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-Anti-atherogenic properties of Date vs. Pomegranate polyphenols, in-vitro and in-vivo, in atherosclerotic mice: beneficial role for the combination

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Running Head: Pomegranate and Date anti-atherogenic properties

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Summary

Pomegranate hydrolysable tannins polyphenols and date fruit and seeds phenolic acids are potent antioxidants and anti-atherogenic agents, and thus we questioned in the present study the possible extended benefit of their combination over the individual fruit, in-vivo in the atherosclerotic apolipoprotein E KO (E^0) mice.

In-vitro studies revealed that the date seeds extract contains more polyphenols than Amari or Hallawi date extracts, and possesses a most impressive free radicals scavenging capacity. Similarly, pomegranate juice (PJ), punicalagin, punicalain, gallic acid, and urolithins A and B were most potent antioxidants. E^0 mice consumed for 3 weeks, 0.5µmol gallic acid equivalents (GAE)/mouse/day, of PJ, of Hallawi extract, of date seeds extract, or of their combination. Consumption of the combination was the most potent treatment as it decreased serum cholesterol and triglyceride levels, and increased serum paraoxonase 1 (PON1) activity. Consumption of the combination also significantly reduced mouse peritoneal macrophages (MPM) oxidative stress, MPM cholesterol content, and MPM LDL uptake. Finally, in the mice aortas, the lipid peroxides content significantly decreased and aorta's PON lactonase activity increased by the combination. We thus conclude that consumption of pomegranate, together with date fruit and date seeds, possesses most beneficial anti-atherogenic effects in E^0 mice serum, macrophages, and aortas, probably due to their unique and different structure.

Key words: pomegranate juice, date fruit, date seeds, macrophages, oxidative stress, E^0 mice.

Introduction

In atherosclerotic patients, as well as in E^0 mice increased oxidative stress was shown

in their serum lipoproteins, and in their arterial or peritoneal macrophages [1, 2].

Macrophage accumulation of oxidized lipid, cholesterol and triglyceride lead to foam

cells formation, the hallmark of early atherogenesis [3]. Macrophage cholesterol

accumulation can result from an increased uptake of LDL, and / or decreased rate of

high-density lipoprotein (HDL) -mediated cholesterol efflux from the cells [3].

Because increased oxidative stress plays an important role in atherogenesis, its

inhibition by nutritional antioxidants was demonstrated to retard the progression of

the disease. Punica granatum L. (pomegranate) was shown to reduce several risk

factors for cardiovascular diseases including: high blood pressure, hyperglycemia,

high blood and arterial wall cholesterol and triglycerides accumulation, increased

oxidative stress and enhanced inflammation [4]. PJ prepared from squeezed whole

fruit, contains several unique polyphenolic antioxidants. PJ soluble polyphenols

include hydrolysable tannins such as the ellagitannins punicalagin, punicalain, gallic

and ellagic acids, as well as anthocyanins and catechins [5]. In-vitro studies

demonstrated that PJ ellagitannins are the most potent antioxidants [6, 7]. PJ

supplementation to E^0 mice [8, 9] or pomegranate extract supplementation to SR-

B1/E^0 double knockout mice [10], were both shown to reduce coronary artery

atherosclerosis, secondary to reduction in oxidative stress and inflammation. PJ

consumption also decreased blood oxidative stress in humans [9, 11]. Furthermore, PJ

consumption by patients with carotid artery stenosis reduced common carotid artery

intima-media thickness, blood pressure, and LDL oxidation [12]. In addition to the

most potent polyphenolic antioxidants, pomegranate contains also phytosterols such

as β-sitosterol, which is a mild hypocholesterolemic agent due to its effects on

cholesterol absorption and cholesterol biosynthesis rate [13]. Indeed, pomegranate

extract consumption decreased atherogenicity of human monocyte-derived

macrophages (HMDM) in simvastatin-treated hypercholesterolemic patients [14].

Pomegranate ellagitannins undergo extensive metabolism by the gut microbiota to

produce urolithins which are well absorbed [15]. In addition to pomegranate, another

ancient fruit, the Date (Phoenix dactylifera L.) have been also ascribed to have

medicinal properties [16]. Date fruit consumption (mainly the Hallawi variety) by

healthy subjects, despite their high sugar content, demonstrated impressive beneficial

effects on serum triglyceride, and on oxidative stress [17].

Date fruits and date seeds are rich sources of dietary fibers, phenolics, certain

essential vitamins and minerals [18-20]. Date phytochemicals investigations have

revealed that the fruits contain phenolics and phenolic acids, anthocyanins, sterols,

carotenoids, procyanidins and flavonoids [18]. In-vitro studies revealed that the date

fruits possess free radical scavenging capacity, inhibit LDL oxidation, and stimulate

cholesterol efflux from macrophages [18]. Date seeds consumption by Wistar rats also

significantly reduced liver and serum oxidative stress [21]. In addition, date seeds

extract supplementation to hypercholesterolemic rats, increased serum paraoxonase

catalytic activities [22]. The effects of pomegranate polyphenols and their

metabolites, and that of date fruits or seeds on free radicals scavenging activity, on

LDL oxidation , and on cholesterol and triglycerides accumulation was not studied in

details yet. Since the various phenolics and their metabolites in pomegranate and in

date differ in their structure and in their biological activities, the combination of

pomegranate and date constituents could possibly provides added values over their

unique anti-atherosclerotic properties.

