Promoting Stone Fruits for Protection Against the Metabolic Syndrome

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Abstract

In this report we studied the effects of stone fruit polyphenols against some components of the metabolic syndrome. We used cell models including adipocytes to determine effects in differentiation, fat accumulation and inflammation representing events in fat tissue. In addition, we studied inflammation in macrophage cells and Human umbilical vein endothelial cells (HUVEC) associated to atherosclerosis events. The inflammatory response in all these cells is a complex event, which is closely interrelated among them.
Initially we characterized the phenolic profiles of different stone fruits (nectarines, peach and plums) finding 4 major phenolic groups including chlorogenic acid derivatives, anthocyanins, quercetin derivatives and catechins. Profiles and amounts present are dependent on fruit type and variety. We selected chlorogenic acid and a rich anthocyanin extract (RAE) which contained a mixture of anthocyanins, quercetins and catechins derivatives to perform the bioassays in this study.

Results indicated that stone fruit polyphenols have multiple functions and could potentially work against the metabolic syndrome in different fronts simultaneously. For example, RAE inhibited adipogenesis or lipid accumulation by modulating key transcription factors, c/EBPα Pparγ and Pparα reduced the inflammatory response in adipocytes or fat cells by modulating transcription factor NFkB and gene expressions of different pro-inflammatory cytokines. On the other hand, chlorogenic acid inhibited the inflammatory response in macrophages and HUVEC cells by modulating transcription factor NFkB and gene expressions of different pro-inflammatory cytokines and adhesion molecules associated to chronic inflammation and atherosclerosis.

These results are promising and indicate that stone fruit polyphenols have potential health promoting properties against the metabolic syndrome.

Introduction

Metabolic Syndrome is the name for a group of risk factors linked to overweight and obesity that increase the chance for heart disease and other health problems such as diabetes and

stroke. It is estimated that 47 million Americans have Metabolic Syndrome of which two of its root causes are obesity and physical inactivity (American, 1998) which are usually associated with proinflammatory and pro-oxidant state with associated increases in the pro-inflammatory cytokines and stress related proteins.

For the past few years the US population has been considered among the highest in the world in regards to overweight and obesity, with 64% of adults being overweight and 26% obese. Furthermore, it is estimated that 75% of adults in the United States are projected to be overweight and 41% obese by 2015. These numbers plus the fact that in the U.S., cardiovascular diseases have increasingly become the leading cause of mortality, justify the need for studying metabolic syndrome. Consequently, in recent years the U.S. market has seen an increase in the consumption of healthier products with the idea of protection against these degenerative diseases.

It is thought that as obesity (visceral fat accumulation) takes place, adipose tissue secretes various cytokines and chemokines, some of which are desirable such as adiponectin (antidiabetic and antiarteriosclerotic effects), visfatin (antidiabetes) and leptin (feeding regulatory effects) and others undesirable including TNF-α (causes diabetes), PAI-I (causes arteriosclerosis, thrombogenesis), angiotensinogen (angiotensin II, hypertension), interleukin-6 (IL-6, inflammation and causes diabetes) and MCP-I (chemotaxis). These undesirable disease-causing adipocytokines, like TNF-α causing insulin resistance and PAI-I associated with clot formation, may be exacerbated in the adipose tissue as macrophages invade them producing even more formation of TNF-α, MCP-1, IL-6 and NO in a feedback loop and causing a state of chronic inflammatory reactions (activation of inflammatory signaling molecules such as JNK, NFK-β, others) resulting in further exacerbation of the metabolic syndrome. Many of these circulating chemicals will affect the vascular endothelial tissue as well causing inflammation which can be exacerbated by LDL oxidation and infiltration initiating atherosclerosis and thrombosis mediated by platelet aggregation (Kawada et al., 2009).

Under this complex scenario, targeting obesity-related inflammatory components could be a useful strategy for the prevention and amelioration of the development of obesity related pathologies. Our first year report focused on inhibiting inflammatory events in fat cells, endothelial cells (HUVEC cells) and macrophages. Our report for year one confirms that stone fruit phenolics show the ability to inhibit fat accumulation as well as to inhibit inflammation in these cell models.

In this annual report (1st year) we accomplished the following objectives:

a) Characterization of phenolic profiles from selected varieties of nectarine, plum and peach fruit.

b) Study of the anti-inflammatory properties of stone fruit phenolics using Human umbilical vein endothelial cells (HUVEC), macrophages and fat cells (3T3-L1 adipocyte cells).

The present annual report is divided in 4 sections including 1) the characterization of phenolic profiles of selected varieties of stone fruits and extracts, 2) adipogenesis and anti-inflammatory studies using fat cells, 3) anti-inflammatory studies using macrophages and 4) anti-inflammatory studies using Human umbilical vein endothelial cells (HUVEC).

Press release

On June 2, 2010 a press release was set in the internet through Texas A&M University AgriLife. The press release was entitled "Peaches, plums induce deliciously promising death of breast cancer cells". The press release can be found in the following website,

http://agnews.tamu.edu/showstory.php?id=1942

As of 9/23/2010, the press release showed 116 clips that had a total circulation of **11,921,886** and a value of **$124,501.**

1) Characterization of phenolic profiles of selected varieties of nectarine, plum and peach fruit

Materials & Methods

LC-MS analysis of stone fruit phenolic profiles

2.0 g freeze-dried sample was homogenized with 8.0 mL methanol. Tubes were capped and stored for 24 h at 4 °C. Extracts were centrifuged at 29,000 g for 15 min. The clear supernatant was filtered with a 0.22 µm nylon filter. Individual compounds were identified based on retention time, UV spectra and their mass per charge ratio using LC-MS. Chromatographic seperation were performed on a LCQ Deca XP Max LC-MS/MS system (Thermo Finnigan, CA) equipped with an autosampler, a Surveyor 2000 quaternary pump and a UV 2000 PDA detecter, using a 150 × 2.00 mm Synergi 4µ Hydro RP 80A column (Phenomenex, Torrance, CA) and a guard column of the same chemistry. Individual compounds were identified based on retention time, UV spectra and their mass per charge ratio using LC-MS as described previously. Elution gradient were formed with solvent A [1% formic acid -water] and solvent B [acetonitrile]. Separations were achieved by a linear gradient with A and B: 0 min 93% A, 2 min 88% A, 15 min 86% A, 20 min 82% A, 28 min 76% A, 35 min 60% A. The flow rate was 200µl/min. The injection volume was 10 µl. Sample was delivered to the LCQ MS by electrospray ionization (ESI) source. Conditions for analysis in positive ion mode were: spray voltage at 5.0 KV, sheath gas flow rate at 50 arbitray units, auxiliary gas flow rate at 3.0 arbitray units, caplillary temperature at 275°C, caplillary voltage at 10 V. Spectra were scanned over a mass range of m/z 180-2000 at 3 scans sec^{-1}.

Helium was used as collision gas and collision energy was set at 30%. MS2 and MS3 analysis were used during the identification.

Plum rich anthocyanin extract (RAE)

Black Splendor plums were harvested from California, received, pitted and stored at -80C until further use. About 250 grams fruit (skin and flesh) were homogenized with 580 ml methanol and kept at 4C overnight. Samples were then centrifuged for 15 minutes at 29000 x g. Supernatant was collected and evaporated at 35C using a rotavapor (Büchi, Switzerland). The remaining aqueous extracts were re-diluted into 0.1% triflouroacetic acid (TFA) and loaded into a SEP Pack C18 cartridge (10g, Waters Corp, Milford, MA) previously conditioned to a pH below 2 with 20 ml of 0.1% TFA in methanol and 20 ml 0.1% TFA. The cartridge was washed with 100 ml of 0.1% TFA and the phenolic compounds were collected with 0.1% TFA in methanol. The methanol was then evaporated using a rotavapor (Büchi, Switzerland) and the remaining aqueous solution was freeze dried (FTS Systems Inc, Stone Ridge, NY) at -50C and 200mmHg of pressure. Plum powder was kept at -20C until use in experiments. The identification was performed by LC-MS using 1.0 mg powder dissolve in 400uL AcN and filtered with a 0.22 µm nylon filter. LC-MS conditions used are those described above.

Results & Discussions

Identification of Phenolics in Angeleno plum, Fire Pearl nectarine and Galaxy peach with liquid chromatography-mass spectrometry (LC-MS)

LC-MS analysis showed 16 compounds in Angeleno plum (dark red skin, yellow flesh), including 2 chlorogenic acid derivates, 2 anthocyanins, 6 flavonoids and 6 procyanidin derivates (Table 1, Figures 1a, b). In Fire Pearl nectarine (red skin, white flesh), 7 compounds were identified, including 2 chlorogenic acid derivates, 1 anthocyanin and 4 flavonoids (Table 2, Figures 2a, b). For Galaxy peach (red skin, white flesh), we found 6 compounds, including 2 chlorogenic acid derivates, 1 anthocyanin and 4 flavonoids (Table 3, Figures 3a, b).

In general, the quantification of the phenolic profiles (using appropriate standards) within the 4 main phenolic categories, chlorogenic acids, anthocyanins, quercetin derivatives and cathechins showed that Angeleno plums contained 12%, 4.3%, 17% and 65%, Fire Pearl had 84%, <1%, 14%, 0% while Galaxy peach showed 85%, 0%, 13.4% and 0%, respectively. These results indicate large variation of the phenolic profiles with peaches and nectarines containing large amounts of chlorogenic acid while plums mainly flavonoids and catechins. Thus, for our biological experiments we decided to test chlorogenic acid (CGA) and an enriched anthocyanin extract (RAE) which contained mainly flavonoids and catechins. This approach allowed us to determine the specific biological significance of the main phenolic components in stone fruits.

Identification of phenolic profile of a stone fruit enriched anthocyanin extract (RAE) with liquid chromatography-mass spectrometry (LC-MS)

Using LC-MS we identified 16 compounds in Black Splendor enriched anthocyanin extract (RAE) powder, including 1 chlorogenic acid derivate, 2 anthocyanins, 7 flavonoids and 6 procyanidin derivates (Table 4, Figures 4a, b). The relative contents of chlorogenic acids, anthocyanins, flavonoids and procyanidins were 4.3%, 44.2%, 32.7% and 18%, respectively, confirming that the obtained RAE extract is enriched in flavonoids and catechins. This RAE extract obtained from Black Splendor plums was used for our biological assays and compared with chlorogenic acid.

Table 1. Phenolic profile in Angeleno plum

No.	t_R	λ_{max}	$[M-H]^+$	Compound
1	5.43	231, 246, 295, 324	355	Neo-chlorogenic acid
2	5.98	244, 280	579	procyanidin dimer
3	7.23	246, 280	291	catechin
4	7.96	231, 246, 295, 324	355	chlorogenic acid
5	8.48	244, 280	867	procyanidin dimer
6	8.79	244, 280	867	procyanidin trimer
7	9.29	244, 279, 515	449	cyanidin 3-*O*-glucoside
8	10.23	244, 280, 516	595	cyanidin 3-*O*-rutinoside
9	11.02	247, 280	291	*epi*-catechin
10	11.95	244, 280	867	procyanidin trimer
11	22.73	246, 352	611	rutin
12	23.85	247, 352	465	quercetin 3-*O*-glucoside
13	25.45	269, 347	435	quercetin-*O*-pentoside
14	25.97	269, 347	567	quercetin *O*-pentosylepentoside
15	26.77	252, 351	435	quercetin-*O*-pentoside
16	27.61	246, 346	449	quercetin-*O*-rhamaose

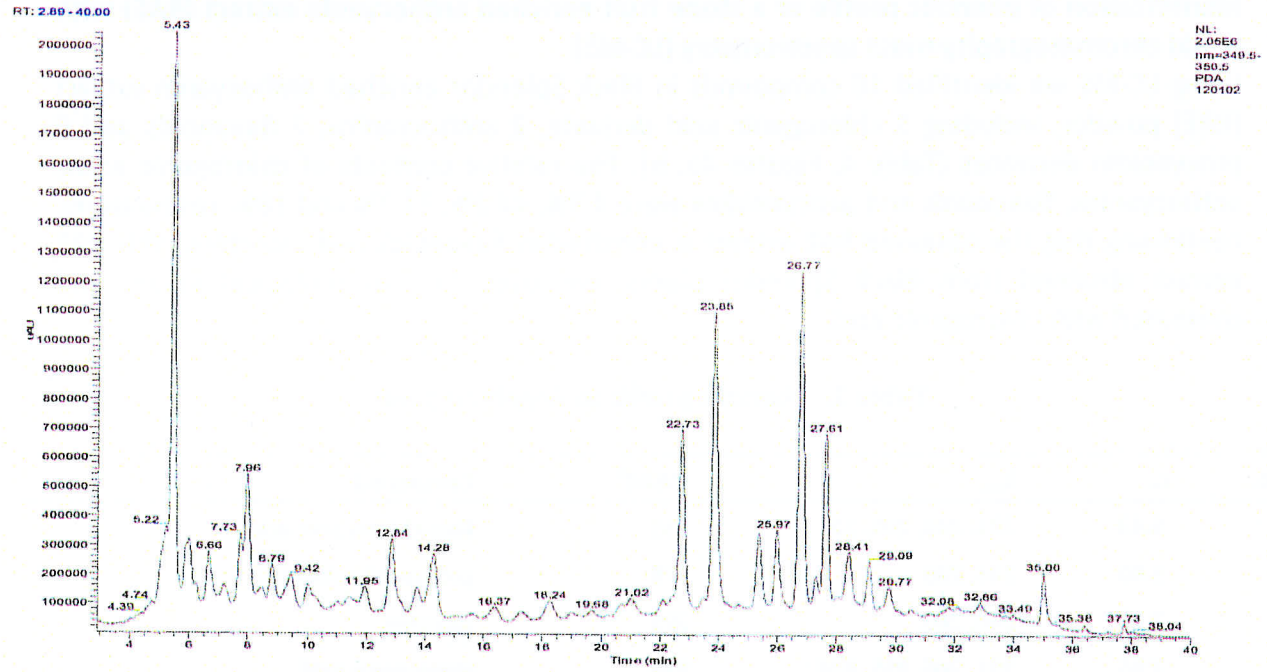


Figure 1a. HPLC Chromatogram of Angeleno plum at 350 nm

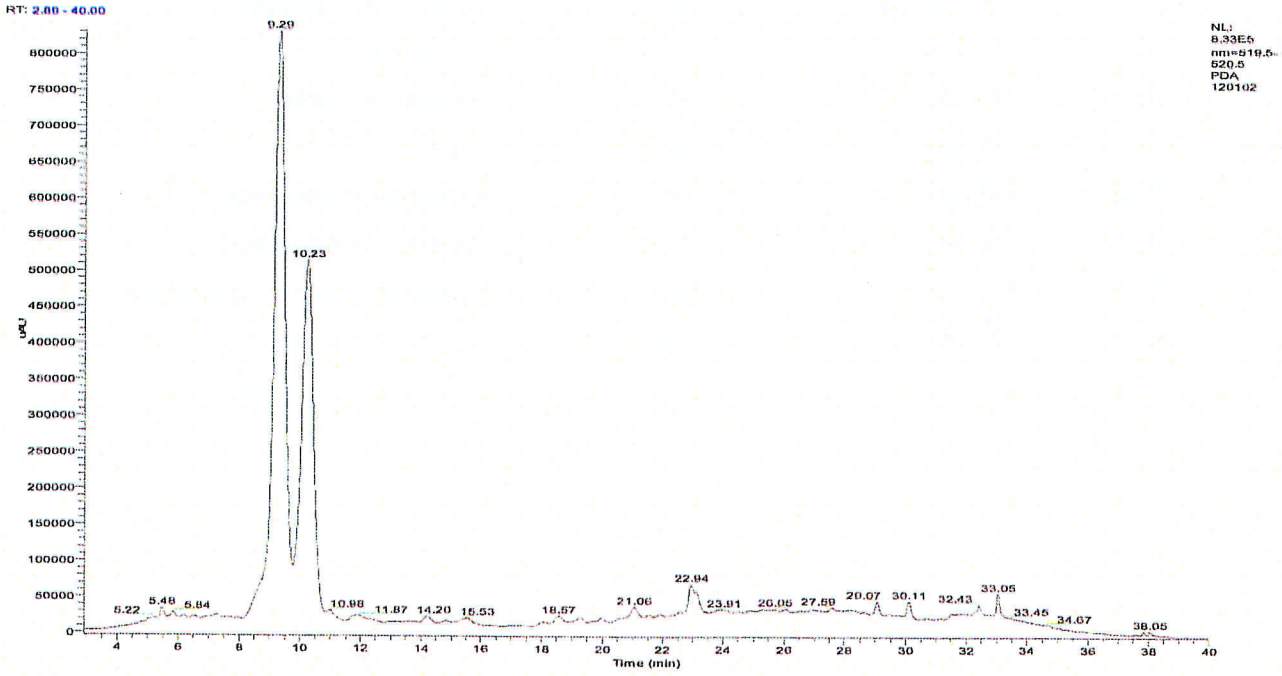


Figure 1b. HPLC Chromatogram of Angeleno plum at 520 nm

Table 2. Phenolic profile in Fire Pearl nectarine

No.	t_R	λ_{max}	$[M-H]^+$	Compound
1	4.87	231, 246, 295, 324	355	Neo-chlorogenic acid
2	6.96	231, 246, 295, 324	355	chlorogenic acid
3	9.29	244, 279, 515	449	cyanidin 3-*O*-glucoside
4	20.93	246, 352	611	rutin
5	21.41	246, 346	465	quercetin 3-*O*-galactoside
6	22.22	247, 352	465	quercetin 3-*O*-glucoside
7	23.08	277, 349	659	

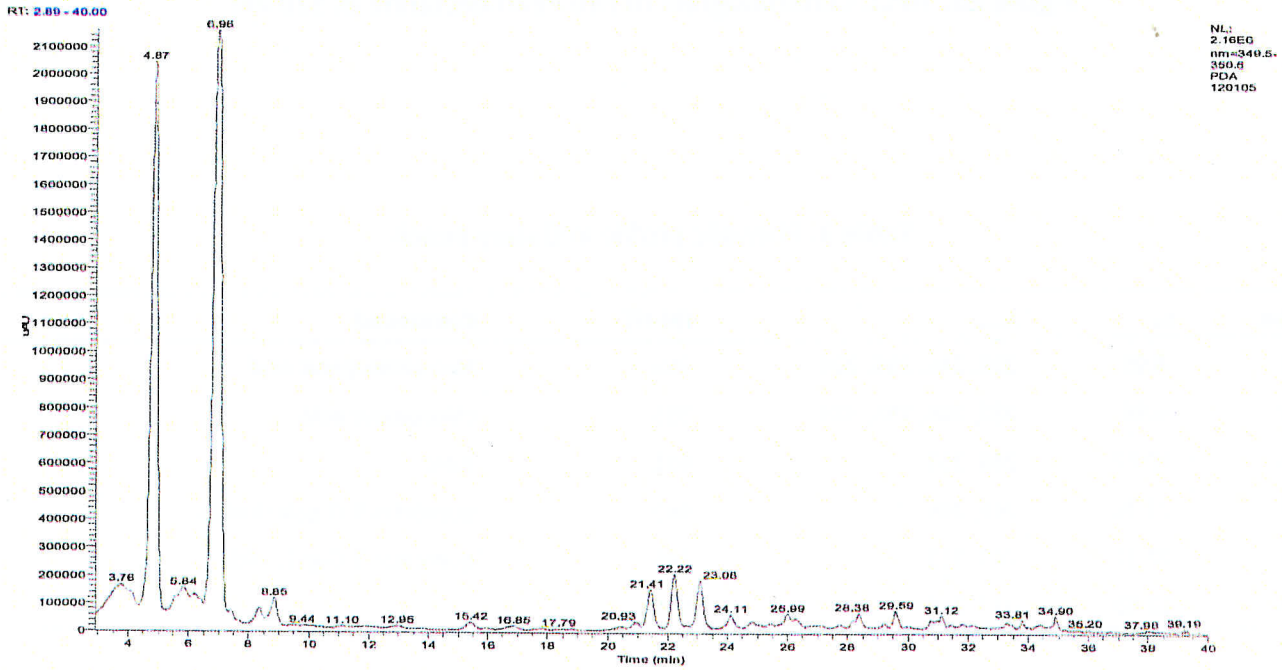


Figure 2a. HPLC Chromatogram of Fire Pearl nectarine at 350 nm

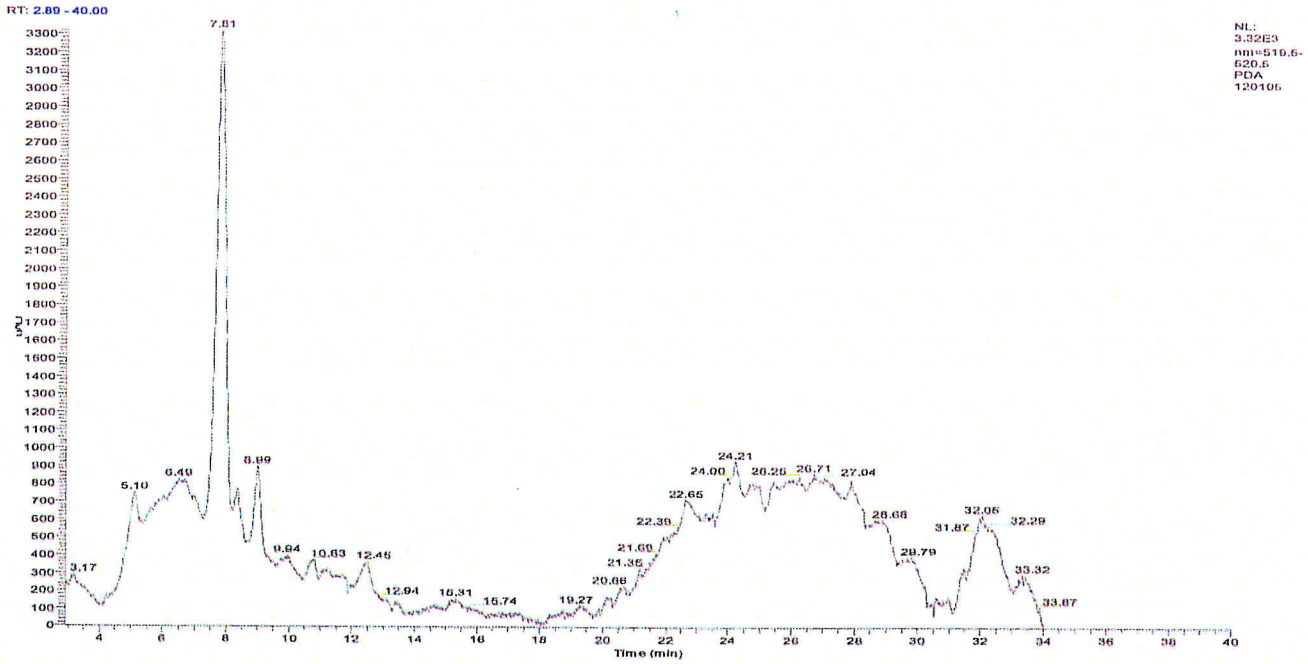


Figure 2b. HPLC Chromatogram of Fire Pearl nectarine at 520 nm

Table 3. Phenolic profile in Galaxy peach

No.	t_R	λ_{max}	$[M-H]^+$	Compound
1	4.95	231, 246, 295, 324	355	Neo-chlorogenic acid
2	6.98	231, 246, 295, 324	355	chlorogenic acid
3	20.87	246, 352	611	rutin
4	21.36	246, 346	465	quercetin 3-*O*-galactoside
5	22.17	247, 352	465	quercetin 3-*O*-glucoside
6	23.03	277, 349	659	

