Anti Cutaneous Aging Effect of Red Djulis (*Chenopodium formosanum)* Extract on Gene Expression of Human Dermal Fibroblast

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Abstract: Red djulis (*Chenopodium formosanum*) is a native cereal plant in Taiwan; it contains abundant polyphenols, betalian and dietary fiber. The appearance of red djulis is bright red. Therefore, it is also called the "ruby of cereals". The antioxidative activity of red djulis extract is well-understood. However, the antiaging function still remains unclear. This study examined the potential of red djulis extract for enhancing collagen secretion and preventing cutaneous aging using red djulis extracts. The red djulis extracts are comprised of an abundant active component that can effectively enhance the ability of collagen secretion of dermal fibroblasts, prevent the glycation of collagen and resist the damage of ultraviolet light exposure. After fibroblast treatment with red djulis extracts, *TGM1*, *KRT1*, *KRT10* and *SOD2* genes were up-regulated significantly by 2.3, 4.3, 4.4 and 27.3 times, respectively, compared to those of the control group. Additionally, it can increase *COL1A2* gene expression by 43% and decrease *MMP9* gene expression 33%. Therefore, it was demonstrated that red djulis extracts affect gene expressions related to the skin barrier, antioxidation and collagen. Moreover, we found positive effects on skin barrier integrity, endogenous antioxidant activity and skin collagen-preservation. The preparation of the *red djulis extracts* is environmental friendly and can promote the economic value of *Chenopodium formosanum*; thus, the proposed extract is suitable for applications in the development of food products, especially beverages, skin care and cosmetic products.

Keywords: *Chenopodium formosanum*; human dermal fibroblast; UV exposure; antioxidant activity; anti-aging; red djulis

1. Introduction

Collagen, one of the primary extracellular matrix (ECM) components of the dermis, is the most abundant protein in mammals, comprising about 30% of total protein[1-4]. Collagens co-polymerize to form extended mechanically stiff fibrils which confer tensile strength to the tissue providing the elasticity of skin[5]. However, the overall collagen content per unit area of the skin surface is known to diminish approximately 1% per year leading to gradual loss of skin elasticity (sagging) with age (intrinsic aging)[6, 7]. Additionally, chronic sun exposure leads to marked degradation of skin collagen structure resulting in wrinkle formation in photo aged skin (extrinsic aging)[8]. Therefore, in addition to sun exposure prevention, novel and effective anti-aging ingredients for skin care have drawn a lot of attention[9, 10].

Red djulis is a native cereal plant in Taiwan. The whole plant is colorful and traditionally called the "ruby of cereals" for its bright red grain color (Figure 1). Besides the high content of dietary fiber and starch, red djulis also possesses high protein levels and abundant essential amino acids, which make it a nutritious food. Djulis is particularly rich in lysine, which increases calcium absorption to help build collagen, and acts as a building block for the fibrils and fibers of collagen[5, 8, 11]. What is more, the batalain pigment in djulis is considered to possess not only good coloring potential but also positive physiological benefits for human health, such as melanoma cell growth inhibition and

46 boosting of antioxidant activity[11-14], through antioxidant molecules such as betacyanin,
47 mesembryanthin, and other polyphenols and flavonoids[11, 12]. Chyau et al., revealed the role of the
48 ethanolic extracts of red djulis *(Chenopodium formosanum)* and its bioactive compounds in preventing
49 adipogenesis in 3T3-L1 adipocytes[15]. Specifically, treatment with red djulis extracts under UVB
50 light exposure can induce an accumulation of betacyanins and flavonoids in the immortal
51 keratinocyte cell line (HaCat) for protection against UV-induced damage[14]. In addition, red djulis
52 extracts have been reported to have antioxidant and moisturizing effects, and can stimulate the
53 proliferation of fibroblasts and keratinocytes in the course of wound healing and regeneration[14, 16].
54 Therefore, djulis extracts contain an abundance of antioxidants and may act as an active ingredient
55 for skin care product[17, 18]. Therefore, this *in vitro* study using genetic analysis aims to determine
56 the anti- Cutaneous aging effect of red djulis extract and exploring the feasibility of application in the
57 biomedical field.

2. Materials and Methods

58

2.1. Preparation and antioxidant analysis of red djulis extract

59

60 The red djulis used in the present study (*Chenopodium formosanum*) was purchased from
61 Pingtung County, Taiwan. The whole grain red djulis (unhulled) stored at 4 °C prior to use. To
62 achieve optimal extraction conditions, before carrying out the extraction step of the present study,
63 2.5g of grounded red djulis was combined was 25mL of water to form a mixture, and extracted with
64 water at different temperatures for 30 minutes. The ratio of grounded red djulis to water was 1:10 (w
65 / v), and the temperatures used for extraction were 25°C, 50 °C, and 70 °C, respectively. The mixture
66 was then centrifuged at 4600 X g for 20 minutes to separate solid substances. The supernatant was
67 collected after centrifugation and final filtration before subjecting the red djulis extract to analysis.
68 Further, sonication at 40 kHz was applied to facilitate red djulis extraction. Finally, the red djulis
69 extract was disinfected and sterilized at 121°C for 15 minutes, stored in a sealed container at 4 °C and
70 kept out of light.
71 The antioxidant analysis of red djulis extract was applied using total phenol content by the Folin-
72 Ciocalteu colorimetric method. Four mL of 2% sodium carbonate water solution and 5mL of Folin-
73 Ciocalteu reagent was added to red tubes with 1 mL djulis extract or standard solution. The standard
74 solution was gallic acid at a concentration of 200 to l000 mg/mL. (7.5% in water) in order to create
75 basic conditions (pH ~10) for the redox reaction between phenolic compounds and the Folin-
76 Ciocalteu reagent. After incubation for 90 min at room temperature, the absorbance was read at 750
77 nm by a microplate reader (BioTek Instruments, Winooski, VT), against the blank. After incubation
78 at 25 ° C for 30 minutes, absorbance was measured as 750 nm by the microplate reader (BioTek
79 Instruments, Winooski, VT), against the blank. Total phenolic content was expressed as the
80 equivalent of gallic acid.
81 The total flavonoid content of crude extract was determined by the aluminium chloride
82 colorimetric method[19]. Fifty μL of red djulis extract was increased to 1 mL with methanol, mixed
83 with 4 mL of distilled water and then 0.3 mL of 5% $NaNO_2$ solution; 0.3 mL of 10% $AlCl_3$ solution
84 was added after 5 min of incubation, and the mixture was reacted for 6 min. Two mL of 1 mol/L
85 NaOH solution were added, and the final volume of the mixture was brought to 10 mL with double-
86 distilled water. The mixture was reacted for 15 min; absorbance was measured at 500 nm. The total
87 flavonoid content was calculated from a calibration curve, and the result was expressed as mg rutin
88 equivalent per g dry weight.