Experimental

Materials. Fluorescein-isothiocyanate (FITC), 2', 7'-dichlorofluorescin diacetate

(DCFH-DA), 1, 1-diphenyl-2-picryl-hydrazyl (DPPH), phenyl acetate, thiobarbituric

acid, and dihydrocumarin were all purchased from Sigma-Aldrich (St. Louis, MO,

USA). BBL ^{TM} thioglycolate medium brewer modified was purchased from Becton,

Dickinson and Company Sparks (MD, USA). Phosphate buffered saline (PBS),

Dulbecco's Modified Eagle's Medium (DMEM), fetal calf serum (FCS), penicillin,

streptomycin, nystatin, L-glutamine, and sodium pyruvate were all purchased from

Biological Industries (Beth Haemek, Israel).

Pomegranate juice (PJ) was obtained from PomWonderful, Los Angeles, CA, USA,

and Punicalain, Punicalagin, Urolithin A and Urolithin B were a generous gift from

Dr. Daneel Ferrara, University of Mississippi, USA. PJ concentrate was diluted x5

with double distilled water (DDW) to get single strength PJ. Stock solutions of

5mg/ml (in 50% ethanol) were prepared from punicalain, punicalagin, urolithin A and

urolithin B.

Date extract preparation. Date fruits for this research were provided by

HADIKALIM, Israel Date Growers' Cooperative Ltd. Date fruit extracts - pitted fruits

were minced with Moulinex HV8 Mincer ME60514A. Ten g minced fruits were

homogenized in cold 50% ethanol using mortar and pestle. The extraction solution

was added stepwise to a final fruit to solvent ratio of 1:2 (w/w). The homogenate was

centrifuged for 10 min at 12,000 x g and 4 °C (Sorvall Instruments RC5C), and the

clear supernatant collected. Date seeds extract - 'Medjool' date seeds were washed in

DDW to remove residual pulp, air-dried and pulverized (KRUPS GX4100 Electric

Spice Herbs and Coffee Grinder). Twenty mL of cold 50% ethanol were added to 4 g

seed powder; the suspension was gently mixed for 1 h, and then allowed to settle for

10 min. The upper liquid phase was decanted, and centrifuged as described earlier.

The clear supernatant was collected.

RP-HPLC Analysis of Phenolics. Date extracts were filtered through a 0.45 μm filter

before injection. Samples were analyzed with a LaChrom Merck Hitachi HPLC

system, consisting of a Pump L7100, a column oven L7350, and a mixer-degasser L-

7614, coupled with a diode array detector with a 3D feature (Multiwavelength

Detector, Jasco MD- 2010 Plus), an interface (Jasco LC-Net II/ADC), and scientific

software (EZChrom Elite Client/Server version 3.1.6 build 3.1.6.2433) that provides

real time data acquisition and postrun data manipulation and integration capabilities.

Extract (20 μL) was injected using a manual injector (Rheodyne, Rohnert Park, CA)

and loaded onto a PurospherStar RP-18 end-capped column (250 mm × 4 mm

LichroCART cartridge, 5 μm particle size) with an end-capped Lichrospher100 RP-18

guard column (4 mm × 4 mm LichroCART cartridge, 5 μm particle size). The binary

mobile phase consisted of 0.1% phosphoric acid, pH 2.4 (solution A), and acetonitrile

(solution B). Elution was carried out at a flow rate of 1 mL min−1 with the following

gradient outline: 1−10 min, 5−15% solution B; 10−30 min, 15−30% solution B;

30−40 min, 30−100% solution B. The column was then washed and equilibrated by

10-min postruns with 100% and 5% B, respectively. Each run was monitored in real

time by three display modes simultaneously: contour plot, chromatogram display at a

chosen wavelength (usually 280 nm), and absorption spectra (200−650 nm). The oven

temperature was set at 40 °C, and the pressure was 158 atm. Acetonitrile was HPLC

grade (LiChrosolv Merck); column-filtered water was further distilled with a Corning

Megapure System, MP-6A, and passed through a 0.20 μm Nylon membrane.