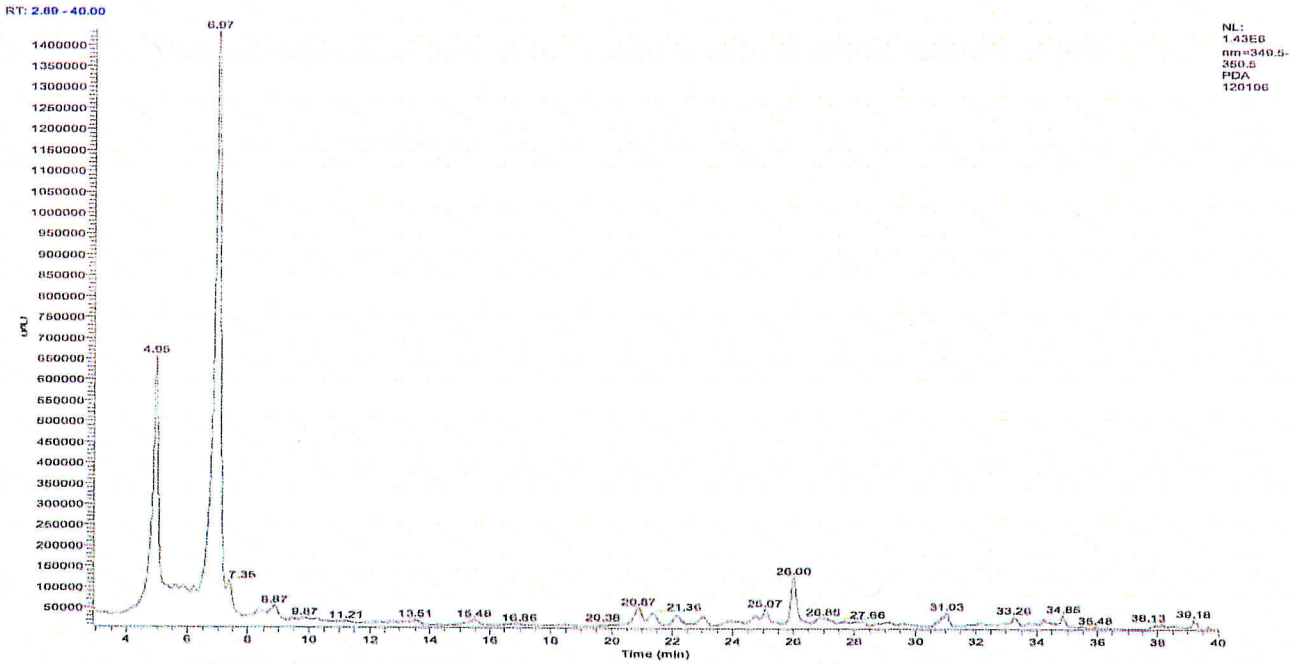


Figure 3a. HPLC Chromatogram of Galaxy peach at 350 nm

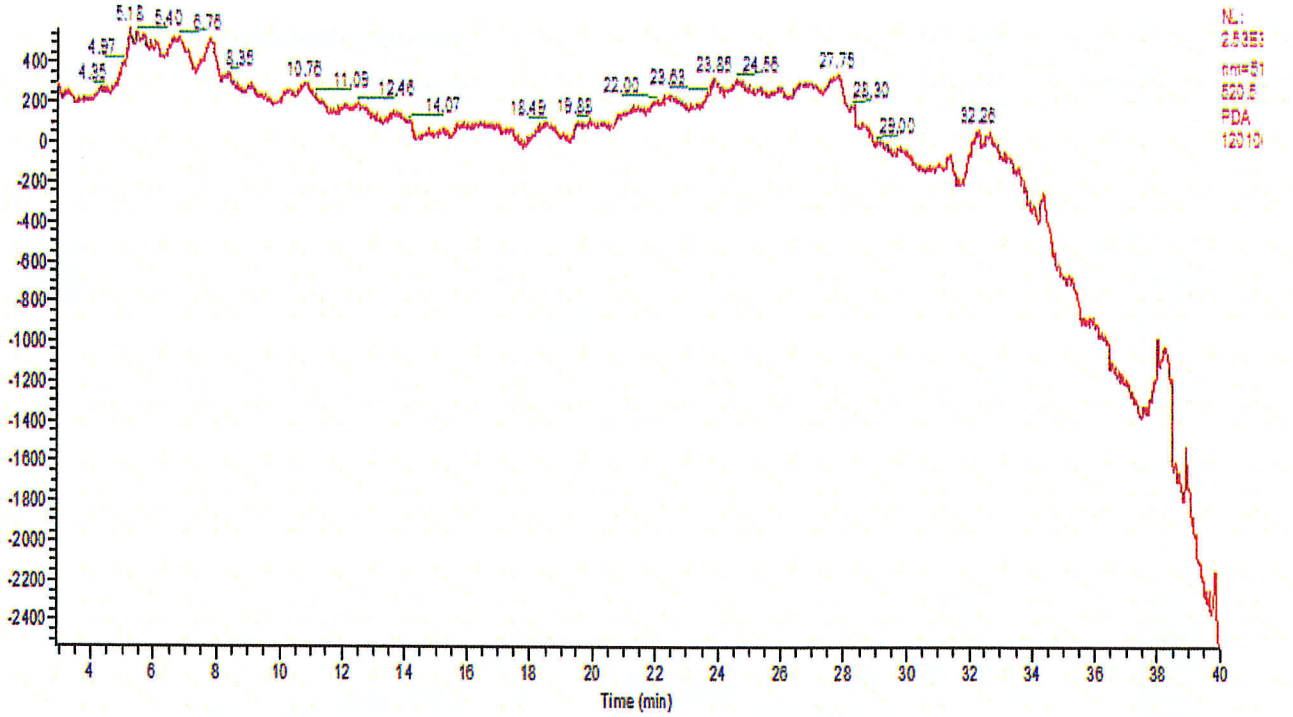


Figure 3b. HPLC Chromatogram of Galaxy peach at 520 nm

Table 4. Phenolic profile of a stone fruit enriched anthocyanin extract (RAE)

No.	t_R	λ_{max}	$[M-H]^+$	Compound
1	4.89	231, 246, 295, 324	355	Neo-chlorogenic acid
2	5.45	244, 280	579	procyanidin dimer
3	6.63	244, 281	867	procyanidin trimer
4	7.00	246, 280	291	catechin
5	7.05	244, 269	867	procyanidin trimer
6	8.50	244, 279, 515	449	cyanidin 3-*O*-glucoside
7	9.34	244, 280, 516	595	cyanidin 3-*O*-rutinoside
8	10.00	247, 280	291	*epi*-catechin
9	12.03	244, 280	867	procyanidin trimer
10	22.05	246, 352	611	rutin
11	22.78	246, 346	465	quercetin 3-*O*-galactoside
12	23.15	247, 352	465	quercetin 3-*O*-glucoside
13	24.63	269, 347	435	quercetin-*O*-pentoside
14	25.38	269, 347	567	quercetin *O*-pentosylepentoside
15	26.07	252, 351	435	quercetin-*O*-pentoside
16	26.89	246, 346	449	quercetin-*O*-rhamaose

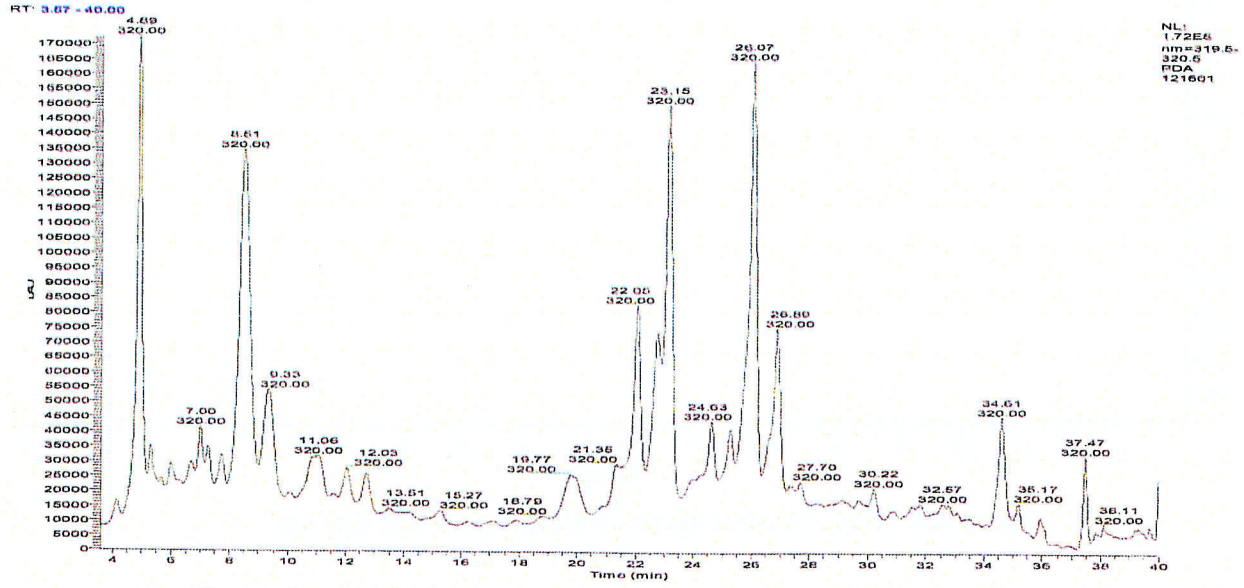


Figure 4a. HPLC Chromatogram of a stone fruit RAE at 320 nm

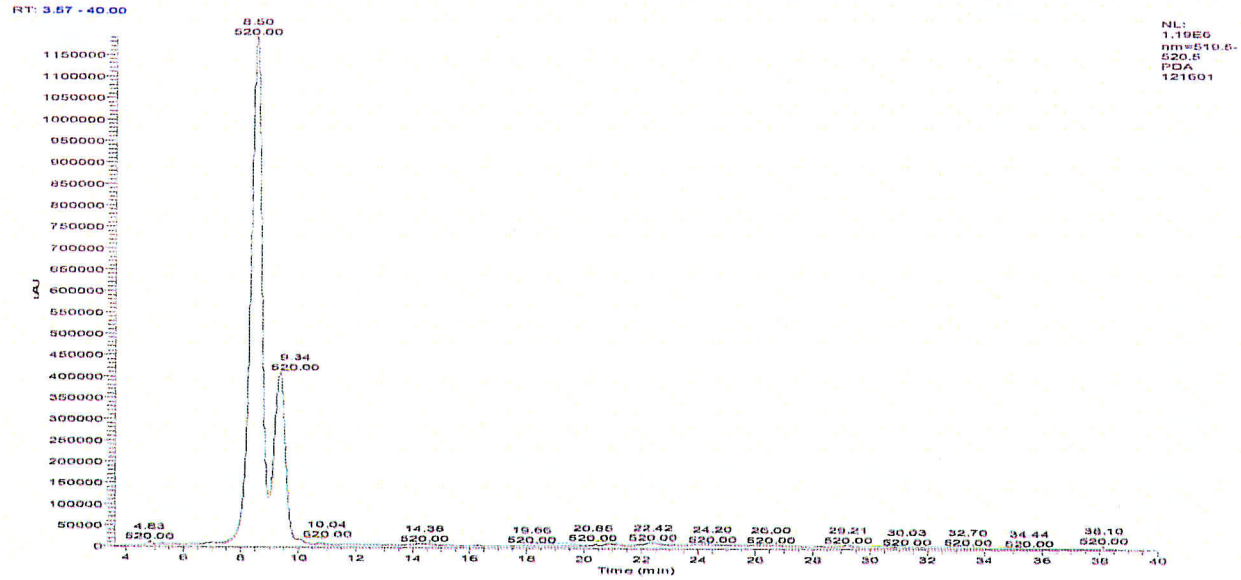


Figure 4b. HPLC Chromatogram of a stone fruit RAE at 520 nm

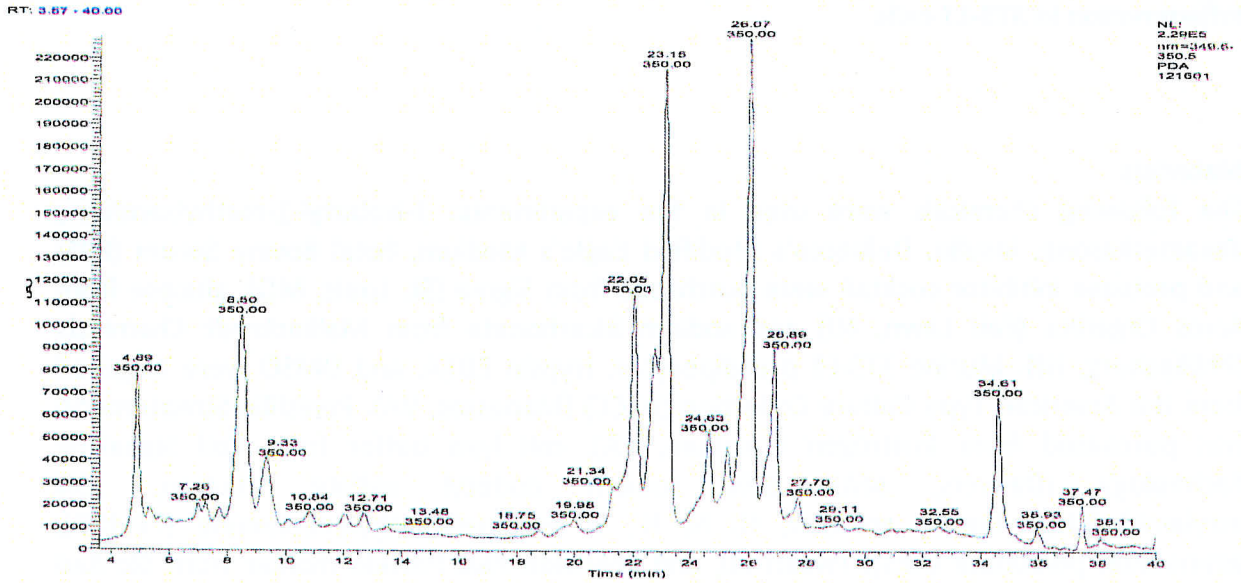


Figure 4c. HPLC Chromatogram of a stone fruit RAE at 350 nm

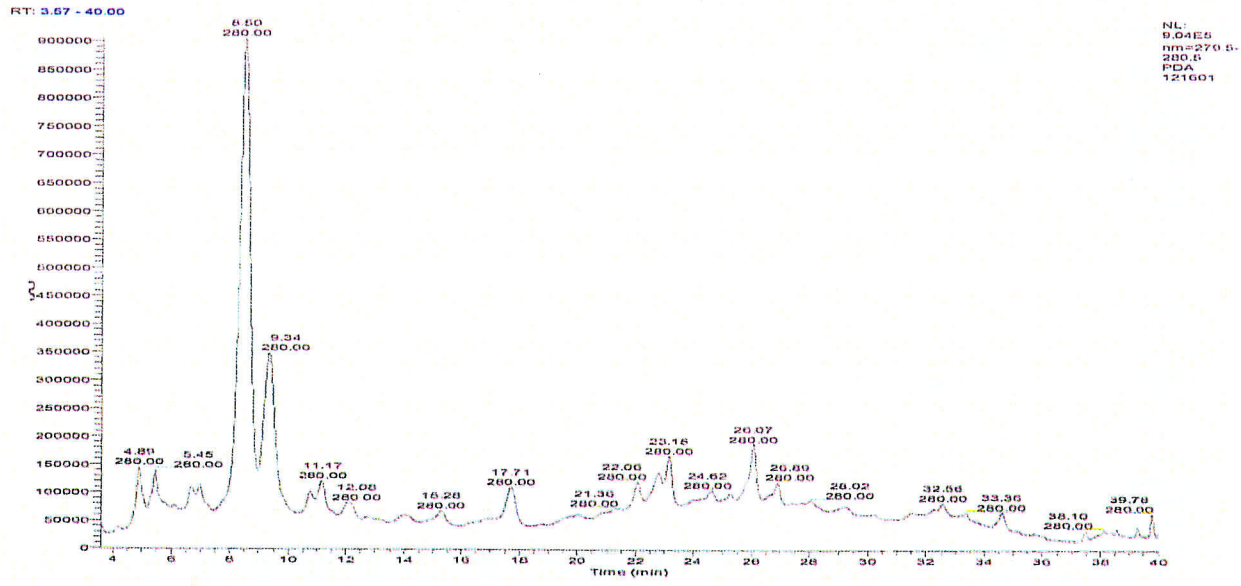


Figure 4d. HPLC Chromatogram of a stone fruit RAE at 280 nm

2.) Effect of a Plum Rich anthocyanin extract and Chlorogenic Acid on Adipogenesis and Inflammation in 3T3-L1 cells

Materials & Methods

Materials

The following chemicals were used in the experiments: 3-isobutyl-1-methylxanthine, dexamethasone, insulin, Dulbecco's Modified Eagle's Medium, Fetal Bovine Serum (FBS) and protease inhibitor cocktail were purchased from Sigma (St. Louis, MO), glucose from Acros Organics (Fair Lawn, NJ) and sodium bicarbonate from Mallinckrodt Chemicals (Phillipsburg, NJ). Murine 3T3-L1 preadipocytes, trypsin EDTA, and DMSO were acquired from the American Type Culture Collection (ATCC) (Manassas, VA). Penicillin-Streptomycin was purchased from Invitrogen (Carslbad, CA), cell lysis buffer from Cell Signalling Technology (Danvers, MA), 10% sodium dodecyl sulfate solution, 30% acrylamide/bisacrylamide solution, N, N, N', N'-tetramethylethylenediamine (TEMED), ammonium persulfate (APS), Tween 20, and Precision Plus Protein marker from Bio-Rad Laboratories (Hercules, CA). Laemmli's loading buffer was purchased from Fermentas Inc. (Glen Burnie, MD), polyvinylidene fluoride (PVDF) membranes from Millipore Corp. (Billerica, MA), pure Chlorogenic acid from MP Biomedicals LLC (Solon, OH), antibodies for PPARγ (sc-7196), C/EBPα (sc-61), PPARα (sc-9000), p-NF-κB p65 (sc-33039), NF-κB p65 (sc-109), and β-actin (sc-47778) from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-rabbit-HRP polyclonal secondary antibody (A120-101P) was from Bethyl Laboratories (Montgomery, TX).Water used was nanopure grade. All the primers were purchased through IDT integrated DNA technology (San Diego, CA).

Cell Viability Assay

Cytotoxic effects of plum rich anthocyanin extract (RAE) and Chlorogenic acid were evaluated in 3T3-L1 preadipocytes using the MTS assay (Promega Corp., Madison, WI), according to the manufacturer's instructions. The quantity of formazan product was measured at 490 nm and is directly proportional to the number of living cells in culture. The assay was performed in a 96-well plate with a density of 8,000 cells/well. The 3T3-L1 pre-adipocytes were left with DMEM/10% FBS medium for 24 h before starting the treatments. Treatment of Chlorogenic acid was added at concentrations from 0 to 300 µM in serum-free medium, and the cell viability was measured at 24 and 48 h. For plum rich anthocyanin extract (RAE), the cell viability and proliferation was determined at 24, 48 and 72 h post-incubation with a range of concentrations from 0 to 400 µM based on Chlorogenic acid equivalents determined by the Folin assay. Solutions of Chlorogenic Acid and Plum RAE were done using serum-free DMEM medium.

Cell Culture, differentiation and treatments for adipogenesis studies

3T3-L1 cells were seeded at a density of 10,000 cells/well in 6-well plates. Pre-adipocytes were cultured in DMEM medium supplemented with a 10% of fetal bovine serum (FBS) and induced to differentiation 2 days after they reached 100% confluency (day 0). To induce differentiation, growth medium was supplemented with 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 1 µM dexamethasone and 10 µg/mL insulin, for 48 h (day 2). At day 2, medium was replaced with fresh DMEM 10% FBS medium, supplemented with insulin (10 µg/mL) for two additional days (day 4). After day 4, the insulin supplemented medium was replaced with fresh DMEM 10% FBS medium, and this medium was replaced every two days until reaching complete maturity of the cells (day 10). The treatments with Chlorogenic acid and Plum RAE started from day 0 until day 10. To evaluate the effect of Chlorogenic acid on adipogenesis, the cells were treated with 50, 100 and 250 µM of the pure compound. The same concentrations were used on the treatments with plum RAE, based on chlorogenic acid equivalents determined by the Folin assay. In both cases, serum-free DMEM medium was used to dissolve the compounds. Protein and RNA samples were taken at days 4 and 10 to measure the protein and gene expression of different markers on the early and late stage of the adipogenesis process.

Quantification of lipid content

Intracellular lipid content was measured using a commercially available kit (AdipoRed, Lonza Wakersville, Inc., Wakersville, MD). AdipoRed is a solution of the Nile Red stain, which fluoresces and enables the quantification of intracellular lipid droplets. To evaluate the fat accumulation, pre-adipocytes were cultured under the same conditions described previously and 6,000 cells/well were seeded in 12-well plates, differentiated after 2 days of 100% confluency and treated with pure Chlorogenic acid or plum rich anthocyanin extract (RAE) at concentrations of 50,100 and 250 µM. The treatments with Chlorogenic acid or plum RAE started from day 0 until day 10. On day 10, cells were washed with 2 mL PBS (pH 7.4), then 2 mL PBS were left per well and 60 µL AdipoRed were added, plates were at 37°C for 10 min and fluorescence readings were measured by scanning wells with excitation at

485 nm and emission at 560 nm in a plate reader (Synergy HT, Bio-Tek Instruments, Inc., Winooski, VT). Measurements were expressed as relative fluorescence units (RFU).

