetes and associated complications and for cosmeceutical utilization, we initiated this study to develop Maplifa^{TM}, as a standardized red maple leaves extract. The total polyphenol content of Maplifa^{TM} were determined in gallic acid equivalents (GAEs) and its major phenolics were identified by HPLC-UV methods by comparison to authentic standards previously isolated from maple species by our laboratory. Lastly, Maplifa^{TM} were evaluated by using a series of bioassays for its nutraceutical and cosmeceutical applications.

MATERIALS AND METHODS

Chemicals and General Experimental Procedures

High performance liquid chromatography (HPLC)-UV was performed on a Hitachi Elite LaChrom system consisting of a L2130 pump, L-2200 autosampler and a L-2455 diode array detector all operated by EZChrom Elite software. All solvents were ACS or HPLC grade and were obtained from Sigma-Aldrich through Wilkem Scientific (Pawcatuck, RI, USA). Unless otherwise stated, all reagents including the Folin-Ciocalteu reagent, gallic acid, ascorbic acid, butylated hydroxytoluene (BHT), diphenylpicrylhydrazyl (DPPH) reagent, α-glucosidase (EC 3.2.1.20) from *Saccharomyces cerevisiae*, p-Nitrophenyl-α-D-glucopyranoside (pNPG), aminoguanidine (AG), bovine serum albumin (BSA), glucose, mushroom tyrosinase, L-tyrosine, 4-hydroxyphenyl β-D-glucopyranoside (arbutin) and kojic acid were purchased from Sigma-Aldrich (St Louis, MO, USA). The authentic maple standards in the HPLC-UV analysis were previously isolated by our group (*17-19*).

Plant Material

Leaves of the red maple (*Acer rubrum*) species were collected on the Kingston Campus of the University of Rhode Island (Kingston, RI, USA) in August 2013, and identified by Mr. J. Peter Morgan (URI Senior Gardener). Voucher specimens (11/6/09LPMCL2) are deposited in the Heber W. Youngken Jr. Medicinal Garden and Greenhouse at the College of Pharmacy, University of Rhode Island.

Extraction of Red Maple Leaves and Preparation of Maplifa™

The extraction of red maple leaves and preparation of the proprietary Maplifa™ extract was according to standardized protocols developed in our laboratory (*20*).

Briefly, dried red maple leaves (100.0 g) were soaked 95 % ethanol (3 x 400 mL) at room temperature. The solvent-leaf slurry was allowed to stir for 12 h. The red maple leaves extract (RMLE; 50.0 g) was obtained after solvent removal *in vacuo*. Then RMLE was subjected to a column chromatographic method with XAD-16 Amberlite resin as the stationary phase. The RMLE (50.0 g) was absorbed onto the XAD-16 resin column (45 x 3 cm) and eluted with a water/ethanol solvent system to remove chlorophyll and other plant pigments and to yield the Maplifa™ extract (11.4 g) after solvent removal *in vacuo*.

Determination of Total Polyphenol Contents of Maplifa™

The total phenolic contents of the Maplifa™ were determined according to the Folin-Ciocalteu method and were measured as gallic acid equivalents (GAEs) as reported previously by our laboratory (*20*). Briefly, Maplifa™ was diluted 1:100 methanol/H2O (1:1, v/v), and 200 µL of sample was incubated with 3 mL of methanol/H2O (1:1, v/v) and 200 µL of Folin-Ciocalteu reagent for 10 min at 25 °C. After this, 600 µL of a 20% Na2CO3aqueous solution was added to each tube and mixed by vortex. Mixture was further incubated for 20 min at 40 °C. After incubation, the samples were immediately cooled in an ice bath to room temperature. Samples and standard (gallic acid) were processed identically. The absorbance was determined at 755 nm, and final results were calculated from the standard curve obtained from a Spectramax plate reader.

Analytical HPLC-UV Analyses of Maplifa™

A Luna C18 column (250 × 4.6 mm i.d., 5 µm; Phenomenex) with a flow rate at 0.75 mL/min and injection volume of 20 µL for all samples (Maplifa™ and authentic standards of compounds 1-7) was used. A gradient solvent system consisting of solvent A (0.1% aqueous trifluoroacetic acid) and solvent B (methanol) was used as follows: 0–

40 min, from 10% to 46% B; 40-60 min, 46–80% B; 65–67 min, 100% B; 67–68 min, from 100% to 10% B; 68-75 min, 10% B. Figure 2 shows the HPLC-UV profiles of Maplifa™.

Identification of Gallotannins Present in Maplifa™

The major compounds present in Maplifa™ were identified by comparison the retention time of HPLC-UV chromatographic profiles of Maplifa™ and authentic standards that were previously isolated by our group from the red maple species (*10*, *11*, *21*). Apart from these standards, two compounds (**6a** and **6b**) were isolated and identified (by NMR spectroscopic data) from RMLE as follows. Briefly, RMLE (1.0 g) was subjected to a Medium Pressure Liquid Chromatography (MPLC) column with ODS resin. Then MPLC column was eluted by a combination of water and methanol mobile phase (water:methanol, 7:3, v/v). Subfractions 13-15 were further purified by semi-preparative HPLC to obtain isomers **6a** and **6b**. Their NMR spectroscopic data were in agreement with published report (*22*).

Quantification of the Levels of the Major Gallotannins Present in Maplifa™

The contents of identified polyphenols in Maplifa™ were determined by the HPLC-UV analytical method that previously reported by our group. Briefly, a stock solution of 1 mg/mL of a ginnalin A was prepared in DMSO and then serially diluted to afford samples of 0.5, 0.25, 0.125, 0.0625, 0.03125 mg/mL concentrations, respectively. Each sample was injected in triplicate and a linear six-point calibration curve (r^2= 0.9976) was constructed by plotting the mean peak area percentage against concentration. All HPLC-UV analyses were carried out with 20 µL injection volumes on a Luna C18 column and monitored at a wavelength of 280 nm. A gradient solvent system consisting

of solvent A (0.1% aqueous tri-fluoroacetic acid) and solvent B (methanol, MeOH) was used with a flow rate at 0.75 mL/min as follows: 0–50 min, 10%–55% B; 50-51 min, 55%–100% B; 51-52 min, 100% B; 52-53 min, 100%–10% B. The concentration of ginnalin A in the Maplifa^{TM} was quantified based on the standard curves. The concentrations of other compounds (**1-3** and **5-6**) in Maplifa^{TM} were quantified based on the percentages of the HPLC area under curve (AUC).

Biological Evaluations

Antioxidant Assay (DPPH)

The antioxidant potential of the isolated compounds was determined on the basis of their ability to scavenge the diphenylpicrylhydrazyl (DPPH) radical according to previously reported methods from our laboratory. Ascorbic acid (vitamin C) and the synthetic commercial antioxidant, butylated hydroxytoluene (BHT), were used as positive controls. The assay was conducted in a 96-well format using serial dilutions of 50 μL aliquots of test compounds, vitamin C and BHT. After this, 100 μL DPPH (80 mg/L) was added to each well. Absorbance was determined after 30 min reaction in dark at 515 nm by a micro-plate reader (SpectraMax M2, Molecular Devices Corp., operated by SoftmaxPro v.4.6 software, Sunnyvale, CA, USA), and the scavenging capacity (SC) was calculated as $SC\% = [(A_0 - A_1)/A_0] \times 100$, Where A_0 is the absorbance of the reagent blank and A_1 is the absorbance of the test samples.

Anti-*α*-Glucosidase Enzyme Assay

A mixture of 50 μL of test samples and 100 μL of 0.1 M phosphate buffer (pH 6.9) containing yeast *α*-glucosidase solution (1.0 U/ml) were incubated in 96 well plates at 25 ℃ for 10 min. After pre-incubation, 50 μL of 5 mM p-nitrophenyl-*α*-D-

glucopyranoside solution in 0.1 M phosphate buffer (pH 6.9) were added to each well at timed intervals. The reaction mixtures were incubated at 25 ℃ for 5 min. Before and after incubation, absorbance were recorded at 405 nm by a micro-plate reader (SpectraMax M2, Molecular Devices Corp., operated by SoftmaxPro v.4.6 software, Sunnyvale, CA) and compared to that of the control which had 50 μL buffer solutions in place of the samples. The α-glucosidase inhibitory activity were expressed as percentage of inhibition and were calculated as follows: $\text{inhibition}\% = 100 \times [(C_5 - C_0) - (S_5 - S_0)] / (C_5 - C_0)$. Where C_0 is the absorbance of the reagent blank in 0 min, S_0 is the absorbance of the samples in 0 min, C_5 is the absorbance of the reagent blank in 5 min and S_5 is the absorbance of the samples in 5 min.

Anti-tyrosinase Enzyme Assay

Inhibitory effects of gallotannins and a maple leaf extract on mushroom tyrosinase was evaluated spectrophotometrically using L-tyrosine as a substrate according to a method described previously with minor modification (*23*). Tyrosinase inhibition assays were performed in 96-well microplate format using SpectraMax M2 microplate reader (Molecular Devices, CA). Briefly, testing samples were dissolved in 10% DMSO at a concentration of 5.0 mg/mL and then diluted to different concentrations with phosphate buffer (0.1 M, pH 6.8). Each well contained 40 μL of sample with 80 μL of phosphate buffer solution, 40 μL of tyrosinase (100 units/mL), and 40 μL L-tyrosine (2.5 mM). The mixture was incubated for 20 min at 37 ℃, and absorbance was measured at 490 nm. Each sample was accompanied by a blank containing all components except L-tyrosine. Arbutin and kojic acid were used as positive controls. The results were compared with a control consisting of 10% DMSO in place of the sample. The

percentage of tyrosinase inhibition was calculated as follows: [(ΔAcontrol − ΔAsample)/ ΔAcontrol] × 100

Anti-AGEs Assay (BSA-Glucose Assay)

The assay was performed as previously reported by our group (*24*). Briefly, BSA (50 mg/mL) was nonenzymatically glycated by incubation under sterile conditions in 1.5 M phosphate buffer (pH 7.4) at 37 ℃ for 7 days in the presence of 0.8 M glucose. Fluorescence of samples was measured at the excitation and emission maxima of 330 and 410 nm, respectively, versus an unincubated blank containing the protein, glucose, and inhibitors. The % inhibition by different concentrations of inhibitor was calculated as [(ΔAcontrol − ΔAsample)/ ΔAcontrol] × 100. Aminoguanidine (AG, 10mM) was used as a positive control.

RESULTS AND DISCUSSION

Preparation and Determination of Polyphenol Contents in Maplifa™

Our laboratory has reported that ginnalin A is a major bioactive compound which is predominantly present in various plant parts of the red maple species (*21*). In order to develop a sustainable source (namely, leaves) of ginnalin A and other bioactive compounds, red maple leaves were extracted using 95 % ethanol and subjected to an XAD-16 resin column to remove chlorophyll and other plant pigments. This standardized preparation protocol resulted 11.4 % yield of a proprietary extract, named Maplifa™, starting from the crude leaves material which was obtained as an off-white free flowing dried powder after solvent removal *in vacuo*. Because we intended to develop Maplifa™ for potential nutraceutical and cosmeceutical applications, instead of using laboratory extracting solvents such as methanol or acetone, we used an

ethanol/water (95% :5%; v/v) combination which are preferred extraction solvents to meet FDA regulatory requirements. In addition, the total polyphenol contents of Maplifa™ were determined as 61.27 % GAEs which was higher than the crude red maple leaves extract (as 45.30 % GAEs). This phenomenon was possibly due to the removal of a large fraction of nonpolar or non-phenolic components including chlorophylls and plant lipids during the XAD-16 column chromatography.

Identification of the Major Phytochemicals (Gallotannins) Present in Maplifa™

The HPLC-UV chromatogram of Maplifa™ is shown in Figure 2. The major compounds in Maplifa™ were identified as maplexin B (**1**) (*18*), ginnalin B (**2**), ginnalin C (**3**), ginnalin A (**4**) (*17*), maplexin F (**5**) (*11*) and two isomers (**6a** and **6b**), 6-*O*-digalloyl-2-*O*-galloyl-1,5-anhydro-D-glucitol (**6a**) and 2-*O*-digalloyl-6-*O*-galloyl-1,5-anhydro-D-glucitol (**6b**) (*22*) (Chemical structures shown in Figure 1.). The chemical structures of **6a** and **6b** were elucidated based on their spectroscopic data, including ^{1}H-NMR and ^{13}C-NMR, which were in agreement with previous reports (*22*). Compounds **1-5** were identified by HPLC-UV chromatography profiles by comparison to authentic standards. As shown in Table 1, the HPLC-UV retention time of each peak was compared with that of the authentic standards that were previously isolated from maple species. All peaks had identical retention time as comparing with authentic standards isolate from red maple barks and twigs (all t_R variations were less than 1 min). In addition, the content level (as of weight percentage) of each compound in Maplifa™ was determined and shown in Table 1. Ginnalin A was the predominant compound present in Maplifa™ (56.41%) suggesting that Maplifa™ might be able to display similar activities compared to ginnalin A alone.

Biological Evaluation of Maplifa™

Maplifa™ was evaluated for anti-oxidant, anti-tyrosinase, anti-α-glucosidase and anti-AGEs activities as summarized in Table 2. In the DPPH assay, Maplifa™ had an IC_{50} value of 78.2 ppm, which was more active more the positive control, a synthetic antioxidant, BHT (IC_{50} = 330.5 ppm). It has been reported that polyphenolic compounds possess potent free radical scavenging capacity and many bioactivities of maple species extracts were greatly associated with their polyphenol contents (*25*). Based on this anti-oxidant data, Maplifa™ can be regarded as a polyphenol-rich antioxidant extract of red maple leaves. Furthermore, Maplifa™ also showed a moderate inhibitory effect against tyrosinase enzyme. Although other maple species extracts have been reported as inhibitors for tyrosinase and melanogenesis, further investigations such as cellular based assays would be necessary to evaluate the skin whiting potency of Maplifa™ (*26*, *27*). In addition, Maplifa™ was assayed for its anti-diabetic activities including anti-α-glucosidase and anti-AGEs. The enzyme α-glucosidase is responsible for hydrolysis of oligosaccharide into monosaccharide such as glucose, so it is considered as a target for the postprandial-hyperglycemia. Maplifa™ showed superior anti-α-glucosidase inhibitory activity compared to the positive control, acarbose (IC_{50} = 37.8 vs. 91.6 ppm, respectively). This result was not unexpected since compound **5** has been previously identified as a potent α-glucosidase inhibitor from the red maple bark (*19*) and other compounds in Maplifa™ may also contribute to the activity synergistically. Lastly, Maplifa™ showed a promising inhibitory effect against the formation of AGEs (Figure 3). It was approximately 10-fold more potent than aminoguanidine which was used as the positive control. Reactive oxygen species (ROS) and reactive carbonyl species (RCS) are

the two factors that accelerate the formation of AGEs, so Maplifa™ might inhibit AGEs formation via serving as trapping agent of free radicals and RCS.

CONCLUSIONS

Maplifa™ was developed as a proprietary natural product extracted from red maple leaves containing a standardized amount of bioactive ingredients. This study provided preliminary findings of the health beneficial properties of Maplifa™ including anti-oxidant, anti-tyrosinase, anti-α-glucosidase and anti-AGEs effects. Further investigation of Maplifa™ is warranted to evaluate the skin-whitening effect at cellular and molecular level and the mechanisms of its anti-diabetic activities. Overall, Maplifa™ was developed as a sustainable source (using leaves) for bioactive compounds from the red maple species with nutraceutical and/or cosmeceutical potential.

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FIGURE AND TABLE LEGEND

Figure 1. Chemical structures of maplexin B (**1**), ginnalin B (**2**) and ginnalin C (**3**) ginnalin A (**4**), maplexin F (**5**) and 6-*O*-digalloyl-2-*O*-galloyl-1,5-anhydro-D-glucitol (**6a**) and 2-*O*-digalloyl-6-*O*-galloyl-1,5-anhydro-D-glucitol (**6b**) identified from Maplifa^{TM}. All the compounds were identified based on comparing their HPLC-UV peak retention time (t_R, min) with that of the authentic standards.

Figure 2. HPLC-UV chromatograms of Maplifa^{TM} showing the presence of major polyphenolic compounds. Peak **1** = maplexin B; Peak **2** = ginnalin B; Peak **3** = ginnalin C; Peak **4** = ginnalin A; Peak **5** = maplexin F; Peak **6a**/**6b** (co-eluting) = 6-*O*-digalloyl-2-

O-galloyl-1,5-anhydro-D-glucitol (**6a**) and 2-*O*-digalloyl-6-*O*-galloyl-1,5-anhydro-D-glucitol (**6b**), respectively. Chromatograms were monitored at a wavelength of 223 nm.

Figure 3. Inhibitory effect of Maplifa™ against the formation of AGEs. 10 mg/ml BSA and 100 mM D-fructose were incubated at 37 ℃ in 0.2 M phosphate buffer, pH 7.4. Prior incubation, each sample was treated with different concentrations of Maplifa™ or aminoguanidine serving as positive control. After incubating for 21 days, the formation of fluorescence AGEs in each solution was determined at excitation and emission wavelengths of 370 nm and 435 nm respectively. Each fluorescence reading was then compared to the intensity of negative control solution.

Table 1. The HPLC-UV retention times (min) of major compounds present in Maplifa™ as comparing with of those authentic standards. The contents of identified compounds were determined by standard curve of authentic standards.

Table 2. Biological evaluation of Maplifa™ showing 50% inhibitory concentrations (IC_{50} in ppm) in the antioxidant (DPPH radical scavenging), anti-tyrosinase, anti-α-glucosidase and anti-AGEs (BSA-glucose) assays. Values are means ± standard deviations. [a]Positive controls, ascorbic acid and BHT. [b]Positive control, kojic acid and arbutin. [c]Positive control, acarbose. [d]Positive control, aminoguanidine.

Figure 1.

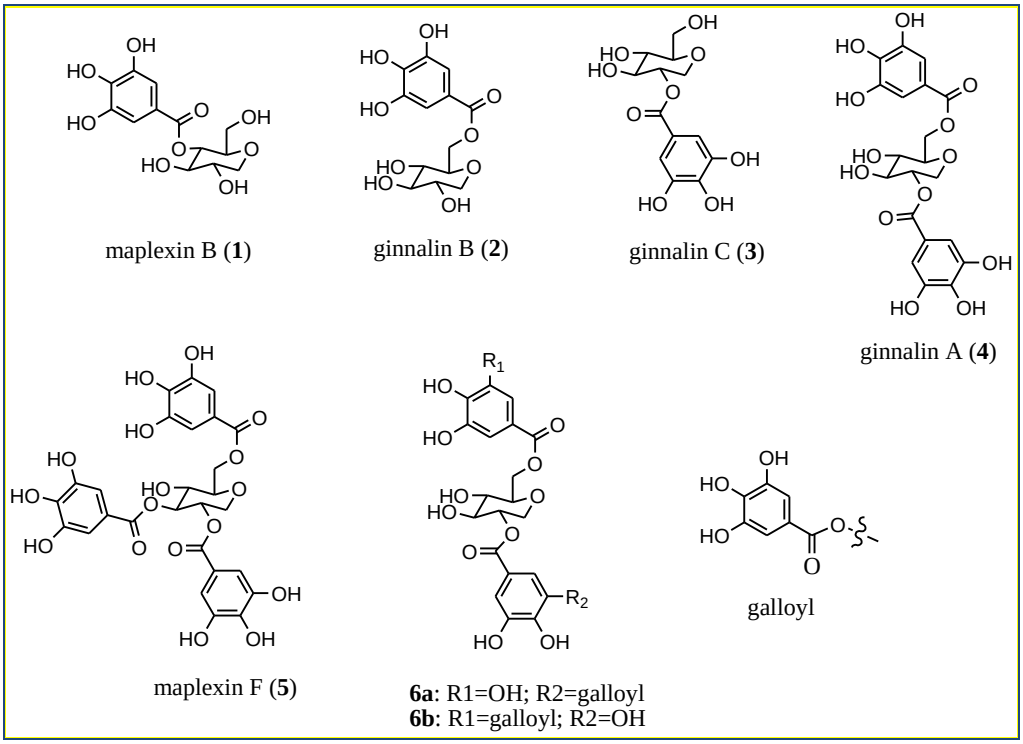

Figure 2.

