ADVANCED GLYCATION END PRODUCTS FORMATION INHIBITION THROUGH STANDARDIZED CRUDE EXTRACT OF PUNICA GRANATUM L. STEM BARK

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I would like to dedicate the thesis to my beloved parents, my dearest Bahar and Melika, and especially my love and wife, Maryam, for always loving, understanding, encouraging and supporting me. Words cannot express my gratitude for having them as my family.

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ABSTRACT

Formation of advanced glycation end products (AGEs) under hyperglycemic condition in diabetes mellitus results in micro/macro-angiopathy disorders. Juice, leaves, or peel of pomegranate have shown antioxidant or antiglycation effects. Pomegranate stem barks which are hugely wasted during the pruning season could be a good source of phyto-based anti-AGEs. This study evaluated standardized pomegranate stem barks extract in term of antioxidant activity, antiglycation potential and also its effect on lipid formation and glucose consumption in 3T3-L1 cells.Various extraction conditions were performed including types of solvents, time and type of extraction methods. Phytochemical analysis of extracts was carried by high-performance liquid chromatography-pulsed amperometric detector (HPLC-PAD), gas chromatography-mass spectroscopy and spectrophotometric methods. Evaluation of antioxidant activity was performed using 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and metal chelating activity. Anti glycation activity of the extract was evaluated in bovine serum albumin (BSA)/glucose or BSA/methylglyoxal (MGO) bioassay systems in presence or absence of samples. Antiglycation property was measured by determination of the level of formation of fructosamine, protein carbonyl and AGE or loss of thiol group. Also, effect of extract on glucose consumption and lipid formation in 3T3-L1 cell line in media containing MGO was investigated in vitro. The result showed that eight hour extraction with methanol using Soxhlet extraction (SM8) was the best extraction process in term of total polyphenolic compounds (59.69 ± 2.913 mg gallic acid equivalent (GAE)/g dry weight (DW), DPPH scavenging capacity [half maximal effective concentration (EC_{50}) 14.99 ± 1.18 mg/L], $ABTS^{\bullet+}$ radical scavenging equal to 2.636 mM trolox equivalent antioxidant capacity (TEAC)/100 g DW and metal chelation activity (EC_{50} 888.1±48.38). Standardization of SM8 extract by HPLC-PAD showed gallic acid as 0.19% and catechin 0.03% of the extract. SM8 extract reduced formation of AGE significantly ($p<0.01$) by 77% in concentration of 250 µg /ml. Moreover, it reduced protein carbonyl (60.2%) and fructosamine formation (33.99 %) and simultaneously inhibited thiol group loss (by 1.84 folds). The SM8 extract increased glucose consumption (by 1.95 folds) in 3T3-L1 cells in glycemic condition. In conclusion, it is recommended that pomegranate stem bark extract as a potential source of raw material to be further investigated for the development of health supplement with AGEs inhibitory properties.