Cell Culture, differentiation and treatments for inflammation studies

3T3-L1 cells were seeded at a density of 10,000 cells/well in 6-well plates. Pre-adipocytes were cultured in DMEM medium supplemented with a 10% of fetal bovine serum (FBS) and induced to differentiation 2 days after they reached 100% confluency (day 0). To induce differentiation, growth medium was supplemented with 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 1 µM dexamethasone, and 10 µg/mL insulin, for 48 h (day 2). At day 2, medium was replaced with fresh DMEM 10% FBS medium, supplemented with insulin (10 µg/mL) for two additional days (day 4). After day 4, the insulin supplemented medium was replaced with fresh DMEM 10% FBS medium, and this medium was replaced every two days until reaching complete maturity of the cells (day 10). The treatments with Chlorogenic acid or plum rich anthocyanin extract (RAE) started at day 10 for a period of time of 72 h. To evaluate the effect of Chlorogenic acid on inflammation, the cells were treated with 50, 100 and 250 µM of the pure compound. The same concentrations were used to study the effects with plum RAE. After 71 h of treatment, 100 ng/mL of lipopolysaccharide (LPS), an acute inflammation inducer, was added to the cells for 1 h, to induce the inflammatory response. Protein and RNA samples were taken right after completing the 72 h treatment.

SDS-PAGE and Western Blotting

For adipogenesis studies, proteins from cells were collected at days 4 and 10 post differentiation induction. In case of inflammation studies, protein samples from mature adipocytes were collected after 72 h of treatment with chlorogenic acid or plum rich anthocyanin extract (RAE). All cells were harvested with a commercial cell lysis buffer (Cell Signaling Technology, Inc., Danvers, MA) following the manufacturer's instructions. Briefly, each well was washed twice with ice cold PBS, added 125 µL lysis buffer, containing a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Cells were then scraped, left at -80 ºC overnight, centrifuged at 14,000 rpm at 4 ºC and supernatant was stored at -80 ºC. The total protein was quantified using the BCA Protein Assay Kit (Pierce, Thermo Fisher Scientific, Inc., Rockford, IL) and 40 µg of protein equivalent volume were mixed with Laemmli's loading buffer, boiled, and loaded on a gel together with a pre-stained, broad-range, molecular weight protein marker (Fermentas, Glen Burnie, MD). Proteins were separated by electrophoresis using 10% polyacrylamide gels; the stacking gel was run at 75 V for 15 min and protein separation (resolving gel) was run for 2.5 h at 95 V. The gels were transferred by wet blotting onto PVDF membranes at 100 V for 1.5 h at 4 °C. The membranes were then blocked with 5% skim milk in tris buffered saline with 1% Tween-20 (TBS-T) for 1.5 h with gentle shaking; three 5-min washes with TBST were performed consecutively. For adipogenesis studies, membranes were incubated with a specific primary antibody against PPARγ (1:1000), PPARα (1:2000), c/EBPα (1:4000). For inflammation studies, membranes were incubated with a specific primary antibody against NFκB (1:1000) and p- NFκB (1:2000). For internal control, membranes were incubated with a conjugated HRP primary antibody against β-actin at a dilution of 1:6000. After the incubation with the primary antibody, the membranes were washed three times with TBS-T and incubated for 1

h with the secondary antibody conjugated with horseradish peroxidase (HRP) at 1:8000 dilution. Specific bands were developed using a SuperSignal West Femto enhanced chemiluminescence (ECL) Western blotting detection kit (Pierce, Thermo Fisher Scientific, Inc., Rockford, IL) after 30 s incubation and signals were captured by a CCD Camera (Cascade II:512, Photometrics,Tucson, AZ) using the WinView/32 software (Version 2.5, Princeton Instruments, Trento, NJ). Bands were measured using densitometry with ImageJ software (NIH, Bethesda, MD).

RNA isolation and reverse transcription

Total cellular RNA was extracted from 3T3-L1 cells, after the treatments described previously, using the RNeasy mini kit (Qiagen, Valencia, CA) following the manufacturer instructions. RNA concentration was measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Willmington, DE), and volume was adjusted to 1µg RNA. Equal aliquots were loaded on 1% agarose gels, stained with ethidium bromide, to check for RNA integrity. For cDNA synthesis, 1 µg RNA was reverse-transcribed into cDNA using the SuperScript III first-strand synthesis supermix (Cat. No. 18080-004, Invitrogen, Carlsbad, CA), following the manufacturers protocol. Briefly, oligo dT (300 ng), 10 mM dNTPs mix (500 µM) and PCR grade water were mixed with 1 µg RNA. Mixture was heated to 65 °C for 5 min followed by ice incubation for at least 1 min. Added first strand buffer, 5mM dithiotreitol (DTT), 40 units RNAse out, and 200 units SuperScript III and incubated at 55 °C for 60 min, and reaction was inactivated by heating to 70 °C for 15 min. cDNA samples were stored at -20°C until further analysis.

Real-time PCR

Gene expression was quantified by real-time PCR using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), following the manufacturer's instructions. DNA amplification was carried out using a 7,900 HT Sequence Detection System (Applied Biosystems, Foster City, CA), and the detection was performed by measuring the binding of the fluorescence dye SYBR Green to double-stranded DNA. Reactions were performed in a 20 µL volume containing 25 ng cDNA with 0.3 µM primers; all the primer sets are listed in table 1 and were provided by Integrated DNA Technologies (IDT, Coralville, IA). After 10 min polymerase activation at 95 °C, 40 cycles with 95°C for 15s (denaturation), 60°C for 1 min (annealing/extension) were performed. Fluorescence was measured at the end of the 60°C extension period. To confirm amplification specificity, the PCR products were evaluated by a melting curve analysis to exclude primer dimers and non-specific amplification. The relative expression of each gene was normalized by β-actin, and was calculated following the comparative Ct method (ΔΔCt). The following equation was used to calculate the relative mRNA expression.

$$\Delta\Delta C_t = \Delta C_{t,\,sample} - \Delta C_{t,\,reference}$$

Where, $\Delta C_{t,\,sample}$ is the Ct value for any sample normalized to the internal housekeeping gene; whereas $\Delta C_{t,\,reference}$ is the Ct value for the calibrator or control, also normalized to the internal housekeeping gene. For the $\Delta\Delta C_t$ calculation to be valid, the amplification efficiencies of the target and the internal reference were approximately equal. The fold

determination of the relative mRNA expression for each target gene was calculated according to the following equation:

$$\text{Fold determination} = 2^{-\Delta\Delta Ct}$$

Statistical analysis

Data represent the mean of triplicates ± S.E. (standard error). Statistical significance was assessed by t-test ($P \leq 0.05$). Tests were conducted using SPSS 15.0 (SPSS Inc., Chicago, IL).

Table 1. Sequences of primers used in the present study.

Primer	Sequence
PREF-1-F	5'- CAG CGG CTA TGG GCT CAC CT -3'
PREF-1R	5'- TGT TGC TCG GGC TGC TGA A -3'
PPARγ-F	5'- GAT GCA CTG CCT ATG AGC ACT T -3'
PPARγ-R	5'- AGA GGT CCA CAG AGCTGA TTC C -3'
Adiponectin-F	5'- TCC TGG AGA GAA GGG AGA GAA AG -3'
Adiponectin-R	5'- TCA GCT CCT GTC ATT CCA ACA T -3'
Leptin-F	5'- GAG ACC CCT GTG TCG GTT C -3'
Leptin-R	5'- CTG CGT GTG TGA AAT GTC ATT G -3'
COX-2-F	5'- ACATCGATGTCATGGAACTG -3'
COX-2-R	5'- GGACACCCCTTCACATTATT -3'
iNOS-F	5'- ACATCGACCCGTCCACAGTAT -3'
iNOS-R	5'- CAGAGGGGTAGGCTTGTCTC -3'
TNF-α-F	5'- ACT GGC AGA AGA GGC ACT CC -3'
TNF-α-R	5'- CGA TCA CCC CGA AGT TCA -3'
IL-6-F	5'- TGA CAA CCA CGG CCT TCC CT -3'
IL-6-R	5'- AGC CTC CGA CTT GTG AAG TGG T -3'
β-Actin-F	5'- CCC AGG CAT TGC TGA CAG G -3'
β-Actin-R	5'-TGG AAG GTG GAC AGT GAG GC -3'

Results & Discussions

Effect of a plum rich anthocyanin extract (RAE) and Chlorogenic acid on pre-adipocytes cell viability. To determine the cell viability, the MTS assay was used and performed as described in materials and methods. Plum RAE showed a minimal time-dependent cytotoxic effect at concentrations in the range of 0 - 400 µM, with the exception of a cytotoxic observed at 72 h at 400 µM (figure 1). Based on these results, concentrations from 0 to 250 µM were selected to continue the studies in adipogenesis and inflammation. In this range of concentrations, the treatment with plum RAE is not affecting the cell viability neither the cell proliferation. Similar to plum RAE, Chlorogenic acid showed only a slight time-dependent cytotoxic effect at elevated concentrations (figure 2). For the studies on

adipogenesis and inflammation with this compound, concentrations from 0 to 250 µM were used to ensure that the treatment is not affecting the cell viability and proliferation.

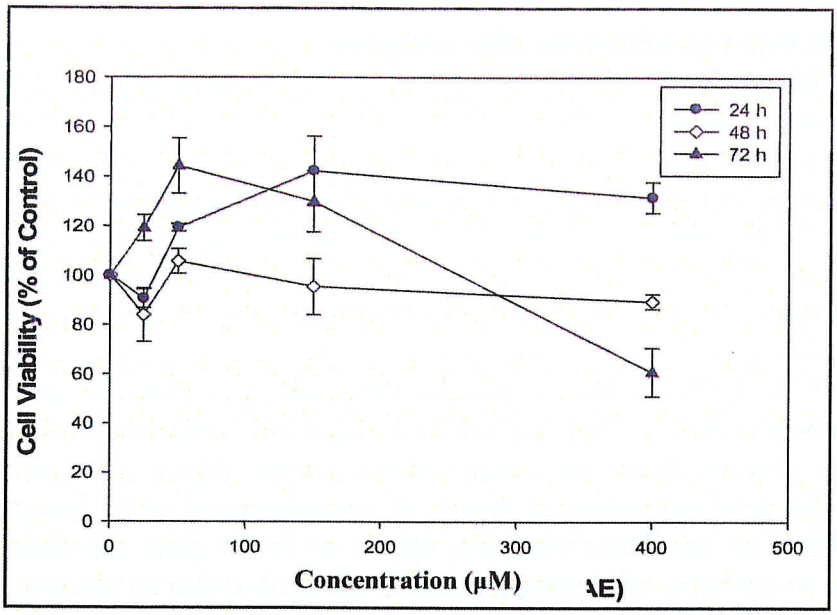


Figure 1. Effect of plum RAE on cell cytotoxicity in 3T3-L1 pre-adipocytes. Cells were incubated with 0-400 µM of Plum RAE for 24, 48 and 72 h at 37°C in a humidified 5% CO_2 incubator. Reported values are relative means ± S.E. compared to the control (n= 3).

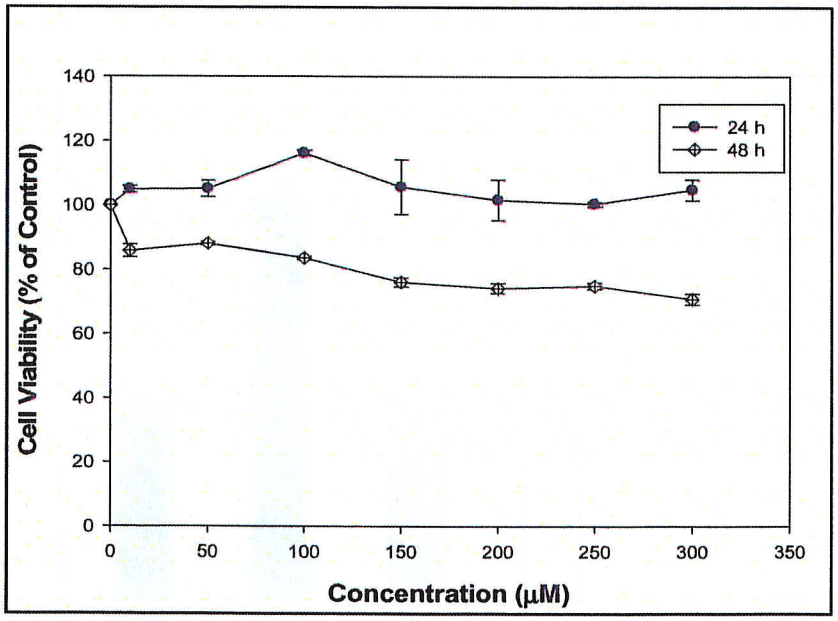


Figure 2. Effect of Chlorogenic acid on cell cytotoxicity in 3T3-L1 pre-adipocytes. Cells were incubated with 0-300 µM of Chlorogenic acid for 24 and 48 h at 37°C in a humidified 5% CO_2 incubator. Reported values are relative means ± S.E. compared to the control (n= 3).

Effects of plum RAE over pre-adipocytes differentiation

To obtain mature adipocytes from 3T3-L1 pre-adipocytes, it is necessary to induce the cells to the differentiation process in presence of insulin, dexametasone and IBMX. These compounds are able to induce an increase in the expression of many important proteins, like c/EBPα and Pparγ, which are keys in regulating the basic metabolic functions of the future adipocytes (1, 2). Pref- is synthesized as an epidermal growth factor (EGF) and is highly expressed in pre-adipocytes, but its expression is abolished during the differentiation process into adipocytes (3). Previous studies reported that the overexpression of Pref-1 or treatment with soluble Pref-1 in pre-adipocytes results in the inhibition of adipocytes differentiation (4, 5, 6, 7). To evaluate a possible differentiation-inhibitory effect of plum RAE, the gene expression of Pref-1 was measured at day 4 after differentiation induction to determine possible changes in the early stage of this process (figure 3). Plum RAE had a slight effect over the gene expression of Pref-1. At concentrations of 50 and 250 µM the treated cells exhibited no significant inhibitory effects on Pref-1 gene expression while at 100 µM there was an increase, suggesting a possible cell cycle arrest on the pre-adipocytes at this concentration. To test this possibility, Pref-1 gene expression was measured in the samples at day 10 after differentiation induction and undetectable amount of pref-1 was found (data not shown). This result indicates that the cells in all treatments are fully differentiated and the slight effects over Pref-1 observed after the treatment with plum RAE were not enough to inhibit the differentiation process.

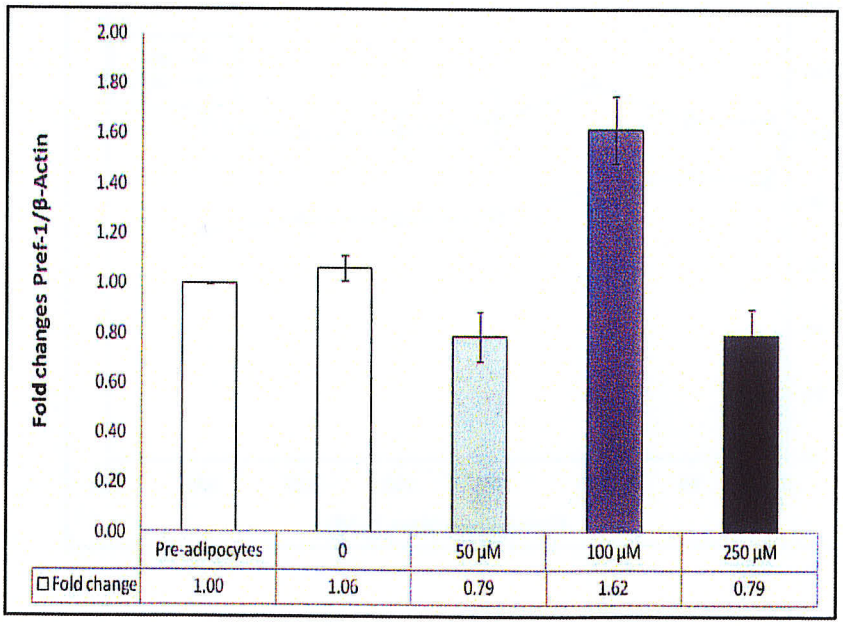


Figure 3. Gene expression of the transcription factor Pref-1 in 3T3-L1 cells after 4 days of treatment with plum RAE. Cells were induced to differentiation (day 0), and incubated with 0, 50, 100 and 250 µM of plum RAE from day 0 to 4 after differentiation induction. Pre-adipocytes were positive controls. Reported values are mean ± S.E (n= 3).

Other important markers for the initiation of differentiation process are c/EBPα and Pparγ. Pparγ is a very important transcription factor in adipocytes, responsible of the induction of many genes involved in lipid metabolism and glucose uptake and metabolism. This transcription factor is able to activate c/EBPα to initiate the differentiation process in pre-adipocytes. c/EBPα and Pparγ are key adipogenic factors, because they can activate the expression of hundreds of genes responsible for terminal adipocytes differentiation (8). At day 4 after differentiation induction, we found a significant increase on the protein expression of transcription factor Pparγ in all tested concentrations (figure 4). The same behavior was observed for Pparγ gene expression, but without significant changes. This result could be directly correlated with the slight increase observed in the protein expression of c/EBPα (figure 5). At 100 µM highest gene and protein expression levels were observed for Pparγ and c/EBPα, respectively.

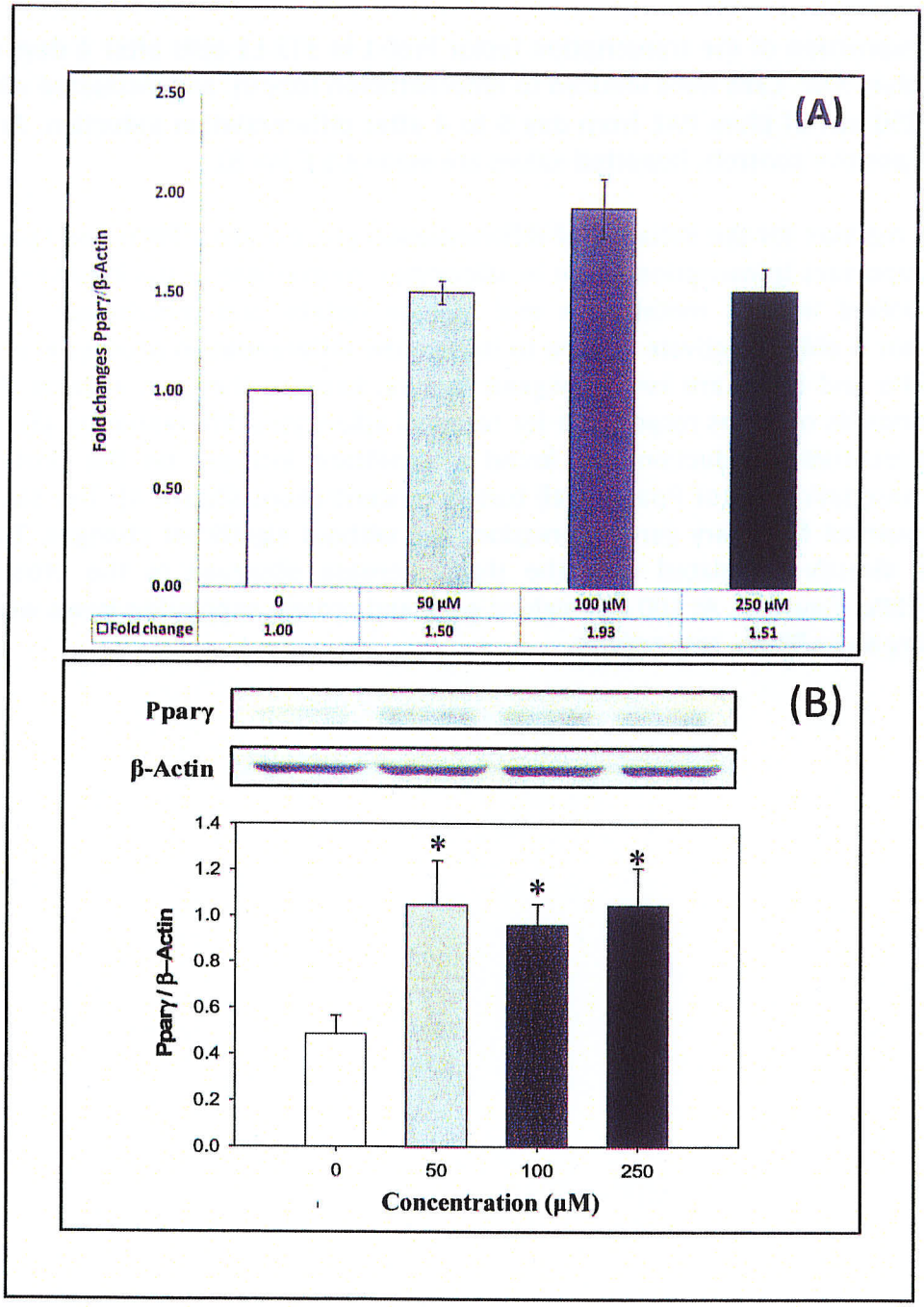


Figure 4. Expression of the transcription factor Pparγ in 3T3-L1 cells after 4 days of treatment with plum RAE. Cells were induced to differentiation (day 0), and incubated with 0, 50, 100 and 250 µM of plum RAE from day 0 to 4 after differentiation induction. Gene and protein expression are shown in (A) and (B), respectively. Reported values are mean ± S.E (n= 3). * Significantly different compared to the control, performed by t-test ($P \leq 0.05$).