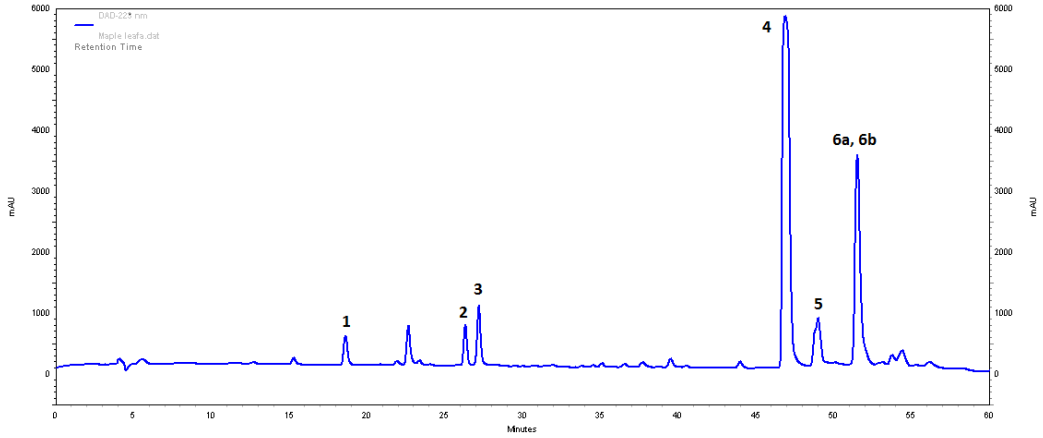

Figure 3.

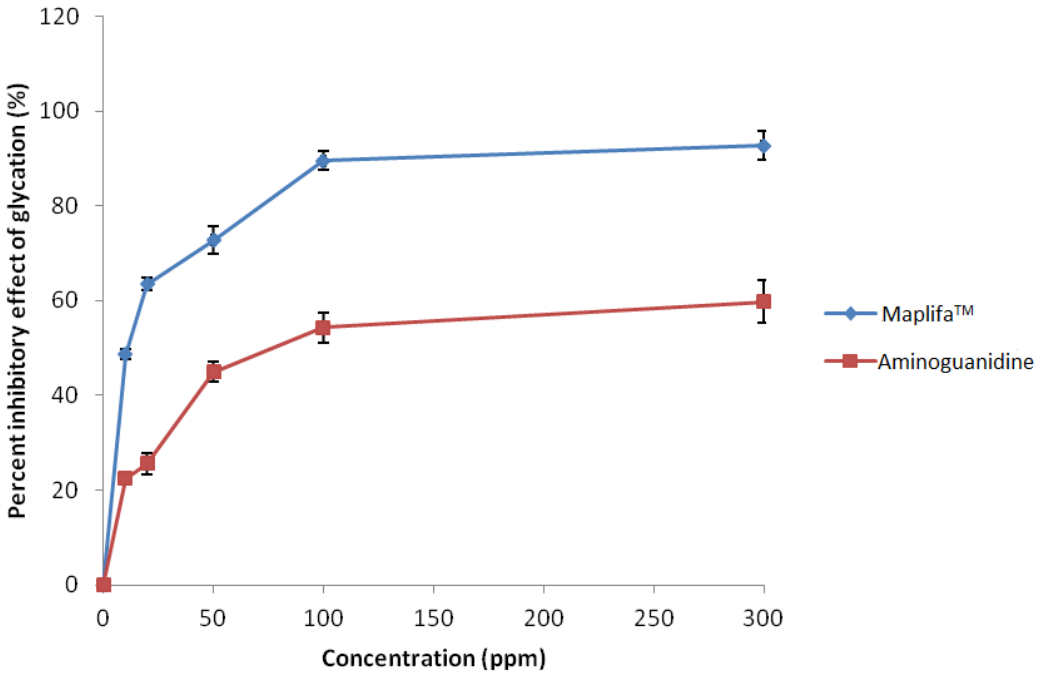

Table 1.

Peak	t_R (min)	standards (t_R, min)	Content (weight %)
1	18.84	18.84	6.48
2	26.55	26.5	5.66
3	27.41	27.53	5.28
4	47.13	47.09	56.41
5	49.22	50.01	4.83
6a, 6b*	51.74	52.67	12.1

*compounds isolated and identified by NMR data

Table 2.

	Anti-oxidant	Anti-tyrosinase	Anti-α-glucosidase	Anti-AGEs
	DPPH			BSA-glucose assay
Maplifa™	78.2 ±2.6	154.5 ±5.4	37.8 ±3.3	8.6 ±0.7
BHT[a]	330.5 ±8.4	–	–	–
ascorbic acid[a]	10.9 ±0.7	–	–	–
kojic acid[b]	–	3.4 ±1.3	–	–
arbutin[b]	–	17.1 ±2.4	–	–
acarbose[c]	–	–	91.6 ±3.8	–
aminoguanidine[d]	–	–	–	83.9 ±2.1

Manuscript-II

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Comparison of Inhibitory Activities and Mechanisms of Maple Gallotannins against *α*-Glucosidase

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Running Head: Inhibitory mechanisms of maple gallotannins against α-glucosidase

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ABSTRACT

Bioactive gallotannins have been researched extensively for α-glucosidase inhibitors. Our previous phytochemical studies on the red maple (*Acer rubrum*) species lead to the identification of a series of gallotannins with potent α-glucosidase inhibitiory activties. In the present study, we aimed to provide insights into the ligand-enzyme interactions and the binding mechanisms of gallotannins and the α-glucosidase enzyme. The inhibitory effects of four gallotannins: ginnalin A (GA), ginnalin B (GB), ginnalin C (GC) and maplexin F (MF) from the red maple species, as well as a synthetic gallotannin, maplexin J (MJ), were evaluated for α-glucosidase inhibitory activity. The type of GA, MF and MJ was determined as noncompetitive mode. The interactions between gallotannins with potent inhibitory activities (MF and MJ) and α-glucosidase were further elucidated by using spectroscopic means. The results revealed that gallotannins inhibited α-glucosidase by forming stable ligand-enzyme complexes which consequently lead to the quenching of their intrinsic fluorescence statically. In addition, fluorescent probe 1,1′-bis(4-anilino-5-napththalenesulfonic acid (bis-ANS), was used to explore the regions that gallotannins bound to. Meanwhile, the FTIR and circular dichroism spectra showed that gallotannins bound to α-glucosidase and induced conformational changes to the enzyme. These findings were further discussed in the context of structure activity relationship (SAR) for the potential of developing structural gallotannin analogs as potent α-glucosidase inhibitors.

Keywords: α-glucosidase, gallotannins, inhibition, mechanisms, interaction.

INTRODUCTION

In mammals, enzymes involved in the carbohydrate processing pathways are therapeutic targets for various diseases including diabetes, cancer and viral infections (*1-3*). Among these enzymes, glucosidases (EC 3.2.1.20) play a crucial role in the mediation of carbohydrate metabolism by catalyzing the cleavage of the glycosidic linkage of oligosaccharides or glycoconjugates to release mono-saccharides (*4*). Inhibition of glucosidase results in a retardation of glucose absorption and consequently decreases the postprandial blood glucose level. Therefore, development of *α*-glucosidase inhibitors as a class of antihyperglycemic drugs for the type II non-insulin-dependent diabetes mellitus (NIDDM) has drawn considerable research attention (*5*). In fact, several synthetic *α*-glucosidase inhibitors (AGI) including acarbose, miglitol and voglibose are currently used as clinical treatments for NIDDM (Figure 1A). However, applications of these AGIs as therapeutic agents suffered due to a lack of efficacy and undesirable side effects such as flatulence, diarrhea and stomachache (*6*). On the other hand, plant-based natural products are a promising resource of possible alternative AGIs since they possess a variety of chemical structures and bioactivities. The genus *Acer* (family, Aceraceae), commonly known as maple, consists of more than 120 species and are widely distributed in Asia, Europe and North America. Among these species, the *Acer rubrum* (red maple) is indigenous to North America and has been used as for ethnomedicinal purposes by the indigenous peoples of eastern North America to treat many ailments such as sore eyes and skin disorders (*7*). Phytochemical and biological investigations have shown that extracts and their purified compounds from red maple displayed biological activities including anti-oxidant, anti-bacterial and anti-cancer

effects (*8-10*). Interestingly oral administration of red maple bark extract to mice was shown to decrease the elevation of their blood glucose level after carbohydrate ingestion (*11*). As maple phytochemicals seemed to be effective in decreasing mouse postprandial blood glucose levels, it was important to understand the mechanisms of this specific bioactivity.

As a continuous effort of exploring plant natural products with biological activities, our laboratory isolated a number of new gallotannins from *Acer* (maple) species, namely maplexins A-I (*12, 13*). These compounds feature different numbers and positions of galloyl groups attached to a 1,5-anhydro-D-glucitol moiety. These compounds, in particular maplexins E-I, were shown to be potent AGIs, 20-fold more potent than acarbose. Moreover, the structure and activity relationship (SAR) studies revealed that maplexins showed superior inhibitory activity against α-glucosidase as the number of the galloyl groups on 1,5-anhydro-D-glucitol increased (*12, 13*). Furthermore, our laboratory synthesized maplexin J, a tetragalloyl-glucitol (contains the maximum number of 4 galloyl groups) and substantiated our SAR observations on α-glucosidase inhibition. It is logical to question how maplexins bind to α-glucosidase and what types of interactions between the ligands and enzyme lead to the inhibitory effects. This information is essential for the design and development of effective maple-gallotannin-based AGIs and the effective utilization of red maple species extracts as potential anti-diabetic therapeutics.

The natural products based α-glucosidase inhibitors such as 1-deoxynojirimycin, resveratrol and oxyresveratrol can be distinguished by their inhibitory types. Moreover, the mechanisms of their inhibitory effects on α-glucosidase could also be demonstrated

by a series of physicochemical features such as binding to the catalytic domain, modifications of hydrophobicity surface and conformational changes of enzyme that induced by the binding ligands (*14*). In this study, enzymatic kinetics along with biophysical tools including fluorescence spectroscopy and circular dichroism (CD) were used to elucidate the inhibitory mechanisms of maple gallotannins against α-glucosidase.

MATERIALS AND METHODS

Chemicals. α-Glucosidase (EC 3.2.1.20) from *Saccharomyces cerevisiae*, p-Nitrophenyl-α-D-glucopyranoside (pNPG) and bis-8-anilinonaphthalene-1-sulfonate (bis-ANS) were purchased from Sigma–Aldrich (St. Louis, MO). All other chemicals were analytical reagent grade and purchased from Sigma–Aldrich, unless otherwise specified. Maplexin F was previously isolated from our laboratory (*13*) and maplexin J was synthesized in our laboratory as described below.

Synthesis of Maplexin J. Trisilyl-protected gallic acid (Previously synthezied in our lab, 187 mg, 0.4 mmol) and glucitol (, 10.4 mg, 0.06 mmol) were dissolved in dry dichloromethane (2 mL). *N*,*N*′-diisopropylcarbodiimide (61.4 mg, 0.5 mmol) was added followed by 4-dimethylaminopyridin (74.4 mg, 0.06 mmol). The mixture was stirred at room temperature under nitrogen for 96 h. Water was added to the reaction mixture, and the solution was extracted (× 3) using ethyl acetate. The combined organic layer was washed with brine solution, dried over anhydrous sodium sulfate, and concentrated. The crude product was purified using silica gel column chromatography to yield compound **1**. The esterification products were isolated using silica gel chromatography with a gradient solvent system of hexanes:ethyl acetate starting with hexanes at 100%. The deprotection

of compound **1** was accomplished in the presence of tetra-*n*-butylammonium fluoride. Compound **1** (50 mg, 0.023 mmol) was dissolved in dry tetrahydrofuran (2 mL). Tetra-*n*-butylammonium fluoride (61.1 mg, 0.23 mmol) was added, and the mixture was stirred at room temperature under a nitrogen atmosphere for 10 min. The crude product was purified using reverse-phase HPLC to yield the final product (10 mg, 52.3%) which was characterized and assigned the common name of maplexin J.

***α*-Glucosidase Inhibitory Assay.**

A mixture of 50 μL of test samples and 100 μL of 0.1 M phosphate buffer (pH 6.9) containing yeast α-glucosidase solution (1.0 U/ml) were incubated in 96 well plates at 25 ℃ for 10 min. After pre-incubation, 50 μL of 5 mM p-nitrophenyl-α-D-glucopyranoside solution in 0.1 M phosphate buffer (pH 6.9) were added to each well at timed intervals. The reaction mixtures were incubated at 25 ℃ for 5 min. Before and after incubation, absorbance were recorded at 405 nm by a micro-plate reader (SpectraMax M2, Molecular Devices Corp., operated by SoftmaxPro v.4.6 software, Sunnyvale, CA, USA) and compared to that of the control which had 50 μL buffer solutions in place of the samples. The α-glucosidase inhibitory activity were expressed as inhibition % and were calculated as follows: $\text{inhibition}\% = 100 \times [(C_5 - C_0) - (S_5 - S_0)] / (C_5 - C_0)$. Where C_0 is the absorbance of the reagent blank in 0 min, S_0 is the absorbance of the samples in 0 min, C_5 is the absorbance of the reagent blank in 5 min and S_5 is the absorbance of the samples in 5 min (*15*).

Kinetics of Enzyme Inhibition.

The inhibition types of ginnalin A and maplexins F and J were determined from Lineweaver–Burk plots, using methods reported in literatures with minor modification.

Typically, two concentrations of each sample around the IC_{50} values were chosen. Under each concentration, α-glucosidase activity was tested by different concentrations of pNPG glycoside (1 to 1000 μM). The enzyme reaction was performed under the above-mentioned reaction condition. The mixtures of the enzyme and the inhibitor were dissolved in 50 mM phosphate buffer (pH 6.8), and pre-incubated at 37 ℃ for 30 min, and then the substrate was added. The enzymatic reaction was carried out at 37 ℃ for 60 s, and monitored spectrophotometrically by measuring the absorbance at 405 nm. Inhibition types of the inhibitors were determined by Double-reciprocal plots (*16*).

Instrinsic Fluoescence Measurements.

α-Glucosidase (2 μM) was pretreated with various concentrations of MF or MJ (0–60 μM) for 20 min at 37 ℃. The intrinsic fluorescence spectra (300–400 nm) were measured using a micro-plate reader (SpectraMax M2, Molecular Devices Corp., operated by SoftmaxPro v.4.6 software, Sunnyvale, CA, USA) with the excitation wavelength (λ_{ex}) of 280 nm, and emission (λ_{em}) spectra were acquired by scanning from 300 to 340 nm (*17*).

Hydrophobic Analysis of α-Glucosidase Using Bis-ANS.

α-Glucosidase (2 μM) was incubated in the presence of various concentrations of gallotannins (0-80 μM) at 37 ℃ for 5 min. Bis-ANS (5 μM) was then added, and fluorescence was measured after incubation at 37 °C for 15 min (λ_{ex} = 400 nm, λ_{em} = 440−600 nm) (*18*).

ATR-FTIR Measurements.

The FTIR spectra of α-glucosidase (2 μM) and its maplexins complex were measured in the range of 2000–1400 cm^{-1} in sodium phosphate buffer, pH 6.8 at room

temperature. All spectra were recorded by using the ATR mode with the resolution of 4 cm^{-1} and 60 scans. The final concentration of maplexins was 40 μM. The corresponding absorbance of free maplexins and buffer solutions were recorded and subtracted with the same instrumental conditions. The curve-fitted results of amide I band and all data were analyzed using Omnic software (version 7.2, Nicolet Instrument Co, Madison, WI, USA), and the curves were fitted using Origin software (version 8.0, Origin Lab, Northampton, MA, USA) (*19*).

Circular Dichroism (CD) Spectroscopy.

Far UV CD measurements (190-240 nm) were conducted on a Jasco J-810 spectropolarimeter equipped with a Peltier temperature controller at 25 ℃. Briefly, 2 μM of α-glucosidase was treated with various concentrations of a gallotannins (0-80 μM). The samples were dissolved in 200 μL of a sodium phosphate buffer (0.1 M, pH 6.8) and placed in a 1.0 mm path length cell. The spectra were collected and corrected by subtraction of a blank 0.1 M sodium phosphate buffer (pH 6.8), reduction of noise and smoothing (*20*). The changes of secondary structure of α-glucosidase were estimated according to a method of DichroWeb program, an online server for protein secondary structure analyses from CD spectroscopic data (*21*, *22*).

RESULTS

Comparison of α-Glucosidase Inhibitory Effects by Gallotannins.

As shown in Table 1, the inhibition of α-glucosidase by maple gallotannins was enhanced by increasing the number of galloyl groups that attached to the 1,5-anhydro-D-glucitol moiety. The mono-galloyl substituted gallotannins (ginnalin B and C) did not

show the inhibitory activities on glucosidase (IC_{50} > 1000 μM), and the di-galloyl gallotannin (GA) only showed a moderate inhibitory activity at an IC_{50} value of 216.43 μM. However, the activities of maplexin F (MF) and maplexin J (MJ), were significantly more potent (IC_{50} = 13.70 and 4.27 μM, respectively) at inhibiting α-glucosidase.

To further elucidate the inhibitory characteristics of GA, MF and MJ, the enzyme kinetics were determined by using Lineweaver-Burk plots (Figure 2). The Lineweaver-Burk plot of each gallotannin at different concentrations generated straight lines that had the same intersections on the *X*-axis suggesting that all three gallotannins were typical noncompetitive α-glucosidase inhibitors. This means that GA, MF and MJ bound to a site on α-glucosidase other than the catalytic domain of the enzyme (*23*).

Gallotannins Binding Quenched the Intrinsic Fluorescence of α-Glucosidase.