ABSTRAK

Pembentukan hasil akhir glikation lanjutan (AGEs) berlaku dalam keadaan hiperglisemik diabetes melitus menyebabkan gangguan angiopati makro dan mikro. Jus, daun atau kulit buah delima telah dilaporkan menunjukkan kesan antioksidan dan anti glikation. Sisa kulit batang pokok delima dari musim cantasan ladang pokok delima mungkin merupakan sumber bahan asas-fito anti AGEs yang bernilai. Kajian ini menilai ekstrak kulit batang pokok delima terpiawai dari segi, aktiviti antioksidan, potensi anti glikation dan kesannya terhadap pembentukan lipid dan penggunaan glukosa dalam kultur sel 3T3-L1. Pelbagai keadaan pengekstrakan telah dijalankan termasuk jenis pelarut, masa dan kaedah pengekstrakan. Analisa fitokimia ekstrak dijalankan menggunakan kaedah kromatografi cecair berprestasi tinggi-pengesan amperometrik denyut (HPLC-PAD), kromatografi gas- spektroskopi jisim dan spektrofotometrik. Penilaian aktiviti antioksidan telah dijalankan menggunakan 2,2-difenil-1-pikrilhidrazil (DPPH), 2,2′-azino-bis (3-etilbenztiazolin-6-asid sulfonik) (ABTS) dan aktiviti pengkelatan logam. Aktiviti anti glikation bagi ekstrak telah dinilai menggunakan sistem bioasai bovin serum albumin (BSA)/glukosa atau BSA/metilglioksal (MGO) dengan sampel atau tanpa sampel. Sifat anti glikation diukur dengan mengira tahap pembentukan fruktosamina, karbonil protein dan AGE atau kehilangan kumpulan tiol. Juga, kesan ekstrak terhadap penggunaan glukosa dan pembentukan lipid dalam kultur sel 3T3-L1 mengandungi MGO dikaji secara in vitro. Hasil kajian menunjukkan bahawa ekstrak metanol menggunakan kaedah Soxhlet selama lapan jam (SM8) merupakan kaedah pengekstrakan terbaik dari segi jumlah sebatian polifenolik 59.69 ± 2.913 mg asid galik setara (GAE)/g berat kering (DW), keupayaan hapus sisa DPPH [kepekatan berkesan separuh maksimum (EC_{50}) 14.99 ± 1.18 mg/L], radikal hapus sisa $ABTS^{\bullet+}$ bersamaan dengan 2.636 mM aktiviti kapasiti antioksidan bersamaan trolox (TEAC)/100 g DW dan aktiviti pengkelatan logam (EC_{50} 888.1 ± 48.38). Pempiawaian ekstrak SM8 menggunakan HPLC-PAD menunjukkan nilai asid galik sebanyak 0.19% dan katekin sebanyak 0.03%. Pembentukan AGE menurun secara ketara ($p<0.01$) sebanyak 77% pada kepekatan SM8 250 µg /ml. Tambahan pula, ia menurunkan karbonil protein (60.2%) dan pembentukan fruktosamina (33.99 %) dan sekaligus merencat kehilangan kumpulan tiol (1.84 kali ganda). Ekstrak SM8 meningkatkan penggunaan glukosa (1.95 kali ganda) dalam kultur sel 3T3-L1 berkeadaan glisemik. Sebagai kesimpulannya, adalah dicadangkan bahawa ekstrak kulit pokok delima merupakan bahan mentah berpotensi untuk dikaji secara lebih lanjut sebagai suplemen kesihatan yang mempunyai sifat anti AGEs.

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ABTS	-	2,2-Azino-bis-3-ethylbenzothiazoline-6-sulfonic Acid
AGEs	-	Advanced glycation end products
ALEs	-	Advanced lipoxidation end products
AG	-	Aminoguanidine
BSA	-	Bovine serum albumin
CEL	-	Carboxyethyllysine
CML	-	Carboxymethyllysine
DEX	-	Dexamethasone
3-DG	-	3-deoxyglucosone
DMEM	-	Dulbecco's modification of Eagle's medium
DNPH	-	2,4-dinitrophenylhydrazine
DPPH	-	2,2 diphenyl-2-picrylhydrazyl
DTNB	-	5,5'-dithio-bis-(2-nitrobenzoic acid)
ELISA	-	Enzyme linked immunosorbent assay
EDTA	-	Ethylenediaminetetra acetic acid
FBS	-	Fetal bovine serum
FCS	-	Fetal calf serum
Glu	-	Glucose
GO	-	Glyoxal
GOLD	-	Glyoxal-lysine dimer
HPLC	-	High performance liquid chromatography
HT	-	Hydrolysable tannins
HRP	-	Horseradish peroxidase
IBMX	-	3-isobutyl-1-methylxanthine
LDL	-	Low density lipoprotein
MGO	-	Methylglyoxal

MMPs	-	Matrix metalloproteinases
MOLD	-	Methylglyoxal lysine dimer
MTT	-	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NBT	-	Nitro-blue tetrazolium
NFK-B	-	Nuclear Factor-kappa –B
NIST08	-	National Institute of Standards and Technology
ORO	-	Oil red O
PSBE	-	Pomegranate stem bark extract
PCC	-	Protein carbonyl content
PNU	-	Precinorm Universal Control
PPAR	-	Peroxisome proliferator-activated receptors
PPU	-	Precipath Universal Control
PS	-	Penicillin streptomycin
PSBE	-	Pomegranate stem bark extract
RAGE	-	Receptor for AGEs
RCS	-	Reactive carbonyl species
ROS	-	Reactive oxygen species
SDS-PAGE	-	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SM8	-	Soxhlet method 8 extract
SOD	-	Superoxide dismutase
TCA	-	Trichloroacetic acid
TEAC	-	Trolox equivalent antioxidant capacity
TEMED	-	Tetramethylethylenediamine
TFC	-	Total flavonoid content
TPC	-	Total phenolic content
TNF- α	-	Tumour necrosis factor-α
UV/VIS	-	Ultraviolet-Visible spectrophotometry
VCAM-1	-	Vascular cell adhesion molecule
WHO	-	World Health Organization