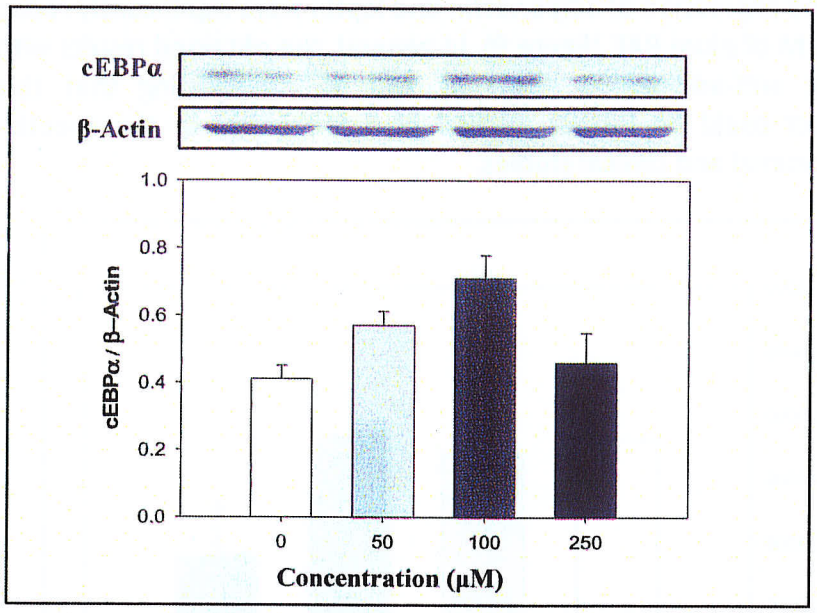


Figure 5. Protein expression of the transcription factor c/EBPα in 3T3-L1 cells after 4 days of treatment with plum RAE. Cells were induced to differentiation (day 0) and incubated with 0, 50, 100 and 250 μM of Plum RAE from day 0 to 4 after differentiation induction. Reported values are mean ± S.E (n= 3).

Effects of Plum RAE on maturation and lipid accumulation of 3T3-L1 cells

In mature adipocytes, many genes related with the lipid and glucose uptake and metabolism are highly expressed, and its expression is carefully regulated to maintain the cell metabolism homeostasis (9). To study the effect of plum RAE over adipocytes metabolism and fat storage, intracellular lipid accumulation was measured using the adipoRed assay. Plum RAE showed a significant reduction on lipid accumulation at 250 μM (figure 6). Lower concentrations do not affect significantly the intra-cellular lipid accumulation, neither the expression of the other markers measured for this study. The reduction on lipid accumulation at 250 μM could be correlated with the significant reduced levels on protein expression of c/EBPα and Pparγ (figures 7and 8A). The reduction on these transcription factors, are likely decreasing the glucose uptake and consequently decreasing lipid accumulation. Similar to c/EBPα and Pparγ, the protein expression of Pparα was also reduced (figure 8B).

On the other hand, mature adipocytes are able to synthesize several adipokines to regulate the metabolism of cells in other organs of the body (10). Two of the most important adipokines secreted by mature adipocytes are adiponectin and leptin. Some functions of adiponectin are related with insulin-sensitizing and stimulation of fatty acids oxidation, while leptin acts like a satiety factor regulating body weight by suppression of appetite and stimulating the energy expenditure (11). Similarly to the transcription factors c/EBPα, Pparγ

and Pparα, the gene expression of adiponectin and leptin were significantly reduced on cells treated with 250 µM of plum RAE (figure 9). In general, the obtained results with plum RAE indicate a strong anti-adipogenic effect at 250 µM, suggesting that this enriched anthocyanin extract could be further studied as a preventive or therapeutic treatment against obesity in animal and clinical studies.

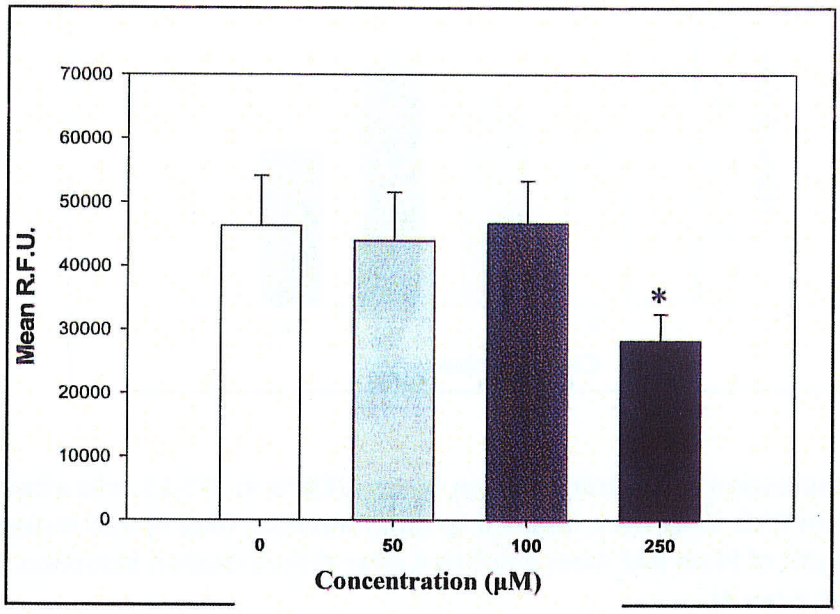


Figure 6. Effect of plum RAE on lipid content in mature 3T3-L1 cells. Cells were incubated with 0, 50, 100 and 250 µM of plum RAE from day 0 to 10 after differentiation induction. Reported values are mean ± S.E of relative fluorescent units obtained using the AdipoRed assay as described in materials and methods (n= 9). * Significantly different compared to the control, performed by t-test ($P \leq 0.05$).

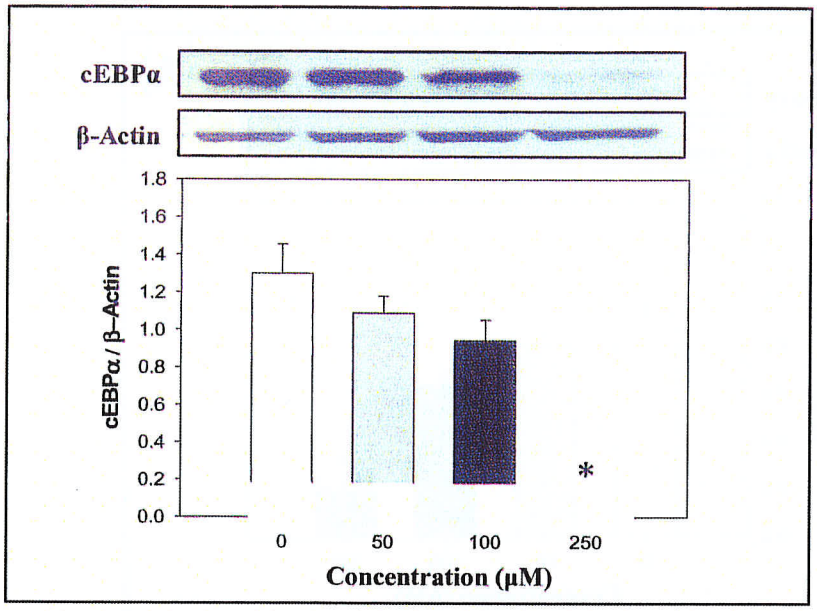


Figure 7. Protein expression of the transcription factor c/EBPα in 3T3-L1 cells after 10 days of treatment with plum RAE. Cells were induced to differentiation (day 0), and incubated with 0, 50, 100 and 250 μM of plum RAE from day 0 to 10 after differentiation induction. Reported values are mean ± S.E (n= 3). * Significantly different compared to the control, performed by t-test ($P \leq 0.05$).

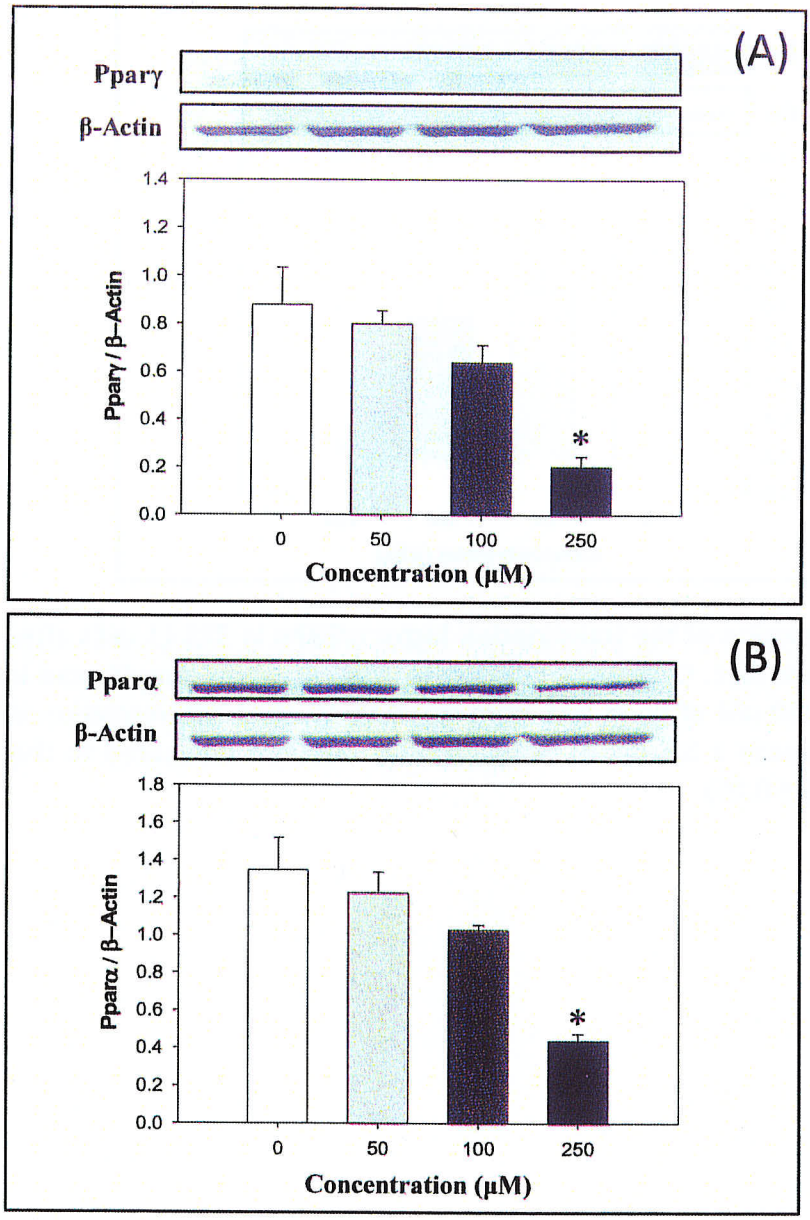


Figure 8. Protein expression of the transcription factor Pparγ (A) and Pparα (B) in 3T3-L1 cells after 10 days of treatment with plum RAE. Cells were induced to differentiation (day 0), and incubated with 0, 50, 100 and 250 μM of plum RAE from day 0 to 10 after differentiation induction. Reported values are mean ± S.E (n= 3). * Significantly different compared to the control, performed by t-test ($P \leq 0.05$).

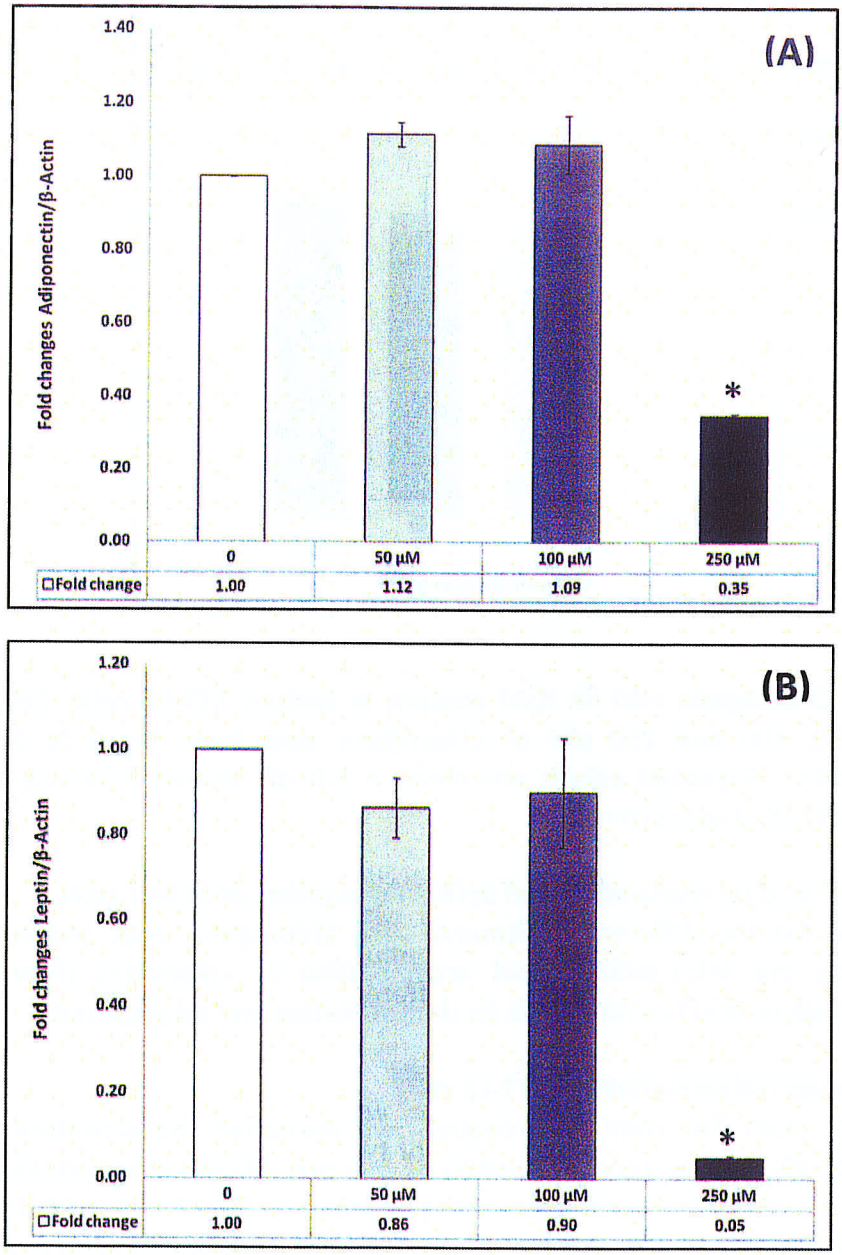


Figure 9. Gene expression of the adipokines Adiponectin (A) and Leptin (B) in 3T3-L1 cells after 10 days of treatment with plum RAE. Cells were induced to differentiation (day 0), and incubated with 0, 50, 100 and 250 µM of plum RAE from day 0 to 10 after differentiation induction. Reported values are mean ± S.E (n= 3). *Significantly different compared to the control, performed by t-test ($P \leq 0.05$).

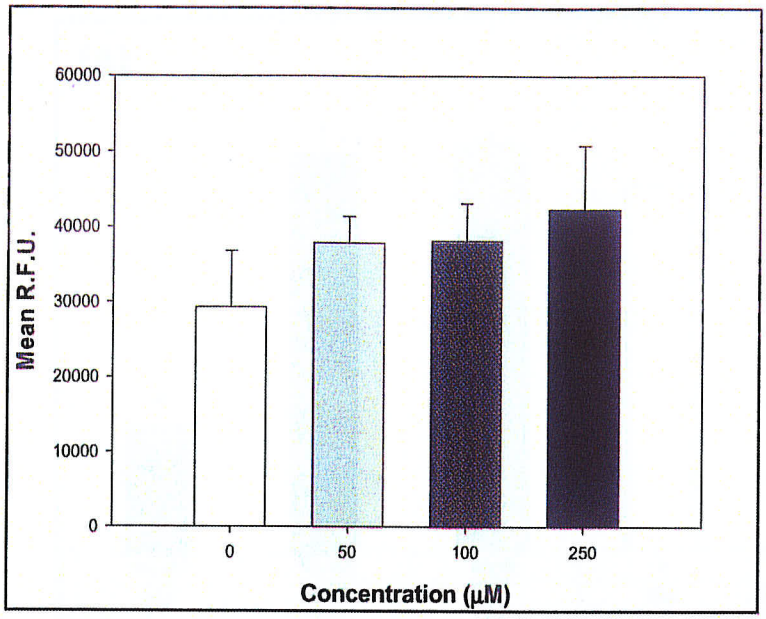


Figure 10. Effect of Chlorogenic acid on lipid content in mature 3T3-L1 cells. Cells were incubated with 0, 50, 100 and 250 µM of Chlorogenic acid from day 0 to 10 after differentiation induction. Reported values are mean ± S.D of relative fluorescent units obtained using the AdipoRed assay (n= 9).

Effects of Chlorogenic acid on maturation and lipid accumulation of 3T3-L1 cells

The results obtained for the different treatments with Chlorogenic acid, showed non-significant effects on the intra-cellular lipid accumulation in adipocytes (Figure 10), suggesting that chlorogenic acid has no effects on differentiation and adipogenesis.

Effects of plum RAE over inflammation on 3T3-L1 cells

To test the effect of plum RAE over inflammation, we measured the activation of the transcription factor NFκB and the gene expression of two pro-inflammatory cytokines IL-6 and TNF-α. We also measured the gene expressions of enzymes COX-2 and iNOS. In figure 11, are presented changes on protein expression of NFκB, and its activated form p-NFκB. In control cells, LPS induced a significant increase in p-NFκB. Cells treated with plum RAE, showed a concentration-dependent protective effect against LPS and at 100 and 250 µM we found a significant reduction in the amount of p-NFκB. No significant differences were found at 50 µM. Gene expression results for IL-6 and TNF-α were in agreement with the reduction on the levels of p-NFκB, showing a significant reduction at all the tested concentrations compared to the control (figure 12C and D, respectively). Similarly, the gene expression for enzyme COX-2 was reduced (figure 12A), suggesting a possibly decrease on Prostaglandin E_2 (PGE_2) production. This is a well studied inflammatory mediator, which synthesis is induced by TNF-α through the activation of COX-2 (12).

iNOS is an enzyme that catalyzes the production of nitric oxide (NO) which activation is regulated by TNF-α through the NFκB pathway. TNF-α is able to induce the expression of iNOS (with a subsequent increase in NO production) in various tissues, including adipose tissue (13). Like COX-2, the gene expression of iNOS was reduced in cells treated with plum RAE (figure 12B). This reduction on COX-2 and iNOS gene expression could be directly related to the TNF-α gene expression decrease. These important anti-inflammatory effects observed on the cells treated with plum RAE could be potentially useful to develop a new strategy of treatment for different pathologies related to chronic inflammation, like in metabolic syndrome.

Effects of Chlorogenic acid over inflammation on 3T3-L1 cells

In our study, we found interesting effects over NFκB protein expression and activation. On cells treated with 50, 100 and 250 μM of Chlorogenic acid and exposed to LPS, a significant decrease was observed in the amount of p-NFκB compared with control cells exposed to LPS (figure 13). Cells treated with Chlorogenic acid, but non-exposed to LPS showed no significant changes compared with the control cells without LPS. In addition, the expression of p-NFκB between the samples treated with Chlorogenic acid with and without LPS co-incubation is very similar, without significant changes. The effects over gene expression of COX-2 and IL-6 are shown in figure 14A and 14C, respectively, and no significant differences were found.

On iNOS gene expression, we observed a significant decrease in cells treated with 50 and 250 μM of Chlorogenic acid and exposed to LPS. Finally, we observed a significant decrease on the expression of TNF-α at 100 μM in presence of LPS. In general, the results observed for the Chlorogenic acid treatments are not as clear compared to those observed with plum RAE. However, these results indicated that Chlorogenic acid is able to act like a regulator molecule of transcription factor NFκB, and its effect is strong enough to keep similar levels to control cells, even in the presence of LPS. Like in the present study, other researchers observed similar results with Chlorogenic acid treatments in other cell lines, but the mechanisms or intracellular pathways involved in the response still remain unclear (14, 15).

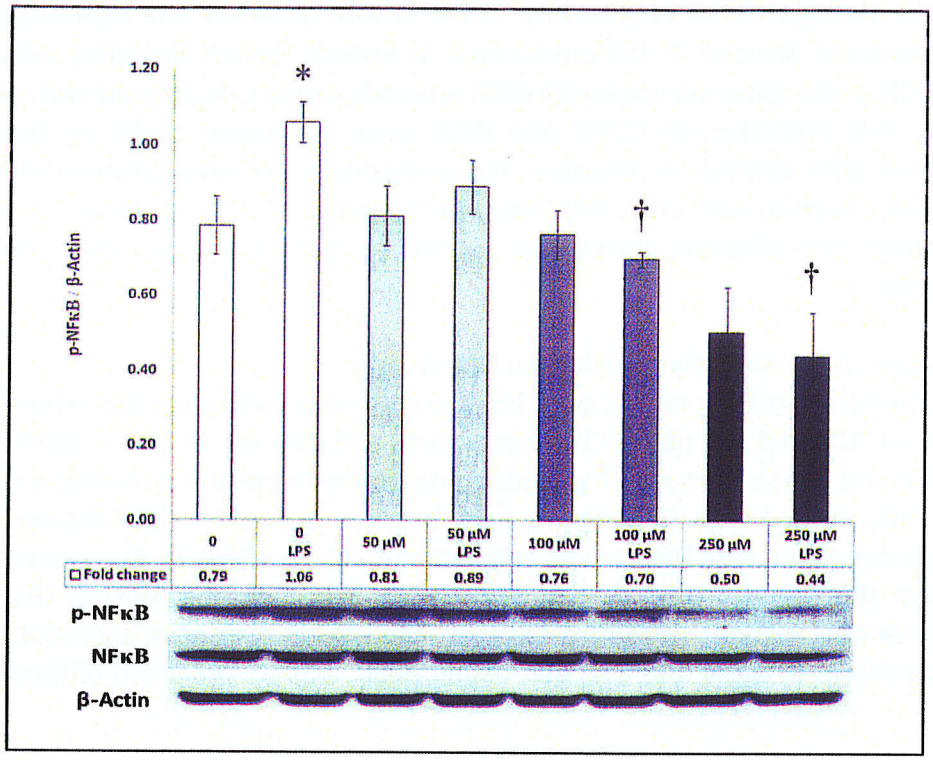


Figure 11. Effect of plum RAE on protein expression of NFκB. Mature 3T3-L1 adipocytes were treated for 72 h with 0, 50, 100 and 250 μM of plum RAE and co-incubated with 100 ng/mL of LPS for the last 1 h. Results are expressed as mean ± S.E. (n= 3). *Significantly different between samples with and without LPS ($P \leq 0.05$), by t-test. † Significantly different between control and treatments with LPS ($P \leq 0.05$), by t-test.