Both MF and MJ displayed potent inhibitory activity in a non-competitive model indicating that these gallotannins bound a non-catalytic domain of α-glucosidase. Therefore, an intrinsic fluorescence assay was carried out to further explore the interaction between ligands and α-glucosidase. As shown in Figure 3, the α-glucosidase fluorescence emission spectra showed a peak at 312 nm, which was largely attributed to the tryptophan residues of α-glucosidase. Neither MF nor MJ show any intrinsic fluorescence. The fluorescence intensity of α-glucosidase was gradually quenched when it was co-incubated with maplexins at various concentrations. Maplexins clearly quenched α-glucosidase fluorescence at a comparable level (both MF and MJ had a quenching ratio of 21%) when their concentration was increased to 60 μM, suggesting that ligands (MF and MJ) interacted with α-glucosidase which further leaded to the variation of tryptophan residues.

Gallotannins Binding Reduced the Hydrophobicity of *α*-Glucosidase.

Noncovalent fluorescent probes have been well developed for the study of protein conformation (*24*, *25*). External fluorescent probes such as bis-8-anilinonaphthalene-1-sulfonate (bis-ANS) are sensitive to the protein microenvironment and selectively binds to the hydrophobic surface of protein. Therefore, bis-ANS probe was utilized to assess the *α*-glucosidase hydrophobic surface. As shown in Figure 4, after co-incubated with *α*-glucosidase for 20 min at 37 ℃, MF and MJ were able to decrease the fluorescence of bis-ANS-enzyme complex in a concentration-dependent manner. Both ligands clearly reduced the fluorescence intensities at concentration of 80 *μ*M, suggesting that MF and MJ could decrease the hydrophobic surface of *α*-glucosidase. Although at a low concentration (20 *μ*M), MJ also induced a large change in hydrophobic surface. Because hydrophobic interactions are an important part of the enzyme active site, it is reasonable to suggest that MF and MJ inhibit *α*-glucosidase activity by inducing poor hydrophobic surface interactions in the enzyme active site (*25*).

FTIR Spectroscopic Analysis of *α*-Glucosidase.

In order to study the impact of the ligands on the conformational changes of *α*-glucosidase, ATR-FTIR spectra of *α*-glucosidase and *α*-glucosidase-ligands complex were obtained. It has been known for the use of infrared spectroscopy for the study of proteins and the focuses have been placed on the amide I and II bands which represent different vibrations of the amino acid residues from proteins. As shown in Figure 5 A, free *α*-glucosidase had a strong peak of amide I region (1700-1600 cm^{-1} and 1600-1500 cm^{-1}, mainly contribution of C=O stretch and C−N stretch, respectively) (*19*, *26*). Co-incubation of *α*-glucosidase with ligands, for example maplexin F as shown in Figure 5

B, induced the shift of amide I band (1637 cm^{-1}) and amide II band (1564 cm^{-1}) to 1633 cm^{-1} and 1541 cm^{-1}, respectively. The similar amide band shift pattern was also observed in the MJ-α-glucosidase complex FTIR spectra suggesting that maplexins F and J interacted with the C=O and C−N groups in the α-glucosidase subunit and might further induce the change of carbonyl hydrogen binding pattern.

Change in Conformation of α-Glucosidase Induced by Maplexins F and J.

In order to further examine the effects of ligands on the secondary structure of α-glucosidase, circular dichroism (CD) spectra of free α-glucosidase and maplexin-α-glucosidase complex were acquired. In the Figure 6, CD spectra of free α-glucosidase exhibited two characteristic negative bands at 208 and 222 nm indicating that the major secondary protein structure present in the α-glucosidase was α-helix. When 20 or 40 μM of ligands were co-incubated with α-glucosidase, they had the identical CD spectra as that of free α-glucosidase, however, the CD spectra of ligands-enzyme complex significantly altered when the concentrations of maplexins reached to 60 or 80 μM indicating that the conformation of enzyme protein was affected by the ligands in a concentration dependent manner. As summarized in Table 2, maplexins bind to the enzyme protein and mainly resulted in the loss of α-helix conformation. For instance, when 80 μM of MF and MJ were induced to α-glucosidase, it lost 14.8 and 15.0% of α-helix conformation respectively, which might lead to the lost of its biological function.

DISSCUSSION AND CONCLUSSION

Red maple (*Acer rubrum*) is one of the *Acer* (maple) species that is widely distributed in eastern North America and it is known for its sap which is used for the

production of maple syrup. It has been used by the indigenous peoples of eastern North America as food resource or herbal medicine (*27*). Recent phytochemical and biological studies lead to the discovery of bioactive compounds from red maple species that potentially could be used as therapeutic approaches for diabetes (*11-13*). Among these compounds, maplexins (F and J), as the representatives of a large group of gallotannins, showed 20-30 times more potent inhibitory effects against α-glucosidase than acarbose (a clinically used AGI drug). However, the mechanisms of action of maplexins on α-glucosidase were unknown. Therefore, we initiated this study to explore the interactions between maplexins and α-glucosidase by using kinetic assay and a set of spectroscopic methods. Firstly, the inhibition type of GA, MF and MJ against α-glucosidase was determined as noncompetitive mode suggesting that these gallotannins bonded to a specific site of enzyme-substrate complex rather than the enzyme catalytic domain. This binding pattern distinguishes maple gallotannins from the other known clinically used drugs, such as acarbose, which is known as a competitive inhibitor that directly binds to the active site of α-glucosidase (*6*). This finding was in agreement with previous studies that demonstrated a few of other gallotanins, including pentagalloyl glucose (PGG), are noncompetitive α-glucosidase inhibitors (*28*). In addition, it was demonstrated that increasing of numbers of galloyl groups on the glucitol core could enhance inhibitory activities of maplexins and this SAR effects were also observed in other glucose-based gallotannin α-glucosidase inhibitors (*29*). Interestingly, MJ as a fully galloyl substituted glucitol gallotannin (four galloyl groups, IC_{50} =4 μM) seemed to be more potent than PGG, a gallotannin with a glucose core and maximum numbers of galloyl groups (five galloyl groups, IC_{50} = 130.71 μM). It is speculated that the absence of galloyl group at

the glucitol C-1 position facilitated the formation of a favorable binding site for maplexins and further enhanced their inhibitory effects against α-glucosidase.

In order to get further insight of the interaction between maplexins and α-glucosidase, fluorescence quenching assay was performed. Since the intrinsic fluorescence of α-glucosidase mainly contributed from tryptophan residues and its intensity could be quenched by maplexins in a concentration dependent manner, it confirmed that maplexins could directly bind to α-glucosidase and possibly lead to the variation of microenvironment of tryptophan residues. This observation was consistent with many previously demonstrated ligands-protein interactions.

Hydrophobic surface is another important factor for maintaining the normal functions of proteins. It has been established that enzyme activity suffers from the exposure of hydrophobic surface to water, which usually impede the formation of the active center (*30*). Fluorescent probes such as 4,4'-dianilino-1,1'-binaphthyl-5,5'-disulfonic acid (bis-ANS) have been used to monitor the hydrophobic surface of α-glucosidase (*31*). The bis-ANS probe can specifically bind to the hydrophobic area of the enzyme and then generate fluorescent signal. Therefore, the decreased fluorescent intensity of probe-ligands-enzyme complex indicated that maplexins could reduce the hydrophobic surface of enzyme.

FTIR spectroscopy has been widely used to study the conformation of protein and it is often complemented by other physical tools such as circular dichroism (CD) for detailed secondary structural changes. The FTIR spectra clearly showed that maplexins interacted with the C=O and C−N groups of α-glucosidase subunits and lead to the peak shift of amide I band. In addition, the secondary structures of α-glucosidase were

monitored by CD spectra and it demonstrated that both maplexins F and J significantly decreased the α-helix conformation. Since it is known that α-helix is a rigid secondary structure and loss of it may result in the destabilization of enzyme conformation (*32*). In a word, maplexins inhibited α-glucosidase activity by binding to the enzyme-substrate complex and consequently changed the hydrophobic surface and enzyme secondary conformation.

In summary, we have determined the type of inhibition of maple gallotannins GA, MF and MJ. Two potent α-glucosidase inhibitors, MF and MJ, were further studied for the ligands-enzyme interaction by using several biophysical tools. It has been showed that maplexins could affect enzyme activity by decreasing the hydrophobic surface of enzyme as well as reducing the α-helix conformation. The inhibitory mechanisms of maplexins provide valuable information towards the understanding of interactions between maple gallotannins and α-glucosidase. This is critical for the potential application of maplexins or maple species extracts as therapeutics for the NIDDM. Further investigation of the anti-diabetic properties of maple gallotannins will be focused on: 1) the possible synergistic anti-α-glucosidase effects of bioactive compounds that are totally present in the red maple extracts and 2) *in vivo* studies that evaluate the efficacy of anti-diabetic property of maplexins. These information are crucial for the development of red maple leaf extracts as potential nutraceutical and dietary management for type 2 diabetes

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FIGURE AND TABLE LEGEND

Figure 1: (A) Chemical structures of known clinical used α-glucosidase inhibitors: acarbose, miglitol and voglibose. (B) Chemical structures of five gallotannins: ginnalin A, ginnalin B, ginnalin C, maplexin F and maplexin J.

Figure 2: Lineweaver-Burk plots of the kinetics of GA (A), MF (B) and MJ (C) on α-glucosidase. Two concentrations of ligands that near to the IC_{50} were co-incubated with α-Glucosidase at 37 ℃ for 30 min, then pNPG were added with final concentrations from 1-1000 μM.

Figure 3: Intrinsic fluorescence of α-glucosidase quenching induced by MF (A) and MJ (B). α-Glucosidase (2 μM) was co-incubated with MF (0-60 μM) or MJ (0-60 μM) at 37 ℃ for 20 min. Spectra were acquired from 300 to 340 nm.

Figure 4: Fluorescence intensity of bis-ANS-α-glucosidase complex. The changes of fluorescence intensity were induced by MF (A) and MJ (B) at different concentrations.

Figure 5: The ATR-FTIR of free α-glucosidase (A) 2 μM of α-glucosidase and 40 μM of MF (A) or MJ (B) complex.

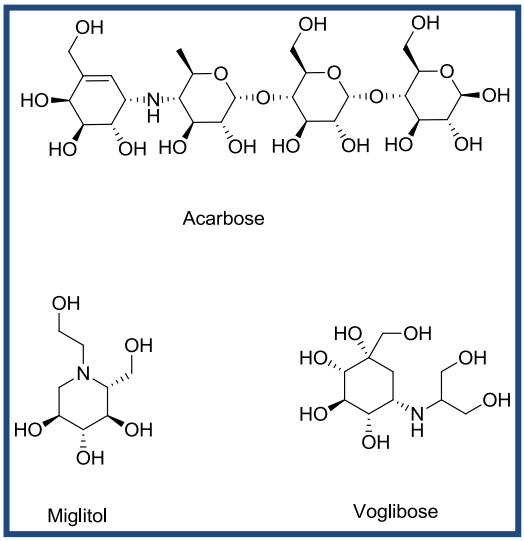
Figure 6: Circular dichroism spectra of the α-glucosidase-maplexins complex. α-Glucosidase (2 μM) were co-incubated with MF or MJ at 20-80 μM at 37 ℃ for 20 min.

Table 1: α-Glucosidase inhibitory activities of compounds[a]

Table 2: The effect of maplexins (MF and MJ) on the secondary structure of α-glucosidase.

Figure 1.

(A)



(B)

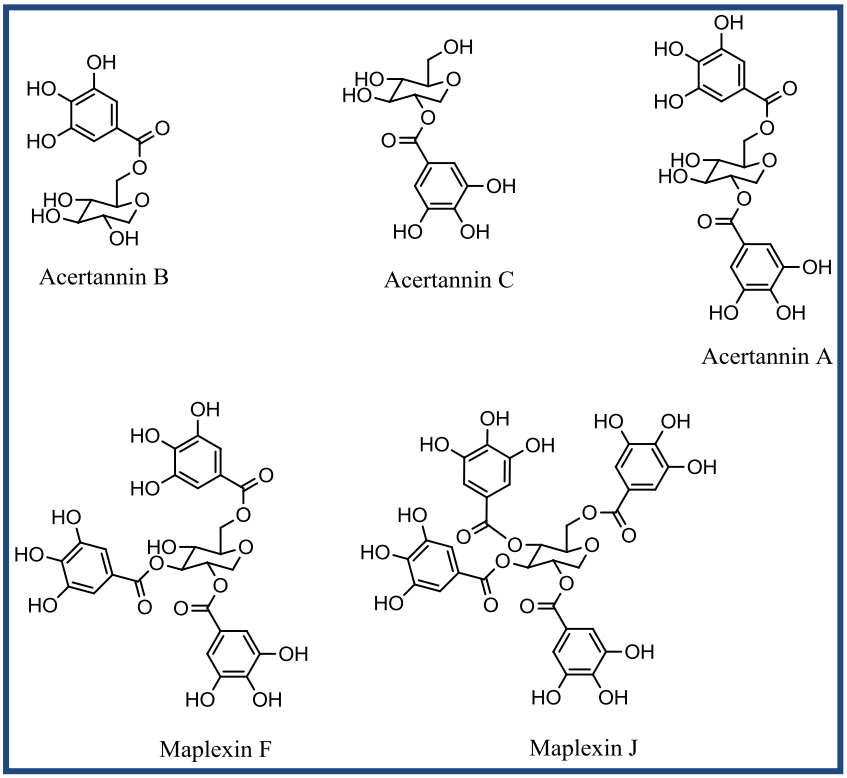
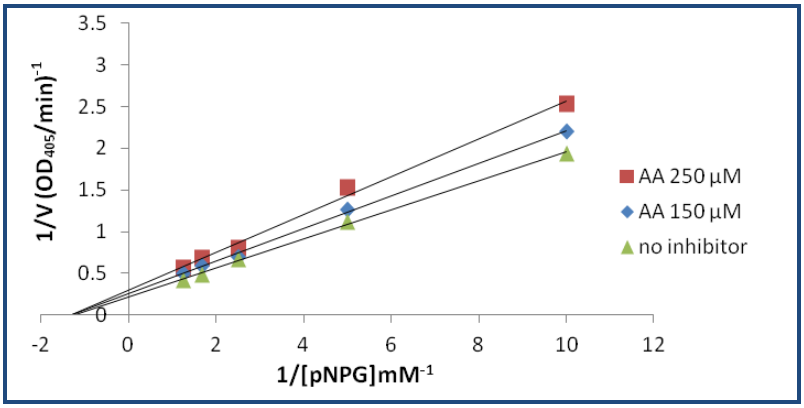


Figure 2.

(A)



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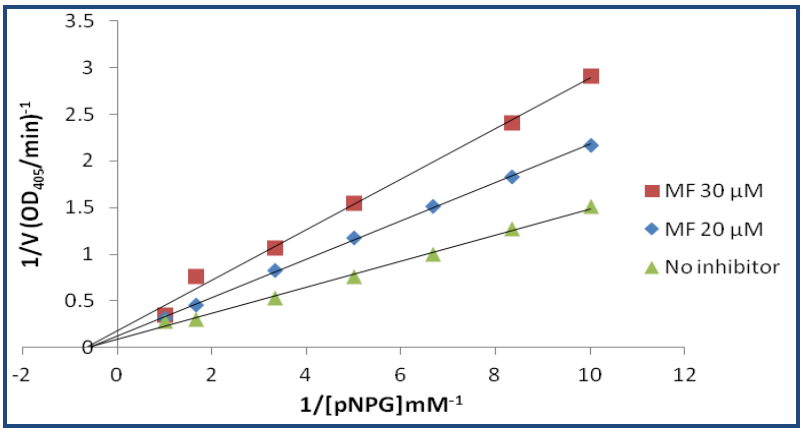

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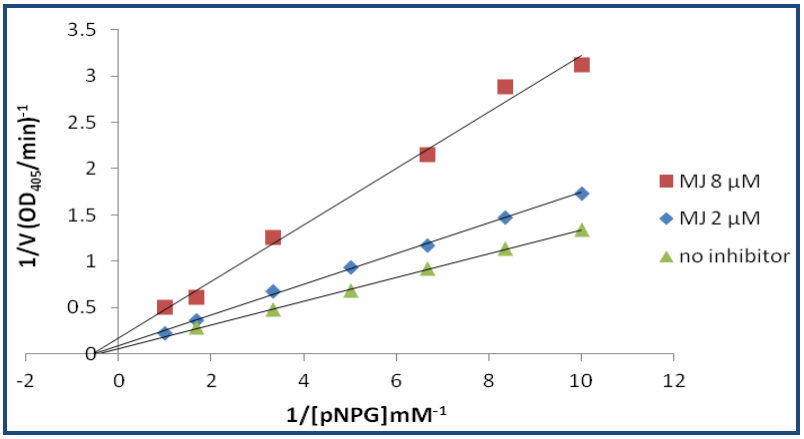
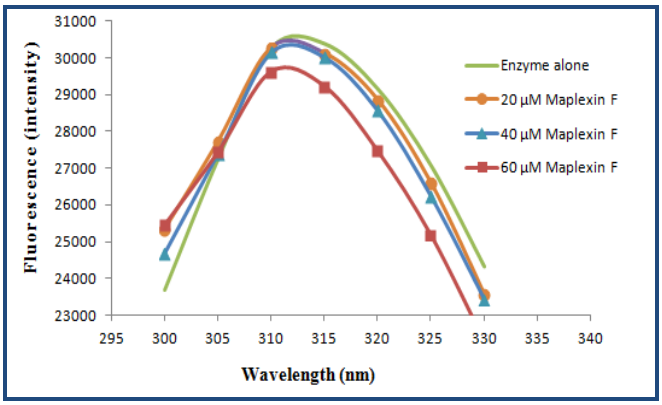


Figure 3.