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CHAPTER 1

INTRODUCTION

1.1 Research Background

Basic source of energy for cells is glucose. However, imbalance of glucose level in bloodstream (hypoglycaemia or hyperglycemia) is an origin of many metabolic disorders. Diabetes, *Diabetes mellitus*, is a factorial metabolic chronic disease characterised by elevated blood glucose (hyperglycaemia) and insulin deficiency (type I) or resistance (type II) (World Health Organozation, 2011). According to World Health Organization (WHO) report, it is estimated that diabetic cases raise from 285 million in 2010 to 438 million in 2030. Consequently, global expenditures will increase from $418 billion to at least $561 billion (World Health Organozation, 2011).

Glycation is a mechanism in which carbonyl group of a reducing sugar binds to an amino group of a protein without enzymatic control (Zhang et al., 2009). In diabetic condition with prolong hyperglycemia glycation is occurred in an elevated level. Intermediate compounds with α–dicarbonyl structure such as methylglyoxal, 3-deoxyglucosone and glyoxal play a distinguished role as advanced glycation end product (AGEs) precursors to form stable crosslinked proteins or AGEs which result in protein dysfunction (Gugliucci and Menini, 2002, Pazdro, 2010). Accumulation of AGEs in tissues promote disorders such as elasticity and ionic problems in kidney, atherosclerotic lesions of arterial walls, chronic renal failure and amyloid fibroids in hemodialysis-related amyloidosis, stiffening, angiogenesis, and extracellular matrix

accumulation physiology of AGEs (Mendez et al., 2010, Beisswenger, 2010). AGEs contribute to the progression of diabetes complications therefore inhibition of formation of AGEs reduces development of the diabetic complications.

There are natural and synthetic approaches to control AGEs formation. However the synthetic medicines are more specific to target but possess some side effects. In contrast, the natural products have beneficial multifunctional properties and if being used in right concentration have no serious side effects. Aminoguanidine (AG), phenacylthiazolium bromide, ALT-71, and thiazolidine are some synthetic AGEs inhibitor compounds. Nowadays, AG is only used for in vitro and animal study, not human, because of very harmful side effects and refuse in clinical trials. Todays, there is rising interest in application of naturally occurring inhibitors as antiglycation agents (Tsuji-Naito et al., 2009). There is a pile of evidence that show antiglycation activities of plants phytochemicals in vivo and in vitro. Antiglycation inhibitory effects of a plant is mostly related to its polyphenolic compounds (Peng et al., 2008b). Noticing that, there are many different phytochemicals with thousands of structures spread in plant species, it is reasonable to search for finding the more specific compounds for various diseases.

Pomegranate is a plant native to southern regions of Caspian Sea in Iran, Afghanistan, India and the Mediterranean region. Pomegranate fruit is a valuable source of vitamins and minerals and also is rich in bioactive compounds. The use of pomegranate plant parts like roots, bark, fruit, juice and the leaves as medicine has ancient root in traditional medicine system in Iran, China, India and Unani traditional medicine. In folk medicine bark and fruit rind are administered for treatment of diseases such as dysentery, diarrhea, piles, bronchitis, biliousness and as an anthelmintic (Bagri et al., 2009). Several studies have been conducted in related to the therapeutic properties of this plant. In pomegranate the major group of phytochemical that exert antidiabetic activities are polyphenols, which may be able to improve hypoglycemia through mechanisms such as, the inhibition of glucose absorption in the gut or of its uptake by peripheral tissues (Huang et al., 2006b).