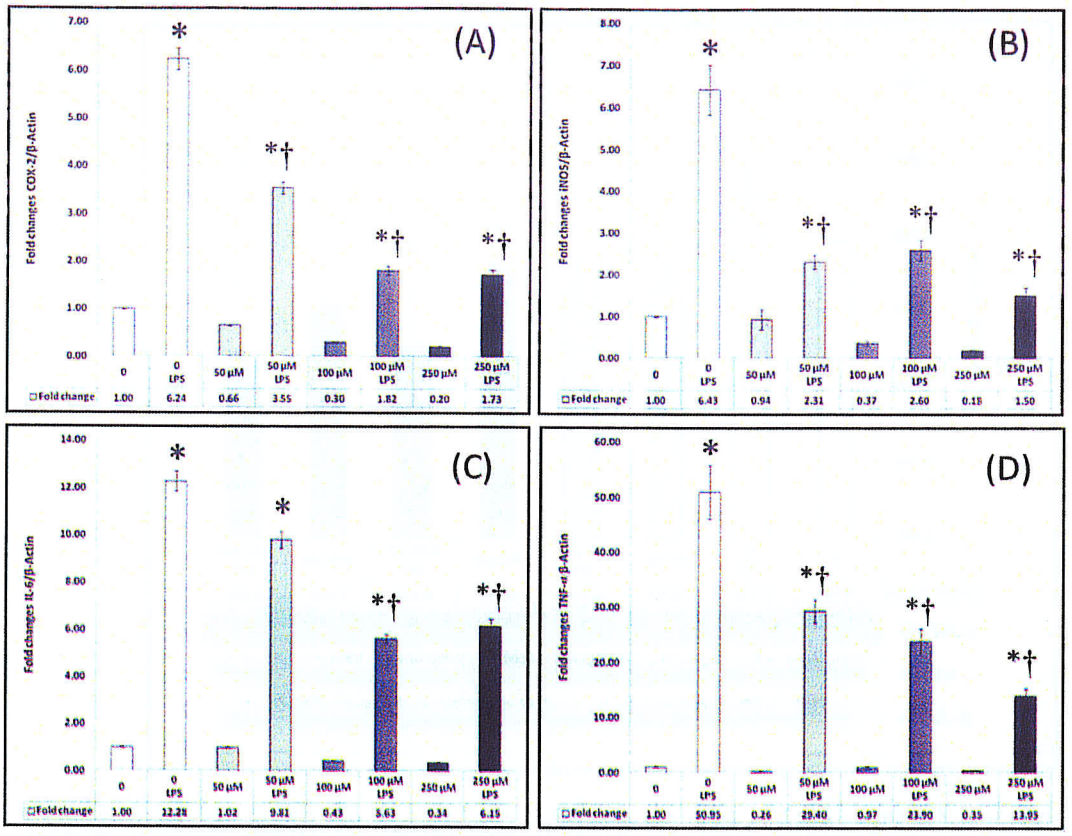


Figure 12. Gene expression for several inflammation markers after 72 h of treatment with plum RAE. Gene expression by qPCR of COX-2 (A), iNOS (B), IL-6 (C) and TNF-α. Results are mean ± S.E. (n= 3). *Significantly different between the samples with and without LPS ($P \leq 0.05$), by t-test. † Significantly different between control and treatments with LPS ($P \leq 0.05$), by t-test.

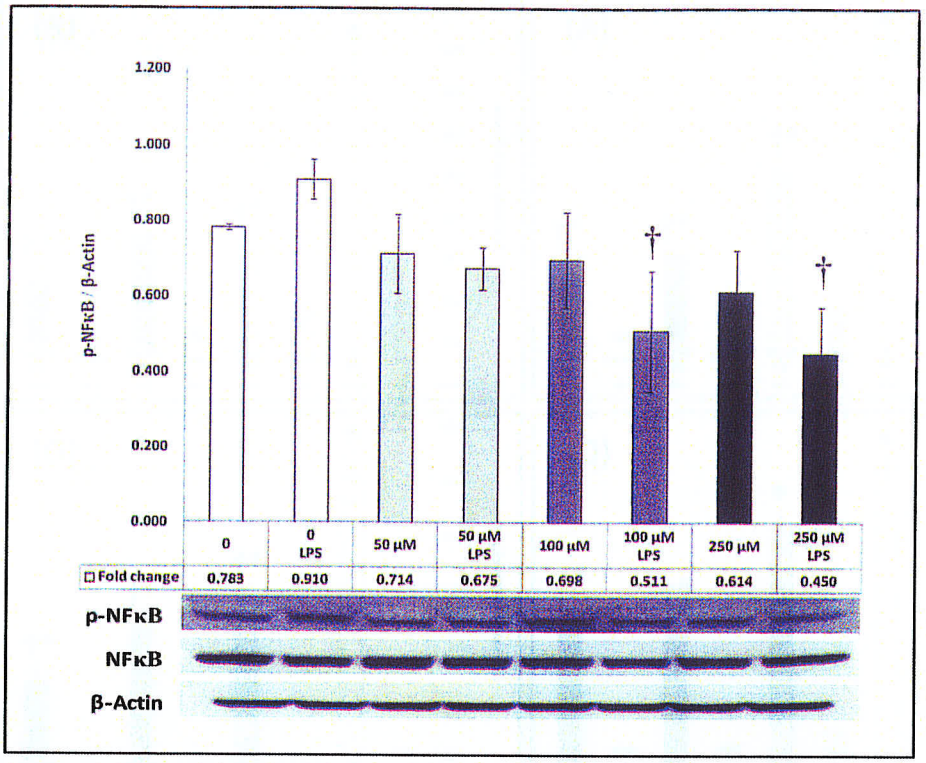


Figure 13. Effect of Chlorogenic acid on protein expression of NFκB. Mature 3T3-L1 adipocytes were treated for 72 h with 0, 50, 100 and 250 µM of Chlorogenic acid and co-incubated with 100 ng/mL of LPS for the last 1 h. Results are expressed as mean ± S.E. (n= 3). † Significantly different between control and treatments with LPS ($P \leq 0.05$), by t-test.

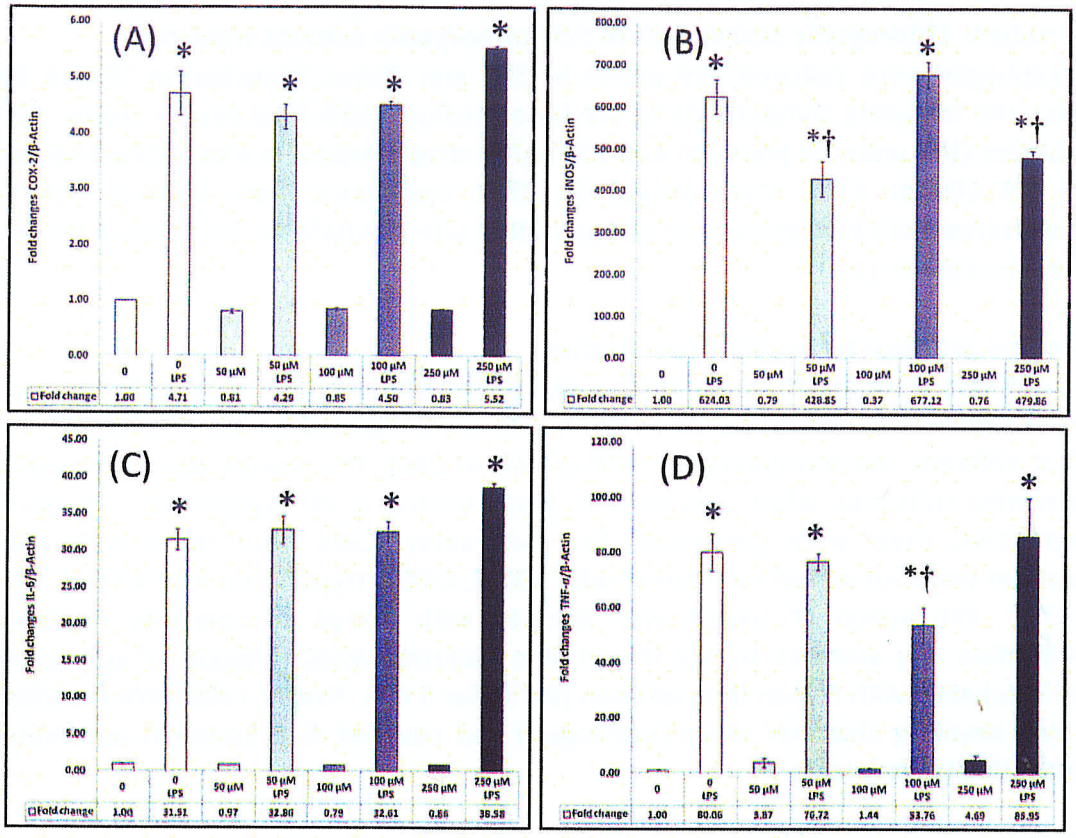


Figure 14. Gene expression for several inflammation markers after 72 h of treatment with Chlorogenic acid. Gene expression by qPCR of COX-2 (A), iNOS (B), IL-6 (C) and TNF-α. Results are mean ± S.E. (n= 3). *Significantly different between the samples with and without LPS ($P \leq 0.05$) by t-test. † Significantly different between control and treatments with LPS ($P \leq 0.05$) by t-test.

3) Effect of a Plum Rich anthocyanin extract and Chlorogenic Acid on Inflammation in Macrophages

Materials& Methods

Materials

The Macrophages RAW 264.7 (cell line TIB-71™) was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cell culture reagents were purchased from Sigma Chemical Co., St. Louis, MO. Chlorogenic acid was obtained from MP Biomedicals (Solon, Ohio). Plum rich anthocyanin extract (RAE) was obtained in our laboratory, and the stock solutions were prepared by dissolving it in dimethyl sulfoxide (Sigma Chemical Co., St. Louis, MO) and used immediately.

Cell culture, Chlorogenic acid and plum rich anthocyanin extracts treatment

Macrophages were cultured and grown in 100 mm dishes (Nunclon) in DMEM (Sigma), adding the following components to the base medium: 10% fetal bovine serum (FBS) and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin) in a humidified atmosphere with 5% CO_2 at 37°C. Macrophages at 100% confluence were treated with various concentrations of Chlorogenic acid and plum RAE (20–100 µM) for 24 h before the LPS (100 ng/mL) treatment (1 h).

Cell count protocol for proliferation studies

Macrophages were plated in Multidish 6 well plates (Nunclon) at 6×10^4 cells per well; they were maintained in culture for 24 h. After, cells were exposed for 24, 48 and 72 h to plum RAE at different concentrations (0 – 400 µmol/L of CAE). The powder extract was dissolved in dimethyl sulfoxide, which was used as blank control in all experiments. At least three independent trials were performed for each assay. Cells from three wells for each treatment were harvested by treating with 500 µL 0.05% trypsin and 0.02% EDTA for 6 min at 37 °C. The action of trypsin was stopped with 500 µL of complete medium. Cell proliferation was assessed by the trypan blue exclusion assay. Aliquots of cell suspension were incubated with trypan blue solution (0.4%) for 5 min. Finally, cells were transferred to the cell counting chamber slides (Invitrogen) and counted in a Countess automated cell counter (Invitrogen, USA).

RNA extraction and reverse transcription

Total cellular RNA was extracted from macrophages after treatment for 24 h and LPS addition for 1 h, using the RNeasy mini kit (Qiagen, Valencia, CA). RNA concentration was measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Willmington, DE). For cDNA synthesis, 1 µg RNA was reverse-transcribed into cDNA using the SuperScript III first-strand synthesis supermix (Invitrogen, Carlsbad, CA), following the manufacturers protocol. Every cDNA samples were stored at -20°C until further analysis.

Real-time quantitative RT-PCR

Gene expression in macrophages was quantified by real-time quantitative RT-PCR using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), following the manufacturer's instructions. DNA amplification was carried out using a 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA). The primer sets are listed in Table 1 and were provided by Integrated DNA Technologies (IDT, Coralville, IA). The relative expression of each gene was normalized by ⍰-Actin, and was calculated following the comparative Ct method (ΔΔCt), also known as the $2^{-\Delta\Delta Ct}$ method.

Table 1. Sequences of primers used in gene expression studies.

Primer	Sequence
COX2-F	5'- ACATCGATGTCATGGAACTG -3'
COX2-R	5'- GGACACCCCTTCACATTATT -3'
IL-1□-F	5'- GAGCACCTTCTTTTCCTTCATCT -3'
IL-1□-R	5'- GATATTCTGTCCATTGAGGTGGA -3'
iNOS-F	5'- ACATCGACCCGTCCACAGTAT -3'
iNOS-R	5'- CAGAGGGGTAGGCTTGTCTC -3'
TNF-□-F	5'- ACTGGCAGAAGAGGCACTCC -3'
TNF-□-R	5'- CGATCACCCCGAAGTTCA -3'
□-Actin-F	5'- CCCAGGCATTGCTGACAGG -3'
□-Actin-R	5'- TGGAAGGTGGACAGTGAGGC -3'

Western immunoblotting

The macrophages were harvested with a cell lysis buffer (Cell signaling), following the manufacturer's instructions. Each well was washed with ice cold PBS (Sigma), added 100 μL lysis buffer containing a protease inhibitor cocktail. Cells were then scraped, left incubated at -80 ºC overnight, centrifuged at 14,000 rpm at 4 ºC for 15 min and an aliquot from the supernatant was stored at -80 ºC. The total protein was quantified using the BCA Protein Assay Kit (Pierce, Thermo Fisher Scientific, Inc., Rockford, IL) and 50 μg of protein were loaded on a gel with a pre-stained, broad-range, molecular weight protein marker (Fermentas). Proteins were fractionated by electrophoresis using 10% polyacrylamide gels. The gels were transferred by wet blotting onto PVDF membranes (Millipore). The membranes were blocked with 5% skim milk in Tris-buffered saline with 1% Tween-20 (TBS-T) for 1,5 h with gentle shaking; four times 5 min washes with TBST were performed consecutively. Membranes were then incubated with a specific primary antibody against p-NFkB (1:2000) and b-Actin (1:5000). The membranes were washed four times with TBS-T and incubated for 1 h with the secondary antibody conjugated with horseradish peroxidase (HRP) at 1:7000 dilution. Specific bands were developed using a SuperSignal West Femto enhanced chemiluminescence (ECL) Western blotting detection kit (Pierce, Thermo Fisher Scientific, Inc., Rockford, IL) after a 60 s incubation where the signals were captured by a CCD Camera (Cascade II:512, Photometrics, Tucson, AZ) using the WinView/32 software (Version 2.5, Princeton Instruments, Trento, NJ). Bands were measured and quantified using densitometry with ImageJ software (NIH, Bethesda, MD)

Results & Discussions

Effects of a plum rich anthocyanin extract on proliferation of Macrophage

To assess the effect on proliferation and determine the IC_{50} exerted by plum RAE on macrophages, cells were treated with increasing concentrations (20–400 µM) for 24, 48 and 72 h. Results showed a dose-dependent proliferation inhibition of Macrophages with cytotoxic effects at concentrations only above 100 □M plum RAE (Figure 1). The IC_{50} was 200

□M and the RAE concentrations selected for the inflammation experiments were in the range 20-100 □M.

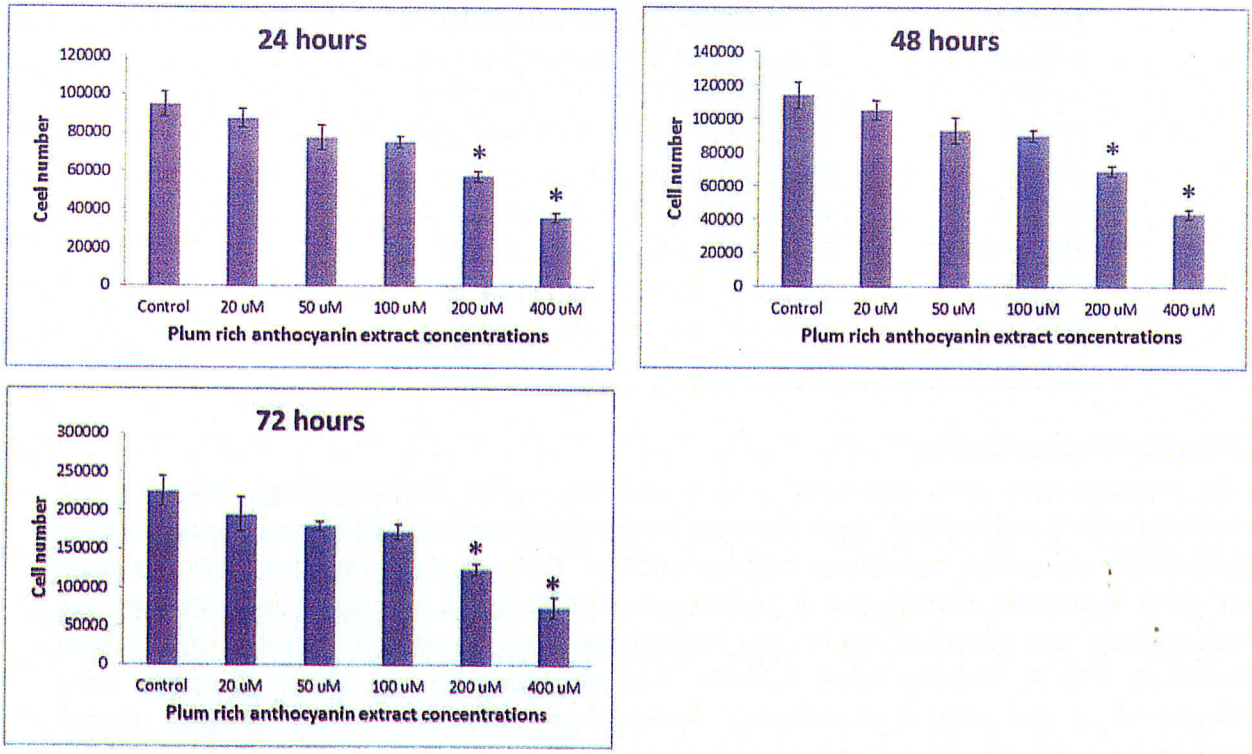


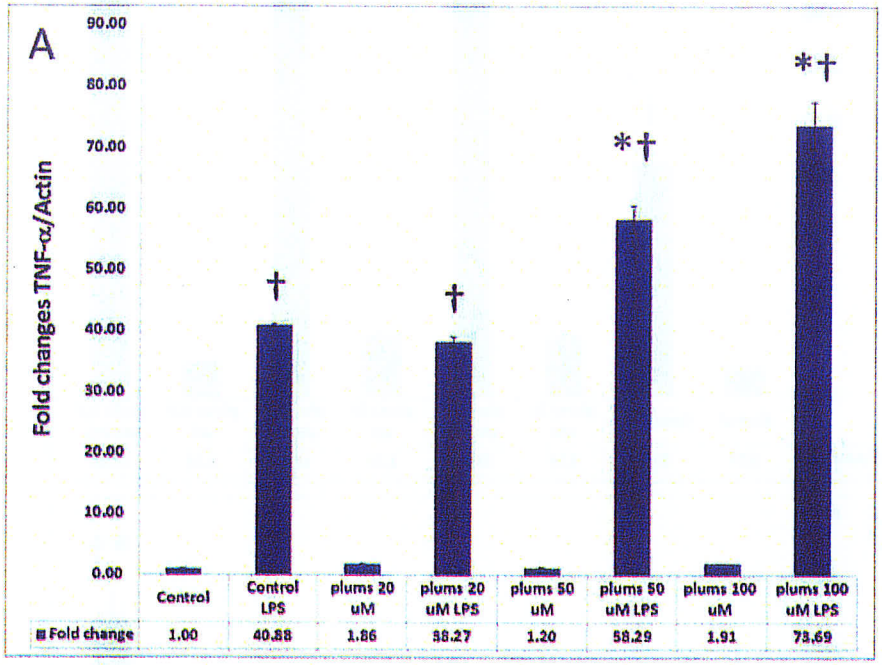
Figure 1: Plum rich anthocyanin extract effects on proliferation of Macrophages RAW 264.7. Reported viable cell numbers measured with the trypan blue assay after 24, 48 and 72 h incubation. All data are shown as mean ± SE values. * Significantly different compared to the control ($P<0.05$) by t-student.

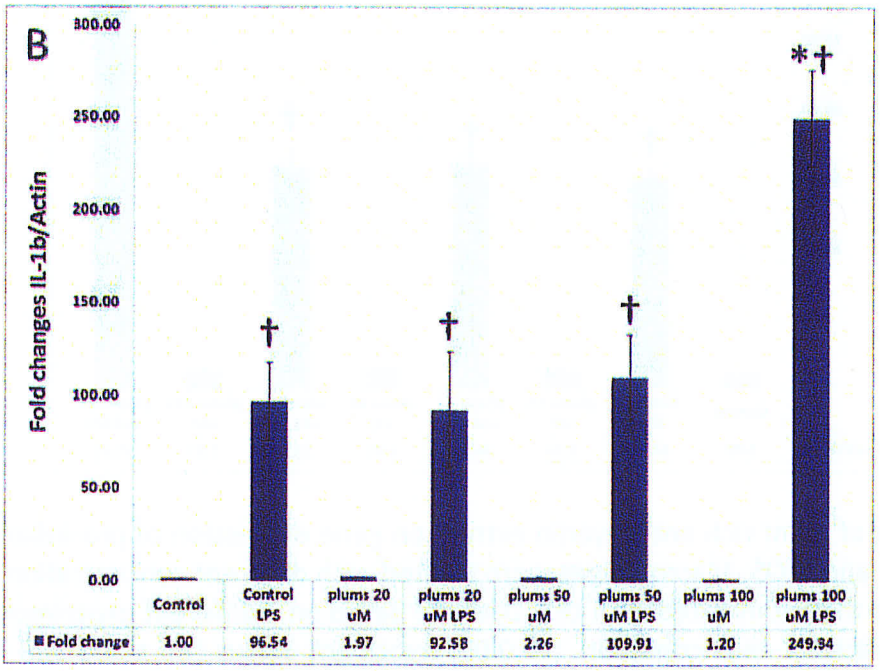
Effects of a plum RAE and Chlorogenic acid on gene expression

Macrophages are versatile cells that play many roles. As scavengers, they rid the body of worn-out cells and other debris. They are foremost among the cells that "present" antigen, a crucial role in initiating an immune response (1). They activate human granulocytes (neutrophils, eosinophils and basophils) which can lead to acute neutrophilic inflammation (2). They also induce the synthesis and release of other pro-inflammatory cytokines such as interleukin 1 (IL-1) and TNF-α from fibroblasts and macrophages (3).