Phenolics Identification and Quantification. A phenolics standard library was

constructed as follows: Each authentic standard (50−100 μg mL−1 in methanol) was

injected separately, and the data acquired by the photodiode array detector with the

3D feature were incorporated into the system phenolic standard library. The library

included catechin; epicatechin; catechin gallate; epicatechin gallate; gallocatechin;

epigallocatechin; gallocatechin gallate; epigallocatechin gallate; caffeic, chlorogenic,

o-coumaric, p-coumaric, 3-hydroxybenzoic, 4-hydroxybenzoic, ellagic, ferulic, gallic,

hydrocaffeic, protocatechuic, salicylic, sinapic, syringic, vanillic, and tannic acid;

kaempferol-3- glucoside; pyrogallol; quercetin-3-β-glucoside; procyanidin B2; 3-

mono- and 3,5-diglucosides of delphinidin; cyanidin and pelargonidin; and malvidin

3-glucoside chloride. Peak assignment was performed by the software on the basis of

UV/vis absorbance spectra and the retention times of the phenolics standards. Each

peak was tested for purity by a three point purity test and for similarity by a library

search comparing the peak spectrum to that of the standards. High similarity index

and a common retention time with the standard were considered a positive

identification; a similar UV/vis absorption spectrum but a different retention time was

considered a partial identification (e.g., derivative of the phenolic compound with the

similar absorption spectrum). Under the conditions employed in this study, the

relative standard deviation for the retention times in three repetitive runs was in the

range of 0.2−1.9%. Individual phenolic compounds were quantified from the

corresponding chromatogram peak area calculated by the software and standard

calibration curves. Calibration curves (linear, $R2 = 0.999$) were constructed with

authentic standards of caffeic acid, gallic acid, quercetin-3-β-glucoside, and salicylic

acid at six concentrations in the range of 1−200 μg mL−1 and of cyanidin 3-glucoside

at four concentrations in the range of 5−500 μg mL−1 . Sample concentrations of

gallic acid derivatives were expressed in terms of GAE; monohydroxybenzoic acid

derivatives in salicylic acid equivalents; hydroxycinnamic acid derivatives in caffeic

acid equivalents; flavonols in quercetin-β-glucoside equivalents; and anthocyanins in

cyanidin-3- glucoside equivalents. The concentrations were determined from three

repetitive runs.

Table 1: Date extracts soluble phenolics concentration

	Amari Date Extract (µg GAE/gram)	Hallawi Date Extract (µg GAE/gram)	Date Seeds Extract (µg GAE/gram)
Gallic Acid	33.3±1.8	37.0±1.9	37.0±11.0
Salicylic Acid	3.0±0.7	17.9±1.9	Non detectable
Hydrocinnamic Acid	68.6±2.3	12.5±0.8	29.0±10.0
Flavonols	14.1±1.1	5.8±0.7	Non detectable
Procyanidins	Non detectable	Non detectable	139.0±33.0

Values are mean ± SD (n=3)

In-vitro determinations

Total soluble phenolics content. Total soluble polyphenols content in PJ, in

pomegranate fractions and in date extracts was measured colorimetrically with Folin-

Ciocalteau 2N phenol reagent [23]. Aliquots of 100 µL were added to 900 µL of

reaction solution consisting of 200 µL freshly prepared 10-fold diluted Folin-

Ciocalteau reagent, 100 µL 20% Na_2CO_3 and 600 µL of DDW. Calibration curves

were constructed with gallic acid (0, 5, 10, 25, 50, 75 and 100 µg/mL), and the results

were expressed as µg GAE /mL.

Free radical scavenging capacity. The free radical-scavenging capacity of : PJ,

of pomegranate fractions and of the date extracts was analyzed by the DPPH assay

[24]. DPPH is a long-lived free radical that is widely used to monitor the free

radical scavenging abilities of various antioxidants. The DPPH radical has a deep

violet color due to its unpaired electrons, and radical scavenging can be followed

spectrophotometrically by the loss of absorbance at 517nm, as the pale yellow

non-radical form is produced. The tested antioxidants were mixed with

100µmol/L DPPH (in ethanol) and the change in optical density at 517nm was

monitored after 5 minutes.

Isolation of lipoproteins. Fresh plasma was derived from 3 healthy

normolipidemic volunteers (Rambam Hospital Helsinki Committee number

30572-10 - RBM). These volunteers received Mediterranean diet, but they did not

consume any antioxidants. These plasma samples were inactivated (30 minutes at

56°C) and then pooled together. The LDL and HDL fractions were isolated from

the pooled plasma sample by discontinuous density gradient ultracentrifugation

[25]. The LDL was separated at d=1.063g/mL, and the HDL at d=1.210g/mL.

Both lipoproteins were dialyzed against 150 mmol/L NaCl, 1 mmol/L Na2EDTA

(pH 7.4) at 4°C, and then sterilized by filtration (0.45µM), kept under nitrogen in

the dark at 4°C, and used within 2 weeks. The LDL and HDL protein

concentration was determined by the Lowry method [26]. We used the same LDL

and HDL fractions in all the experiments.

Copper ion-induced LDL oxidation. Prior to oxidation, the LDL was dialyzed

against EDTA-free, PBS solution, pH 7.4, at 4°C. LDL (100µg of protein/mL)

was incubated with 5µmol/L of CuSO4 for 1.5 hours at 37°C. At the end of the

incubation, the extent of LDL oxidation was determined by measuring the

generated amount of thiobarbituric acid reactive substances (TBARS, 27), and the

amount of lipid peroxides [28]. The TBARS assay was performed at 532 nm,

using malondialdehyde (MDA) for the standard curve [27]. Lipid peroxide

formation was measured spectrophotometrically at 365 nm by their capacity to

convert iodide to iodine [28].