2.2. Cell lines and chemicals

89

90 The human skin fibroblast cell line CCD-966SK (CRL-1881) was derived from the American Type
91 Culture Collection (Manassas, VA, USA). All cell culture media and reagents including MTT [3-(4,5-
92 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] were purchased from Sigma-Aldrich (St.
93 Louis, MO, USA) or Gibco (Thermo Fisher Scientific, Inc., Carlsbad, CA, USA) and were of reagent

94 grade or cell-culture grade. Cells were cultured in MEM with 10% fetal bovine serum, and incubated
95 at 37 °C in a humidified atmosphere of 5% CO2.

96 *2.3. Cell viability*

97 Briefly, cells were seeded into 96-well plates at a density of 5×10^3 cells/well. After 24 h of
98 culturing, the medium was removed and replaced with a fresh medium containing various
99 concentrations (0.25, 0.5, 1 and 2 mg/mL) of red djulis extract. Cell viability was assessed through
100 MTT assay. Briefly, 15 μl of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)
101 (Sigma; 4 mg/ml) was added and the cells were incubated for an additional 4 hours. The medium was
102 removed and 50 μl /well of DMSO was added to resolve formazan crystal. The plate was placed on a
103 shaker and incubated for 10 min and the absorbance was measured at 570 nm. Cell viability in
104 response to treatment was calculated as: Cell viability (%) = (OD $_{sample}$/ OD $_{control}$) × 100%.

105 *2.4. Quantification of gene expressions by real-time PCR*

106 The treated CCD-966SK fibroblasts or human primary epidermal keratinocytes were harvested,
107 and total RNA was isolated from cells using an RNA purification kit (Geneaid, Taiwan). DNA-free
108 total RNA was reversely transcribed to cDNA using a SuperScript™ Reverse Transcriptase kit
109 (Invitrogen, Life Technologies Co., CA, USA). Quantitative real-time PCR was conducted using an
110 ABI StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, Inc., CA, USA) and the SYBR
111 Green Master Mix (KAPA Biosystems, MA, USA) for was used transcript measurements. The reaction
112 mixture was cycled as follows: One cycle at 95 °C for 20 s, followed by 40 cycles of 95 °C (1 s), 60 °C
113 (20 s), and plate reading was conducted after each cycle. The melting curves of the PCR products
114 were analyzed during the quantitative real-time PCR. The gene-specific primers used in this study
115 are listed in Table 1. Real-time PCR reactions were performed using the ABI StepOnePlusTM system
116 with KAPA SYBR FAST ABI Prism (KAPA). Antioxidant-related genes (SOD2 and CAT) and
117 collagen-related genes (COL1A1 and TIMP1) were detected (Table.1). GAPDH was used as the
118 reference gene to normalize relative expression. The GAPDH gene was used as a normalization
119 control. Data were analyzed using the ABI StepOne™ Software v2.2.3 (Thermo Fisher Scientific, Inc.,
120 Carlsbad, CA, USA). All PCR assays were performed in duplicate three times.

121 *2.5. Wound healing assay*

122 In vitro wound scratch assay was performed as described in Yarrow et al.[20] with
123 modifications. Briefly, CCD-966SK cells were grown to 80% confluence in 24-well plates. The
124 scratching was performed by scraping with a sterile 1-ml pipette tip across the center of the well,
125 followed by incubation with media only (mock) or with 2 mg/ml of SGE in culture media for 17 h.
126 The wound closures were subsequently photographed under a microscope (Eclipse Ti-U, Nikon
127 Corporation, Tokyo, Japan) using a CCD digital camera. Cell migration was analyzed by Image J
128 (image processing and analysis in Java: https://imagej.nih.gov/ij/download.html) and expressed as
129 percentage of wound coverage by cells moving into the scratched wound area. Cell migration data
130 were expressed as the percentage of wound coverage.

