

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

SHAHPOUR KHANGHOLI

A thesis submitted in fulfilment of the
requirements for the award of the degree of
Doctor of Philosophy (Bioprocess Engineering)

Faculty of Chemical and Energy Engineeringg
Universiti Teknologi Malaysia

OCTOBER 2017

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.

ACKNOWLEDGEMENTS

In first and foremost, I wish to express my most sincere appreciation to my supervisor, Prof. Dr Fadzilah Adibah Abdul Majid, for the opportunity of being part of her research laboratory member and also for the guidance, challenges and support that she provided during my research which allowed me to further my knowledge, experience and career. I was taught to carry out the research in a scientifically logical way, and be enthusiastic and optimistic in the face of any challenges. I truly appreciate my co-supervisors Prof. Ramlan Aziz and Assoc. Prof. Farediah Ahmad for their advice on my research during my whole Ph.D. study. I also thank the Tissue Culture Engineering Research Group members for their friendship, support and guidance throughout my study especially Hassan Fahmi Bin Ismail, Patrick Ong Yit Han, Teh Liam Chee, Norsamsiah binti Muhamad, Mohsen Marvi Beigi, Hala A. K. Rasheed. I also thank Dr. Hamid Kashefi for his advice and counsel on statistical analysis. I also would like to thank Institute of Bioproduct Development and applied physiology laboratory for providing materials and equipment for performing some part of the study. I would also like to thank the Universiti Teknologi Malaysia (UTM) for its favorable academic atmosphere which makes me enjoy my research. I also would like to acknowledge Shahed University for financial supporting me to pursue a graduate degree. Last but not least, my deepest gratitude is reserved for my dearest family. This journey would not have been possible without the support of my beloved wife. I think they would be delighted to see the completion of this thesis. To them, my appreciation never ends.

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^{•+} 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 μ g /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-etilbenziazolin-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^{•+} bersamaan dengan 2.636 mM aktiviti kapasiti antioksidan bersamaan trolox (TEAC)/100 g DW dan aktiviti pengkelatan logam (EC_{50} 888.1 ± 48.38). Pempiwaaian 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 μ g /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.

TABLE OF CONTENTS

CHAPTER	TITLE	PAGE
	DECLARATION	ii
	DEDICATION	iii
	ACKNOWLEDGMENTS	iv
	ABSTRAK	v
	ABSTRACT	vi
	TABLE OF CONTENTS	vii
	LIST OF TABLES	xiv
	LIST OF FIGURES	xvi
	LIST OF ABBREVIATIONS	xxi
 1	 INTRODUCTION	 1
	1.1 Research Background	1
	1.2 Problem Statement	4
	1.3 Objectives of the Study	6
	1.4 Scope of the Study	6
	1.5 Significant of the Study	7
 2	 LITERATURE REVIEW	 8
	2.1 Introduction	8
	2.2 Maillard Reaction and AGEs	8
	2.3 Formation of Advanced Glycation End Products	10
	2.3.1 Fluorescent Crosslinking AGEs	14
	2.3.2 Non-Fluorescent Crosslinking AGEs	15
	2.3.3 Non-Fluorescent Non-Crosslinking AGEs	15
	2.4 AGEs and Oxidative Stress	16

2.5	Role of AGEs in Diabetes Complications	17
2.5.1	Atherosclerosis	18
2.5.2	Diabetic Retinopathy	18
2.5.3	Diabetic Nephropathy	19
2.5.4	Diabetic Neuropathy	20
2.5.5	Wound Healing in Diabetes	20
2.6	Intervention of AGEs in Pathogenesis System	21
2.7	Anti-Glycation Strategies	23
2.7.1	Synthetic Inhibitors of Glycation	24
2.7.2	Natural Inhibitors of Glycation	25
2.8	Pomegranates (<i>Punica granatum</i> L.)	26
2.8.1	Taxonomy and Distribution	27
2.8.2	Phenolic Compounds of Pomegranate	29
2.8.2.1	Flavonoids	33
2.8.2.2	Tannins	36
2.9	Therapeutic Properties of Pomegranate	37
2.10	Therapeutic Effects of Stem Bark	41
2.11	Antiglycation Potential of Pomegranate	42
3	MATERIALS AND METHODS	45
3.1	Introduction	45
3.2	Chemicals and Reagents	45
3.3	Plant Material	46
3.4	Quality Assessment	47
3.4.1	Macroscopic Assessment	47
3.4.2	Preliminary Phytochemical Screening	47
3.4.2.1	Carbohydrates	47
3.4.2.2	Protein Detection	48
3.4.2.3	Tannin Detection	48
3.4.2.4	Alkaloids Detection	48
3.4.2.5	Ash Value	49
3.4.3	Fourier Transform Infrared Spectrophotometer (FTIR)	49