J774A.1 macrophage cell line. J774 A.1 murine macrophage cells were purchased

from the American Tissue Culture Collection (ATCC, Manassas, VA, USA). The

cells were grown in DMEM containing 5% FCS. The J774A.1 cell line lifespan is

infinite, and the population doubling time is 17 h. We used cells only up to 10

passages. All macrophage experiments were carried out with the same number of

cells. The split ratio was 1:6, and we seeded 0.75×10^6 cells /ml in a 12 wells plate, and

performed the experiment the next day of cell seeding.

Cholesterol efflux from J774A.1 macrophages. J774A.1 macrophages were

incubated with [3H]-labeled cholesterol (2μCi /mL) in DMEM with no addition for 1h

at 37°C, followed by cell wash in ice-cold PBS (x3), and a further incubation in the

absence [basal, non-specific loss of cholesterol from the cells to the media (DMEM

with no addition)], or presence of 100μg of HDL protein/mL for 3h at 37°C in

DMEM medium [29]. Cellular and medium [3H]-labels were quantified, and basal or

HDL-mediated cholesterol efflux were calculated as the ratio of [3H]-label in the

medium / ([3H]-label in the medium+ [3H]-label in the cells). Net HDL-mediated

cholesterol efflux data were corrected for the basal, non-specific loss of cholesterol to

the media.

In-vivo studies

Mice Studies. E^0 male mice were fed with chow diet. At the age of 6 weeks the mice

(n=20) were divided into 5 groups that consumed for 3 weeks in their drinking water

0.5μmoL GAE/mouse/day of: PJ, date seeds extract, Hallawi extract, or their

combination. The placebo – treated mice consumed similar volume of 50% ethanol.

The mice weight after the treatment was: placebo mice 28±1 gr, PJ-treated mice 29±2 gr, date seeds extract-treated mice 30±1 gr, Hallawi extract treated mice 30±2 gr, and the combination-treated mice 32±1 gr. At the end of the study the mice were injected intraperitoneal (IP) with thioglycolate to induce inflammatory reaction and the differentiation of blood monocytes into macrophages in the peritoneum. Three days post-injection blood samples, peritoneal macrophages and aortas were harvested from the mice. This protocol was approved by the Committee for the Supervision of Animal Experiments and complied with the Guide for Care and Use of Laboratory Animals, the Technion-Israel Institute of Technology, Haifa, Israel (IL0460408).

<u>Serum analyzes</u>

Serum lipids and glucose concentrations. Serum cholesterol, triglyceride and glucose concentrations were determined using commercially available diagnostic kits.

Serum PON1 arylesterase activity. Arylesterase activity was measured using phenyl acetate as the substrate [30]. Initial rates of hydrolysis were determined spectrophotometrically at 270 nm. The assay mixture included 5 μL serum (diluted 1:10), 1.0 mmol/L phenylacetate, and 1 mmol/L $CaCl_2$ in 50 mmol/L Tris HCL, pH 8.0. The E_{270} for the reaction is 1,310 M^{-1} cm^{-1}. One unit of arylesterase activity is equal to 1 μmoL of phenylacetate hydrolyzed/min/mL.

Serum lipid peroxidation. Serum samples were diluted x4 in PBS, and were incubated with or without 100mol/L of 2.2'-azobis, 2-amidinopropane hydrochloride (AAPH, Wako, Japan) for 2h at 37°C [31]. The extent of lipid peroxidation was measured by the TBARS assay [27].

Mouse peritoneal macrophages (MPM). MPM were harvested from the peritoneal fluid of the E^0 mice, 3 days after IP injection into each mouse of 3mL of aged thioglycolate (40g/L) in saline. The cells ($10-20x10^6$/mouse) were washed and

centrifuged three times with PBS at 1000 xg for 10 min, then resuspended at

10^9cells/L in DMEM containing 5% FCS, 1×10^5 units penicillin/L, 100mg

streptomycin/L and 2mmol/L glutamine. The dishes were incubated in a humidified

incubator ($5\% CO_2$, 95% air) for 2h, washed with DMEM to remove non adherent

cells, and the analyses to measure cellular oxidative stress were immediately

performed.

MPM analyses

MPM cholesterol mass. Lipids from the mice MPM (2×10^6) were extracted with

hexane: isopropanol (3:2, v: v) and the hexane phase was evaporated under nitrogen.

The amount of cellular total cholesterol was determined using a commercial kit

(CHOL, Roche Diagnostics GMbH, Mannheim, Germany). The remaining cells in the

dish were dissolved in 0.1 moL/L NaOH, and an aliquot was taken for measurement

of cellular protein by the Lowry assay [26].