131 *2.6. Antiglycosylation analysis*

132 AGE formation was determined by using collagen and fructose as glycation reactants. The
133 modification procedure was described as in previous study by Peter-Katalinić (2005)[21]. Collagen
134 solution (60 mg/mL) and fructose (1.5 M) were prepared in 0.2 M potassium phosphate buffer (pH
135 7.4, containing 0.06% sodium azide). For each reaction of glycation, 250 μL of collagen solution, 250
136 μL of fructose solution, and 250 μL of deionized water diluted sample or control (3mM
137 aminoguanidine) were mixed. 100 μL of the above mixture was withdrawn immediately to a well of
138 a 96-well plate and subjected to intensity determination by a spectrofluorometer (Paradigm
139 microplate reader, Beckman Coulter, Fullerton, CA, USA) set at 360 nm of excitation and 460 nm of
140 emission. The data were defined as Fluorescence sample 0hr or Fluorescence control 0hr. 450 μL of

141 the remaining mixtures were taken into a 1.5 mL microfuge tube and incubated at 50⁰C for 24hr. After
142 incubation, the reacted solutions were measured with a spectrofluorometer again and the result was
143 defined as Fluorescence sample 24hr or Fluorescence control 24hr. The antiglycosylation activity rate
144 of the sample was calculated as follows:

145 Antiglycosylation activity (%) =

$$\left[1- \frac{\text{Fluorescence sample 24hr- Fluorescence sample 0hr}}{\text{Fluorescence control 24hr- Fluorescence control 0hr}} \right] \times 100\%$$

146 *2.7. Collagen secretion assay*

147 The soluble collagen was determined by the method described by Aramwit et al. (2009). Human
148 skin fibroblast cells (CCD-966SK) were seeded at an initial concentration of $4 \times 10_4$ cells per well of
149 24-well plate in MEM containing 10% FBS. After 24 h, the culture medium was replaced by fresh
150 medium. The sample extracts were diluted with various concentrations with phosphate buffer saline
151 (PBS) and added into each well. Cells without a sample extract served as negative controls. After
152 incubation for 48h at 37°C with 5% CO2, the supernatants were collected. The total amount of soluble
153 collagen type I was assayed using the Sircol® Collagen Assay Kit (Bicolor Life Science Assays,
154 Northern Ireland, UK). Briefly, 100 µL of experimental supernatant was mixed with 1 mL of dye
155 solution at room temperature for 30 min. Then the samples were centrifuged at 15,000 × g for 10 min
156 to form a pellet of collagen. The supernatant was removed and the soluble collagen produced was
157 dissolved in 1 mL of alkali reagent. The resultant alkali reagent solutions were assayed by a
158 spectrophotometer at a wavelength of 540 nm. The amount of collagen was calculated based on a
159 standard curve of soluble collagen (bovine skin collagen type I standard from American disease free
160 animals).

161 *2.8. Collagen immunofluorescence staining*

162 For immunohistochemistry, antigen retrieval was performed by heating sections of 10 mM
163 citrate buffer (pH 6.0) in a microwave oven. Reactions with endogenous peroxidases and proteins
164 were blocked by incubation with 3%H_2O_2 diluted in methanol and 10% normal goat or rabbit serum,
165 depending on the host animal for the secondary antibody. Then incubation with primary antibodies
166 was done overnight at 4 °C. The primary antibodies used were as follows: rabbit anti-collagen I (1:50;
167 Abcam, Cambridge, UK), rabbit anti-collagen III (1:100; Abcam), mouse anti-collagen IV (1:50; Dako,
168 Glostrup, Denmark), rabbit anti-collagen V (1:50; Abcam), and rabbit anti-collagen VI (1:50; Abcam).
169 The EnVision labeled polymer-HRP system (Dako) was used as the secondary antibody and
170 peroxidase activity was visualized with a liquid diaminobenzidine substrate (Dako). Nuclei were
171 counter-stained with hematoxylin. Collagen staining of the nodular lesions was classified into three
172 categories according intensity compared with background staining (strongly positive, weakly
173 positive, and negative).

174 *2.9. Trosinase activity inhibition assay*

175 Trosinase activity inhibition assay was conducted following Chan et al (2011)[22]. Briefly, 100 µl
176 of freshly prepared L-DOPA solution, at different concentrations (2.5, 5.0 and 10.0 mM), was added
177 for dopachrome formation. The relationship between total protein and concentration of L-DOPA for
178 dopachrome formation was observed. The reaction mixture (200 µl/well) consisting of cell-extracted
179 protein and L-DPA in 0.1 M sodium phosphate buffer (pH 6.8) was added into wells of a 96-well plate
180 in triplicate. The plate was incubated at 37°C and absorbance was measured at 475 nm for a time
181 course of up to 4 hours.

182 *2.10. Statistical analysis*

183 The significance of treatment was evaluated using paired t-test with the software Statistical
184 Product and Service Solutions (SPSS® 18.0; SPSS Inc., Chicago, IL). A difference was considered
185 statistically significant when the p value was < 0.05. All results were expressed as mean ± standard
186 deviation (SD).

187 **3. Result and Discussion**

188 *3.1. Optimal extraction condition of red djulis extracts*

189 When evaluating the optimal conditions for total flavones and total phenol content from red
190 djulis, the amount of total flavones and total phenol may be affected by the type of solvent, pH level,
191 solvent-water ratio and extraction time[23]. To avoid the Islamic legal limitations and cost
192 consideration, authors decided to use water extraction. The antioxidant compounds of different
193 extraction conditions are shown in Table 2. The amount of total flavones and total phenol of the
194 unhulled red djulis groups were higher than those of hulled red djulis and the difference reached
195 almost three to four fold. Furthermore, the maximum amount of total flavones and total phenol
196 extract from red djulis was obtained from unhulled red djulis at 50°C for 30 minutes. A previous
197 study revealed that the djulis hull contains numerous antioxidant compounds and could be used to
198 develop functional foods[16, 24]. Therefore, we decided to obtain extracts from unhulled red djulis
199 at 50°C for 30 minutes for further experiments.

200 *3.2. In Vitro Efficacy Evaluation*

201 3.2.1. Cytotoxicity Assessment

202 The effect of red djulis extracts on cell viability of human dermal fibroblasts (CCD-966SK) was
203 analyzed. The results showed that cell viabilities of red djulis extracts with concentrations of 0.1875
204 to 3 mg/mL were not significantly different with those of the control group (100%) (Figure 2). The
205 results indicated that red djulis extracts are not cytotoxic.