(A)



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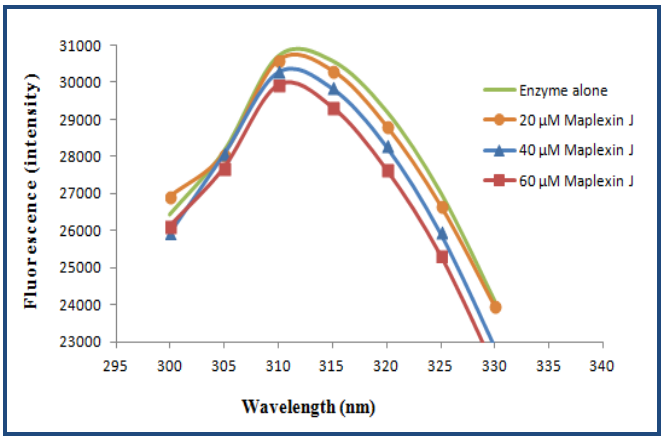

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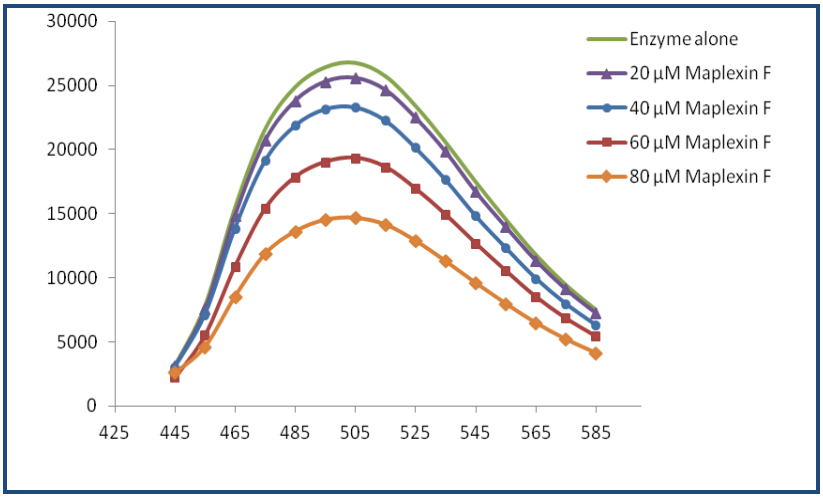
(A)



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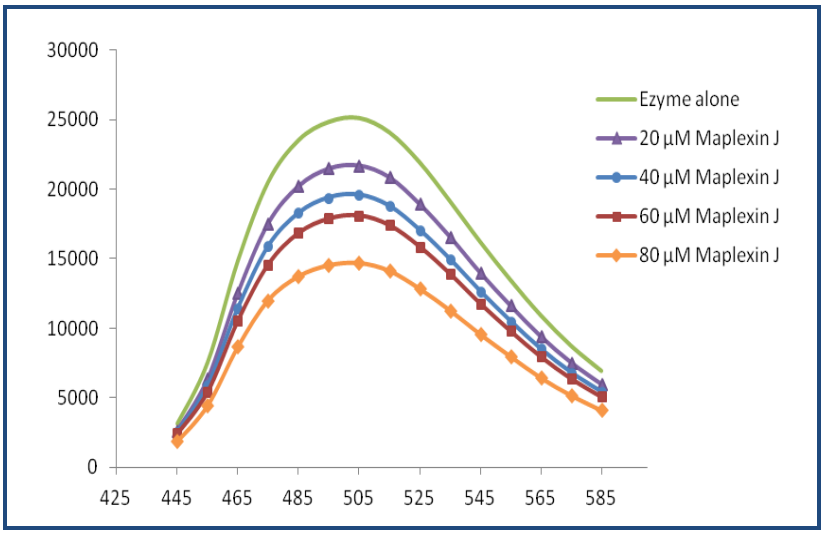
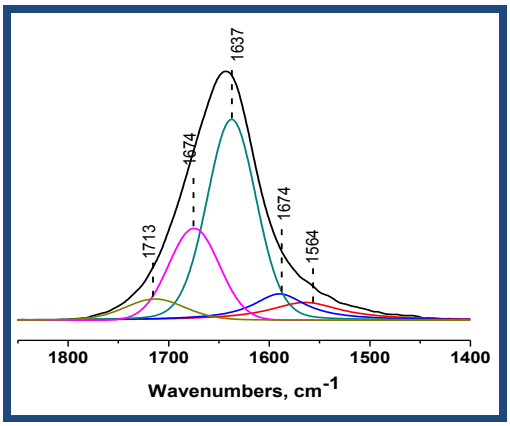


Figure 5.

(A)



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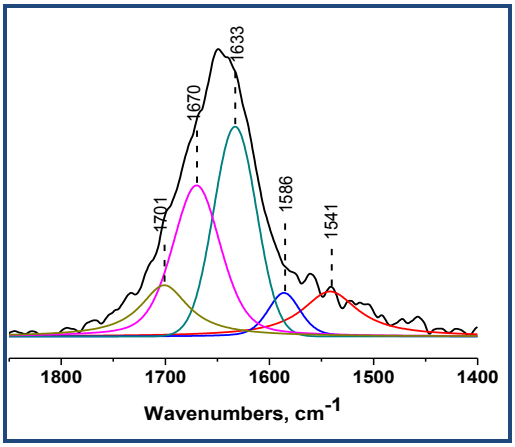

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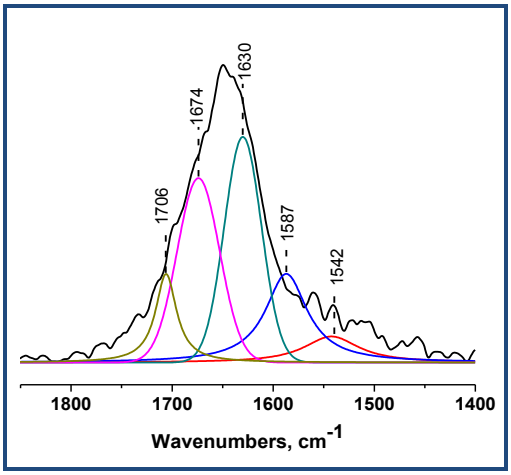
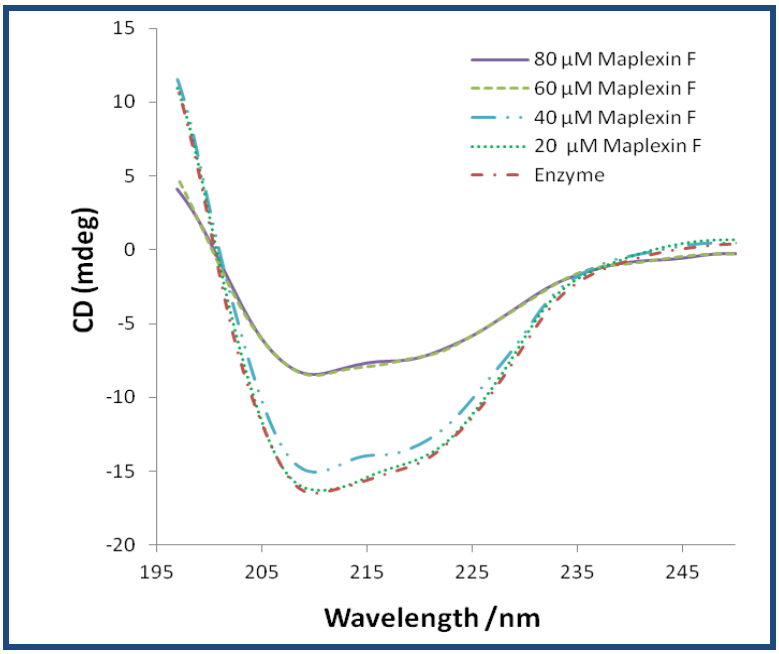


Figure 6.

(A)



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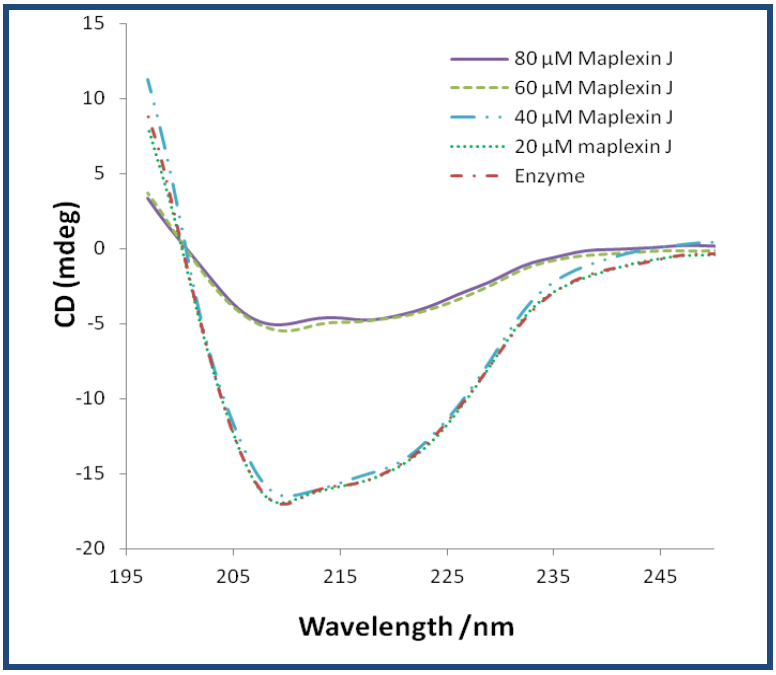

Table 1

Compd.	# of galloyl groups	IC_{50} (μM)	Type of inhibition
ginnalin B	1	> 1000	n.d.
ginnalin C	1	> 1000	n.d.
ginnalin A	2	216.43 ± 3.19	noncompetitive
maplexin F	3	13.70 ± 0.87	noncompetitive
maplexin J	4	4.27 ± 0.13	noncompetitive
acarbose [b]	—	142.40 ± 1.68	competitive[6]
PGG[c]	5	130.71 ± 4.37	noncompetitive[28]

[a] IC_{50} values are shown as mean ± S.D. from three independent experiments; [b] Positive control; n.d. = not determined. [c]PGG (Pentagalloyl glucose, previously isolated in our laboratory) showed an IC_{50}value of 130.71 ± 4.37 μM.

Table 2

Inhibitor	conc. (μM)	α-helix (%)	α-helix loss (%)	β-sheet (%)	β-turn (%)	random coil(%)
free α-glucosidase		42.3	–	33.4	1.2	23.1
MF	20	42.3	0	33.6	1.4	22.7
	40	43	-0.7	36.7	-1.3	21.6
	60	30.9	11.4	35.8	9.8	23.5
	80	27.5	14.8	40.1	6.5	25.9
MJ	20	44.1	-1.8	32.5	0.9	22.5
	40	43.6	-1.3	35.4	-0.7	21.7
	60	26.9	15.4	40.8	4.9	27.4
	80	27.3	15	39.2	8	25.5

Manuscript-III

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Inhibitory Effects and Mechanistic Study of Maple Gallotannins on the Formation of Advanced Glycation Endproducts

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Running Head: Maple gallotannins inhibit formation of AGEs

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ABSTRACT

Glycation is a spontaneous process typically initiated with a condensation reaction between a reducing sugar and a protein, leading to the formation of Advanced Glycation Endproducts (AGEs). Glycation is a spontaneous non-enzymatic process leading to the formation of Advanced Glycation Endproducts (AGEs). The reaction is typically initiated through a condensation reaction between a reducing sugar and a protein. The objective of this study was to evaluate the inhibitory effects of a series of maple gallotannins, namely ginnalins A-C and maplexins F and J, at individual stages of protein glycation. Using a combination of analytical methods, including fluorescence spectroscopy, HPLC-FL, Circular Dichroism, MALDI-TOF and BSA-fructose assay, early and middle stages of protein glycation inhibition by maple gallotannins were determined. In the BSA-fructose assay, maplexin F showed the most potent inhibitory effect against the formation of AGEs with an IC_{50} value of 15.8 μM. In addition, MALDI-TOF analysis revealed that the maple gallotannins were able to reduce the number of fructose that adducted to BSA protein indicating that they were able to inhibit the production of Amadori products. Moreover, maple gallotannins were effective in the G.K. peptide-ribose assay suggested that the protein cross-linking formation at the late stage of glycation could be prevented by maple gallotannins. Lastly, the spectroscopy data revealed that maple gallotannins were able to reduce the conformational changes of BSA protein that induced by glycation.

Keywords: maple gallotannins, glycation, FL-HPLC, MOLDI-TOF-MS, G.K. peptide, circular dichroism

INTRODUCTION

The Maillard reaction, also referred to as glycation, is a non-enzymatic process including a reducing sugar and an amino containing molecule such as protein, lipid or nucleotide (*1*). The process of glycation includes three distinguishable phases: the initial stage, the intermediate stage and the late stage. In the initial phase of glycation, the carbonyl group of a reducing carbohydrate condenses with the free amino group on a protein to reversibly form glycosylamine. Glycosylamine can be further converted to a more stable aldimine, or Amadori product (*2*). Under appropriate conditions, these Amadori products can be degraded to generate more reactive dicarbonyls such as methylglyoxal (MGO), glyoxal (GLY) and 3-deoxyglucosone (3-DG) (*3, 4*). These dicarbonyl compounds can in turn continue to react with unmodified proteins and/or Amadori products. In the late stage of glycation, the Amadori product could undergo dehydration, cyclization, oxidation, and rearrangement to form a polymorphic group of compounds collectively known as Advanced Glycation Endproducts (AGEs) (*5, 6*).

The accumulation of AGEs *in vivo* has been implicated as a major pathogenic factor in diabetic chronic complications such as renal failure, cataract formation, Alzheimer's disease and cardiovascular disease (*7-9*). Most of the glycation related chronic complications are due to the AGE accumulation on long-lived proteins such as collagen and lens crystallins. Glycation can also induce the formation of reactive oxidation species (ROS), causing oxidation stress and tissue damage (*10*).

Aminoguanidine (AG) is a synthetic chemical that acts as anti-glycation agent by preventing the formation of reactive oxygen species, however due to potential side effects did not propagate to clinical use (*11*). Polyphenolic compounds from plant sources, such

as curcumin and resveratrol, have drawn considerable research interests for their biological activities including anti-oxidant and anti-AGEs formation effects (*12, 13*). Moreover, polyphenols isolated from many maple (*Acer*) species including ginnalins A-C (Figure 1) are known for their free radical scavenging capacity and other bioactivities (*14, 15*). Recently, a series of maple gallotannins, namely maplexins E-I, as well as a synthetic analogue maplexin J (Figure 1), were reported for their anti-diabetic effects as potent anti-α-glucosidase inhibitors in a structure dependent manner (*16, 17*). Due to aforementioned biological and SAR activities, it is interesting to evaluate maple gallotannins for further anti-diabetic potential. However, to our best knowledge, maple gallotannins have never been reported for the anti-AGEs formation effects. Therefore, we initiated this study to evaluate the inhibitory effects of maple gallotannins in the early, middle and late stages of protein glycation.

MATERIALS AND METHODS

Chemicals

Bovine serum albumin (BSA), sodium azide, D-fructose, methylglyoxal (MGO), aminoguanidine (AM), 1,2-phenylenediamine (PD), 2,3-dimethylquinoxaline (DQ), sinapic acid were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). HPLC-grade acetonitrile and trifluoroacetic acid (TFA) were procured from Thermo Fisher Scientific (Rockford, IL, USA). ZipTip pipette tips with C_4 resin and 0.22 μm filter unit were obtained from EMD Millipore Co. (Billerica, MA, USA).

BSA-Fructose Assay

The BSA-fructose assay was used to evaluate the inhibitory ability of PGG on early and middle stage of BSA glycation according to the methods described previously (*18*). Briefly, bovine serum albumin was glycated using fructose as a glycating agent. The reaction mixtures contained 20 μM BSA with 20 mM fructose in a final volume of 3 mL of the 20 mM sodium phosphate buffer, pH 7.0 containing 0.2 M NaCl and incubated at 37 ℃ for different time intervals (7–21 days).

Preparation of Reaction Mixtures

In brief, stock solutions of BSA (50 mg/ml) and D-fructose (200 mM) were prepared in 0.2 M phosphate buffer, pH 7.2. Blank solutions included either BSA alone (10 mg/ml) or D-fructose alone (100 mM) in 0.2 M phosphate buffer. Negative control solutions included 10 mg/ml BSA and 100 mM D-fructose in the absence of maple gallotannins. The negative control mixtures was then mixed with different amount of maple gallotannins, resulting in the final concentrations of 1, 5, 10, 20, 50, 100, 200 and 400 μM. 10 mg/ml BSA and 100 mM D-fructose incubated with different concentrations of aminoguanidine served as positive control solutions. Unless otherwise indicated, all reaction mixtures were prepared in triplicate and incubated at 37 ℃ in a shaking water bath for a total length of 21 days. During incubation, aliquots were drawn from the reaction mixtures on day 3, day 7, day 14 and day 21 and stored at -20 ℃ until analysis. Analysis of the mixtures was performed on samples that were thawed, centrifuged and purified by ZipTip pipettes, EMD Millipore Co. (Billerica, MA, USA).

Fluorescence Spectroscopy

Prior to analysis, 40 μl of each sample was diluted 5 fold with 0.2 M phosphate buffer, pH 7.2 and then transferred to 96-well black fluorescence reading plates.

Fluorescence measurements for monitoring glycated products were then carried out with a Spectra Max M2 spectrometer (Molecular Devices, Sunnyvale, CA, USA) at excitation and emission wavelengths of 340 nm and 430 nm, respectively. The above excitation and emission values were optimal for detecting all of the BSA AGEs.

MALDI-TOF Mass Spectrometry

Intact BSA samples were purified and concentrated using C_4 ZipTips and analyzed on a Bruker Autoflex MALDI-TOF mass spectrometer. Prior to analysis, the purified protein samples (0.7 µL) were each mixed with a 50% aqueous acetonitrile solution (0.7 µL) of saturated α-cyano-4-hydroxy-cinnamic acid containing 0.05% TFA as matrix, spotted onto a stainless steel sample plate and allowed to air dry. Samples were analyzed on a Bruker Autoflex MALDI-TOF mass spectrometer with instrument settings optimized for intact protein analysis. Spectra were acquired in linear TOF mode with a 550 nsec delay in the m/z range from 25,000 to 75,000. Each spectrum was the sum of 5000 single laser shots randomized over multiple positions within the same spot. Analysis of data was performed using FlexAnalysis and ClinProTools software (Bruker Daltonics Inc., Billerica, MA, USA).

High Performance Liquid Chromatography

Each HPLC run was performed in triplicate using a Hitachi Elite LaChrom system consisting of a L2130 pump, L-2200 auto sampler, and a L-2455 Fluorescent Detector. All the instruments were operated on the EZChrom Elite software. All AGE species were separated on a Shodex (New York, NY, USA) RSpak C_{18} reverse phase HPLC column (5 µm×4.6 mm×150 mm) with a 415 Å pore size. Mobile phase A consisted of 0.1% trifluoroacetic acid (TFA) and 1% acetonitrile (ACN) in water. Mobile phase B

included 0.1% TFA and 95% ACN in water. A linear gradient from 20% to 60% of mobile phase B was applied at a constant flow rate of 1.0 ml/min over 25 minutes. The formation of AGEs was monitored using a programmable fluorescence detector with excitation and emission wavelengths set at 340 nm and 430 nm, respectively. Prior to analysis, all solvents were degassed by sonication for 15 min and all samples were filtered by a 0.22 μm membrane.

G.K. Peptide-Ribose Assay

GK-peptide-ribose assay was performed with slight modifications from the previously published method (*13*). Briefly, GK peptide (40 mg/ml) was incubated with 100 mM ribose in 100 mM sodium phosphate buffer, pH 7.0 and the testing compounds were added to the final concentrations of 0-300 μM. After incubation for 6 h, the fluorescence of the mixture was read at 340 nm excitation wavelength and 420 nm emission wavelengths using a Spectra Max M2 spectrometer (Molecular Devices, Sunnyvale, CA, USA).

Circular Dichroism Experiments

The CD analysis was performed with Jasco J-720 spectropolarimeter (Tokyo, Japan) using quartz cuvettes with 1 mm path length. Interpretation of results was performed by the Spectra Manager software. Prior to spectral acquisition, the concentration of BSA in each sample was adjusted to 1 mg/ml with the 0.2 M phosphate buffer, pH 7.2. The CD spectral signatures were obtained in the far-ultraviolet region (200-250 nm) employing a total of 8 consecutive scans for each sample. The bandwidth in each case was adjusted to 1 nm.