MPM uptake of FITC-labeled LDL. LDL was conjugated to fluoroisothiocyanate

(FITC) for cellular lipoprotein uptake studies [32]. MPM were incubated at 37°C for

3h with FITC-conjugated LDL at a final concentration of 25µg of protein/mL. The

uptake of the lipoprotein was determined by flow cytometry. Measurements of

cellular fluorescence determined by FACS were done at 510 nm to 540 nm after

excitation of the cells at 488 nm with an argon ion laser. Cellular fluorescence was

measured in terms of mean fluorescence intensity (MFI).

Macrophage oxidative status-DCFH assay. MPM were washed (x1) with PBS and

then further incubated with 10µmol/L of DCFH-DA, for 30 minutes at 37°C [33]. The

cells were washed (x2) with PBS, and then the adherent cells were detached by gentle

scraping. Measurements of cellular fluorescence determined by FACS were

performed at 510 nm to 540 nm after excitation of the cells at 488 nm with an argon

ion laser. Cellular fluorescence was measured in terms of MFI.

MPM PON2 lactonase activity. Lactonase activity was measured in intact cells

using dihydrocumarin (DHC) as a substrate. DHC (1mmoL/L) was added to the cells

in 1mL of 1 mmol/L $CaCl_2$ in 50 mmol/L of Tris HCL, pH 8.0. One unit of lactonase

activity = 1 μmoL of DHC hydrolyzed/min. DHC was incubated under similar

conditions in a cell-free system in order to measure non enzymatic hydrolysis. These

values were subtracted from the values observed with the cells. The remaining cells in

the dish were dissolved in 0.1 moL/L NaOH, and aliquots were taken for

measurement of cellular protein by the Lowry assay [26]. Results are given as units

/mg cell protein.

Aortas' analyses

The mice aortas were rinsed with PBS and homogenized in mL of PBS on ice. After

centrifugation at 1000xg, the supernatant was collected and analyzed for protein

concentration by the Lowry method [26].

Aortic Content of lipid peroxides. Lipids were extracted from the aortas'

homogenate (100μL) with hexane: isopropanol (3; 2, v: v). The hexane phase was

evaporated under nitrogen, and the amount of lipid peroxides was determined by the

El-Saadani method [28].

Aortic cholesterol content. Lipids were extracted from the aortas' homogenate

(100μL) with hexane: isopropanol (3; 2, v: v). The hexane phase was evaporated

under nitrogen, and the amount of cholesterol was determined using a commercial kit

(CHOL, Roche Diagnostics GMbH, Mannheim, Germany).

Aortic triglyceride content. Lipids were extracted from the aortas' homogenate (100μL) with hexane: isopropanol (3; 2, v: v). The hexane phase was evaporated under nitrogen, and the amount of triglyceride was determined (after addition of 50μL DMSO) using a triglyceride assay commercial kit (Sigma, Cat no. TR0100).

Aortic PON lactonase activity. The assay was performed in 96 well UV plates, in a total volume of 200μL per well. Lactonase activity was measured using dihydrocumarin (DHC) as the substrate. Initial rates of hydrolysis were determined spectrophotometrically at 270nm, for 10 minutes (every 15 seconds). The assay mixture included 1mmol/L $CaCl_2$ in 50 mmol/L Tris HCl, pH 8.0, containing 10μL of the aortas' homogenate and 1mmol/L DHC. Nonenzymatic hydrolysis of DHC was subtracted from the total rate of hydrolysis. One unit of lactonase activity equals 1μmoL of DHC hydrolyzed/min/mL.

Statistic*s*. Three experiments (each one in triplicates) were performed in order to achieve statistical significance. Statistical analyses used the Students't test for comparing differences between the 2 groups. Results are given as mean ±SD.

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Results

In-Vitro Studies

a. Anti-oxidative properties of Amari Date extract, Hallawi Date extract or Date Seeds extract, and their ability to affect macrophage cholesterol efflux

As shown in Fig. 1A, the total polyphenols concentration in the Amari, or the Hallawi date extracts was almost similar, whereas in the date seeds extract, it was 10 fold higher. Next, we analyzed the anti-oxidative properties of the above extracts: their ability to scavenge free radicals and to inhibit copper ions-induced LDL oxidation. The Hallawi date or Amari date extracts (0-5µg GAE/mL) were not able to reduce the optical density of the free radicals generator DPPH, indicating almost no free radicals scavenging capacity (Fig. 1B). In contrast, the date seeds extract (0-5µg GAE/mL) possesses impressive free radicals scavenging capacity, as it dose – dependently decreased DPPH absorbance at 517nm by up to 57% (Fig. 1B). We have then pre-incubated the LDL with the above extracts (0-5µg GAE/mL), followed by the addition of copper ions. The extent of LDL oxidation was determined by the TBARS (Fig. 1C), or by the lipid peroxides (Fig. 1D) assays. The Amari date extract did not affect the extent of LDL oxidation, whereas the Hallawi date extract and more so, the date seeds extract significantly decreased the LDL associated aldehydes (TBARS) levels by up to 47% or 83%, respectively (Fig. 1C). Similarly, LDL-associated lipid peroxides levels were decreased by Hallawi and Seeds by up to 76% or 99%, respectively (Fig. 1D). Finally, we have pre-incubated J774A.1 macrophage cell line for 20h with 2 or 5µg GAE/mL of the above date extracts, and control cells were incubated with similar volume of 50% ethanol. The extent of HDL-mediated cholesterol efflux from the macrophages was minimally increased, by up to 7%, or by up to 13% in the date seeds extract or in the Amari date extract –treated cells,