206 3.2.2. Wound Healing Assay

207 The wound healing rate of red djulis extracts was 2.3 fold greater than that of the control group.
208 After 24 h exposure to the test sample, red djulis extracts were enhanced so that the cell migrates
209 towards the provisional gap (Figure. 3). According to comprehensive cytotoxicity results, the red
210 djulis extracts had low cytotoxicity and enhanced cell migration. These results seem to be correlated
211 with of antioxidant compound of red djulis extracts.
212 Every living cell that undergoes aerobic metabolism produces reactive oxygen species (ROS) as
213 its byproduct. The ROS is removed by antioxidative compounds of red djulis extracts, thus
214 maintaining the healing physiological functions of fibroblast. Our findings are consistent with those
215 of previous studies[14, 25, 26].

216 3.2.3. Gene Expressions of CCD-966SK Fibroblasts

217 Skin-barrier-related genes (*TGM1*, *KRT1*, *KRT10*), antioxidant-related genes (*SOD2*) and
218 collagen-related genes (*COL1A1* and *MMP9*) were selected for analysis of the mRNA expression in
219 human dermal fibroblasts (CCD-966SK) by qPCR. The *TGM1* gene provides instructions for
220 producing an enzyme called transglutaminase 1. This enzyme is involved in the formation of the
221 cornified cell envelope, which is a structure that surrounds skin cells and helps to form a protective
222 barrier between the body and the environment. The *KRT1* and *KRT10* gene encode a member of the
223 type I (acidic) and type II (basic or neutral) cytokeratin families, respectively. They are coexpressed
224 during the differentiation of the epithelial tissues. The *SOD2* gene encodes superoxide dismutase 2
225 (SOD2, mitochondrial), which is a member of the iron/manganese SOD family, catalyzing the
226 dismutation of the superoxide radical into hydrogen peroxide or oxygen. The *COL1A2* (collagen type

227 I alpha 2) encodes the pro-alpha2 chains of type I collagen, which is a fibril-forming collagen found
228 in most connective tissues and is abundant in dermis, bone, cornea and tendon. The *MMP9* gene
229 encodes matrix metallopeptidase 9, which is a class of enzymes involved in the degradation of the
230 extracellular matrix (collagen) [11]. The "photo aging" or "photo-aging effect" refers to aging cells with
231 UV light caused by factors including but not limited to UV light activated protein kinase pathway
232 split via silk (MAP Kinase pathway) and increased phosphorylation of the dermal layer of matrix
233 metalloproteinase (matrix metalloproteinases, MMPs) content. The matrix metalloproteinases
234 decomposes collagen, decreasing the collagen content of the skin; will promote the UV light reactive
235 oxygen species (reactive oxygen species, ROS), such as oxygen ions, free radicals and peroxides such
236 as organic and inorganic produce, other cause denaturation of collagen (denature) and loss of
237 function.
238 　　In the present study, after treatment with red djulis extracts, *TGM1*, *KRT1*, *KRT10* and *SOD2*
239 genes were up-regulated significantly 2.3, 4.3, 4.4 and 27.3 times, respectively, compared to those of
240 the control group (Figure 4). Additionally, the extract can increase *COL1A2* gene expression 43% and
241 decrease *MMP9* gene expression 33% (Figure 5). Therefore, it was demonstrated that red djulis
242 extracts affect the gene expressions related to skin barrier, antioxidation and collagen, and shows
243 positive effects on skin barrier integrity, endogenous antioxidant activity and skin collagen-
244 preservation.

245 *3.4. Stimulation of Collagen Secretion*

246 　　After treatment with red djulis extracts, collagen content in human dermal fibroblast obviously
247 increased based on immunofluorescence staining compared with the control (Figure 6 a).
248 Additionally, the collagen content of the red djulis extract group was about 2 times higher than that
249 of the control group (Figure 6 b). These results revealed that red djulis extracts can promote collagen
250 secretion from skin fibroblast cells.
251 　　Analysis of red djulis extract used in this study show that the extract promotes the secretion of
252 collagen, wherein the vertical axis represents the percentage of collagen secretion rates, with the
253 control group shown as 100% for the sake of reference. According to the findings, with 0.2mg/mL of
254 unhulled treated red djulis extract, collagen secretion in human skin fibroblasts reached 138%
255 compared to the untreated control group. Thus, treatment with red djulis extract led to an increase
256 of 38%; and 0. shelling red djulis extract treated 2mg / mL of collagen secretion rates of human skin
257 fibroblasts 201.4% is reached, the control group protein secretion in human skin fibroblasts treated to
258 extract collagen without red djulis rate more than 2 times. Thus, experiments confirmed that
259 unhulled red djulis extract and hulled red djulis extract can effectively promote the collagen secretion
260 rate of cells.

261 *3.5. Anti- Advanced Glycation End-products (AGEs)*

262 　　Red djulis extracts inhibited the formation and accumulation of Amadori Product (primary
263 glycation products), α-dicarbonyl compounds (secondary glycation products) and Advanced
264 Glycation End-products (AGEs), with inhibition of about 58%, 53% and 80%, respectively, compared
265 with the control (Figure 7). The results showed that red djulis extracts decreased the formation of
266 AGEs and the glycation of collagen. The experiment indicated that red djulis extracts have the ability
267 to protect and maintain the function of collagen in the skin. Moreover, according to our analysis, red
268 djulis extracts inhibit the glycation of collagen. The extract reached an optimal effect at a
269 concentration of 8% (w / w), inhibiting collagen glycation by up to 62.2%.