RESULTS

Effects of Maple Gallotannins on the Protein Glycation (BSA-Fructose Assay)

The inhibitory activities of maple gallotannins (GA, GB, GC, MF and MJ) against the formation of AGEs were shown in Figure 1. The fluorescence of glycated BSA with the treatments of maple gallotannins were compared with the control group, where BSA was only incubated fructose. When maple gallotannins were co-incubated with BSA and fructose complex, the fluorescence intensity significantly decreased in a concentration-dependent manner. As shown in table 1, ginnalin B and C inhibited 50% of AGEs formation at the concentration of 83.2 and 151.3 μM, respectively. Ginnalin A had a comparatively lower IC_{50} value than the other ginnalin's at 30.7 μM. Maplexins F and J acted as the most potent anti-glycative agents with the IC_{50} value of 15.8 and 17.4 μM, respectively. Meanwhile, all the maple gallotannins had superior inhibitory activities at a high concentration (300 μM) than AM as the positive control. For instance, maplexin F inhibited 86.4 % formation of AGEs *vs.* AM of 38.6% inhibition.

Maple Gallotannins Inhibited the AGEs Formation at the Early Stage

The early stage products of the Maillard reaction of BSA with D-fructose were analyzed by using MALDI-TOF mass spectrometry (MALDI-TOFMS). At this stage, D-fructose reacted with the amino groups of an amino acid or a protein to form the Amadori products. The numbers of sugar that were adducted to the BSA could be calculated based on the shifted mass of fructose-adducted BSA and natural BSA. As shown in Table 2, in the absence of maple gallotannins, glycated BSA had a mass shift of 1580 Da indicating that 10 D-fructose were adducted to the BSA protein. Maple gallotannins showed activities in preventing the adduction of fructose to protein. Both GB and GC had less

mass shift (940 Da and 820 Da, respectively) and the number of adducted D-fructose reduced to 5. In addition, the maplexin F treated BSA-D-fructose only had a increasing of 320 Da mass, which was equivalent to the mass of two fructose added to BSA. Among all the maple gallotannins, GA and MJ were the most active ones in the inhibition of Amadori products formation. Only 1 D-fructose was able to be adducted to BSA in the presence of GA or MJ which lead to a mass addition of 140 Da.

Maple Gallotannins Inhibited the AGEs Formation at the Early Stage

The intermediate AGEs fragments could be detected by their intrinsic fluorescence using HPLC-FL chromatograph spectra. Representative AGEs fragment HPLC-FL profiles are shown in Figure 7. The HPLC-FL chromatograph of native BSA that co-incubated with ribose showed several appreciable peaks indicating that BSA protein interacted with carbonyl compounds and their AGEs products were generated (as shown in Figure 7 A). However, peak intensities of ginnalin A treated group were significantly reduced as shown in Figure 7 C. All maple gallotannins were able to decrease the formation of AGEs as comparing with the control group (Figure 8) and the quantitative analysis of production of intermediate AGEs products are shown in Table 3. Maplexin J was the most active gallotannin that decreased the AGEs production to 4.6% followed by MF (7.2%). Other maple gallotannins, GC, GB and GA, also showed comparable inhibition percentage at 13.8%, 10.5% and 9.9%, respectively.

Maple Gallotannins Inhibited the AGEs Formation at the Late Stage

A synthetic peptide (G.K. peptide) containing a lysine residue was co-incubated with ribose in the presence or absence of maple gallotannins. This assay was adopted to evaluate the inhibitory ability of maple gallotannins against the cross-linking of *N*-

acetylglycyllysine methyl ester and ribose as in the late stage of AGEs formation. The fluorescence of G.K. peptide-ribose that co-incubated with maple gallotannins during this protein glycation course were determined and compared with that of G.K. peptide-ribose alone as control. The inhibitory activities of maple gallotannins were in a concentration-dependent manner as shown in Figure 9 (A). Although maple gallotannins merely showed inhibition of peptide-ribose cross-linking at 5 μM (inhibition rate < 20%), their activities clearly increased at 50 μM or higher concentrations. For example, maplexin F, as the most active gallotannin, showed inhibition effects of 40.1, 50.2 and 57.9% at the concentrations of 50, 100 and 300 μM, respectively. The inhibitory effects of maple gallotannins at the highest concentration (300 μM) are shown in Figure 9 (B). Apart from maplexin F, all maple gallotannins at 300 μM nearly inhibited the half of peptide-ribose glycation (ranged from 48.5 to 50.1%), whereas the positive control, aminoguanidine, only showed 6.8% inhibition. Only at a much higher concentration of 5 mM did Aminoguanidine show superior activity (63.1% inhibition) compared to the maple gallotannins.

Conformational changes of BSA induced by AGEs

The formation of AGEs often leads to the conformational changes of native protein. In order to study if maple gallotannins were able to protect BSA protein by maintaining its secondary structures from AGEs formation, the CD spectra of glycated BSA and native BSA were obtained and compared with that of maple gallotannins treated BSA. As shown in Figure 8, the CD spectra of glycated BSA protein clearly showed an alteration in the secondary structure as comparing with intact BSA protein, while maple gallotannins had the protective effects against the conformational changes. Quantitative

analysis of the spectra (Table 4) demonstrated that maple gallotannins maintained BSA protein α-Helix structures in the order of MJ (67.7%) > MF (63.9%) > GA (59.3%) > GB (42.8%) > GC (40.2%), at a concentration of 100 μM.

DISCUSSION AND CONCLUSSION

Maple gallotannins, which structurally feature different numbers and locations of galloyl groups attached to a glucitol moiety, demonstrated numerous bioactivities including anti-oxidant, anti-α-glucosidase and anti-cancer (*14, 16, 17, 19*). Although maple species extracts have been studied for the anti-glycation property (*20*), maple gallotannins as pure compounds have not been evaluated for anti-AGEs effects, nor the mechanisms of inhibitory activity against AGEs formation have been elucidated. Therefore, we initiated this study to investigate the anti-AGEs effects of maple gallotannins at individual stages of AGEs formation. In addition, the activities of mono-galloyl gallotannins (ginnalins B and C), di-galloyl gallotannin (ginnalin A), tri-galloyl gallotannin (maplexin F) and tetra-galloyl gallotannin (maplexin J) were discussed in a SAR context.

All of the gallotannins displayed anti-AGEs effects in a concentration-dependent manner (Figure 2). At the highest concentration (300 μM), tri-galloyl MF had the most potent inhibitory effect (86.3% inhibition) and tetra-galloyl MJ had a comparable activity as 81.7% inhibition. Then the activity order was followed by di-galloyl gallotannin (GA), and GB/GC as mono-galloyl gallotannins. It seemed that the inhibitory activities of maple gallotannins were enhanced as increasing the number of galloyl group on the glucitol. Additionally, a minimum number of two-galloyl groups were critical for the

maple gallotannins to possess potent inhibitory effects. This SAR observation was also further supported by the assays that evaluate the anti-AGEs activities at individual stage.

At the early stage of AGEs development, amino groups on lysine or arginine residue are able to condense with the carbonyl group of a reducing sugar by nucleophilic attack to form an unstable aldimine product (*21*). Schiff base is reactive and can be further rearranged to produce a stable ketosamine product known as the Amadori products. Many biophysical tools, such as MALDI-TOF-MS, are able to elucidate the adduction of reducing sugar to the amino groups of a protein (*22*, *23*). The number of adducted sugar to the BSA protein could be calculated based on the mass shift of the protein. The MALDI-TOF-MS data revealed that GA, MF, and MJ were superior in the interruption of fructose-BSA protein adduction as comparing with mono-galloyl gallotannins (GB and GC) where only moderate activities were observed.

Reactive carbonyl species (RCS) are the driving force in the intermediate phase of AGEs formation. The carbonyl compounds including glyoxal, 3-deoxyglycosone and methylglyoxal are generated in the early stage of AGEs formation. Reactive dicarbonyl compounds further react with Amadori products fragments and generate the intermediate AGEs products with intrinsic fluorescence (*24*). In this study, HPLC equipped with a fluorescent detector was able to characterize the RCS-BSA products by their fluorescent chromophores. All maple gallotannins were active in the FL-HPLC assay indicating that the presence of maple gallotannin hindered the interaction of RCS and BSA protein. However, it is noteworthy that maple gallotannins were not able to trap methylglyoxal as the most active RCS (data not shown). Therefore, maple gallotannins might inhibit the

formation of middle stage AGEs by impeding the catalysis of RCS generation, rather than directly trapping the generated carbonyl compounds.

In the last stage of AGEs formation, reactive RCS could further interact with Amadori products to generate a class of heterogeneous complex including fluorescent cross-linked structures. The synthetic G.K. peptide with lysine residue is feasible to react with ribose to form the cross-linking products. Therefore G.K. peptide-ribose system was adopted to mimic the late stage of AGEs development. Although mono-galloyl gallotannins (GB and GC) were less active than GA, MF and MJ at low concentrations (5 or 10 μM), all gallotannins demonstrated potent inhibitory activities at 300 μM. Since oxidation stress is a pivotal factor in the late stage of AGEs formation, maple gallotannins, which have been reported as potent free radical scavengers, may serve as anti-oxidants to neutralize reactive oxygen species. However, it needs further investigation to evaluate the free radical trapping capacities of maple gallotannins in the glycation course.

Lastly, it is known that the secondary structures are crucial in terms of maintain the protein function and studies have demonstrated that BSA protein loss it α-Helix conformation during protein glycation (*25*). Our CD spectra confirmed that maple gallotannins were able to protect BSA protein by attenuated the conformational changes induced by fructose. In addition, SAR effect was also in agreement with previous observed pattern.

In this study, maple gallotannins were evaluated for the anti-AGEs activity and their mechanisms of actions were studied by examining their inhibitory effects in the early, middle and late stage of AGEs formation. All maple gallotannins displayed anti-

AGEs formation effects by blocking the formation of intermediate Amadori products, and breaking the cross-linking of proteins. Information obtained from this study is important for the utilization of maple species as potential anti-AGEs management.

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FIGURE AND TABLE LEGEND

Figure 1: Chemical structures of aminoguanidine, ginnalins A-C, maplexin F and maplexin J.

Figure 2: Inhibitory effect of AM, GA, GB, GC, MF and MJ against the formation of AGEs. 10 mg/ml BSA and 100 mM D-fructose were incubated at 37 ℃ in 0.2 M phosphate buffer pH 7.4. Prior incubation, each sample was treated with different concentrations of natural products. Aminoguanidine (AM) was served as positive control. After incubating for 21 days, the formation of fluorescence AGEs in each solution was determined at excitation and emission wavelengths of 370 nm and 435 nm respectively. Each fluorescence reading was then compared to the intensity of negative control solution which incubated in the absence of natural products.

Figure 3.1: MALDI-TOF mass spectrum of the +2 ion of A) BSA alone, B) BSA glycated with fructose for 3 days at 37 ℃, C) BSA glycated with fructose in the presence of 100 μM GB for 3 days at 37 ℃, and D) BSA glycated with fructose in the presence of 100 μM GC for 3 days at 37 ℃.

Figure 3.2: MALDI-TOF mass spectrum of the +2 ion of A) BSA alone, B) BSA glycated with fructose for 3 days at 37 ℃, C) BSA glycated with fructose in the presence of 100 μM GA for 3 days at 37 ℃, and D) BSA glycated with fructose in the presence of 100 μM MJ for 3 days at 37 ℃.

Figure 3.3: MALDI-TOF mass spectrum of the +2 ion of A) BSA alone, B) BSA glycated with fructose for 3 days at 37 ℃, C) BSA glycated with fructose in the presence of 100 μM MF for 3 days at 37 ℃.

Figure 4: The shifted m/z value of glycated BSA and treatment group as comparing to the natural BSA. Treatment group: AM, GB, GC, GA, MF and MJ, all the final concentration of each testing compound was 100 µM. Blue bars indicate the calculated fructose that added to the BSA.

Figure 5: HPLC-fluorescence elution profiles of 10 mg/ml BSA and 100 mM D-ribose after 7 days incubation at 37 ℃ with or without any treatment. Negative control solution consisted of BSA and D-ribose alone **(A)**. Positive control sample consisted of BSA and D-ribose in the presence of 100 µM aminoguanidine **(B)**. Experimental groups contained BSA, D-ribose and 100 µM maple gallotannins **(C)**. The HPLC elution profile of the blank solution containing BSA only yielded no fluorescence absorbing peaks suggesting the absence of AGE products in the blank sample after 7 days incubation (data not shown). Repeat chromatographic analysis of reaction mixtures by HPLC revealed no significant differences in the elution profiles of any of the AGE peaks in each of solutions.

Figure 6: Fluorescence intensity of BSA and D-ribose complexes with absence and presence of maple gallotannins.

Figure 7: (A) Inhibitory effects of maple gollotannins on the final stage of protein glucation (G.K. peptide-ribose assay). G.K. peptide (40 mg/ml) was co-incubated with ribose (800 mM) for 9 hours in the absence and presence of maple gallotannins at concentrations from 5 to 300 µM. Aminoguannidine (300 and 5000 µM) were served as positive controls. (B) Inhibitory effects of maple gollotannins at 300 µM on the final stage of protein glycation (G.K. peptide-ribose assay). Fluorescence of samples was

measured at excitation 340 nm and emission 420 nm and results are means ± SD for three independent tests.

Figure 8: Far-UV circular dichroism spectra of native BSA, glycated BSA and maple gallotannins treated BSA protein.

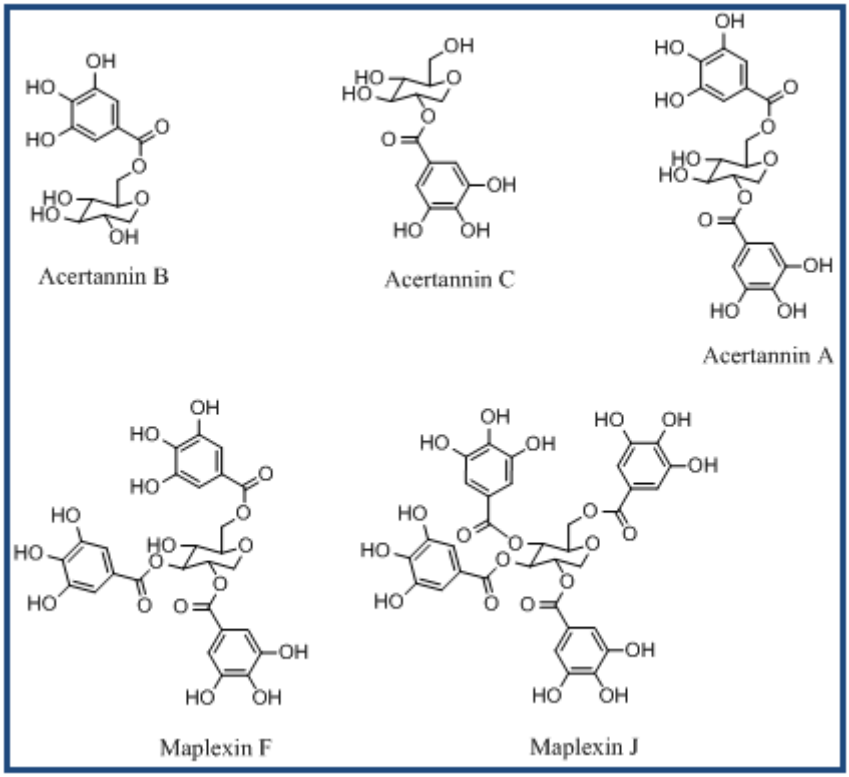
Table 1: Inhibitory effects of maple gallotannins on the formation of AGEs. The activities were expressed in IC_{50} value and the inhibition rate of testing compounds at 300 μM. Aminoguanidine was served as the positive control.

Table 2: The mass shift and possible number of adducted fructose of glycated BSA and maple gallotannins treated BSA protein.

Table 3: The HPLC-FL area under curve value for middle stage AGEs products in the FL-HPLC assay.

Table 4: Secondary structures (α-Helix and β-sheet) of native BSA, glycated BSA and maple gallotannins treated BSA protein.

Figure 1:



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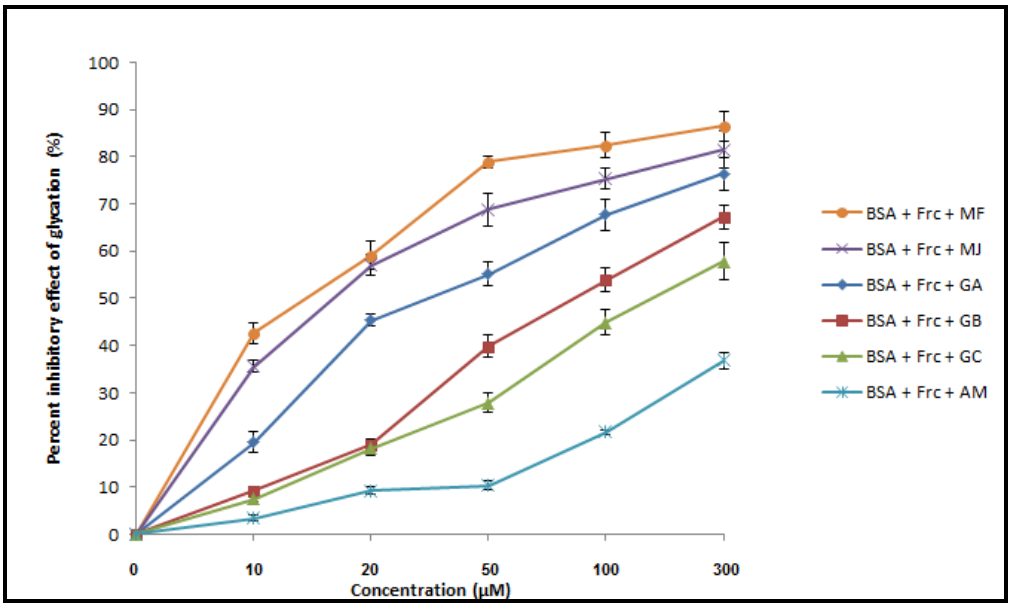