A known function of the macrophages is when a leukocyte enters damaged tissue through the endothelium of a blood vessel, a process known as the leukocyte extravasation; it undergoes a series of changes to become a macrophage (4, 5). Monocytes are attracted to a damaged site by chemical substances through chemotaxis, triggered by a range of stimuli including damaged cells, pathogens and cytokines released by macrophages already at the site (6). For this reason targeting a decrease in chronic or acute inflammation, will decrease the levels of pro-inflammatory cytokines. To obtain some preliminary insight on the mechanism by which plum rich anthocyanin extract and Chlorogenic acid affect LPS-

stimulated macrophages, we investigated their effects on TNF-α, iNOS, COX2 and IL-1b mRNA levels. Results did not show a decrease in mRNA levels on incubation with different concentration of plum RAE, but an unexpected significant increase in mRNA levels with higher concentrations of plum RAE (Figure 2).





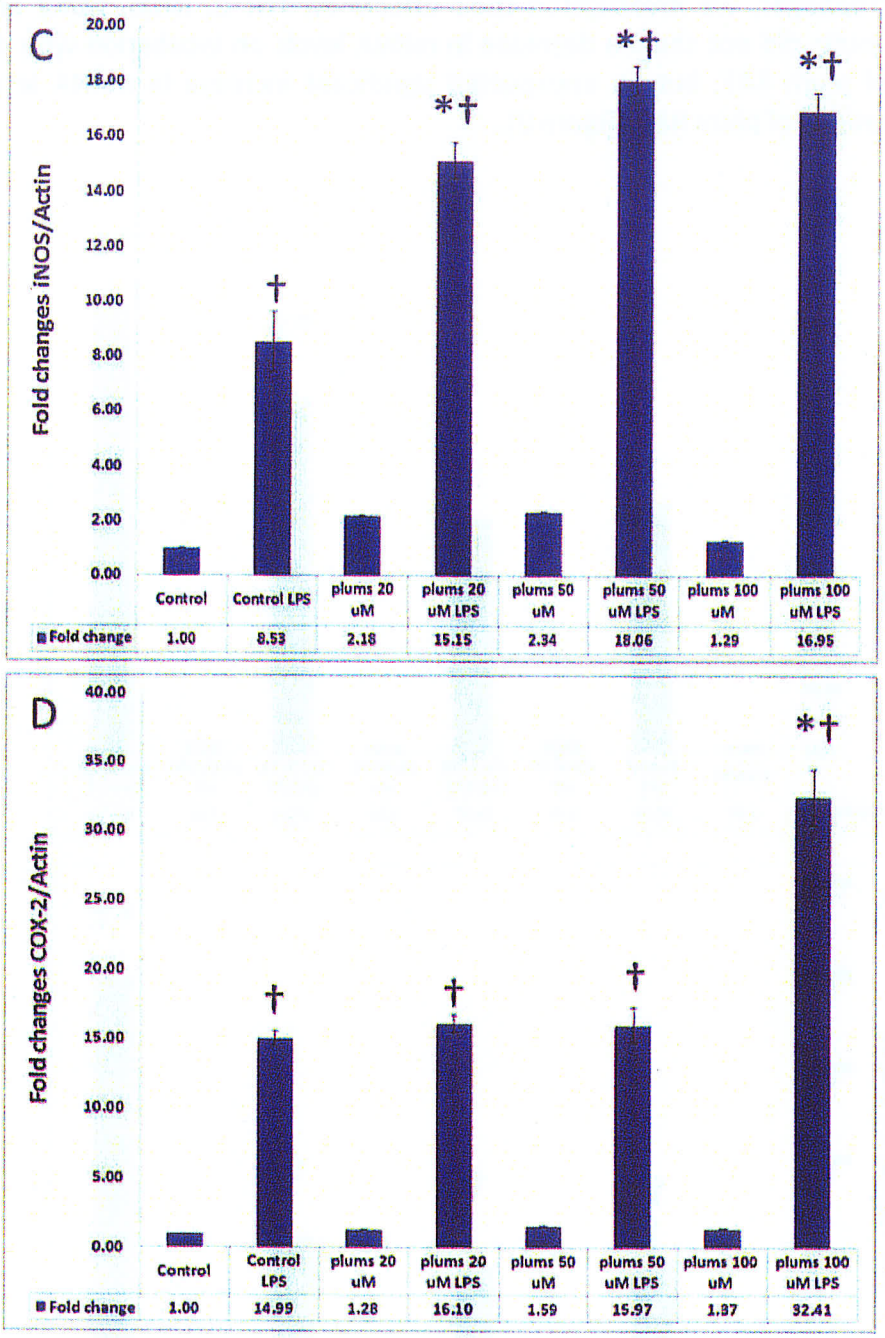
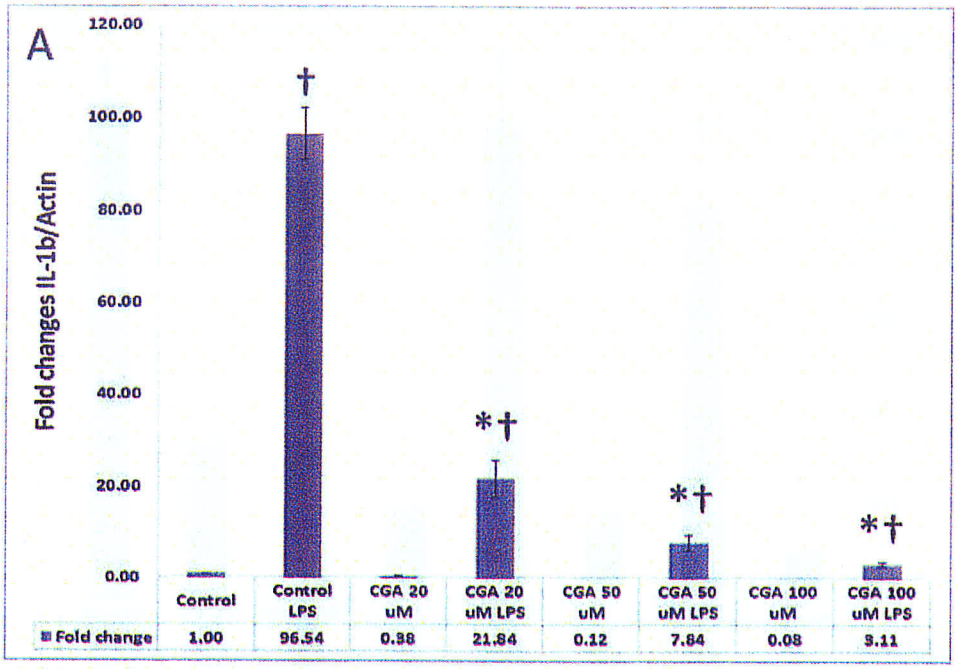


Figure 2. Effects of plum rich anthocyanin extract on gene expression of pro-inflammatory cytokines, COX2 and iNOS. Macrophages were treated with different concentration of plum RAE for 24 h and then stimulated with LPS. Data are based on 3 different experiments, each based on 2 repeats for each condition. *P<0.05, treatment versus LPS alone and †P<0.05, treatment versus treatment plus LPS.


A
Fold changes IL-1b/Actin
120.00
100.00
80.00
60.00
40.00
20.00
0.00
†
*†
*†
*†
Control
Control LPS
CGA 20 uM
CGA 20 uM LPS
CGA 50 uM
CGA 50 uM LPS
CGA 100 uM
CGA 100 uM LPS
Fold change
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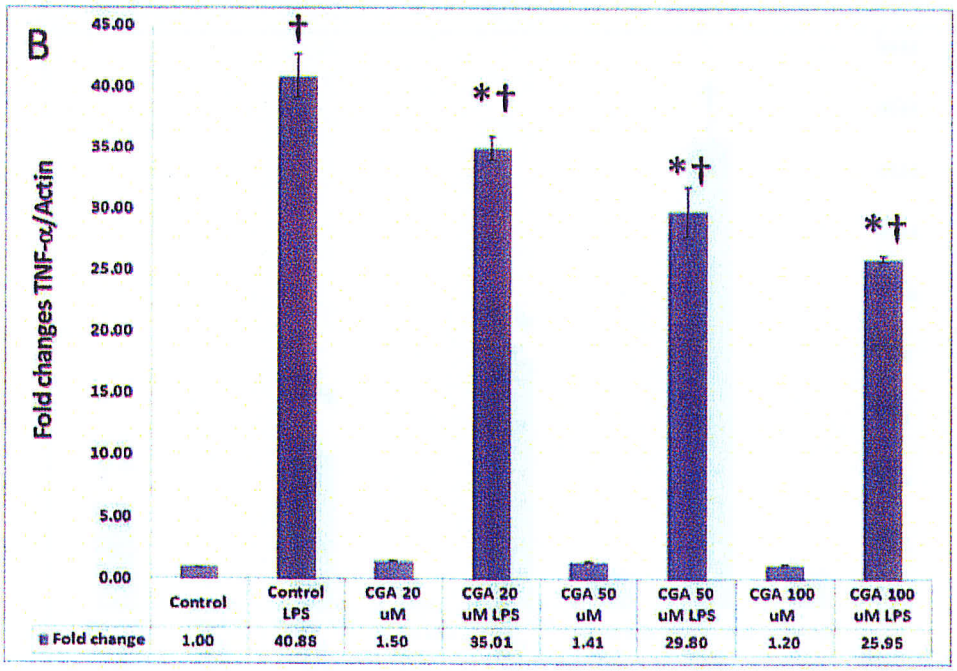
0.38
21.84
0.12
7.64
0.08
3.11


B
Fold changes TNF-α/Actin
45.00
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
†
*†
*†
*†
Control
Control LPS
CGA 20 uM
CGA 20 uM LPS
CGA 50 uM
CGA 50 uM LPS
CGA 100 uM
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Fold change
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1.41
29.80
1.20
25.95

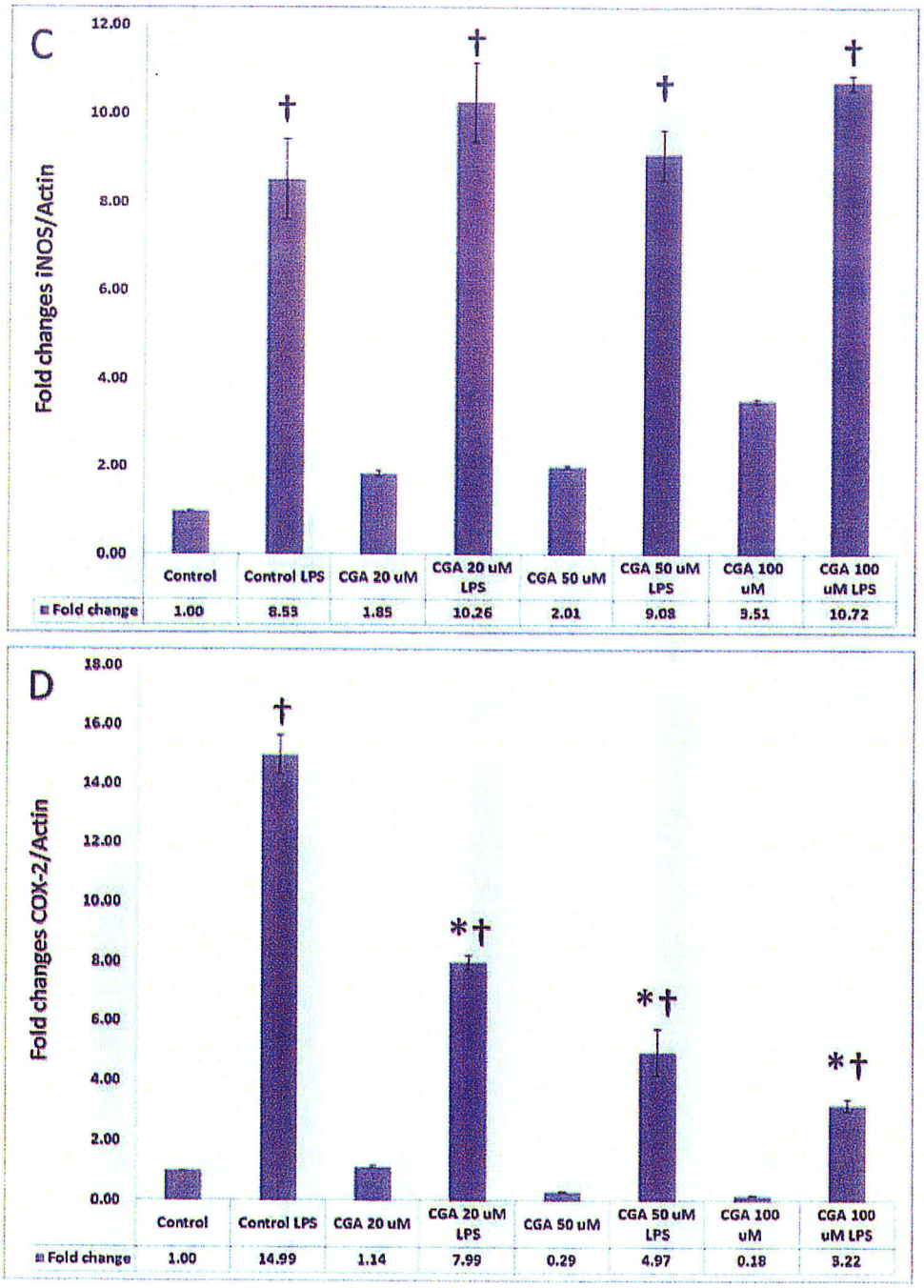


Figure 3. Effects of Chlorogenic acid on gene expression of pro-inflammatory cytokines, iNOS and COX2. Macrophages were treated with different concentration of Chlorogenic acid for 24 h and then stimulated with LPS. Data are based on 3 different experiments, each based on 2 repeats for each condition. *P<0.05, treatment versus LPS alone and †P<0.05, treatment versus treatment plus LPS.

On the other hand, Chlorogenic acid decreased mRNA levels of pro-inflammatory cytokines and COX-2, that functions in the formation of important biological mediators called prostanoids, including prostaglandins, prostacyclin and thromboxane. Pharmacological inhibition of *COX-2* can provide relief from the symptoms of inflammation and pain (7). However, there was no decrease in the mRNA level for iNOS, that functions in making NO (nitric oxide), a molecule that acts as a signal for a variety of cellular functions throughout the body (8), including the triggering of inflammation and dilating of blood vessels (Figure 3). These results indicate that Chlorogenic acid generates positive effect in the gene expression of important biomarkers related to inflammation in macrophages.

Effects of a plum rich anthocyanin extract and Chlorogenic acid in protein expression

Since p65 is a major component in the NFkB complex activation, we examined p65 activation by immunoblotting (Figure 4). Macrophages were incubated with LPS in the presence or absence of plum rich anthocyanin extract and Chlorogenic acid for 1 h. Results from the relative expression of the protein p-NFkB after its exposure to treatments with plum RAE and Chlorogenic acid in Macrophages are shown in Figures 4 and 5. LPS-stimulated Macrophages treated with plum RAE did not show changes in relative protein expression between 20 and 50 □mol/L of RAE, while with 100 □mol/L of RAE there was a significant increase of p-NFkB (Figure 4). LPS-stimulated Macrophages treated with Chlorogenic acid showed a dose dependent decrease in p-NFkB relative protein expression (Figure 5). The decrease in relative expression of the protein p-NFkB is correlated with a decrease in the gene expression of IL-1b, TNF-□ and COX-2. In general, the decrease in transcription factor p-NFkB in LPS-stimulated Macrophages exposed to Chlorogenic acid and the decrease of gene expression of IL-1b, COX-2 and TNF-□ indicate a strong anti-inflammatory effect of this compound present in stone fruits.

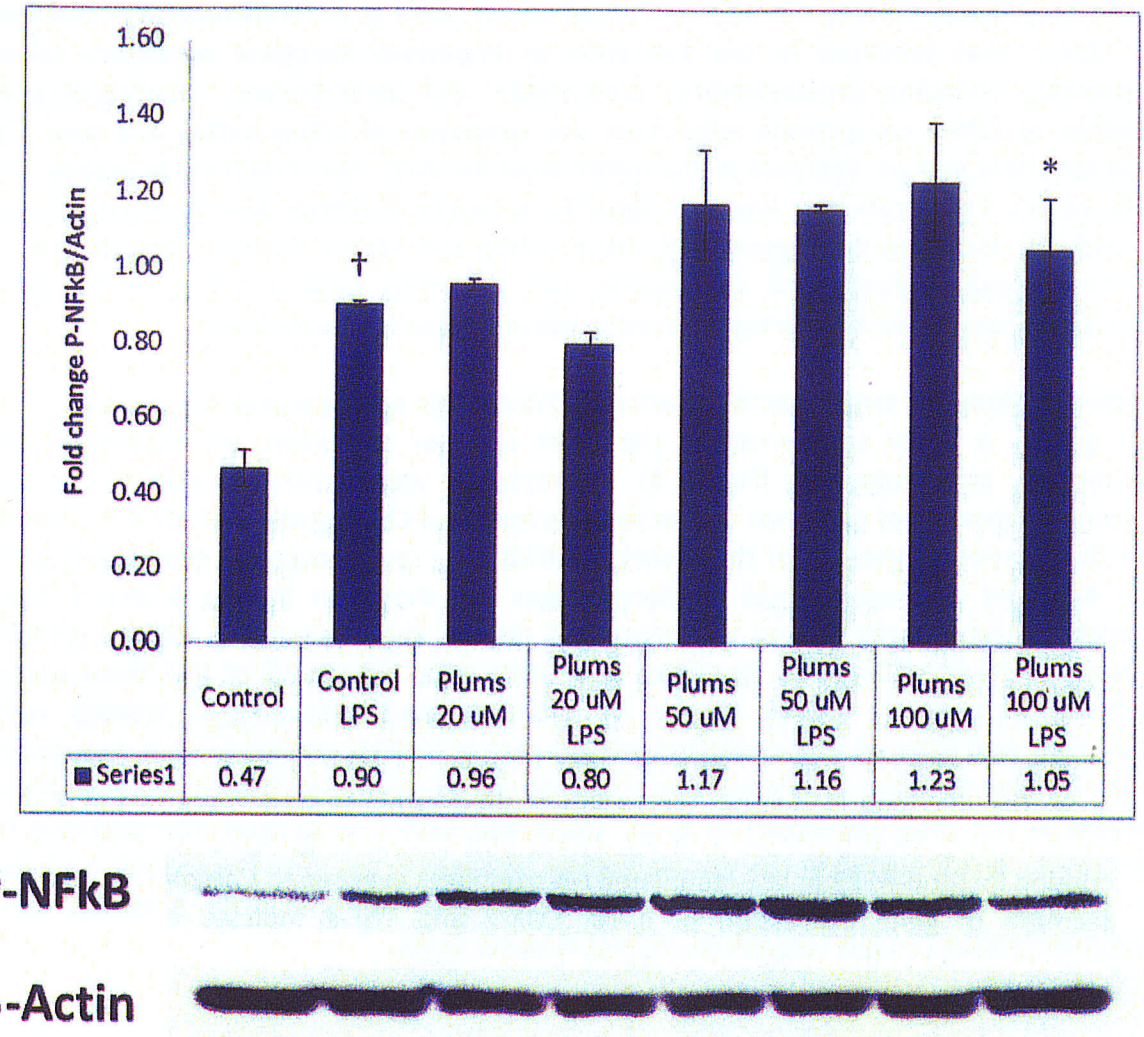


Figure 4. Expression of the transcription factor p-NFkB (p65) at the protein level in Macrophages treated with different concentrations plum rich anthocyanin extract and LPS (100 ng/mL). Data are based on 3 different experiments, each based on 2 repeats for each condition. †$P<0.05$, treatment versus LPS alone and *$P<0.05$, treatment versus treatment plus LPS.

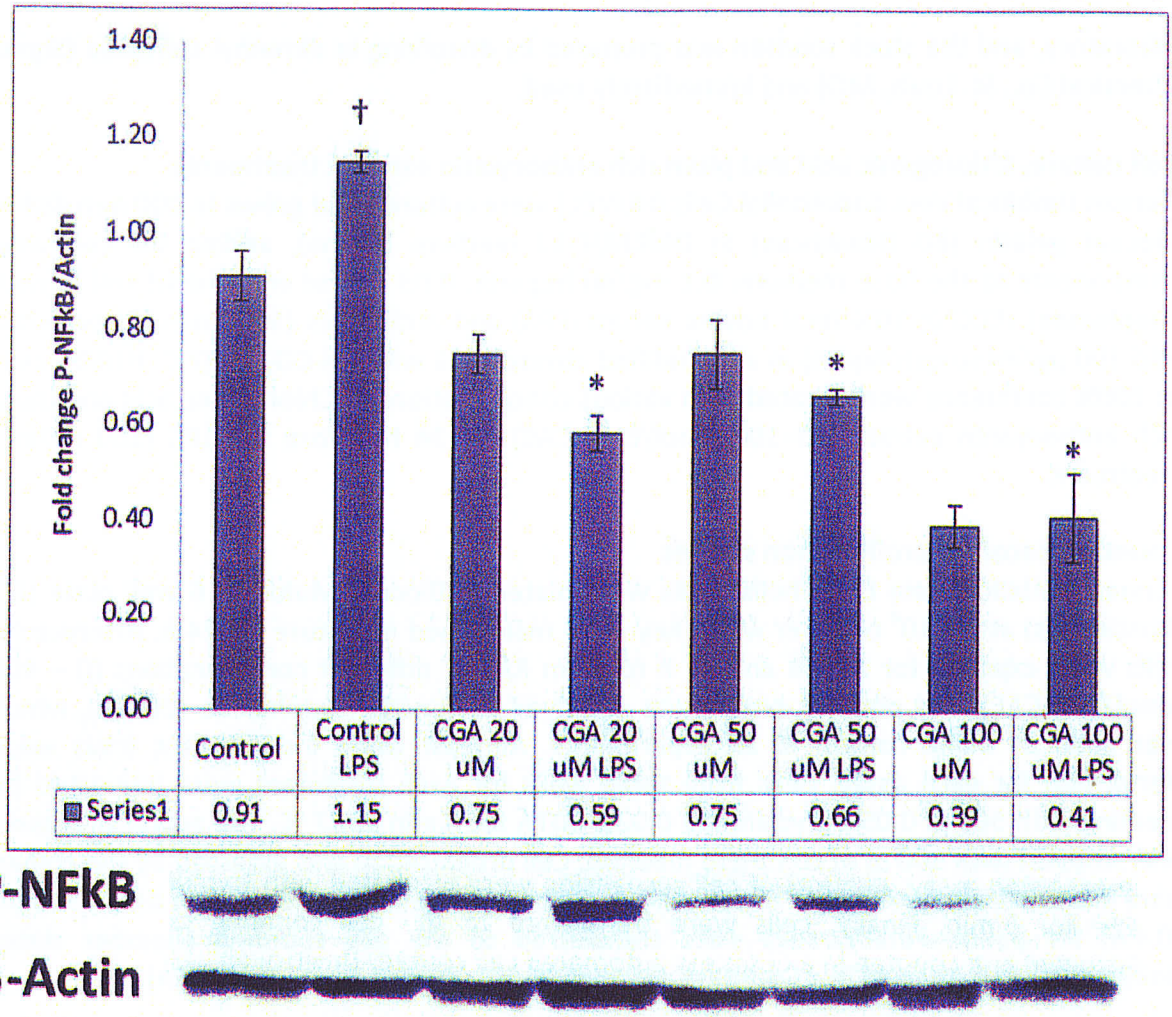


Figure 5. Expression of the transcription factor p-NFkB (p65) at the protein level in Macrophages treated with different concentrations Chlorogenic acid and LPS (100 ng/mL). Data are based on 3 different experiments, each based on 2 repeats for each condition. †$P<0.05$, treatment versus LPS alone and *$P<0.05$, treatment versus treatment plus LPS.