270 *3.6. Red Djulis Extracts Inhibited Melanin Synthesis*

271 　　The result showed that after treatment with different doses of red djulis extracts, the tyrosinase
272 activity was significantly inhibited by 30% and 43% compared with the control group (Figure 8), and
273 the result was dose dependent. Red djulis extracts   also significantly reduced melanin synthesis by
274 about 30% compared to the control group (Figure 8). This study indicated that red djulis extracts had

275   a whitening effect by reducing the activity of tyrosinase, a key enzyme in melanin synthesis, and
276   inhibiting melanin synthesis.

Conclusion

278   In summary, red djulis extracts contain abundant bioactive compounds rich in flavonoids and
279   phenolic 1S. Thus, the extract not only promotes secretion of collagen of dermal fibroblasts, but also
280   effectively inhibits the glycation effect, and may enhance the dermal fibroblasts viability and
281   migration rate. After fibroblasts were treated with red djulis extracts, TGM1, KRT1, KRT10 and SOD2
282   genes were up-regulated significantly by 2.3, 4.3, 4.4 and 27.3 times, respectively, compared to those
283   of the control group. Additionally, the extract can increase the COL1A2 gene expression by 43% and
284   decrease MMP9 gene expression by 33%. Therefore, it was demonstrated that red djulis extracts affect
285   gene expressions related to the skin barrier, antioxidation and collagen. The results show positive
286   effects on skin barrier integrity, endogenous antioxidant activity and skin collagen-preservation.
287   Furthermore, the water soluble red djulis extracts do not harm the environment and could provide
288   flexible formulation for safe anti-aging skin care products, cosmetic raw materials or functional
289   beverages.

Legends

Table 1.	Real-time quantitative PCR primers used in this study.
Table.2	Antioxidative compounds of red djulis extract.
Figure.1	Appearance of red djulis (*Chenopodium formosanum)*
Figure.2	Cytotoxicity test of red djulis extracts at different concentrations.
Figure.3	Wound healing effect of red djulis extracts at different concentrations.
Figure.4	Relative *TGM1*, *KRT1*, *KRT10* and *SOD2* gene expression in fibroblasts with or without red djulis extract treatment (*p<0.5; ***p<0.001).
Figure.5	Relative *COL1A2* and *MMP9* gene expression in fibroblasts with or without red djulis extract treatment (**p<0.01).
Figure.6	Effects of red djulis extracts in fibroblasts with or without red djulis extract treatment: (a) Collagen immunofluorescence staining and (b) collagen secretion.
Figure.7	The effect of red djulis extracts on formation of Advanced Glycation End-products (AGEs).
Figure.8	Effect of red djulis extracts on tyrosinase activity.

291

292   **Table 1.**

Gene Name	Primer Name	Primer Sequence	Gene Function	Reference
TGM1	TGM1-F	5'- GATCGCATCACCCTTGAGTTAC -3'	epidermal barrier structural genes	27
	TGM1-R	5'- GCAGGTTCAGATTCTGCCC -3'		
KRT10	KRT10-F	5'- TCCTACTTGGACAAAGTTCGGG -3'	epidermal barrier structural genes	28
	KRT10-R	5'- CCCCTGATGTGAGTTGCCA -3'		
KRT14	KRT14-F	5'- TTCTGAACGAGATGCGTGAC -3'	epidermal barrier structural genes	29
	KRT14-R	5'- GCAGCTCAATCTCCAGGTTC -3'		

SOD1	SOD1-F	5'- GGTGGGCCAAAGGATGAAGAG -3'	anti-oxidative gene	30
	SOD1-R	5'- CCACAAGCCAAACGACTTCC -3'		
COL1A2	COL1A1-F	ATCAACCGGAGGAATTTCCGT	provides instructions for making part type I collagen	31
	COL1A1-R	CACCAGGACGACCAGGTTTTC		
MMP9	MMP9-F	GGGACGCAGACATCGTCATC	enzymes engaged in the degradation and remodeling of extracellular matrix (ECM)	32
	MMP9-R	TCGTCATCGTCGAAATGGGC		

293

Table 2.

	Extraction Temperature (°C)	Total flavones content (μg/mL)	Total phenolic content (μg/mL)
Unhulled red djulis	25	418±14	326±26
	50	418±4	332±18
	70	421±8	318±20
Hulled red djulis	25	105±7	110±2
	50	116±9	90±18
	70	87±9	73±11

294

295
296

Figure 1.