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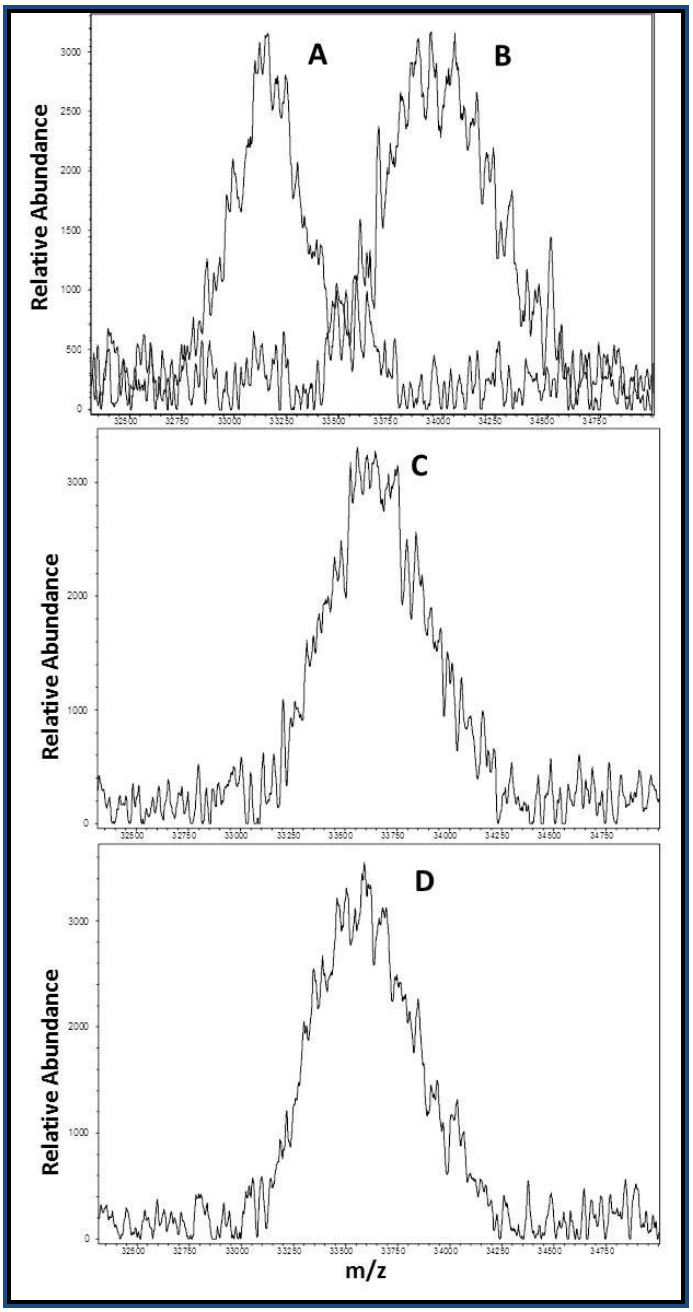

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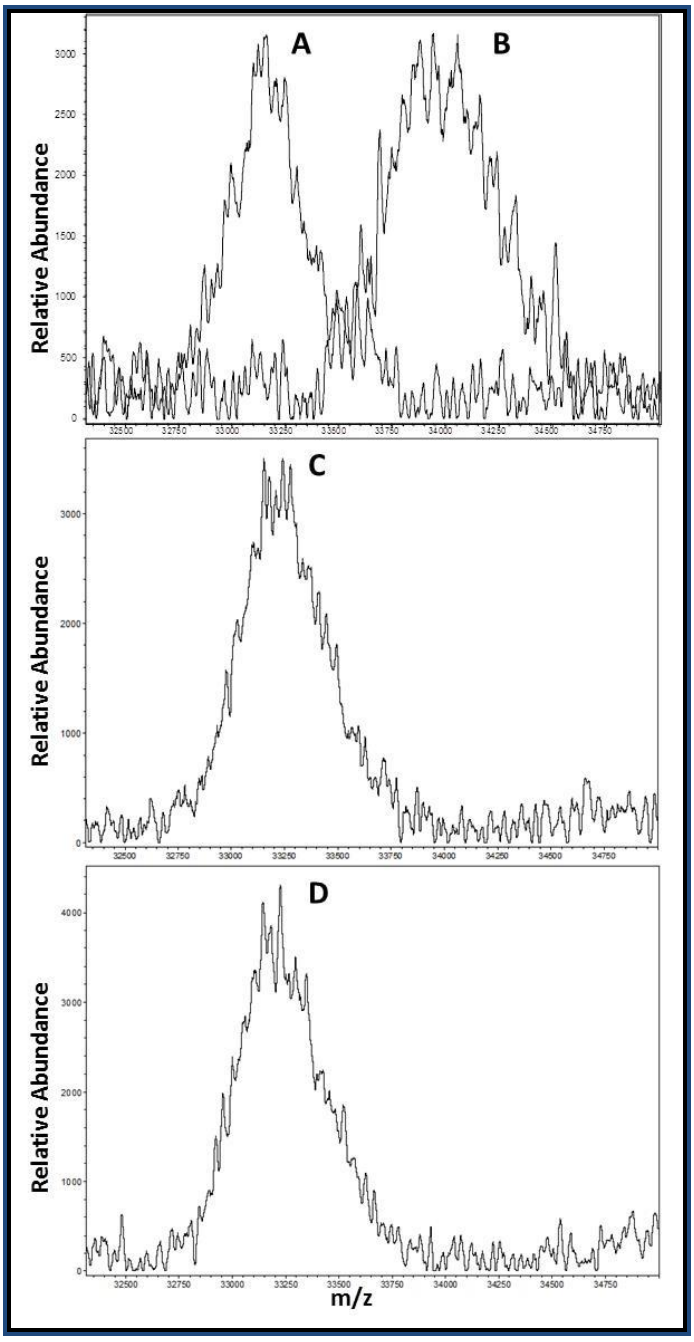

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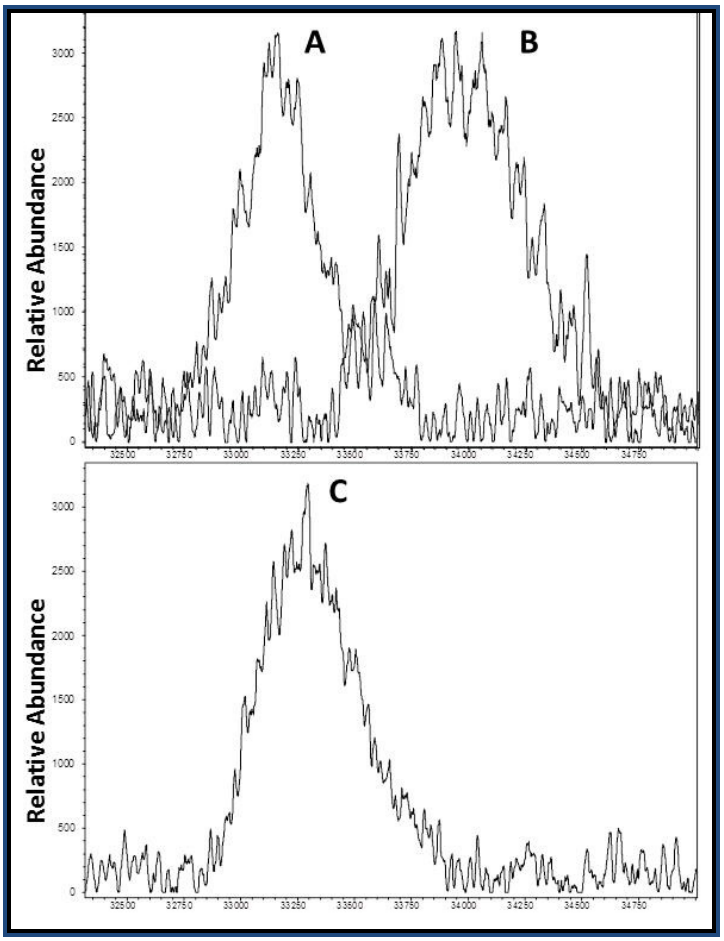

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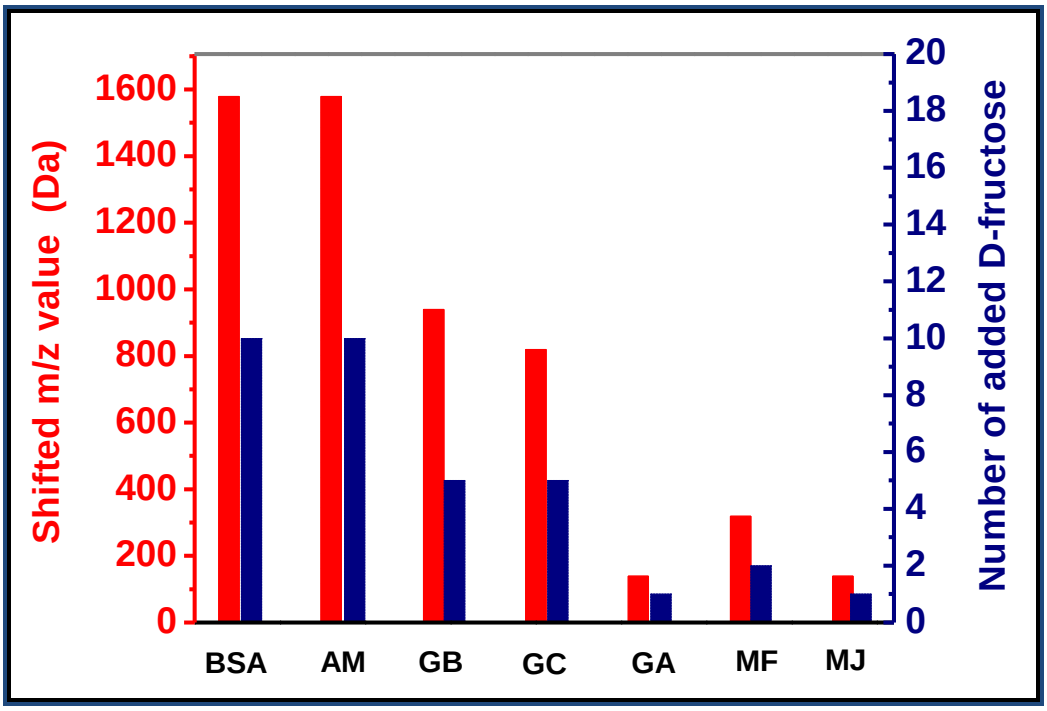

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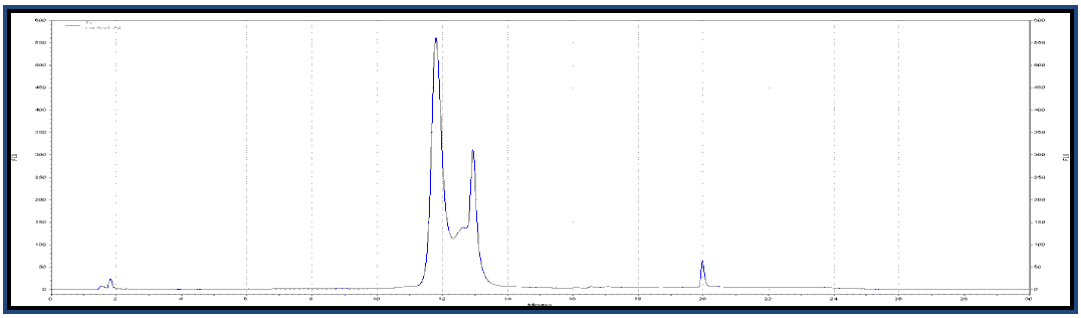
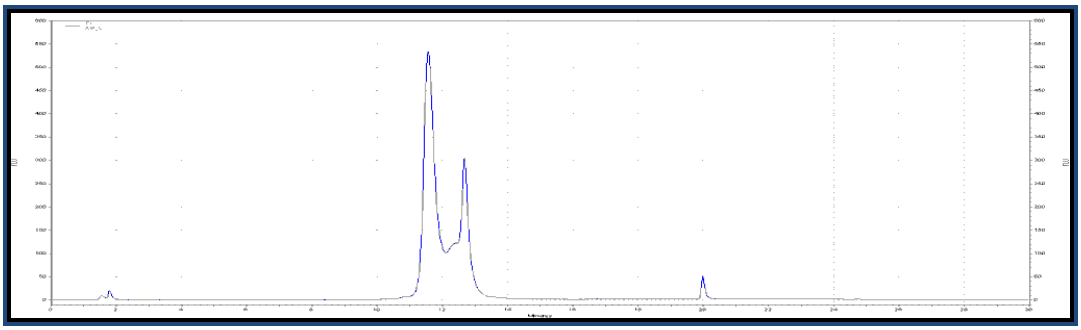


Figure 5(B):



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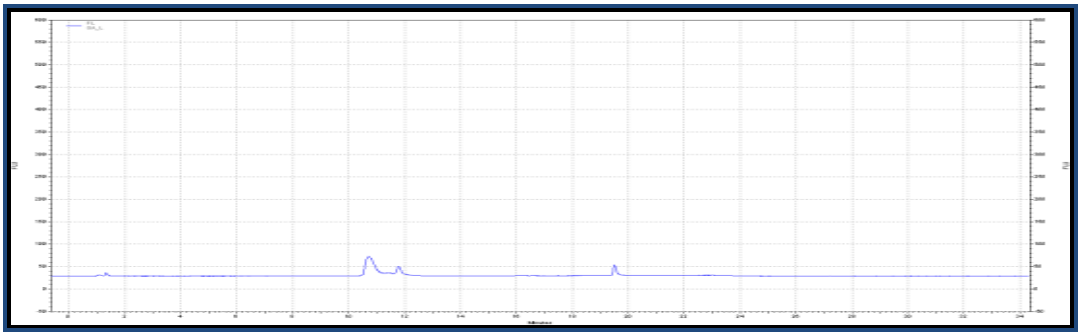

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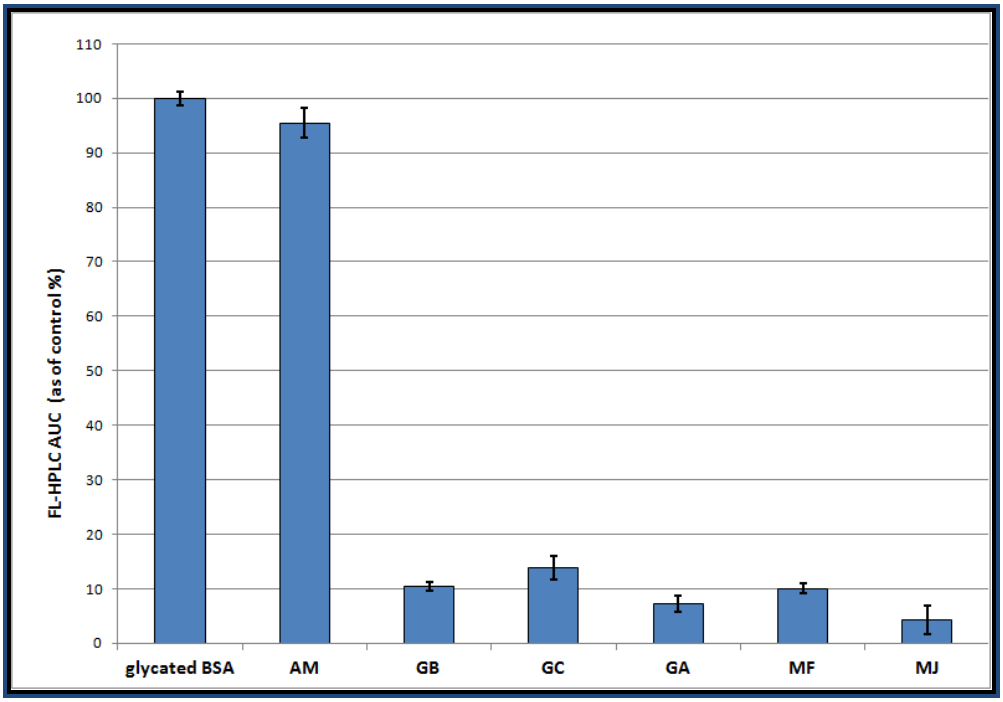
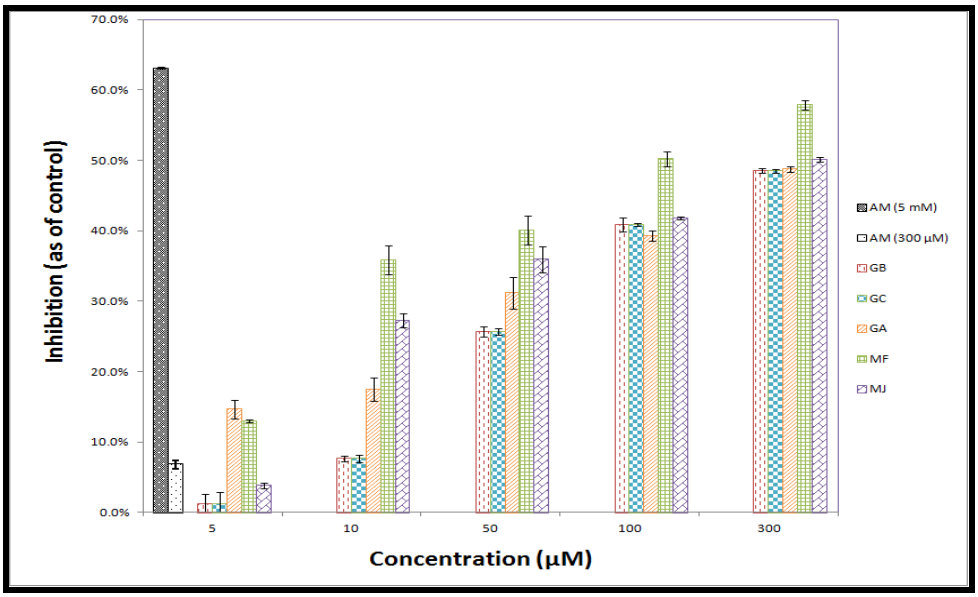


Figure 7(A):



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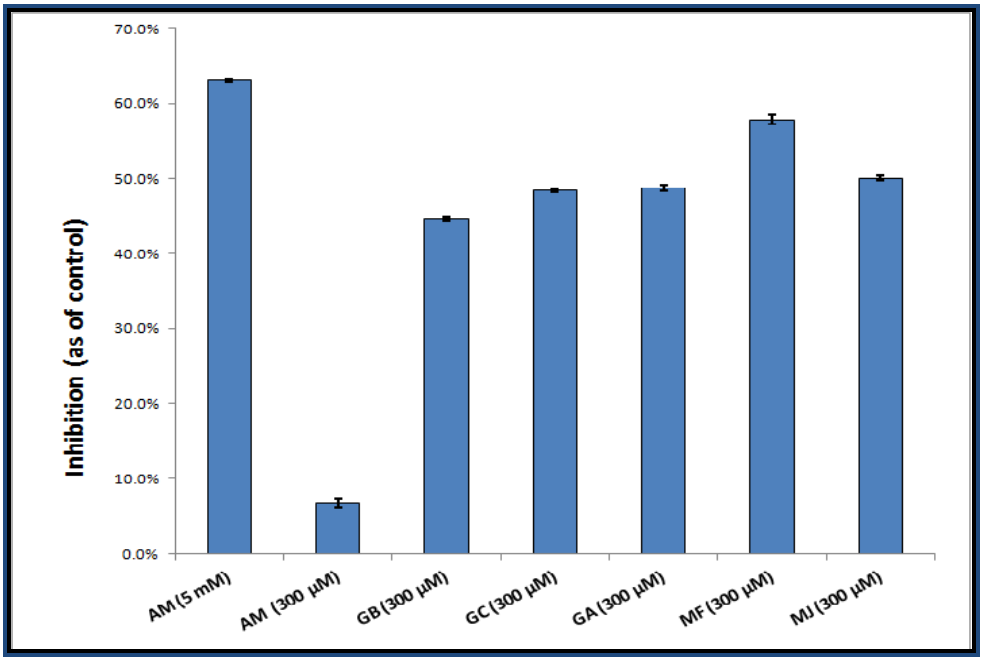