Health benefit of pomegranate phytochemical components such as punicalagin, ellagic acid, galoyllglucose, anthocyanins and tannins have exhaustively been investigated in vitro and in vivo. The major phytochemicals in rind and fruit juice are galloylglucose, punicalin, ellagic acid and gallic acid. However aril juice contain different types of anthocyanins like cyaniding, delphinidin, 3-glucoside, glucoside, 3,5- diglucoside and pelargonidin. Punic acid, sterols γ-tocopherol have been identified in seeds extract. It has indicated that pomegranate seed extract have potential of anti-diarrheal and antioxidant properties (He et al., 2010).

Traditionally, medicinal plants have been used for treatment or prevention of different diseases. Based on researches, different parts of pomegranate including husk, aril, mesocarp (pulp) and leaves contain phenolic compounds including anthocyanins, flavonoids and different types of tannins. Therapeutic effects of these compounds has been documented to play a role as anticancer, lowering blood glucose level, preventing cardiovascular disease and lipid peroxidation (Seeram et al., 2006).

Insulin-mediated signalling pathways have remarkable responsibility in glucose metabolism. Impairment of insulin function causes metabolic disorders and diseases like atherosclerosis, diabetes and obesity due to inducing insulin resistance. Some authors have demonstrated that elevated level of AGEs inside the cell prevents insulin signalling pathway and in this way induces insulin resistance. Glucose regulation in blood is depended to insulin-mediated signalling pathways, thus the degree of glucose consumption in fat cells could be considered as a monitor index to explain the insulin signalling condition (Peng et al., 2010). Consequently, perfect insulin signalling pathways represent cell glucose consumption capability and vice versa. Antioxidants scavenge reactive carbonyl species (RCS) in different mechanisms such as trapping α-dicarbonyl intermediate compounds, metal chelating, activation of enzymatic antioxidant system, or neutralizing free radical molecules. According to the evidence more than 120 polyphenols have been isolated from different parts of pomegranate including root, fruit bark, whole fruit, aril, and flowers (Seeram et al., 2006).

Autoxidation of glucose in the presence of ion metals generates free radicals leading to AGEs formation. Studies have shown that plant extracts or phytochemicals exert antiglycation through several mechanisms such as metal chelating, free radical scavenging, interfering in glucose metabolism, mimicking insulin activity or increasing insulin secretion (Gurav et al., 2007, Okabayashi et al., 1990 , Arun and Nalini, 2002, Eshrat and Hussain, 2002, Sajithlal et al., 1998, Abdullah et al., 2004, Shen et al., 2012). Phytochemicals, depends on their structures, are able (or unable) to trap reactive carbonyl species, scavenge free radicals, prevent autoxidation of glucose or oxidative stress which resulting suppression of reactions leading to AGEs formation (Ardestani and Yazdanparast, 2007). For instance, in an exhaustive research among 115 compounds only 10 compounds were able to reduce AGE formation (Rahbar and Figarola, 2003). Therefore trying to explore natural AGEs inhibitors seems valuable effort.

Different types of synthetic and natural compounds have been introduced and used against AGEs activities and formation. Unfortunately, synthetic compounds usually show unfavorable side effects however, natural compounds are safer to use. The potential of antiglycation activity of a plant is related to structure of its phytochemicals and also the concentration of those phytochemicals in the plant parts (Rahbar and Figarola, 2003, Peng et al., 2008a).

1.2 Problem Statement

The hallmark of diabetes is elevated level of glucose (hyperglycemia). Therefore, in diabetic patients formation of AGEs is higher compare to healthy peoples. AGEs contribute in pathogenesis of diabetes complications. Strategies for inhibition of AGEs effects are based on detoxification, inhibition of formation and prevention of AGEs accumulation by synthetic or natural AGEs inhibitors. Normally, introducing new AGEs inhibitors needs a lot of time and cost which unfortunately, most of the time, they are rejected in clinical trials. Moreover they have undesirable side effects. For example, some antidiabetic medicines which

decrease blood glucose level cause fatness in patients because of increasing glucose intake and depositing in tissues in form of lipid. Therefore, there is a lot of attention to discovery of phytochemicals as the main natural resources with hope to explore potent glycation inhibitors. Considering to pomegranate, as a fruit rich of polyphenols with antioxidant properties, many researchers have been attracted to investigate its antiglycation potential as well.