3.5	Safety Assessment	49
3.5.1	Heavy Metal Test	49
3.5.2	Microbiological Tests	50
3.5.2.1	Enumeration of Yeasts and Moulds	50
3.5.2.2	Escherichia coli Test	51
3.6	Extraction of Pomegranate Stem Bark	51
3.6.1	Extraction Experimental Design	52
3.6.2	Extraction by Soxhlet Method	53
3.6.3	Extraction by Conventional Maceration	55
3.7	Chemical Analysis	55
3.7.1	Determination of Total Phenolic Content	56
3.7.2	Determination of Total Flavonoid Content	56
3.7.3	GC-MS Analysis of SM8	57
3.7.4	HPLC–PDA Analysis of SM8	58
3.8	Determination of Antioxidant Activity of the Crude Extracts	58
3.8.1	DPPH Radical Scavenging Assay	59
3.8.2	Metal Chelating Activity Assay	60
3.8.3	Trolox Equivalent Antioxidant Capacity (TEAC)	61
3.9	Modification of Protein	62
3.9.1	Modification of Bovine Serum Albumin by Glucose	62
3.9.2	Modification of Bovine Serum Albumin by Methylglyoxal	64
3.9.3	Protein Oxidation	66
3.9.3.1	Protein Carbonyl Determination	66
3.9.3.2	Thiol Group Oxidation	67
3.10	Evaluation of Antiglycation Activity	69
3.10.1	Determination of Early Stage Glycation Product	69
3.10.2	Evaluation of Formation of AGEs in BSA- MGO System Using ELISA	70

3.10.3	Evaluation of Formation of AGE in BSA-Glucose System Using ELISA	71
3.11	Evaluation of Protein Glycation Using (SDS-PAGE)	72
3.12	Lipid Accumulation and Glucose Consumption in 3T3-L1 under Hyperglycemic Condition	73
3.12.1	Generation of 3T3-L1 Growth Curve	73
3.12.2	In Vitro Cytotoxicity Evaluation and MTT Viability Assay	73
3.12.3	Evaluation of Lipid Accumulation In 3T3-L1 Cells	74
3.12.3.1	Cell Culture and Differentiation	75
3.12.3.2	Oil-red O Staining and Quantification	76
3.12.4	Effects of SM8 on Glucose Consumption in 3T3L-1 in Hyperglycemic Condition	76
3.12.4.1	Cell Culture	76
3.12.4.2	Procedure of Glucose Determination	77
4	RESULTS AND DISCUSSION	78
4.1	Introduction	78
4.2	Quality Assessment	78
4.3	Fourier Transform Infrared Spectrophotometer (FTIR) Analysis	79
4.4	Safety Assessment	82
4.5	The Extraction Yield and Chemical Analysis of Pomegranate Stem Bark Extract	83
4.5.1	The Effects of Methods, Times and Solvents on Extraction Yield	83
4.5.2	The Effects of Methods, Times and Solvents on Total Polyphenol Content	85

4.5.3	The Effects of Methods, Times and Solvents on Total Flavonoids Content	88
4.5.4	Gas Chromatography-Mass Spectrometry Analysis of Pomegranate Stem Bark	90
4.5.5	The HPLC Standardization of SM8 Extract	95
4.6	Antioxidant Analysis of Pomegranate Stem Bark Extract	99
4.6.1	The Effects of Methods, Times and Solvents on DPPH Radical Scavenging Capacity Assay	99
4.6.2	The Effects of Methods, Times and Solvents on Trolox Equivalent Antioxidant Capacity (TEAC) Assay	105
4.6.3	The Effects of Methods, Times and Solvents on Metal Chelating Activity	107
4.7	Evaluation of Antiglycation Activity of Pomegranate Stem Bark Extract	113
4.7.1	The Effect of Pomegranate Stem Bark Extract on Early Stage Glycation Product (Amadori Products) Formation in BSA-Glucose System	114
4.7.2	Inhibitory Effect of Pomegranate Stem Bark Extract on Intermediate Glycation Products (Methylglyoxal Adducts) Formation in BSA-MGO System	120
4.7.3	Inhibitory Effect of Pomegranate Stem Bark Extract on End Stage Glycation Product in BSA-Glucose System	125
4.8	Effects of Pomegranate Stem Bark Extract on Protein Carbonyl and Thiol Group Contents of BSA Modified by Glucose or Methylglyoxal	131
4.8.1	The Effect of Pomegranate Stem Bark Extract on Protein Oxidation (Protein	