Figure 8:

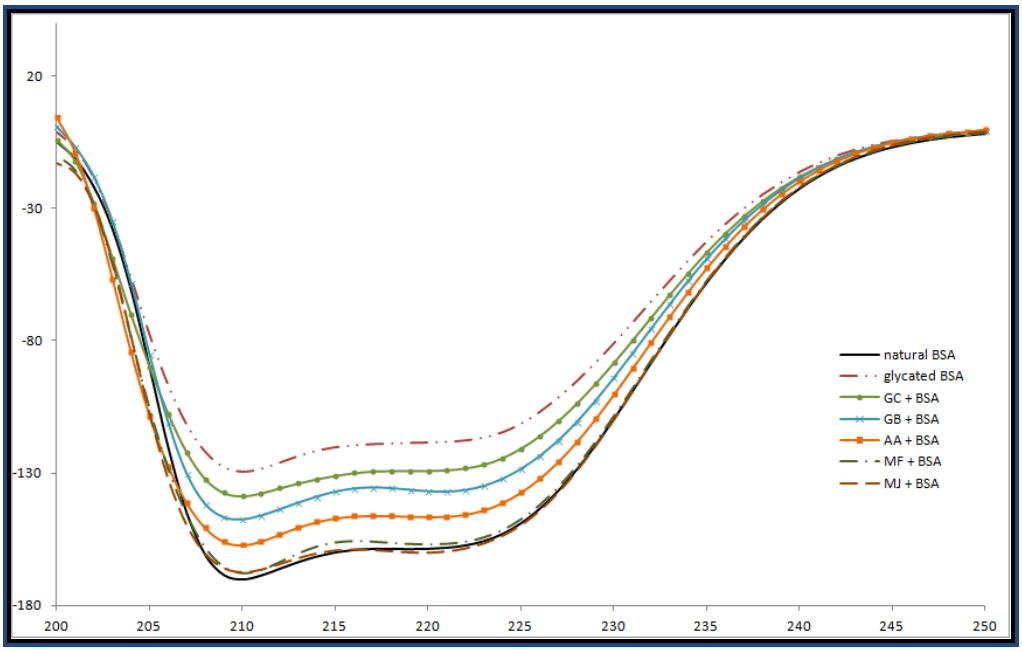

Table 1:

Compd.	# of galloyls	IC_{50} (μM)	Inhibition % (at 300 μM)
GB	1	83.2 ± 2.8	67.1
GC	1	151.3 ± 3.4	58.1
GA	2	30.7 ± 0.6	76.9
MF	3	15.8 ± 1.1	86.3
MJ	4	17.4 ± 0.9	81.7
AG	–	> 300	38.6

Table 2:

Treatment	m/z value of +2 ion (Da)	m/z value of +1 Ion (Da)	Shifted m/z value (ΔDa)	# of adducted D-fructose
BSA	33,170	66,340	0	0
BSA-Frc	33,960	67,920	+1580	10
BSA-Frc + AG	33,960	67,920	+1580	10
BSA-Frc + GB	33,640	67,280	+940	5
BSA-Frc + GC	33,580	67,160	+820	5
BSA-Frc + GA	33,240	66,480	+140	1
BSA-Frc +MF	33,330	66,660	+320	2
BSA-Frc +MJ	33,240	66,480	+140	1

Table 3:

BSA treatment	Peak 1 Area	Peak 2 Area	Peak 3 Area	Peak 4 Area	Total AGEs Area	Relative AGE Area %
Frc	57585823	11725137	23028179	2021731	94360870	100
Frc + AG	54559011	9488533	24054288	1971908	90073740	95.456665
Frc + GB	5188024	0	3166023	1568500	9922547	10.515531
Frc + GC	6063848	1057314	4582415	1389647	13093224	13.875692
Frc + GA	4154347	475608	1317708	853922	6801585	7.2080567
Frc + MF	4494927	799368	2455755	1683915	9433965	9.9977512
Frc + MJ	2022385	336914	1313660	432946	4105905	4.3512793

Table 4.

Secondary structures	natural BSA	glycated BSA	GB	GC	GA	MF	MJ
α-Helix (%)	67.3	34.5	42.8	40.2	59.3	63.9	67.7
β-Sheet (%)	7.7	21	15.1	16.5	10.2	9.4	9

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Title: Cosmetic applications of red maple (*Acer rubrum*) leaf extract and its purified gallotannin constituents: Inhibition of melanogenesis via down-regulation of tyrosinase and melanogenic gene expression in B16F10 melanoma cells.

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Key words: anti-tyrosinase – antimelanogenic – *Acer rubrum* – red maple-phytochemicals

Abstract: Our group has previously reported a series of bioactive gallotannins isolated from the red maple (*Acer rubrum* L.) species, namely ginnalins A-C and new molecules

named maplexins A-I, with potent antioxidant capacities. These natural compounds contain 1, 2 or 3 galloyl groups (from a possible 4 locations) attached to a 1,5-anhydro-D-glucitol moiety. Herein, we initiated a project to investigate the cosmetic skin lightening/whitening applications of Maplifa™, a proprietary extract from the leaves of the red maple species, in enzyme and cell based assays. Maplifa™ contains ca. 45-50% of ginnalin A along with other gallotannins including ginnalin B and C and maplexins. SAR studies showed that increasing the number of galloyl groups attached to the 1,5-anhydro-D-glucitol moiety resulted in greater inhibitory effects on tyrosinase enzyme. Consequently we synthesized maplexin J, the first tetragalloyl-glucitol (contains the maximum number of 4 galloyl groups) and confirmed our SAR observations. Purified ginnalins A-C, as the representative gallotannins in Maplifa™, were assayed for their inhibitory effects on melanin production in B16F10 cells. Ginnalin A (contains 2 galloyl groups) clearly reduced the melanin content at 50 μM whereas ginnalin B and C (contains 1 galloyl group of each) showed only minor anti-melanogenic effects. Lastly, the mechanisms of the inhibitory effects of ginnalins A-C on melanogenesis in B16F10 cells were elucidated by using real-time PCR and Western blot experiments. The results indicated that ginnalins were able to down-regulate the expression of MITF, TYR, TRP-1 and TRP-2 gene levels in a time and dose-dependent manner and significantly reduce the protein expression of TRP-2 gene. The findings in our study indicate that phytochemicals in red maple leaves possess anti-melanogenic effects and thus may have cosmetic skin-whitening applications.

Introduction

In mammals, skin pigments are produced in the melanosomes of melanocytes cells that are situated on the basal layer between the dermis and epidermis.(*1*) The pigmentation plays a crucial role in protecting skin against radiation-induced damage such as exposure to ultraviolet light. However, overproduction or abnormal accumulation of melanin may lead to many skin hyperpigmentation disorders including freckles, age spots, post-inflammatory hyperpigmentation and even melanoma.(*2*) Undesired excessive skin pigmentation is a severe health concern since it can cause negative burdens on patients' psychological well-being. Thus, skin depigmentation remains a compelling research area for the cosmetic industry and the exploration of novel classes of safe and effective melanogenesis inhibitors from natural sources has attracted immense research interest.

The biosynthesis of melanin is collectively regulated by over 100 distinct genes. In mammals, three enzymes, tyrosinase (TYR), tryosinase-related protein-1 (TRP-1) and tryosinase-related protein-2 (TRP-2) are essential for the overall melanin production.(*3*) Tyrosinase performs a pivotal role in the modulation of melanogenesis. It is the rate-limiting enzyme that catalyzes the hydroxylation of L-tyrosine into 3,4-dehydroxyphenylalanine (DOPA) and consequently oxidizes DOPA into DOPA quinone.(*4*) TRP-2, which serves as a DOPA-chrome tautomerase (DCT), further promptly coverts DOPA quinone into 5,6-dihydroxyindol-2-carboxylic acid (DHICA), whereas TRP-1 facilitates the oxidization of DHICA to form carboxylated indole-quinone.(*5*) Two types of melanin are produced: a red/yellow pheomelanin and a black/brown eumelanin. The TYR enzyme is critically involved in both pheomelanin and

eumelanin synthesis while TRP-1 and DCT contribute more in the synthesis of eumelanin.(*6*) Microphthalmia-associated transcription factor (MITF) is a primary transcriptional activator of the melanogenic enzymes and seems to be the principle transcription regulator that mediates the survival, proliferation and differentiation of melanoblasts and melanocytes.(*7*) Given that melanin synthesis in mammal involves multiple-step catalyzation that modulated by a group of enzymes and transcription factors, it is important to understand the biological molecular mechanisms of melanogenesis inhibitors.

Although many synthetic compounds, such as hydroquinone and its derivatives had been conventionally used to suppress the over production of melanin, their application in cosmetics have been restrained because the lack of effectiveness or potential side effects including skin irritation and/or toxicity.(*8, 9*) Therefore, natural products and botanical extracts have emerged as attractive candidates for melanogenesis inhibitory applications since they tend to be safe and have fewer adverse effects than synthetic compounds.

The maple genus (*Acer*) comprises of over 120 species, most of which are found in Asia, with the remaining being native and endemic to North America. Phytochemical and biological investigation of *Acer* species including *A. buergerianum* (China maple) and *A. nikoense* (Japanese maple) resulted in a number of compounds with anti-melanogenic effects in B16F10 cells.(*10, 11*) Interestingly, *A. rubrum* L. (red maple) which is native to eastern North America was traditionally used as a folk medicine by the Native Americans for many ailments including dermatological disorders.(*12*) Moreover, a recent report supports the cosmetic potential of a red maple bark extract but neither the

active compounds nor the mechanisms of action have been identified.(*13*) Furthermore, until now, the leaf extract from the red maple species have never been investigated for its cosmetic application, in particular relating to anti-melanogenic effects, nor have the active compounds therein been identified.

For the past 5 years, our laboratory has been involved in a comprehensive phytochemical and biological program of studies on maple sap, maple syrup, and native North American maple plant parts, mainly the sugar maple and red maple species. This work has resulted in the isolation and identification of over 100 compounds from maple, many of which are new molecules, with anti-oxidant, anti-α-glucosidase and anti-cancer activities.(*14-18*) Here, we developed a proprietary standardized extract from red maple leaves and evaluated the cosmetic applications of the extract, and its major purified chemical constituents, for anti-tyrosinase and anti-melanogenic effects on B16F10 cells.

Materials and methods

Materials: Leaves of *A. rubrum* were collected on the University of Rhode Island (URI) Kingston campus, (RI, USA) by Mr. J. Peter Morgan. A voucher specimen has been deposited in the URI College of Pharmacy Heber W. Youngken Jr. Greenhouse and Herbarium. All solvents were of either ACS or HPLC grade and were purchased from Wilkem Scientific (Pawtucket, RI). Mushroom tyrosinase, L-tyrosine, 4-hydroxyphenyl β-D-glucopyranoside (arbutin) and kojic acid were purchased from Sigma–Aldrich (St. Louis, MO). Ginnalins A-C and maplexins E were isolated from the red maple species as previously reported by our group. Maplexin J, a new synthetic gallotannin analogue containing four galloyl groups on a 1,5-anhydro-D-glucitol moiety, was chemically

synthesized in our laboratory. Unless otherwise specified, all other chemicals were purchased from Sigma–Aldrich. The HPLC analyses were performed on a Hitachi Elite LaChrom system consisting of a L2130 pump, L-2200 auto sampler, a L-2455 Diode Array Detector, and a Phenomenex Luna C_{18} column (250 × 10 mm, S-5 μm). All the instruments were operated on the EZChrom Elite software.

Preparation of Maplifa™: Maplifa™ was prepared by proprietary protocols developed in our laboratory. Briefly, leaves of red maple were dried and macerated in aqueous ethanol to obtain the crude extract which was then further purified on a resin column to remove chlorophyll and other plant pigments. Maplifa™ was obtained as an off-white free flowing powder after solvent removal.

Cell culture and treatment: Murine melanoma B16F10 was purchased from the American Type Culture Collection (Rock-ville, MD). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) medium (Wilkem Scientific, RI) that was supplemented with 10% v/v fetal bovine serum, 1% v/v nonessential amino acids and 1% v/v antibiotic solution at 37 ℃ in 5% CO_2.(*19*) Samples were dissolved at a concentration of 50 mg/mL in dimethylsulfoxide (DMSO) as stock solution and then diluted to the desired final concentrations with growth medium. The final DMSO concentration was less than 0.1%.

Tyrosinase inhibition assay: Inhibitory effects of Maplifa™, ginnalins A-C and maplexins F-J on mushroom tyrosinase were evaluated spectrophotometrically using L-tyrosine as a substrate according to a method described previously with minor modification.(*20*) Tyrosinase inhibition assays were performed in 96-well microplate format using SpectraMax M2 microplate reader (Molecular Devices, CA). Briefly,

testing samples were dissolved in 10% DMSO at a concentration of 5.0 mg/mL and then diluted to different concentrations with phosphate buffer (0.1 M, pH 6.8). Each well contained 40 μL of sample with 80 μL of phosphate buffer solution, 40 μL of tyrosinase (100 units/mL), and 40 μL L-tyrosine (2.5 mM). The mixture was incubated for 20 min at 37 ℃, and absorbance was measured at 490 nm. Each sample was accompanied by a blank containing all components except L-tyrosine. Kojic acid and arbutin were used as positive controls. The results were compared with a control consisting of 10% DMSO in place of the sample. The percentage of tyrosinase inhibition was calculated as follows:

$[(\Delta A\text{control} - \Delta A\text{sample})/ \Delta A\text{control}] \times 100$

Cell viability tests: The MTS assay was carried out as described previously with modifications.(*17*) At the end of either 24, 48 or 72 h of treatment with test samples (in the concentration range of 1-100 μM), 20 μL of the MTS reagent, in combination with the electron coupling agent, phenazine menthosulfate, were added to the wells and cells were incubated at 37 °C in a humidified incubator for 3 h. Absorbance at 490 nm was monitored with a spectrophotometer (SpectraMax M2, Molecular Devices Corp., operated by SoftmaxPro v.4.6 software, CA, USA) to obtain the number of cells relative to control populations. Inhibition of proliferation in the sample treatment cells are expressed as percentage compared to control (0.1% DMSO) cells. Data are presented as mean values ± S.D. and were obtained from three separate experiments.

Cellular melanin contents: The melanin content was determined using a modification of the method described by Yoon *et al*.(*21*) Briefly, B16F10 cells (5×10^4 cells/well) were first seeded in 24-well plates for 24 h and then medium was changed with new DMEM medium containing test samples of various concentrations. After 72 h

incubation, the cells were harvested through trypsinization and washed with phosphate-buffered saline (PBS) twice. Then cells were lyzed with 1 N NaOH containing 10% DMSO and heated at 80 ℃ for 1 h. After samples cooled down to room temperature, the amount of melanin content was spectrophotometrically measured at 400 nm.

Real-time quantitative PCR analysis of MITF, tyrosinase (TYR), TRP-1 and TRP-2 expression: The B16F10 cells were planted in 6-well plates at a density of 2.0×10^5 cells/well. After incubated for 24 h, cells were treated with 2 or 10 μM of ginnalins for 48 or 72h. Total RNA was isolated from cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. One microgram of total RNA was converted to single-stranded cDNA using oligo(dT)18 primers, and mRNA levels were quantified by quantitative real-time PCR using a Roche LightCycler detection system (Roche Applied Science, Mannheim, Germany). Samples were run by using SYBR Green and compared with levels of b2m rRNA as a reference housekeeping gene. Quantitative real-time PCR conditions were optimized for each gene using appropriate forward and reverse primers. All oligonucleotides were synthesized by Invitrogen Inc., CA.(*22*)

Evaluation of MITF, TYR, TRP-1 and TRP-2 expression expression by western blot: Expressions of melanin biosynthesis-related proteins, including *MITF, TYR, TRP-1 and TRP-2 expression* in B16F10 cells were measured by western blot. After incubation with ginnalins for 72 h, proteins from cells were resolved by SDS-PAGE and then transferred to polyvinylidene fluoride membrane. The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline with Tween followed by incubation with primary antibodies overnight. Bands were visualized on X-ray film using an ECL detection kit (Amersham Biosciences, Piscataway, NJ).(*23*)

Statistical analysis: Two-tailed unpaired student's t-test was used for statistical analysis of the data using the Office Excel 2010 software. A p value < 0.05 was considered significant.

Results

Effects of ginnalins A-C on tyrosinase activity: Inhibitory effects of five maple gallotannins, ginnalins A-C (**1**-**3**) and maplexin F and J (**4**-**5**), on mushroom tyrosinase activity were assayed and their IC_{50} values are shown in Table 1. The gallotannins with two to four galloyl groups were more active than acertanins with only one galloyl group. Ginnalin A (**1**), a 1,5-anhydro-D-glucitol moiety with two galloyl groups, showed the high inhibitory activity with an IC_{50} value of 181.9 μM. Similarly, maplexin F (**4**) and maplexin J (**5**), with three or four galloyl groups respectively, showed comparable IC_{50} values of 212.2 and 190.4 μM. However, gallotannins with only one galloyl group, namely, ginnalin B (**2**) and ginnalin C (**3**), only showed weak inhibitory effects (IC_{50} = 1047.3 and 857.8 μM, respectively) on the tyrosinase enzyme.

Cell viability of ginnalins A-C on B16F10 cells: To further investigate the anti-melanogenic activities of ginnalins A-C, present in the Maplifa™ extract, cellular based assay were employed to measure the melanin content on murine melanoma B16F10 cells. In order to determine the non-toxic concentrations of ginnalins for cellular assays, cell viability on B16F10 cells were first evaluated by using MTS assay. Melanoma B16F10 cells were treated with serial concentrations (0, 5, 10, 25, and 50 μM) of ginnalins A-C for 72 h and the cell viabilities were determined by comparing with the untreated control group. All of the ginnalins B and C were non-toxic to B16F10 cells at all concentrations

from 0 to 50 μM (cell viabilities > 90%), while, ginnalin A was non-toxic at low concentrations (5 and 10 μM) but slightly reduced the B16F10 cell viabilities to 87.0% and 89.5% at concentrations of 25 and 50 μM, respectively. According to findings from previous studies that screens for safe and effective melanogenesis inhibitors in cell based assays, 40 μg/mL is a threshold concentration for cellular melanogenesis assays. Thus, all of the test concentrations of the ginnalins in our study were lower than this published threshold and, and therefore can be considered as being non-toxic doses resulting in safe melanogenesis inhibitory agents.

Effect of ginnalins A-C on melanin contents: The contents of biosynthesized melanin in melanoma B16F10 cells that treated with ginnalins A-C at serial concentrations were evaluated by comparing with the control group. Although ginnalins did not show significant inhibitory activities on melanin biosynthesis at low concentrations (5 and 10 μM), ginnalin A demonstrated reduced the formation of melanin at relatively high dosages. The melanin contents in B16F10 that treated with 25 and 50 μM of ginnalin A clearly decreased to 79.1% and 56.7%, respectively, compared to those of in control group, while, ginnalin C slightly reduced melanin level to 89.7% and 68.8% at 25 and 50 μM, respectively. In addition, cells treated with 25 and 50 μM of ginnalin B remained melanin level at 90.0% comparing with the control group.