297

298
<center>**Figure 2.**</center>

299

300
<center>**Figure 3.**</center>

301

302

Figure 4

303

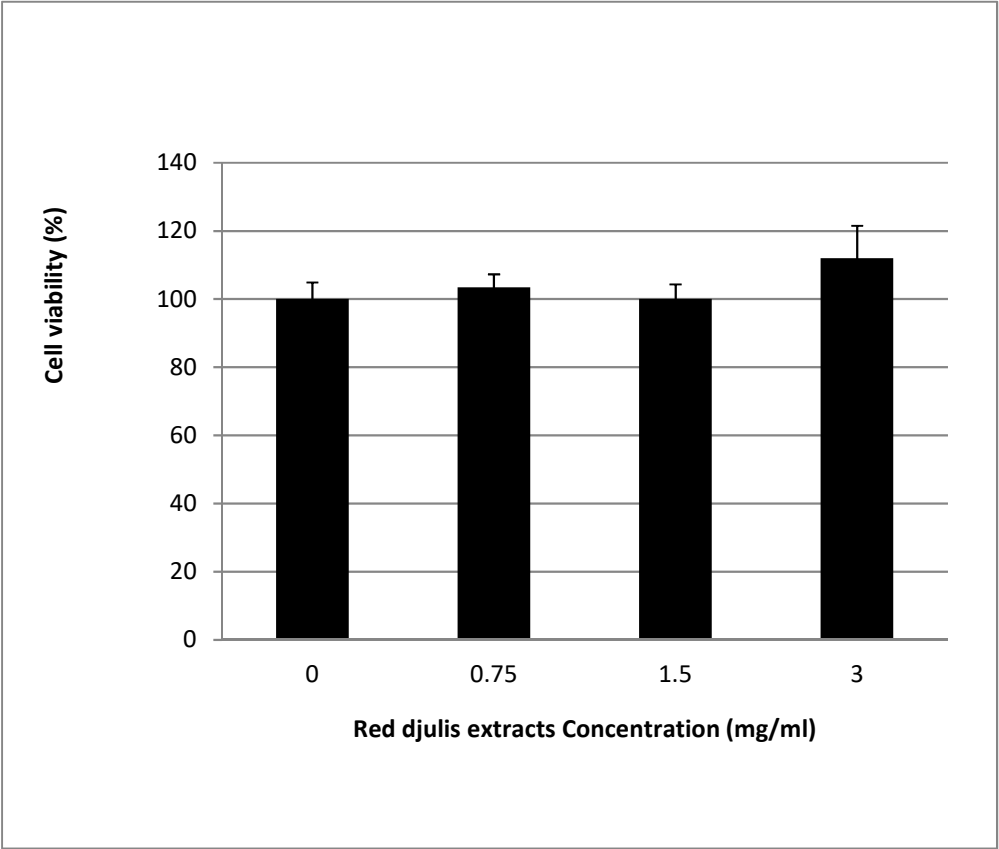