	Carbonyl) in Modified BSA with Glucose	132
4.8.2	The Effect of Pomegranate Stem Bark Extract on Protein Oxidation (Protein Carbonyl) in Modified BSA with Methylglyoxal	139
4.8.3	The Effect of Pomegranate Stem Bark Extract on Preventing Thiol Group Loss in BSA-Glucose System	148
4.8.4	The Effect of Pomegranate Stem Bark Extract on Thiol Group Loss in BSA-MGO	154
4.9	Effects of Pomegranate Stem Bark Extract on Lipid Accumulation and Glucose Consumption in Hyperglycemic Condition	163
4.9.1	Generation of 3T3-L1 Growth Curve	164
4.9.2	Cytotoxicity Evaluation of Test Samples (SM8, Catechin and Gallic Acid)	165
4.9.3	Effects of Pomegranate Stem Bark Extract on Lipid Accumulation in 3T3-L1 Cell	168
4.9.4	Effects of Pomegranate Stem Bark Extract on Glucose Consumption by 3T3L-1 in Hyperglycemic Model	172
5	CONCLUSIONS AND RECOMMENDATIONS	179
5.1	Conclusions	179
5.2	Recommendations	181
	REFERENCES	183
	Appendices A - D	222-223

LIST OF TABLES

TABLE NO.	TITLE	PAGE
2.1	Exogenous sources of AGEs in some common foods and fruits (Hsu and Zimmer, 2010).	23
2.2	Botanical classification of pomegranate.	27
2.3	Phytochemicals in pomegranate (Seeram et al., 2006).	30
3.1	Various combinations of modification of BSA with glucose.	63
3.2	Various combinations of BSA modification with MGO.	65
4.1	Physic-chemical characteristic of pomegranate stem bark.	79
4.2	Qualitative phytochemical screening.	79
4.3	FTIR peak values and functional groups of methanolic extract of Punica granatum L.	81
4.4	Safety assessment according to the presence of microorganisms and heavy metals in pomegranate stem bark.	82
4.5	Extraction yield of pomegranate stem bark under different extraction conditions. Data are presented as percent of three replicate determinations (n=3) (p<0.05).	83
4.6	Distribution of total polyphenolic compounds in pomegranate stem bark extracts obtained by various methods, temperatures and solvents.	86
4.7	Effects of methods, times and solvents on total flavonoid content expressed as catechin.	88
4.8	Phytochemicals identified in the methanolic extracts of the Punica granatum stem bark by GC-MS. P: peak number, RT: retention time, MW: molecular weight.	92

4.9	Effects of methods, times and solvents on DPPH scavenging capacity expressed as IC ₅₀ mg/L.	100
4.10	Correlations and significances between total phenolic compounds, flavonoid contents and DPPH radical scavenging activity for pomegranate stem bark extract prepared using different methods, times and solvents.	104
4.11	TEAC value of extracts obtained under different extraction conditions.	106
4.12	Effects of methods, times and solvents on metal chelating activities of PSBE expressed as IC ₅₀ .	108
4.13	The inhibitory effect of SM8, gallic acid and catechin on the level of fructosamine formation.	118
4.14	The inhibitory effect of catechin (CAT), gallic acid (GAL), pomegranate stem bark extract (SM8) and aminoguanidine (AG) (positive control) on the formation of fructosamine in the hyperglycemic condition.	119
4.15	The effects of SM8, catechin and gallic acid on the level of protein carbonyl in BSA/glucose system in healthy and hyperglycemic conditions.	133
4.16	The effects of SM8, catechin and gallic acid on the level of PCC in BSA-MGO system in three measure points.	146
4.17	The effects of catechin, SM8 and gallic acid on the level of thiol group in BSA-glucose system with normal and hyperglycemic conditions in day 21.	150
4.18	The effects of catechin, SM8 and gallic acid on the level of thiol group in BSA incubated with MGO in concentrations of 100 and 150 mM for 21 days.	155
4.19	Pearson's correlation coefficients of PCC and thiol group level in day 21. BSA was incubated with glucose (5.5 and 250 mM), or methylglyoxal (100 or 150 mM) associated with SM8, catechin or gallic acid in different concentrations.	159
4.20	Summary of cytotoxicity assessment data of catechin, gallic acid and SM8.	167
4.21	Effect of catechin, gallic acid and SM8 on glucose consumption of 3T3-L1 cells grown in media containing 78μM of methylglyoxal during 7 days. Control had no sample test or MGO.	175