respectively (Fig. 1E). In contrast, in the Hallawi date extract –treated cells, HDL-

mediated cholesterol efflux was significantly increased by up to 46% (Fig. 1E).

b. Anti-oxidative properties and ability to stimulate macrophage cholesterol

efflux from macrophages, of PJ, its major polyphenols (Punicalain, Punicalagin,

Gallic Acid), or their metabolites (Urolithin A, Urolithin B)

The total polyphenol concentrations, in PJ, punicalain, punicalagin, gallic acid,

urolithin A or urolithin B are given in Fig. 2A. We analyzed the anti-oxidative

properties of the above fractions: their ability to scavenge free radicals and to inhibit

copper ions-induced LDL oxidation. Urolithin A or Urolithin B (0-5µg GAE/mL)

only minimally decreased the DPPH optical density, by up to 6% or 11%, respectively

(Fig. 2B). In contrast, PJ, punicalain, punicalagin or gallic acid, possess impressive

free radicals scavenging capacities, as they decreased DPPH absorbance after 5

minutes of incubation by up to 86%, 85%, 81% or 93%, respectively (Fig. 2B).

However, all the analyzed fractions, including urolithin A and B significantly

inhibited,, in a dose-dependent manner, copper ions-induced LDL oxidation, as

measured by the TBARS (Fig 2C), or by the lipid peroxides (Fig. 2D) assays. Pre-

incubation of LDL with 0-5µg GAE/mL of urolithin A, urolithin B, PJ, gallic acid,

punicalain or punicalagin, followed by copper ions addition, decreased the amount of

LDL-associated aldehydes by 55%, 70%, 79%, 97%, 95% or 98%, respectively (Fig.

2C), and LDL-associated lipid peroxides by 85%, 84%, 89%, 89%, 92% or 93%,

respectively (Fig. 2D). Finally, we have incubated J774A.1 macrophages for 20h

with 2 or 5µg GAE/mL of the above pomegranate fractions. Control cells were

similarly incubated with similar volume of 50% ethanol. The extent of HDL-mediated

cholesterol efflux rate from macrophages that were pre-treated with urolithin A, or

urolithin B, or punicalain was similar to that observed from control cells (Fig. 2E). In

contrast, pre-incubation of the cells with PJ, or with punicalagin, or with gallic acid

resulted in a significant stimulation of HDL-mediated cholesterol efflux by up to 48%,

33% or 29%, respectively (Fig. 2E).

In-Vivo Studies

Since PJ, as well as Hallawi date extract and date seeds extract demonstrated

impressive anti-oxidative and anti-atherogenic properties in-vitro, we next analyzed

their effects in-vivo, alone and in combination, on the atherosclerotic E^0 mice which

are under high oxidative stress. These mice, at the age of 6 weeks, consumed, for 3

weeks, 0.5µmol GAE/mouse/day, of PJ, or of Hallawi extract, or of date seeds extract,

or of the combination PJ +Hallawi date extract +date seeds extract. The placebo mice

received similar volume of 50%.ethanol (as all extracts were dissolved in 50%

ethanol).

a. The effect of consumption by E^0 mice of Date Seeds extract, or PJ, or Hallawi

Date extract, or their combination, on their serum lipid and glucose

concentrations

PJ consumption had no significant effect on the mice serum cholesterol, triglyceride,

or glucose concentrations (Table 2). Consumption of Hallawi date extract resulted in

an insignificant increment, by 6% or 10% or 16%, vs. placebo, in the mice serum

cholesterol or triglycerides or glucose concentration, respectively (Table 2). In

contrast, upon consumption of date seeds extract, serum cholesterol or triglycerides

concentrations were significantly decreased, by 38% and by 50%, respectively, vs.

placebo (Table 2), and there was no significant effect on serum glucose

concentrations. Consumption of the combination PJ +Hallawi extract +date seeds

extract decreased the serum cholesterol or triglyceride levels by 24% or by 41%,

respectively (Table 2).