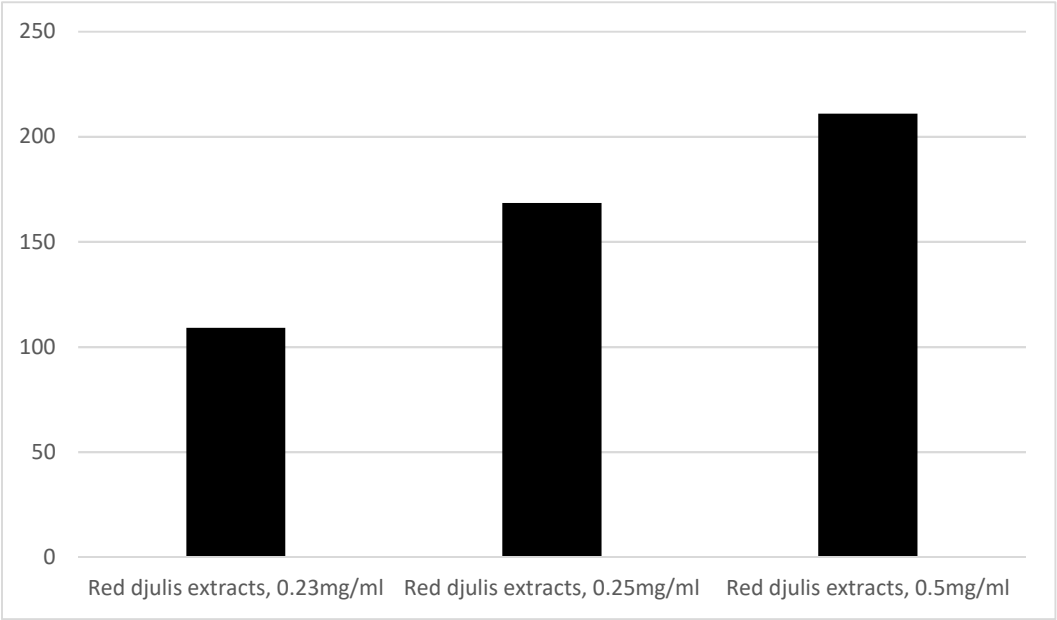
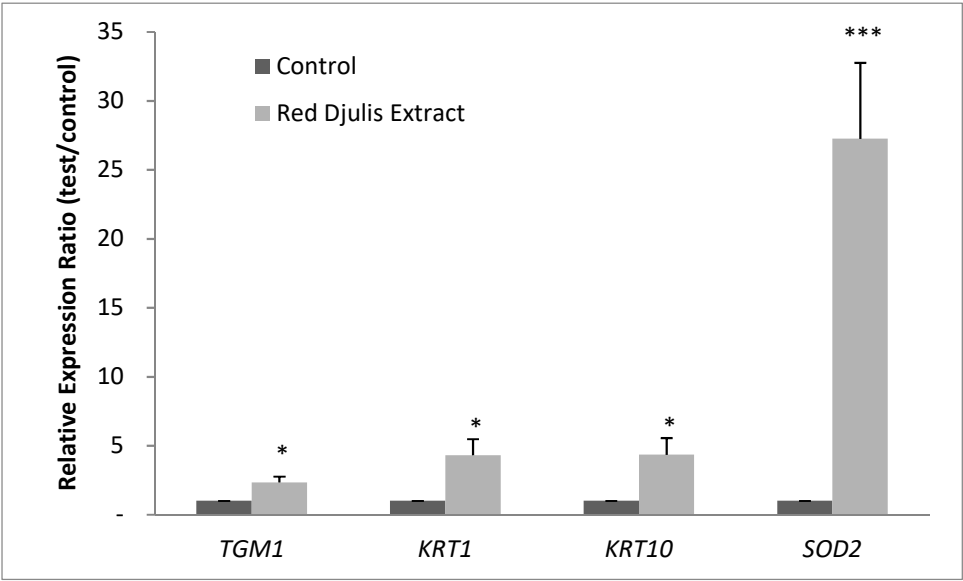
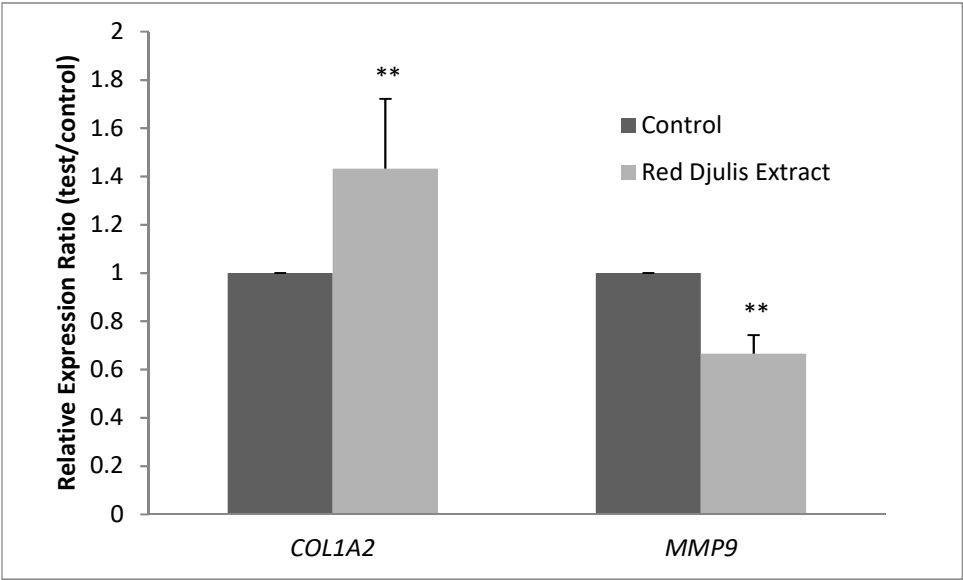
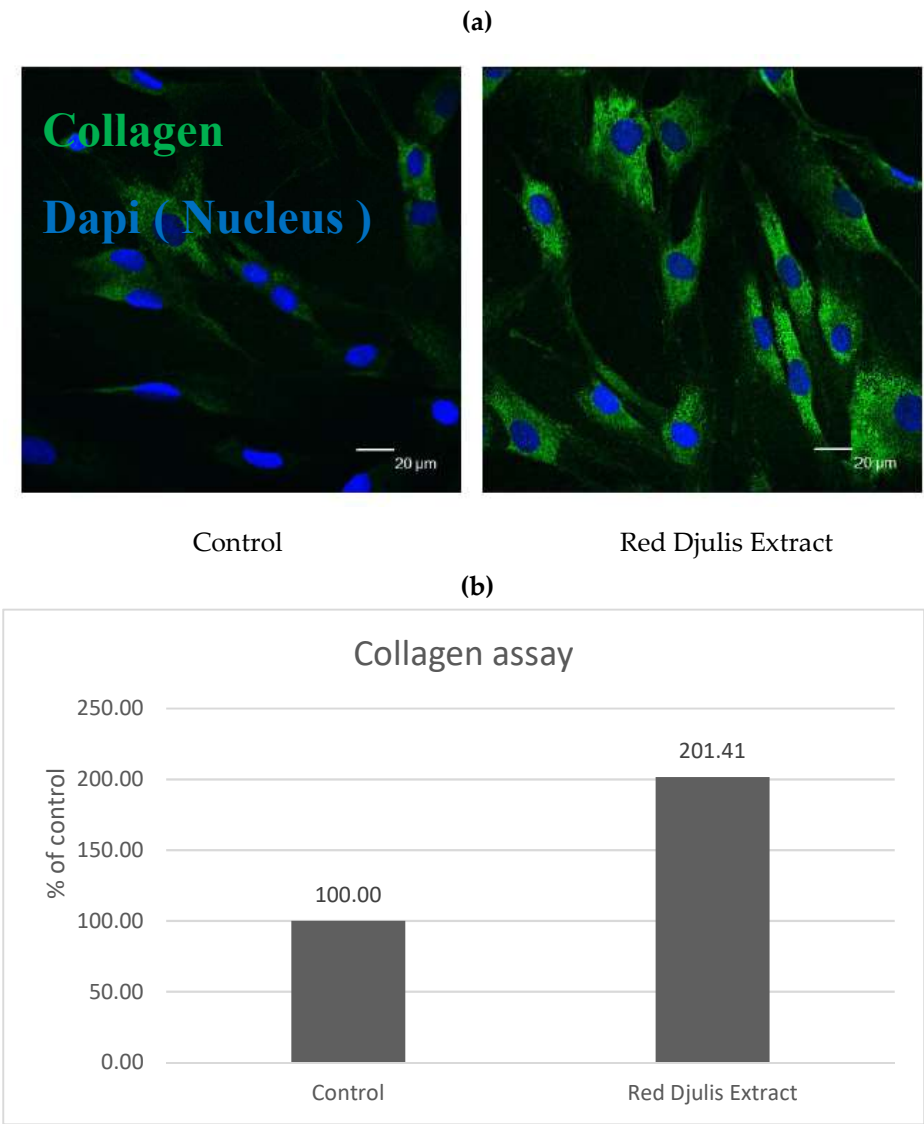
304