LIST OF FIGURES

FIGURE NO.	TITLE	PAGE
2.1	Classical model of Protein glycation pathway leading to AGEs formation.	10
2.2	Formation of some reactive dicarbonyls in Maillard reaction.	12
2.3	Chemical structures of three types of AGEs.	14
2.4	Simplified mechanisms of pathogenesis of AGEs in hyperglycemia condition.	22
2.5	Pomegranate tree (A) and fruit (B).	28
2.6	Molecular structure of some flavonoids (Vermerris and Nicholson, 2008, Urackova, 2008).	34
2.7	The basic structure of flavonoids. The dotted circles show the effective key positions on antioxidant activity of flavonoids (Khangholi et al., 2016).	35
2.8	Classification of tannins with examples. (Khanbabae and Ree, 2001).	37
2.9	Therapeutic effect of pomegranate has been investigated in several aspects (Seeram et al., 2006).	38
3.1	Experimental design to study the effects of extraction methods, times and solvents on pomegranate stem bark extract yield and biological activities.	52
3.2	Soxhlet unit. A, Cooling bath. With adjustable temperature. B, Rotary evaporator and C, soxhlet apparatus.	54
3.3	The DPPH molecular structure in forms of free radical (left) and nonradical (right).	59

3.4	Detection of thiol groups via Ellman's reagent. DTNB reacts with a free sulfhydryl group to yield a mixed disulfide and a TNB ²⁻ (Thermo Fisher Scientific, 2011).	69
4.1	FTIR spectrum of methanolic extract of pomegranate stem bark (SM8).	80
4.2	GC-MS chromatogram of methanolic extract of pomegranate stem bark.	91
4.3	HPLC-PAD analysis of the SM8 extract of pomegranate stem bark. Presence of catechin at retention time of 6.598 min. monitored at 210 nm compared to standard.	96
4.4	Representative HPLC-PAD chromatogram of SM8 extract of pomegranate stem bark displaying the existence of gallic acid detected at 272 nm.	98
4.5	DPPH radical scavenging activity of extracts obtained by different solvents under different extraction times by method of maceration (Panel A) or soxhlet (Panel B).	102
4.6	The effects of different extraction condition on metal chelating activity of pomegranate stem bark extracts.	110
4.7	The effects of samples on reduction of absorbance of NBT in the reaction mixtures containing BSA+Glu _{5.5} mM during the period of the experiment with lengthen the duration of incubation.	115
4.8	The effects of samples on reduction of absorbance of NBT in the reaction mixtures containing BSA+Glu ₂₅₀ mM during the period of the experiment with lengthen the duration of incubation.	116
4.9	The inhibitory effects of catechin, SM8 and gallic acid on the MGO-hydroimidazolone formation compared to the BSA+MGO ₁₀₀ mM (control).	121
4.10	SDS-PAGE Coomassie stained gels profile of BSA incubated with methylglyoxal ₁₀₀ mM wit/without samples.	122