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b. The effect of consumption by E^0 mice of Date Seeds extract, or PJ, or Hallawi Date extract, or their combination on the mice serum oxidative stress and on PON1 activity

PJ consumption did not affect the mice serum susceptibility to oxidation by the free radicals generator AAPH, as measured by the TBARS assay (Fig. 3A). In contrast, consumption of date seeds extract, of Hallawi date extract, or of their combination together with PJ, resulted in a reduction by 27%, 7% or 19%, respectively, in the extent of serum lipid peroxidation vs. placebo mice (Fig. 3A). Since HDL-associated PON1 protects serum lipids from oxidation [34], we have next measured serum PON1 arylesterase activity. Upon consumption of PJ, or of date seeds extract, or of Hallawi date extract, PON1 activity increased by only 9%, 19%, or 9%, respectively, vs. placebo mice, whereas consumption of the combination significantly increased serum PON1 activity by as high as 46% (Fig. 3B).

c. The effect of consumption by E^0 mice of Date Seeds extract, or PJ, or Hallawi Date extract, or their combination, on their MPM atherogenic properties

Consumption of PJ, or of date seeds extract, or of Hallawi extract, or of their combination significantly decreased their MPM oxidative stress as measured by the DCFH assay, with a reduction in the absorbance values by 23%, 19%, 33% or by 25%, respectively vs. placebo MPM (Fig. 4A). Macrophage paraoxonase 2 (PON2) which unlike PON1 is not present in the circulation, was shown to protect cells, including macrophages, from oxidative stress [35]. Just like serum PON1 stimulation by pomegranate or dates (Fig 3B), and more so , by the combination of pomegranate with date fruits + seeds, MPM PON2 activity was also significantly increased by 22% or by 6% after consumption of PJ or Hallawi date extract, respectively, vs. placebo MPM. Interestingly, consumption of date seeds extract had no significant

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effect (Fig. 4B). However, consumption of the combination, significantly increased

MPM PON2 activity, by up to 39% (Fig. 4B). MPM cholesterol content was not

significantly affected by consumption of PJ or date seeds extract alone. In contrast,

consumption of Hallawi date extract or that of the combination, significantly

decreased macrophage cholesterol content vs. placebo MPM by 12% or by 28%,

respectively (Fig. 4C). Finally, we measured the extent of FITC - labeled LDL uptake

by the cells (Fig. 4D). Consumption of date seeds extract, or of PJ, or of Hallawi date

extract alone had no significant effect on LDL uptake by the cells, whereas

consumption of their combination significantly decreased the extent of LDL

macrophage uptake by 16% (Fig. 4D).

d. The effect of consumption by E^0 mice of Date Seeds extract, or PJ, or Hallawi

Date extract, or their combination on their aorta's atherogenic properties

The lipid peroxides levels significantly decreased in the mice aortas, by 14%, vs.

aortas harvested from placebo mice after consumption of the combination, whereas

consumption of date seeds extract, or of PJ, or of Hallawi date extract alone had no

significant effect (Fig. 5A). PJ or Hallawi date extract consumption had no effect on

aortas' paraoxonase (PON) lactonase activity. In contrast, after consumption of date

seeds extract, or of the combination, aortas' PON activity significantly increased, by

22% or by 33%, respectively, vs, aortas from placebo mice (Fig. 5B). Aortas'

cholesterol content significantly decreased by 24%, 15% or by 16% after date seeds

extract, PJ, or the combination, respectively, vs. aortas from placebo mice. In contrast,

the Hallawi date extract had no significant effect (Fig. 5C). Since atherosclerotic

lesions contain substantial amounts of triglycerides, we finally determined the aortic

triglyceride mass. Only consumption of the combination, but not consumption of the

date or pomegranate extracts alone, resulted in a modest, but significant, decrement,

by 8%, in the aortic triglyceride content vs. aortas harvested from placebo mice (Fig.

5D).

Discussion

In the present study we have demonstrated, for the first time, the beneficial anti-atherogenic effects in the atherosclerotic E^0 mice serum, macrophages, and aortas of consuming the combination of PJ together with Hallawi date fruit extract and date seeds extract.

We performed also in-vitro studies in order to analyze the anti-oxidative and anti-atherogenic mechanisms of the date extracts, date seeds, and PJ phenolic compounds. We used concentrations of 2-5μg GAE/ml which corresponds to 10-26μM. These concentrations exceed those that can be reach after the intake of a "normal" food containing ellagitannins, which is in the nM range [36-41]. This is the limitation of the in-vitro studies. The in-vitro study was performed only to compare the anti-oxidative properties of date extracts or pomegranate fractions. But it could be that in vivo the date extracts or pomegranate or their combination will act differently from the in vitro study. In addition, there is no data regarding the concentration of ellagitannins in plasma after date extracts intake, or after intake of date extracts together with pomegranate. Maybe these concentration can reach the low μM values. The anti-oxidative properties of the above samples were analyzed by two parameters: free radicals scavenging capacity, and the ability to inhibit copper ions-induced LDL oxidation. The Amari date extract had no significant free radicals scavenging capacity at the used concentrations, and it also demonstrated minimal ability to inhibit copper ions-LDL oxidation. In contrast, the Hallawi extract significantly inhibited LDL oxidation, mostly through its ability to chelate copper ions. This effect could be attributed to the salicylic acid which is 6 fold higher in the Hallawi vs. Amari extract (Table 1). The date seeds extract had a most impressive free radicals scavenging capacity, and it significantly inhibited LDL oxidation. This effect could be attributed

to the date seeds procyanidins which were not detectable in the Amari or Hallawi

extracts (Table 1).