An exhaustive literature review was done on therapeutic effects of pomegranate on diabetes. Different parts of the plant including leaves, juice, seed or fruit bark (rind, peel) have been examined for biological activities (Seeram et al., 2006). Previous works have shown the effect of pomegranate fruit, flower, fruit bark, leaves or seed on improvement of diabetes. Among different therapeutic effects of pomegranate plant parts on improvement of diabetes, it was not found evidence on the therapeutic effect of pomegranate stem bark on diabetes through inhibition of AGEs formation. Therefore, the study aimed to investigate inhibitory effect of pomegranate stem bark on formation of AGEs. Usually stem barks of trees contain bioactive compounds such as polyphenols (Vekiari et al., 2008). After ends of the experimental work of the current study, Nishida et al. (2015) released a patent on Maillard reaction inhibitor using some plants including pomegranate (Nishida et al., 2015). They have claimed that pomegranate plant part for example root barks, stem bark and fruit, have Maillard reaction inhibitory properties. However, there is no specific detail or information on extraction method, or plant part that they have used for they experiments. Generally, bark contains greater amounts of extractable components including monomeric polyphenol such as flavonoids and polymeric phenolic compounds like tannins and phenolic acids (Anderson et al., 2004). In pomegranate yearly pruning removes a lot of twigs and stems which, at present, have no economical usage. Therefore, if the stem bark shows good potential of antiglycation activity it will be economically valuable because of using of pruning residues to provide functional extract for nutraceutical use. To sum up, antiglycation agents from naturally occurring compounds due to relatively lack or low toxicity or side effects are more promising nominees to be used in functional food or as a nutraceutical or even more, to be developed as a medicine for the treatment of diabetes complications and other AGE-induced diseases.

1.3 Objectives of the Study

Considering the lack of information on effect of pomegranate stem bark extract on AGE formation, the study was designed to follow below specific objectives

i. To determine the most suitable extraction system for pomegranate stem bark with respect to yield of extract, total polyphenolic compounds, total flavonoid content and antioxidant activities.
ii. To develop quality control and standardization of pomegranate stem bark extract.
iii. To examine antiglycation capabilities of pomegranate (*Punica granatum*) stem bark extract on bovine serum albumin (BSA) using glucose and methylglyoxal (MGO) as glycating agents in vitro.
iv. To evaluate the possible anti-obesity activity through adipogenesis and glucose consumption activities of 3T3-L1 cells in hyperglycemic condition in vitro.

1.4 Scope of the Study

The study was conducted to evaluate antiglycation activity of pomegranate stem bark. Therefore some experiments were performed to achieve the listed objectives in previous section. In the first step the plant material was subjected to quality and safety assessment and phytochemical screening. In order to introduce an efficient extraction processing method, plant extracts were obtained using three solvents (methanol 80%, ethanol 80% and pure water) two extraction times (4 and 8 hours) and two extraction methods (soxhlet and maceration). In the next step, extracts were analysed for determination of total polyphenolic compounds, total flavonoids content and volatile compounds and FTIR spectrum. Then antioxidant property of extracts was evaluated using DPPH radical scavenging capacity, trolox equivalent antioxidant capacity test and metal chelating activity. From comparison of the different obtained extracts, the most effective extract in term of antioxidant

assays, polyphenols and flavonoid contents was chosen for further experiments. In vitro antiglycation properties of the selected extract was evaluated by measurement of early, intermediate and advanced glycation products in BSA-glucose and BSA-MGO systems. Measurement of protein carbonyl content, thiol loss and fructosamine content along with ELISA kit for AGEs were used to determine glycation level in BSA-glucose and BSA-MGO systems. Finally, effect of extract on glucose consumption was studied by determination of lipid formation and glucose consumption in 3T3L-1 preadipocytes cells exposing to MGO and different concentrations of extract.

1.5 Significant of the Study

This study will be helpful for some different groups and scientific disciplines as follow

i. Food industry to use the pruning residues (stem bark) for preparing extract and use it as a food supplement.
ii. Antiglycation activity of the stem bark extract may be important for amelioration of diabetes complications. For this reason, present study has a significance to introduce a novel candidate to pharmacologist to consider it for further researches in area of AGEs control.
iii. The present work can be considered as an evidence and base for future in vivo and then clinical experiments on antidiabetic effects of natural occurring compounds such as pomegranate stem bark extract.
iv. This study could causes farmers sell pruning residues instead of burning it and in this way earn some profits**.**