4) Effect of a plum rich anthocyanin extract and Chlorogenic Acid on Inflammation in HUVEC cells

Materials & Methods

Materials

The Human Umbilical Vein Endothelial Cells (cell line CRL-1730™) was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cell culture reagents were purchased from Sigma Chemical Co., St. Louis, MO. Chlorogenic acid was obtained from MP Biomedicals (Solon, Ohio). Plum rich anthocyanin extract (RAE) was obtained in our

laboratory, and the stock solution was prepared by dissolving in dimethyl sulfoxide (Sigma Chemical Co., St. Louis, MO) and immediately used.

Cell culture, Chlorogenic acid and plum rich anthocyanin extracts treatments

Human Umbilical Vein Endothelial Cells (HUVEC) were cultured and grown in 100 mm dishes Biocoat gelatin (BD Biosiences) in DMEM/F-12 medium (Sigma), adding the following components to the base medium: 0.1 mg/ml heparin; 0.03 mg/ml endothelial cell growth supplement (ECGS); 10% fetal bovine serum (FBS) and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin) in a humidified atmosphere with 5% CO_2 at 37°C. HUVEC cells at 100% confluence were treated with various concentrations of Chlorogenic acid and plum rich anthocyanin extract (20–100 µmol/L of CAE) for 24 h before the LPS (100 ng/mL) treatment.

Count protocol for proliferation studies

Human Umbilical Vein Endothelial Cells were plated in Biocoat Multidish 6 well plate (BD Biosciences) at 6×10^4 cells per well; they were maintained in culture for 24 h. Afterwards, cells were exposed for 24, 48 and 72 h to plum RAE at different concentrations (0 – 400 µmol/L of CAE). The powder extract was dissolved in dimethyl sulphoxide (DMSO), which was used as blank control in all experiments. At least three independent trials were performed for each assay. Cells from three wells for each treatment were harvested by treating with 500 µl 0.05% trypsin and 0.02% EDTA for 6 min at 37 °C. The action of trypsin was stopped with 500 µl of complete medium. Cell proliferation was assessed by the trypan blue exclusion assay. Aliquots of cell suspension were incubated with trypan blue solution (0.4%) for 5 min. Finally, cells were transferred to the cell counting chamber slides (Invitrogen) and counted in a Countess automated cell counter (Invitrogen, USA).

RNA extraction and reverse transcription

Total cellular RNA was extracted from HUVEC cells after treatment for 24 h and LPS addition for 1 h, using the RNeasy mini kit (Qiagen, Valencia, CA). RNA concentration was measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Willmington, DE). For cDNA synthesis, 1 μg RNA was reverse-transcribed into cDNA using the SuperScript III first-strand synthesis supermix (Invitrogen, Carlsbad, CA), following the manufacturers protocol. All cDNA samples were stored at -20°C until further analysis.

Real-time quantitative RT-PCR

Gene expression was quantified by real-time quantitative RT-PCR using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), following the manufacturer's instructions. DNA amplification was carried out using a 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA). The primer sets are listed in Table 1 and were provided by Integrated DNA Technologies (IDT, Coralville, IA). The relative expression of each gene was normalized by GAPDH, and was calculated following the comparative Ct method (ΔΔCt), also known as the $2^{-\Delta\Delta Ct}$ method.

Table 1. Sequences of primers used in gene expression studies.

Primer	Sequence
E-Selectin-F	5'- TGCATGGAGGGTTGTTAATGG -3'
E-Selectin-R	5'- GGATGAAAGTGATTAAATTGTGCATAG -3'
ICAM-F	5'- GCTATGCCTTGTCCTCTTG -3'
ICAM-R	5'- ATACACACACACACACACGC -3'
VCAM-F	5'- CAAATCCTTGATACTGCTCATC -3'
VCAM-R	5'- TTGACTTCTTGCTCACAGC -3'
MCP1-F	5'- CAGCCAGATGCAATCAATGC -3'
MCP1-R	5'- GTGGTCCATGGAATCCTGAA -3'
GAPDH-F	5'- GCACCGTCAAGGCTGAGAAC -3'
GAPDH-R	5'- ATGGTGGTGAAGACGCCAGT -3'

Western immunoblotting

The Human Umbilical Vein Endothelial Cells were harvested with a cell lysis buffer (Cell signaling), following the manufacturer's instructions. Each well was washed with ice cold PBS (Sigma), added 100 μL lysis buffer containing a protease inhibitor cocktail. Cells were then scraped, left incubated at -80 ºC overnight, centrifuged at 14,000 rpm at 4 ºC for 15 min and a supernatant aliquot was stored at -80 ºC. The total protein was quantified using the BCA Protein Assay Kit (Pierce, Thermo Fisher Scientific, Inc., Rockford, IL) and 50 μg of protein were loaded on a gel with a pre-stained, broad-range, molecular weight protein marker (Fermentas). Proteins were fractionated by electrophoresis using 10% polyacrylamide gels. The gels were transferred by wet blotting onto PVDF membranes (Millipore). The membranes were blocked with 5% skim milk in Tris-buffered saline with 1% Tween-20 (TBS-T) for 1.5 h with gentle shaking; four times 5 min washes with TBST were performed consecutively. Membranes were then incubated with a specific primary antibody against p-NFkB (1:2000) and b-Actin (1:2000). The membranes were washed four times with TBS-T and incubated for 1 h with the secondary antibody conjugated with horseradish peroxidase (HRP) at 1:7000 dilution. Specific bands were developed using a SuperSignal West Femto enhanced chemiluminescence (ECL) Western blotting detection kit (Pierce, Thermo Fisher Scientific, Inc., Rockford, IL), and after 60 s incubation the signal were captured by a CCD Camera (Cascade II:512, Photometrics, Tucson, AZ) using the WinView/32 software (Version 2.5, Princeton Instruments, Trento, NJ). Bands were measured and quantified using densitometry with ImageJ software (NIH, Bethesda, MD)

Results & Discussions

Effects of a plum rich anthocyanin extract on proliferation of HUVEC cells

To assess the effect on proliferation and determine the IC_{50} exerted by plum RAE on HUVEC, cells were treated with increasing RAE concentrations (20–400 µM) for 24, 48 and 72 h. Results showed a dose-dependent inhibition of proliferation with cytotoxic effects only at concentrations above 100 ?M of plum RAE (Figure 1). The obtained IC_{50} was 200 ?M and the selected range of RAE for further LPS-stimulated HUVEC inflammation studies was 20-100 µM CAE.

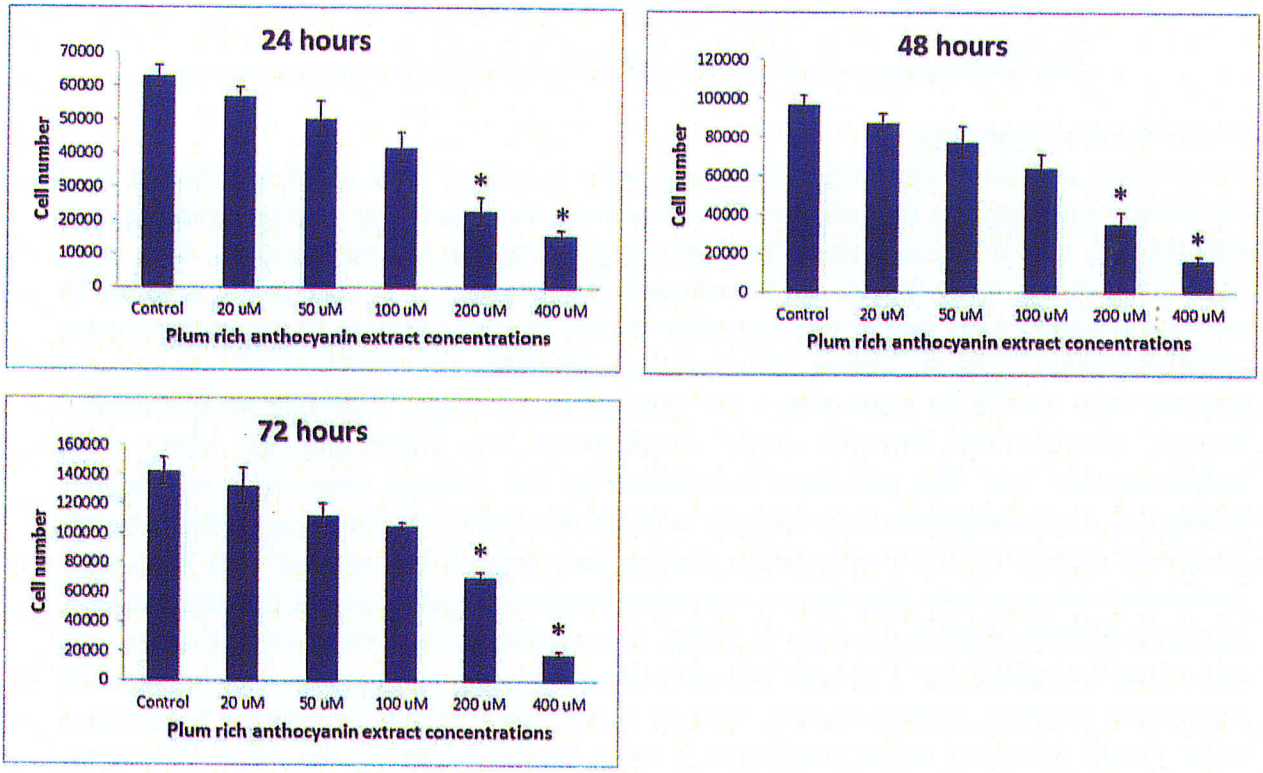


Figure 1: Plum rich anthocyanin extract effects on proliferation of human umbilical vein endothelial cells. Viable cell numbers measured by the trypan blue assay after 24, 48 and 72 h. All data are shown as mean ± SE values. *P<0.05, signicantly different compared to control.

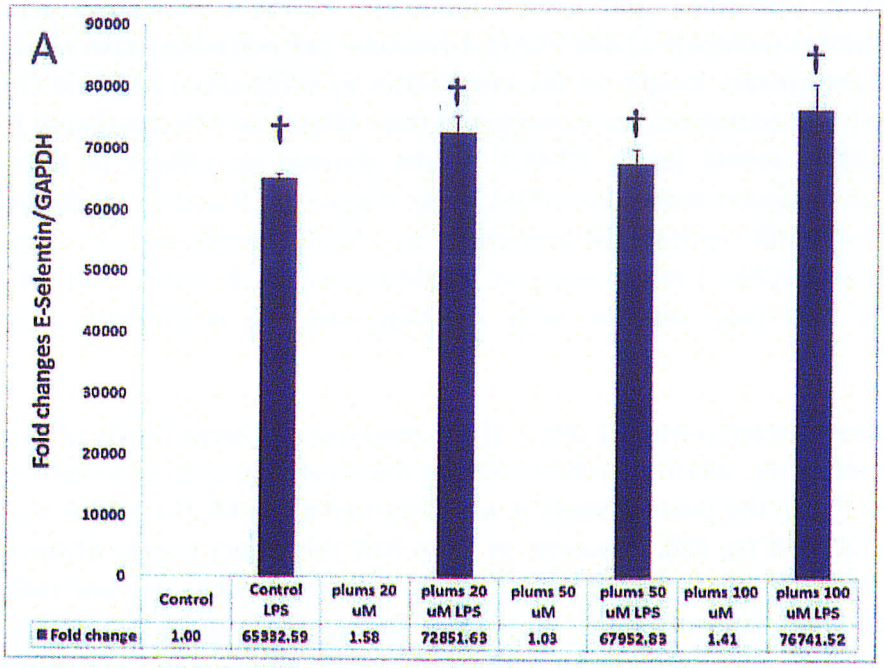
Effects of plum rich anthocyanin extract and Chlorogenic acid on gene expression

Local leukocyte recruitment into the vessel wall is an early step in atherogenesis and it is largely explained by the increased expression of endothelial leukocyte adhesion molecules (1). They regulate the sequential recruitment of blood leukocytes to sites of inflammation by expressing adhesion molecules on their surface which is recognized by ligands on leukocytes (2, 3). Endothelial leukocyte adhesion molecule-1, now termed E-selectin, is one

of these adhesion molecules, the others inducible adhesion molecules are, ICAM-1 (intercellular adhesion molecule-1) and VCAM-1 (vascular cell adhesion molecule-1) (4).
To obtain some preliminary insight on the mechanism by which plum RAE and Chlorogenic acid affect endothelial activation, we investigated their effects on LPS-stimulated E-Selectin, ICAM-1 and VCAM-1 mRNA levels. RT-PCR results showed no effects of different RAE concentrations on adhesion molecules mRNA levels (Figure 2A, B and C), while Chlorogenic acid treatments affected endothelial activation, by clearly decreasing LPS-stimulated E-Selectin, ICAM-1 and VCAM-1 mRNA levels at 20 ?M (Figure 3A, B and C). This effect shows that chlorogenic acid may prevent early atherogenesis, an inflammatory process in endothelial cells.

Monocyte chemoattractant protein-1 (MCP-1) is a member of a large family of chemotactic cytokines, or chemokines, which recruit circulating monocytes to sites of inflammation and vessel wall injury (5). In the present study it was observed a low MCP-1 mRNA level in both treatments (Fig. 2D and Fig 3D). However, in plum RAE this effect is only observed in low concentrations of 20-50 ?M, while for Chlorogenic acid treatments the low mRNA levels were maintained in all concentrations. In general, the decrease in MCP-1 mRNA levels in LPS-stimulated HUVEC cells indicates an anti-inflammatory effect at low concentrations of both RAE and chlorogenic acid. Furthermore, low concentrations of Chlorogenic acid (20 ?M) which decreases significantly E-Selectin, ICAM-1 and VCAM-1 mRNA levels, confirms the anti-inflammatory effects of stone fruit phenolics compounds.

Effects of plum rich anthocyanin extract and Chlorogenic acid on protein expression
Since p65 is a major component in the NFkB complex activation, we examined p65 activation by immunoblotting (Figure 4). HUVEC cells were incubated with LPS in the presence or absence of plum RAE and Chlorogenic acid for 1 h. Results of the relative expression of the protein p-NFkB after its exposure to treatments with RAE and Chlorogenic acid are shown in Figures 4 and 5. LPS-stimulated HUVEC cells treated with plum RAE showed no changes in relative protein expression (Figure 4), while treatments with Chlorogenic acid showed a dose-dependent concentration effect on p-NFkB relative protein expression (Figure 5). The observed decrease in p-NFkB is correlated with the decrease gene expression of VCAM, E-Selectin and ICAM, confirming that chlorogenic acid modulates this transcription factor which regulates inflammatory adhesion molecules. In general, the decrease in transcription factor p-NFkB in LPS-stimulated HUVEC cells by Chlorogenic indicate an anti-inflammatory effect of this compound, present in different stone fruits including plums, peaches and nectarines.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70000
60000
50000
40000
30000
20000
10000
0
Control
Control LPS
plums 20 uM
plums 20 uM LPS
plums 50 uM
plums 50 uM LPS
plums 100 uM
plums 100 uM LPS
Fold change
1.00
65332.59
1.58
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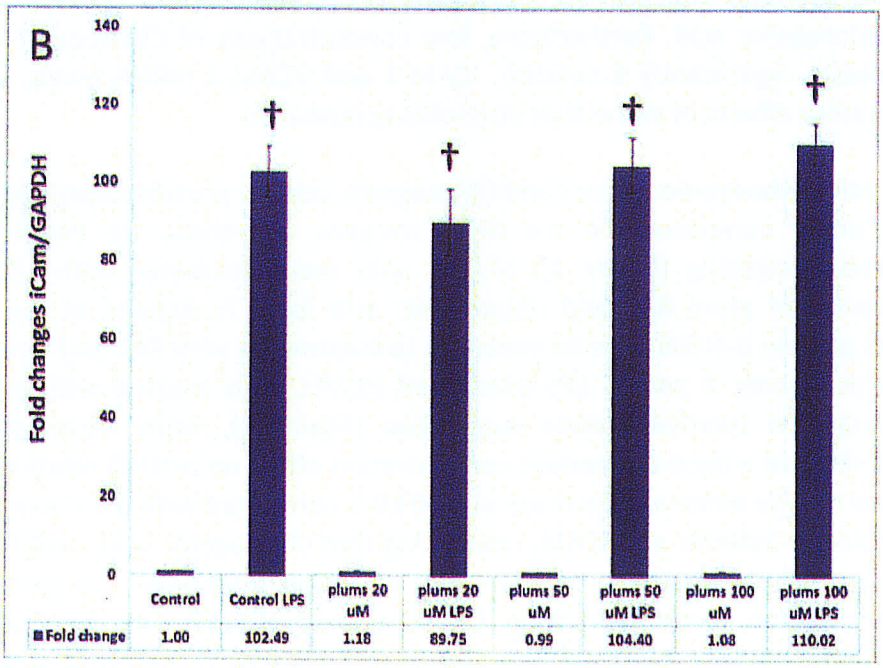
1.03
67952.83
1.41
76741.52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120
100
80
60
40
20
0
Control
Control LPS
plums 20 uM
plums 20 uM LPS
plums 50 uM
plums 50 uM LPS
plums 100 uM
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Fold change
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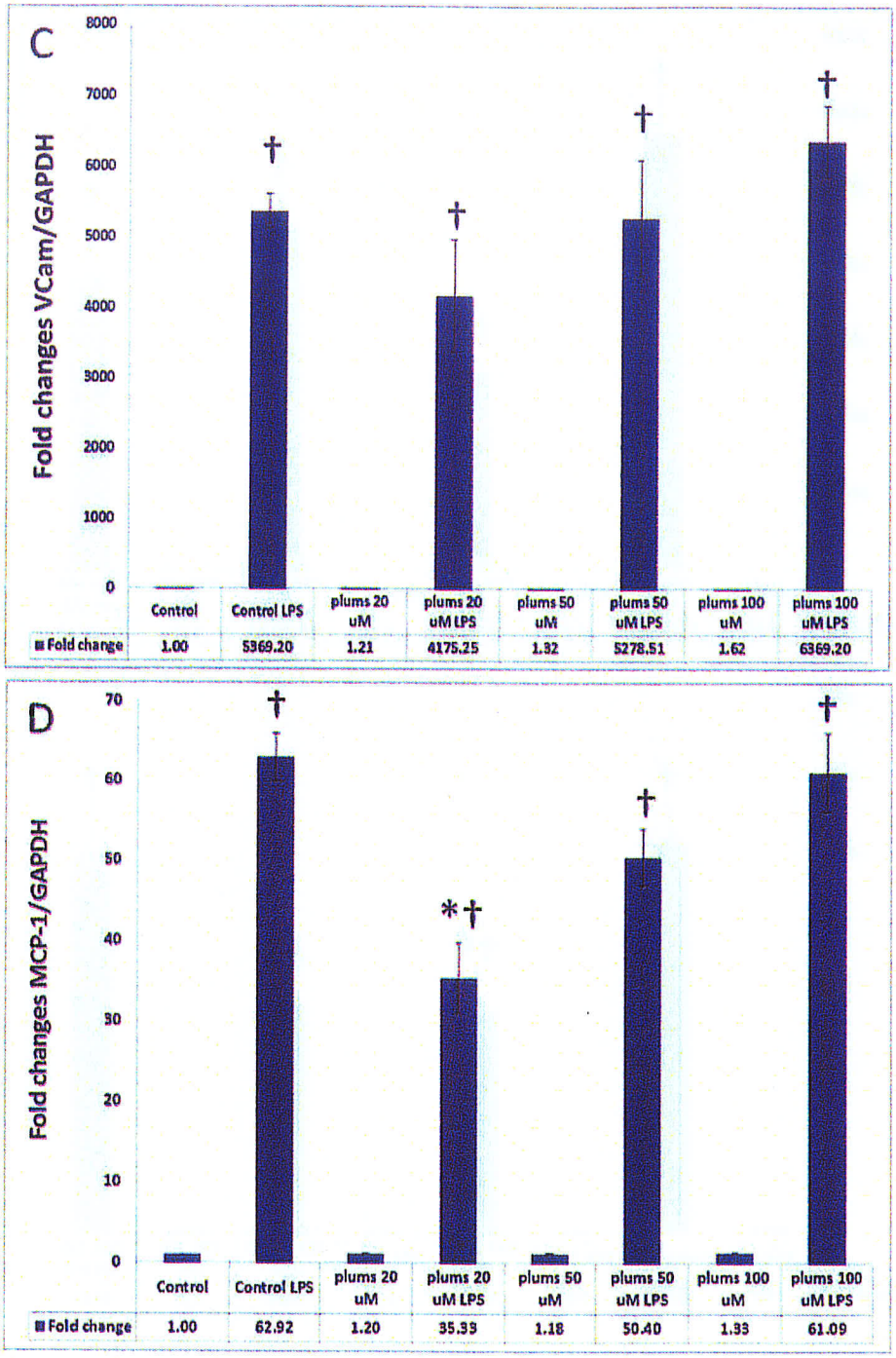
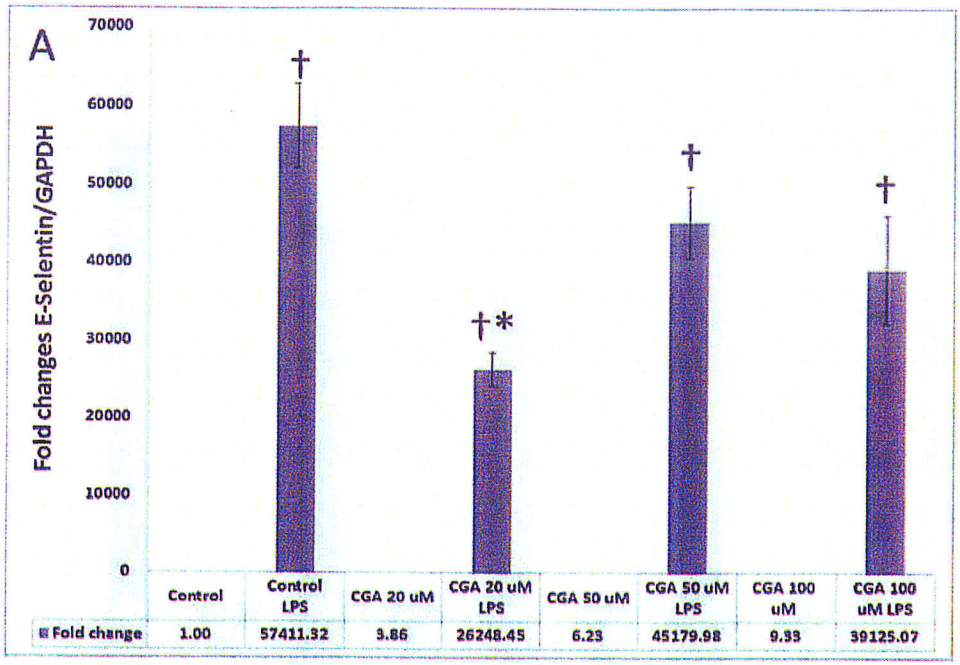


Figure 2. Effects of plum rich anthocyanin extract on inflammatory gene expression adhesion molecules and MCP-1. HUVEC cells were treated with different concentration of plum RAE for 24 h and then stimulated with LPS. Data are based on 3 different experiments, each based on 2 repeats for each condition. *P<0.05, treatment versus LPS alone and †P<0.05, treatment versus treatment plus LPS.