4.11	The inhibitory effects of catechin, SM8 and gallic acid on the MGO-hydroimidazolone formation compared with the BSA+MGO ₁₅₀ (control).	123
4.12	SDS-PAGE Coomassie stained gels profile of BSA incubated with MGO ₁₅₀ mM with/without samples.	124
4.13	The inhibitory effects of catechin, SM8 and gallic acid on CML formation compared to the BSA+Glu _{5.5} (control).	125
4.14	SDS-PAGE Coomassie stained gels profile of BSA incubated with glucose 5.5 mM with/without samples for 30 days.	126
4.15	The effects of catechin, SM8 and gallic acid on the CML formation compared to BSA+Glu ₂₅₀ (control).	127
4.16	SDS-PAGE Coomassie stained gels profile of BSA incubated with glucose 250 Mm with/without samples for 30 days.	128
4.17	The effects of different samples on protein carbonyl formation in BSA-Glu _{5.5} model.	134
4.18	The one-way ANOVA of effects of samples on PCC formation in BSA-Glu _{5.5} model on day21. Results are expressed as mean \pm SEM (n = 3). Same letter on the bars means no significant difference.	135
4.19	The effects of different samples on reduction of PCC in BSA-Glu ₂₅₀ model. Results are expressed as mean \pm SEM (n = 3). ** P < 0.05 when compared to BSA, * P < 0.05 when compared to BSA-Glu ₂₅₀ .	136
4.20	The one-way ANOVA to compare the effects of different samples on protein carbonyl formation in BSA-Glu _{5.5} model on day 21.	137
4.21	Kinetic of protein carbonyl formation in a period of 21 days with three 7 days interval of measurement.	138
4.22	The effects of different samples on protein carbonyl formation in BSA-MGO ₁₀₀ system in a period of 21 days.	140
4.23	One-way analysis of variance of effect of different concentrations of samples on carbonyl content in BSA-MGO ₁₀₀ mM after a period of 21 days.	141

4.24	The effects of different concentrations of samples on protein carbonyl formation in BSA-MGO ₁₅₀ system on day 21.	142
4.25	One-way analysis of variance of effects of different concentration of samples on carbonyl content in BSA-MGO ₁₅₀ mM on day 21.	143
4.26	Kinetic of protein oxidation in a period of 21 days. The protein carbonyl level in BSA-MGO ₁₅₀ is higher than BSA-MGO ₁₀₀ .	144
4.27	The effects of different concentrations of samples on inhibition of protein thiol oxidation in BSA-Glu5.5 model.	149
4.28	One-way analysis of variance of effects of different concentration of samples on inhibition of thiol oxidation in BSA-Glu5.5 on day21.	151
4.29	The effects of different concentration of samples on inhibition of protein thiol oxidation in BSA-Glu250 model in a period of 21 days.	152
4.30	One-way analysis of variance of effects of different concentration of samples on inhibition of thiol oxidation in BSA-Glu250 on day21.	153
4.31	The effects of different concentration of samples on inhibition of thiol oxidation in BSA-MGO ₁₀₀ mM	154
4.32	One-way analysis of variance of effects of different concentrations of samples on inhibition of thiol oxidation in BSA-MGO ₁₀₀ on day 21.	156
4.33	The effects of different concentration of samples on inhibition of protein thiol oxidation in BSA-MGO ₁₅₀ mM on day 21.	157
4.34	One-way analysis of variance of effects of different concentrations of samples on inhibition of thiol oxidation in BSA-MGO ₁₀₀ on day 21	158
4.35	The cumulative effect of SM8 and catechin and gallic acid on thiol group and carbonyl content in BAS-Glucose (Panel A) and BSA-methylglyoxal systems (Panel B).	160

- 4.36 Growth curve of 3T3-L1 cell preadipocytes. Cell counting was continued for 8 days. Results are means \pm Std of two independent experiments. 164
- 4.37 Effects of different concentrations of catechin, gallic acid and SM8 on 3T3-L1 cells viability (MTT assay). Control non-treated cells. 165
- 4.38 Effect of different concentrations of pomegranate stem bark extract, gallic acid and catechin on lipid accumulation during differentiation of 3T3-L1 preadipocytes. 169
- 4.39 The MGO trapping effects of samples on glucose consumption ability of 3T3L-1 cells grown in transition media with or without various concentration of MGO or samples. 174

LIST OF ABBREVIATIONS

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

LIST OF APPENDICES

APPENDIX	TITLE	PAGE
A	List of Publications	222
B	Grid-Following Control Methods	222
C	Modeling and Design of P-I Controller	223
D	Dialog Box and Parameter	223

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 Organization, 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 Organization, 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 prolonged 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. Today, 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, galloylglucose, 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.