Effect of ginnalins A-C on tyrosinase-related gene and protein expression: Melanin biosynthesis involves multi-step pathways. In order to determine the molecular mechanisms of inhibitory effects of ginnalins on melanin synthesis, expression levels of melanogenesis related genes, including MITF, TYR, TRP-1 and TRP-2, in B16F10 cells were analyzed by using RT-PCR. As shown in Fig. 5, after treated with 10 μM of

ginnalin A for 48 h or 72 h, the mRNA expressions of MITF, TYR, TRP-1 and TRP-2 were significantly reduced. Although ginnalin B only slightly decreased the TYR expression by 10.0 and 7.5% at 48 and 72 hours, it significantly decreased the mRNA expressions of MITF, TRP-1 and TRP-2 at 48 and 72 hours. In addition, ginnalin C did not reduce the TRP-1expression level at all time points and only slightly decreased TYR and TRP-2 expression, however, it significantly down-regulated the mRNA expression of MITF at 48 and 72 hours.

Furthermore, the regulation of ginnalins on the protein expressions of melanogenesis related enzymes in B16F10 cells were evaluated by using Western blot. Ginnalins did not show down-regulation effects on the protein expression of melanin biosynthesis related enzymes at 5 and 10 μM at 48 hours, nor reduce the expressions of these proteins in B16F10 cells that co-incubated with ginnalins for 72 hours. However, in the melanoma cells that treated with 10 μM of ginnalins A-C for 72 hours, the protein expression of TRP-2 were decrease by 87.9, 92.0, and 69.5% by ginnalins A-C, respectively (Fig. 6.).

Discussion and conclusion

Previous studies have revealed that a number of maple (*Acer* species) extracts possessed many biological activities including anti-oxidant, anti-microbial, anti-angiogenic and anti-melanogenic.(*10, 11*) Some maple species that are indigenous to Asia including China maple (*Acer buergerianum*) and Japanese maple (*Acer nikoense*), have been reported for their inhibitory effects against melanogenesis on B16 cells and potentials for cosmeceutical applications. Since these bioactivities were largely associated with the phenolic content in the maple extracts, it was suggested that anti-

oxidant capacity of maple polyphenols was critical for the aim of developing maple extracts for the medicinal and/or nutritional uses.(*24*) In addition, standardization of maple extracts is another crucial factor for the application of maple extracts. Although tested maple extracts were mainly obtained from organic solvents extraction, an efficient, environmentally and economically friendly extraction approach is needed to produce maple extracts in consistent quality. Therefore, our laboratory previously developed Maplifa™ as a standardized extract from red maple leaves and we herein present the biological evaluation of major polyphenolic components of Maplifa™, namely ginnalins A-C, for the potential skin-whitening uses.

Ginnalin A, as the predominant phenolic in the Maplifa™ extract, has been known as a strong antioxidant for the free radical trapping capacity.(*14*) Furthermore, ginnalin A has two galloyl groups that attached to the 1,5-anhydro-D-glucitol while both ginnalin B and C only has one galloyl on the same sugar core. Therefore, ginnalins A-C as represent compounds of Maplifa™ were chosen for the further biological assays and possible structure and activity relationship were discussed. Our *in vitro* study showed that ginnalins A-C, maplexins F and J had moderate inhibitory effects against tyrosinase. Generally, the inhibitory activity of gallotannins (GA, MF and MJ) with multiple galloyl group (n>2) were greater than mono- galloyl substituted ginnalins (B and C). However, glucitol moiety with two or more galloyl groups had comparable activity. This phenomenon suggests that maple gallotannins may directly bind to tyrosinase and hinder the formation of active site. In addition, more galloyl groups on the glucitol showed stronger inhibition potentially by increasing the chance of the ginnalins bind to tyrosinase to form hydrogen bonds which may stabilize the ligands-enzyme complex.

In order to further explore the anti-melanogenic property of ginnalins, cellular based assays were employed on the B16F10 murine melanoma cells. Firstly, the cell viability was accessed by using MTS assay to obtain a safe range of ginnalins concentrations for the further experiments. Since anti-melanogenic activity of ginnalins on B16F10 cells should be assayed at the concentrations without inducing significant cytotoxicity, the threshold concentration was set to 50 μM for all testing compounds. This concentration is lower 40 μg/mL (equivalent to 85 μM of ginnalin A or 126 μM of ginnalin B/C) as a general concentration limit for cellular based anti-melanogenesis assay. All ginnalins had cell viability over 90% indicating that ginnalins were non-toxic to the cells. In the melanin content assay, ginnalins A-C all showed a concentration dependant manner in the inhibition of the melanin biosynthesis in B16F10 cells. Similar SAR effects were observed as in the tyrosinase assay, ginnalin A was more potent than other two ginnalins at down-regulating melanin biosynthesis. This activity was supported by examining the mRNA expression of genes that regulate melanin production. The RT-PCR assay demonstrated that in 48 or 72 hours treatments, ginnalin A was the most potent one to decrease the mRNA expression of MITF, TYR, TRP-1 and TRP-2. It had a significant decrease of MITF expression comparing to other melanin synthesis genes indicating that ginnalin A down-regulating melanin biosynthesis by suppression of transcriptional activator of melanin production related enzymes at the mRNA transcriptional level. Lastly, Western blot assay was applied to measure the protein expression of genes MITF, TYR, TRP-1 and TRP-2. A decreased expression level of TRP-2 suggested that ginnalins were able to impede the transformation of DOPA quinone into 5,6-dihydroxyindol-2-carboxylic acid which could further be oxidized to form the

melanin pigments. The RT-PCR and Western blot assays provided useful information towards the molecular inhibitory mechanisms of ginnalins against melanogenesis.

Our finding suggested that ginnalins A-C as the major phytochemicals in Maplifa™ could individually reduce the production of melanin in B16F10 cells. However, it is possible that the polyphenols in the Maplifa™ extract could inhibit the melanin formation in a synergistic manner, especially considering that the biosynthesis of melanin involves with multiple biochemical transformation and is under the regulation of numerous enzymes. Therefore, Maplifa™ might be developed as a novel skin-whitening agent or therapeutic strategy for the treatment of hyperpigmentation. However, the efficacy and safety of Maplifa™ need to be studied in the animal models.

Figure and table legends

Figure 1: Chemical structures of ginnalins A-C (**1-3**) and maplexins F (**4**) and J (**5**)

Figure 2: HPLC-UV chromatograms of Maplifa™, an ginnalin A-riched extract, showing the presence of ginnalins A–C (**1-3**) in the Maplifa™ extract.

Figure 3: Effect of ginnalins A-C (**1-3**) on viability of B16F10 cells. After 72 hours of treatment of serial concentrations (2-50 μM) of ginnalins (**1-3**), the viability of melanoma B16F10 cells were determined by MTS assay. Each value is presented as mean ± S.D. from triplicate independent experiments.

Figure 4: Inhibition of cellular melannin content in B16F10 cells by ginnalins A-C. Cells were treat with of ginnalins A-C for 12 h, and the melanin content were compared to the control group. Each value is presented as mean ± S.D. fro triplicate independent experiments.

Figure 5: The mRNA expression of MITF, TYR, TRP-1 and TRP-2 in ginnalins-treated B16F0 cells. Cells were treated with or without 10μM of ginnalins for 48 h (A) and 72 h (B).

Figure 6: The expression of melanogenesis related protein MITF and TRP-2 in ginnalins-treated B16F0 cells. Cells were treated with or without 10μM of ginnalins for 72 h and protein expression of MITF and TRP-2 were analyzed by Western blotting.

Table 1: Inhibitory activity (IC_{50}) of five phenolics, ginnalins A-C (**1-3**) and maplexins F-J (**4-5**), on tyrosinase enzyme. [a]IC50 are presented as mean ± S.D. from triplicate independent experiments. *Positive controls.

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Figure 1:

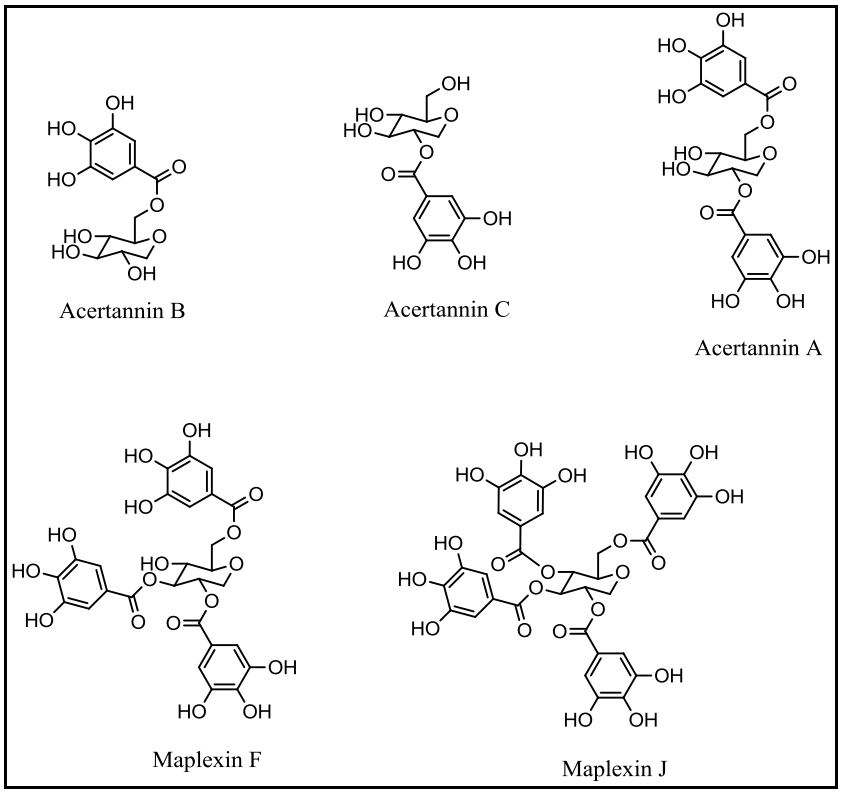


Figure 2:

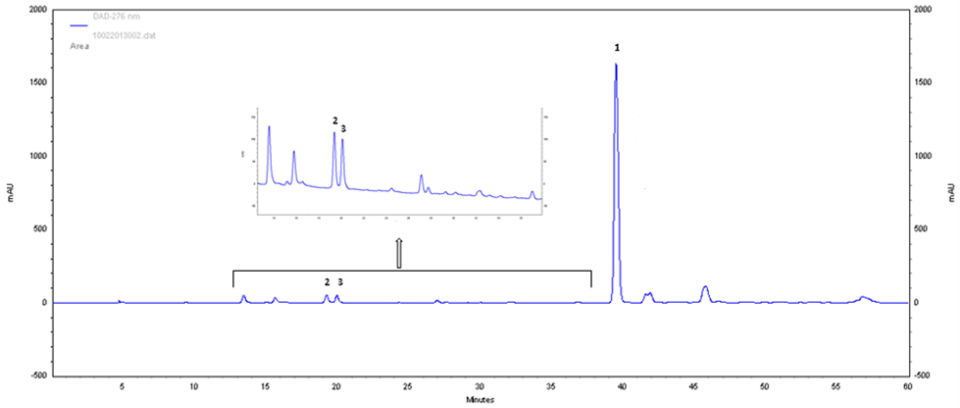
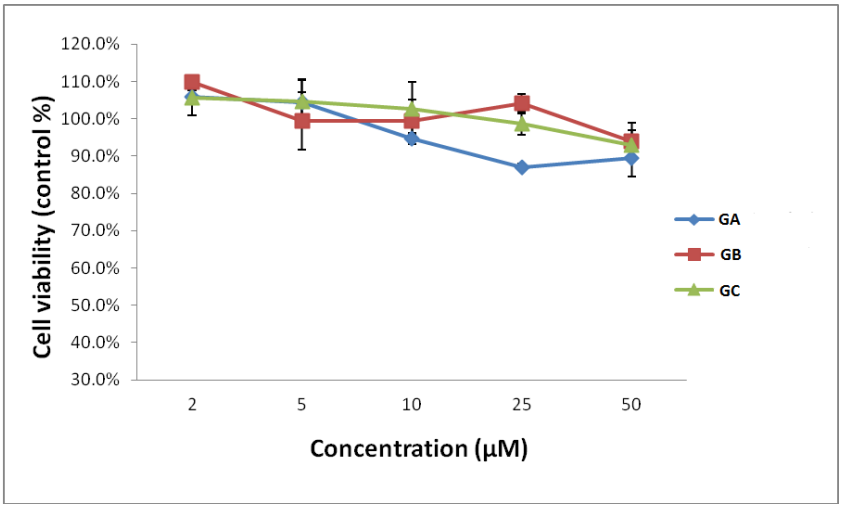


Figure 3:



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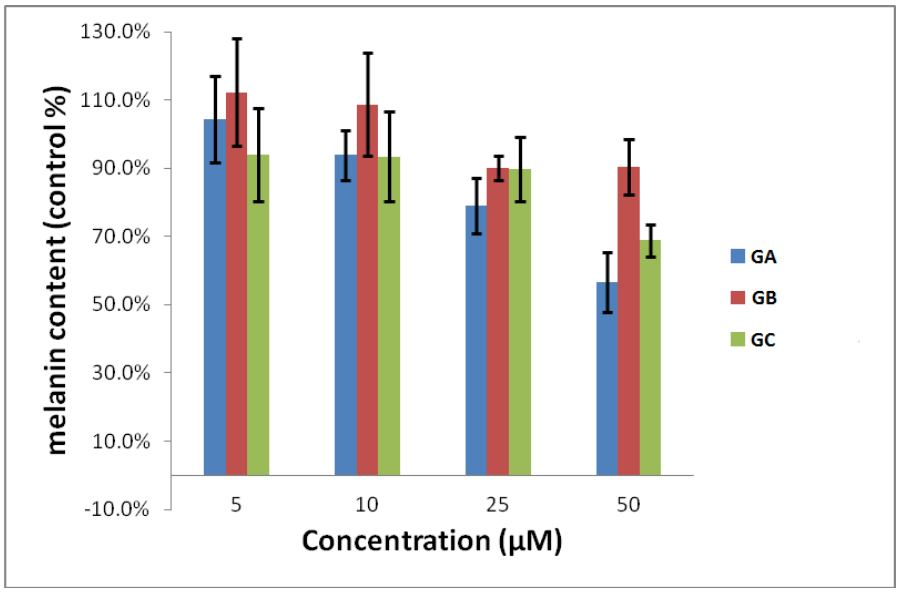
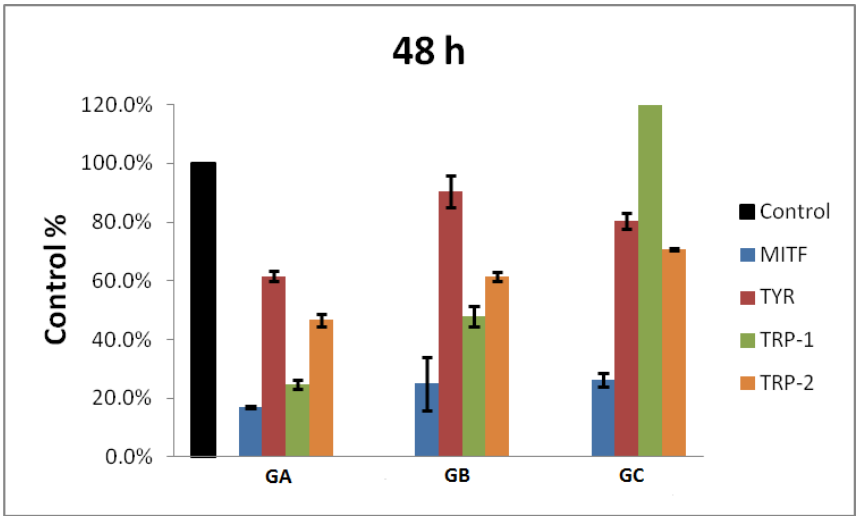
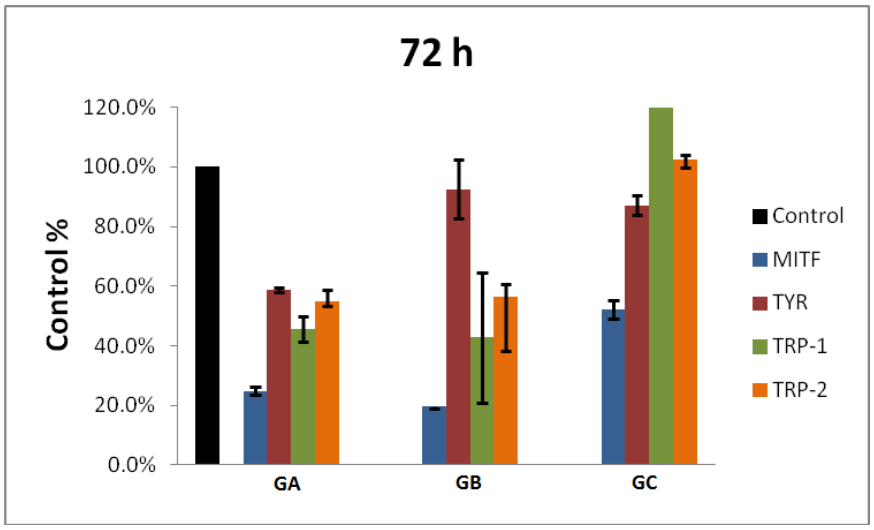


Figure 5:

(A)



(B)



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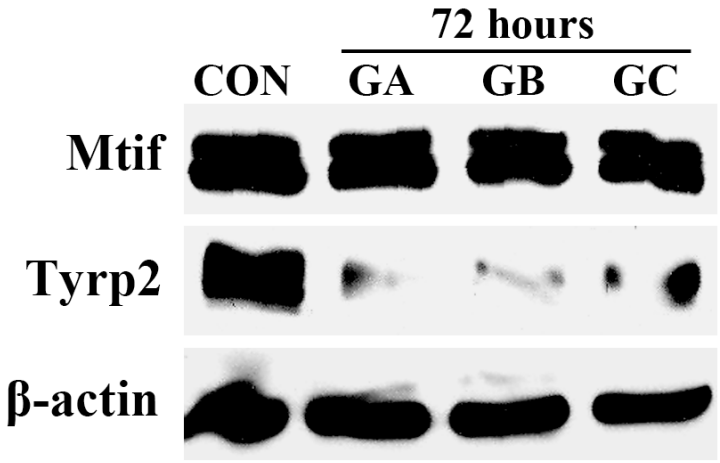

Table 1:

Compd.	# of galloyl group	IC_{50} (μM)[a]
1	2	181.9 ± 3.5
2	1	1047.3 ± 2.7
3	1	857.8 ± 6.9
4	3	212.2 ± 5.5
5	4	190.4 ± 1.7
Kojic acid*	–	21.4 ± 0.1
Arbutin*	–	63.0 ± 0.3