A
Fold changes E-Selentin/GAPDH
70000
60000
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40000
30000
20000
10000
0
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Control
Control LPS
CGA 20 uM
CGA 20 uM LPS
CGA 50 uM
CGA 50 uM LPS
CGA 100 uM
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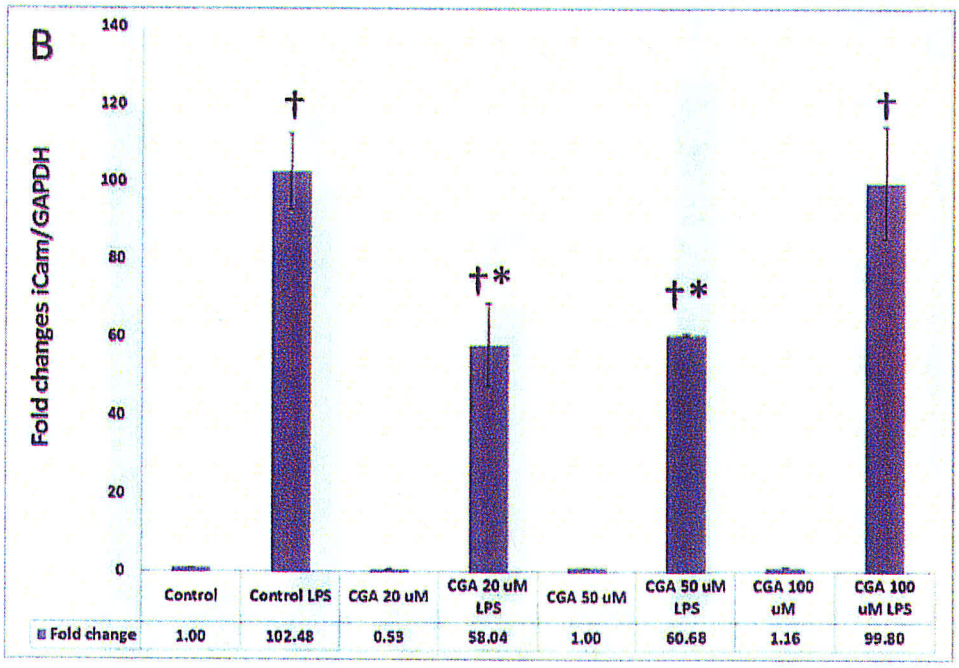
6.23
45179.98
9.93
39125.07


B
Fold changes iCam/GAPDH
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Control
Control LPS
CGA 20 uM
CGA 20 uM LPS
CGA 50 uM
CGA 50 uM LPS
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1.16
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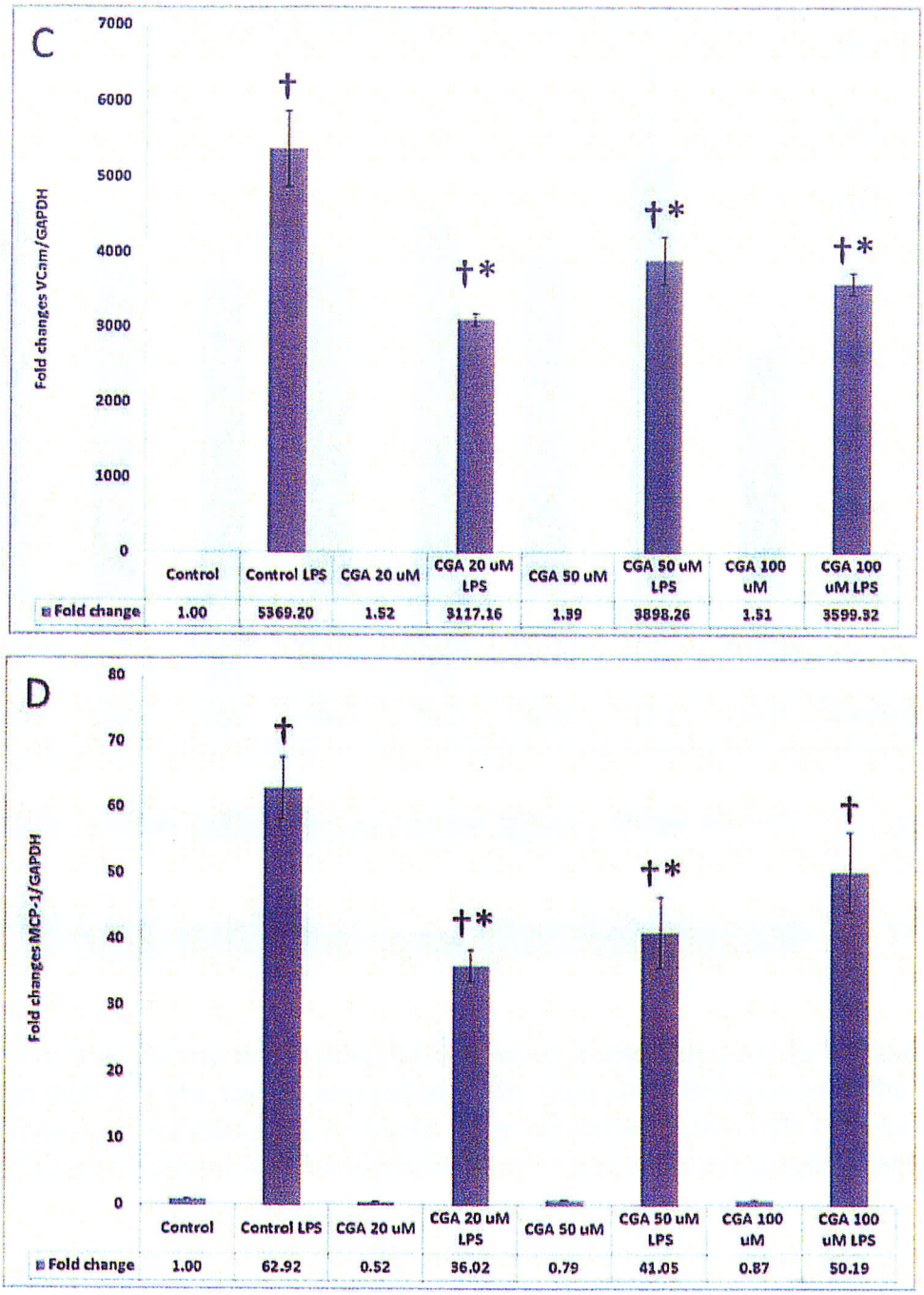
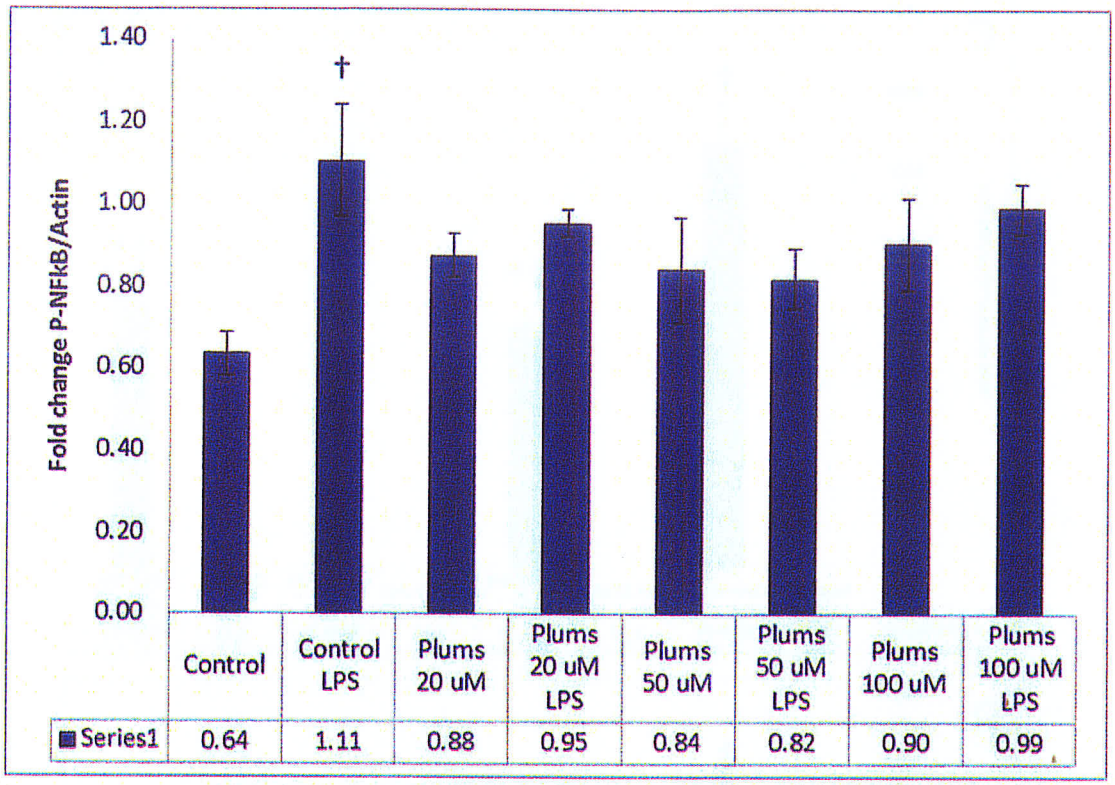


Figure 3. Effects of Chlorogenic acid on inflammatory gene expression adhesion molecules and MCP-1. HUVEC cells were treated with different concentration of Chlorogenic acid for 24 h and then stimulated with LPS. Data are based on 3 different experiments, each based on 2 repeats for each condition. **P*<0.05, treatment versus LPS alone and †*P*<0.05, treatment versus treatment plus LPS.



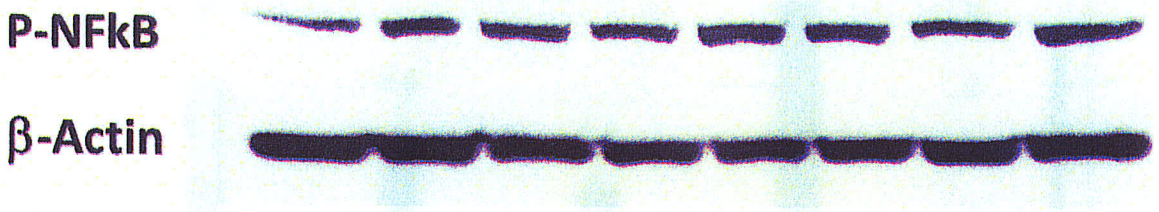


Figure 4. Expression of the transcription factor p-NFkB (p65) at the protein level in HUVEC treated with different concentrations plum rich anthocyanin extract and LPS (100 ng/mL). Data are based on 3 different experiments, each based on 2 repeats for each condition. †$P<0.05$, treatment versus LPS alone and *$P<0.05$, treatment versus treatment plus LPS.

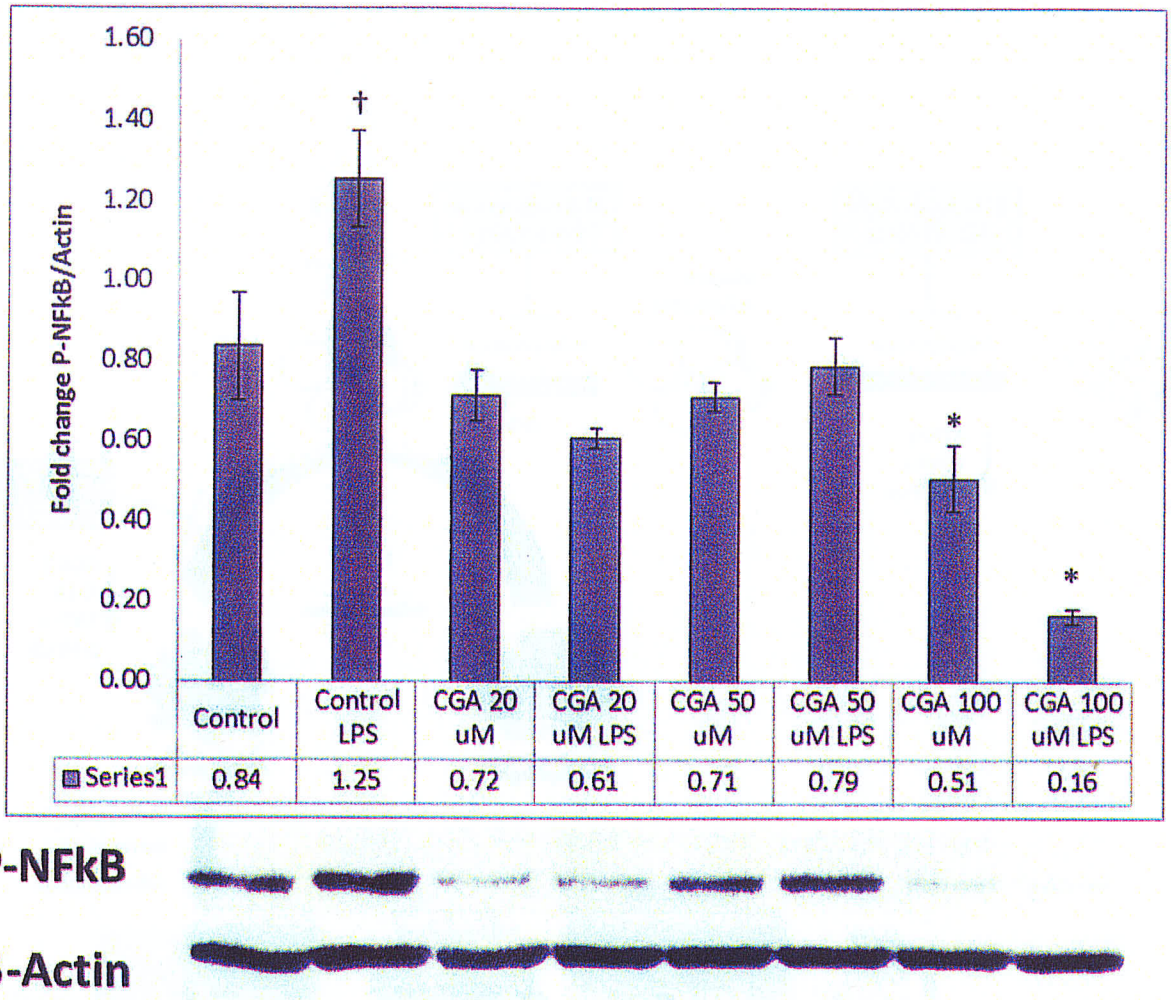


Figure 5. Expression of the transcription factor p-NFkB (p65) at the protein level in HUVEC treated with different concentration Chlorogenic acid and LPS (100 ng/mL). Data are based on 3 different experiments, each based on 2 repeats for each condition. †$P<0.05$, treatment versus LPS alone and *$P<0.05$, treatment versus treatment plus LPS.

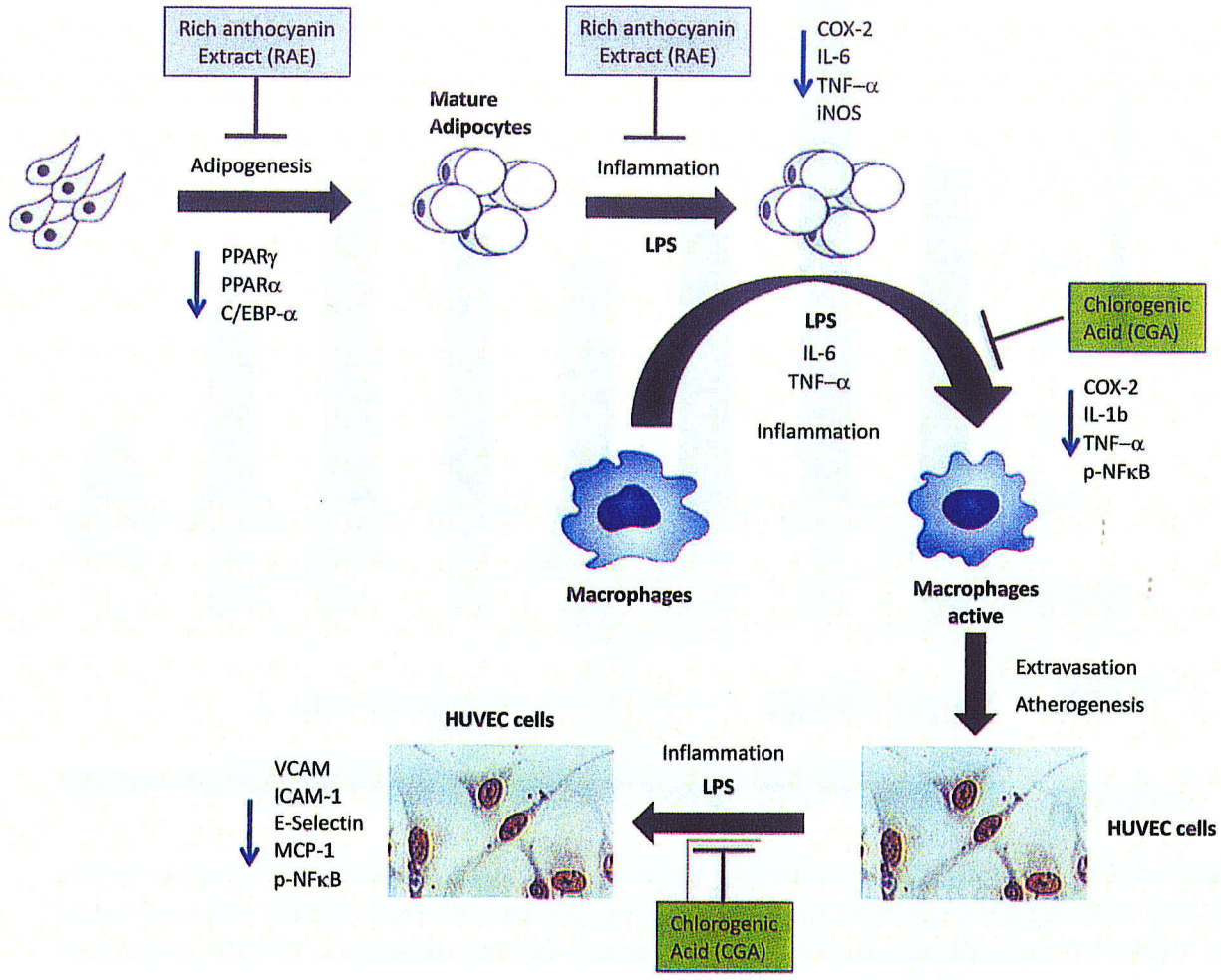


Figure 6. Diagram summarizing the multiple function stone fruit polyphenols exert in inhibiting adipogenesis and ameliorating inflammation in adipocytes, macrophages and endothelial cells.

Summary

In this report we studied the effects of stone fruit polyphenols against some components of the metabolic syndrome. We used cell models including adipocytes to determine effects in differentiation, fat accumulation and inflammation representing events in fat tissue. In addition, we studied inflammation in macrophage cells and Human umbilical vein endothelial cells (HUVEC) associated to atherosclerosis events. The inflammatory response in all these cells is a complex event, which is closely interrelated among them. Initially we characterized the phenolic profiles of different stone fruits (nectarines, peaches and plums)

finding 4 major phenolic groups including chlorogenic acid derivatives, anthocyanins, quercetin derivatives and catechins. Profiles and amounts present are dependent on fruit type and variety. We selected chlorogenic acid and a rich anthocyanin extract (RAE) which contained a mixture of anthocyanins, quercetins and catechins derivatives to perform the bioassays in this study.

Results indicated that stone fruit polyphenols have multiple functions and could potentially work against the metabolic syndrome in different fronts simultaneously (Figure 6). For example, RAE inhibited adipogenesis or lipid accumulation by modulating key transcription factors, c/EBPα, Pparγ and Pparα and reduced the inflammatory response in adipocytes or fat cells by modulating transcription factor NFkB and gene expressions of different pro-inflammatory cytokines. On the other hand, chlorogenic acid inhibited the inflammatory response in macrophages and HUVEC cells by modulating transcription factor NFkB and gene expressions of different pro-inflammatory cytokines and adhesion molecules associated to chronic inflammation and atherosclerosis. These results are promising and indicate that stone fruit polyphenols have potential health promoting properties against the metabolic syndrome.