Figure 5.

305

(a)

Control   Red Djulis Extract

(b)

306   **Figure 6.**

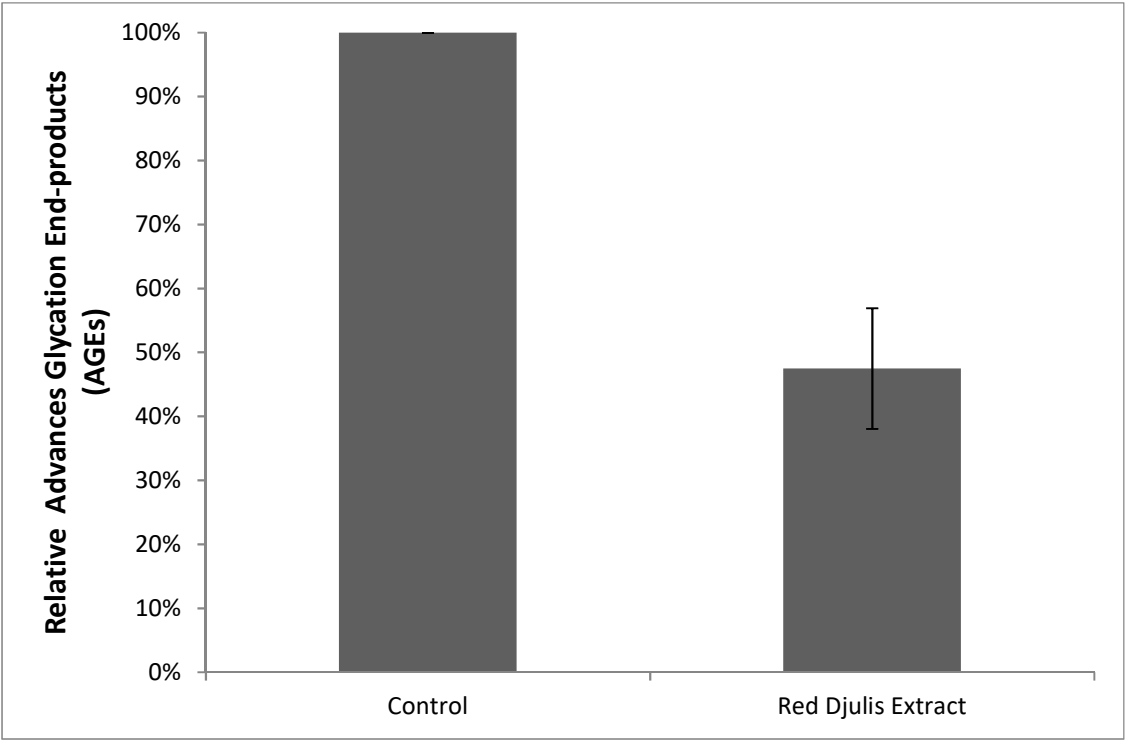
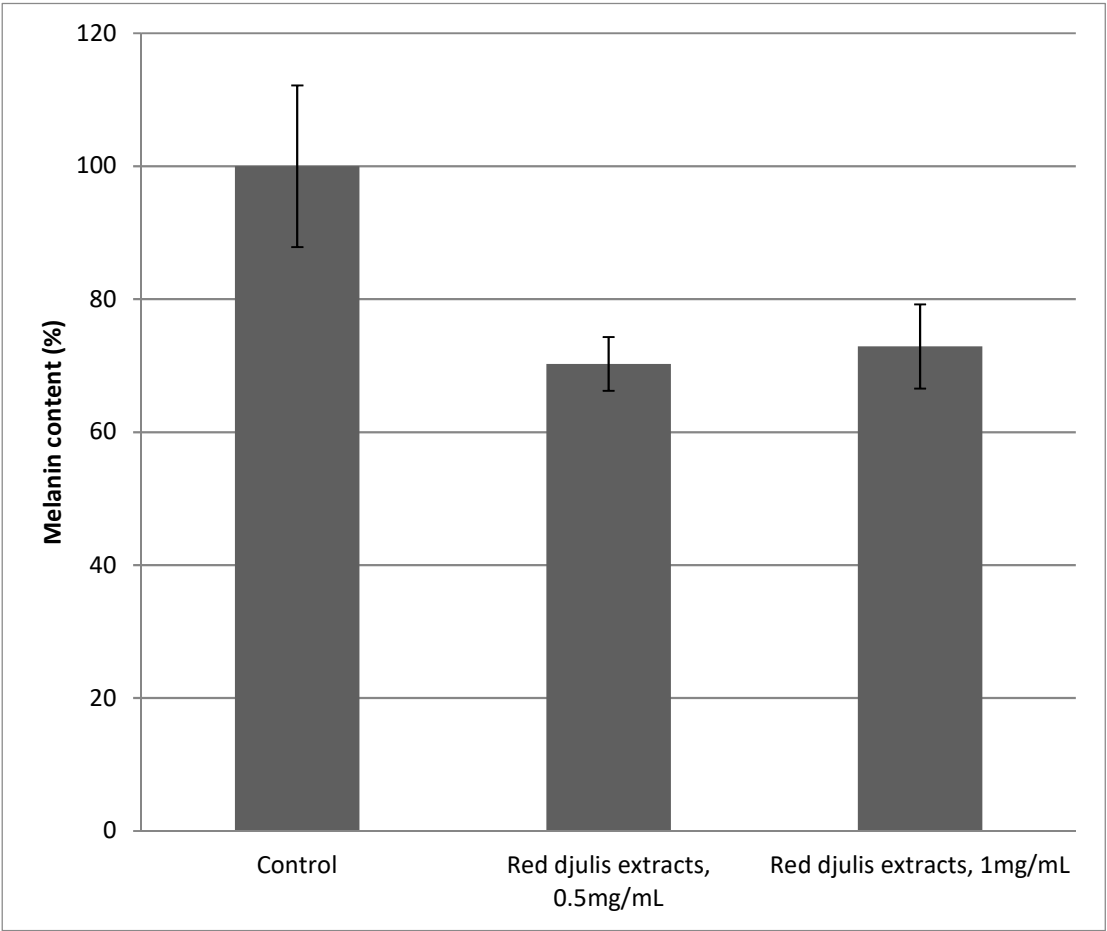



Figure 7.

Figure 8.

Reference

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