PJ and its major polyphenols include punicalagin, punicalain, and gallic acid, all of

them significantly and dose-dependently inhibited LDL oxidation. This effect could

be attributed to their impressive free radicals scavenging capacity. In contrast, the PJ

ellagitannins metabolites urolithin A or urolithin B, possess minimal free radicals

scavenging capacity, though they were able to inhibit LDL oxidation, probably

secondary to chelation of copper ions. Flavonoid interactions with copper ions and

potency to inhibit copper ions-induced LDL oxidation were indeed reported to depend

on the molecule structure properties [42]. The antioxidant activity of urolithins was

correlated with the number of hydroxy groups, as well as, the lipophilicity of the

molecule [43]. We have next determined the effect of J774A.1 macrophages

enrichment with all the above nutrients and their phenolic, on the extent of HDL-

mediated cholesterol efflux from the cells, since decreased cholesterol efflux leads to

macrophage cholesterol accumulation and foam cell formation [3]. We did not use

pre-beta HDL, since its amount in serum in small, and it has short life time in the

circulation [44]. The steady state level of pre-beta HDL in plasma reflects the relative

efficiencies of the major metabolic processes involved in its generation and removal

[44]. Furthermore, HDL is commonly used in cholesterol efflux studies [45, 46].

Hallawi date extract, but not Amari date extract, or date seeds extract, significantly

stimulated HDL-mediated cholesterol efflux from J774A.1 macrophages, and this

effect could be attributed to Hallawi date extract salicylic acid.   PJ, punicalagin and

gallic acid, punicalain or the urolithins, significantly stimulated macrophage

cholesterol efflux by HDL. These findings suggest that phenolic compounds could

interact with the macrophage plasma membrane and, consequently, enhance HDL

binding to- and cholesterol removal from the cells. The less polar compounds may

form hydrogen bonds between macrophage membrane phospholipid polar head

groups and the more hydrophilic phenolic at the membrane/water interface [47]. In

addition, the above molecules may affect the expression of transporters (ABCA1 and

ABCG1) involved in HDL-mediated cholesterol efflux as was recently shown for

Hibiscus sabdariffa leaf polyphenolic extract [48], and for extra virgin oil polyphenols

[49]. We speculate that in cell or tissue cultures polyphenols at the concentrations

used in the current study (up to 30μM) can probably generate a lot of electrophils

which could affect cell signaling. They can increase the protective effect of the

Nrf2/EpRE pathway that results in more reductive / electrophilic environment,

followed by an increment in substrates like glutathione, thioredoxin and NADPH [50].

Since PJ, as well as Hallawi date extract, and date seeds extract demonstrated most

impressive anti-oxidative and anti-atherogenic properties in-vitro, we next analyzed

their effects in-vivo, either alone or in combination, using the atherosclerotic E^0 mice

model [1]. The concentration of 0.5μmol GAE/mouse/day was chosen based on our

previous studies in these mice. Consumption of PJ (0.5μmol GAE/mouse/day) was

not toxic to the mice, and demonstrated impressive anti-atherosclerotic effects [8, 9].

An interesting novel finding is that consumption of the date seeds extract significantly

decreased serum cholesterol and triglyceride levels. Consumption of the combination

also resulted in significant reduction in serum lipid concentration, but this effect was

lower than the effect of date seeds alone. This could be attributed to the fact that PJ

had no significant effect and the Hallawi extract even slightly increased the

cholesterol and triglyceride levels. This effect could be related to the date seeds high

fibers (absorption attenuation) content, as well as to a possible lipids synthesis

inhibition. Similarly, treatment of diabetic rats with fibers – rich date palm leaves

extract significantly decreased serum concentration of total cholesterol and LDL-cholesterol [51]. The date seeds extract vs. PJ or Hallawi date extract had also the major effect on decreasing serum oxidative stress, as was previously shown in rats [21], and on increasing serum PON1 activity, as was previously shown in hypercholesterolemic rats [22]. This could be attributed to the reduction in serum oxidative stress, since PON1 was shown to be inactivated by oxidized lipids [52]. PJ consumption did not decrease the high serum lipid peroxidation observed in the E^0 mice, and it slightly increased serum PON1 activity which is low in these mice. Maybe higher concentration of PJ is needed to get more significant results. However, in all our previous in vivo studies in the apoE KO mice, as well as studies in humans (healthy subjects, diabetic patients, or patients with carotid artery stenosis) , we have demonstrated significant reduction in serum oxidative stress after PJ or pomegranate extracts consumption inspite of the high antioxidant concentrations present in plasma [8, 9, 12, 14]. In these mice or in the atherosclerotic patients which have increased oxidative stress in their serum, the levels of their serum antioxidants were shown to be low.

Pomegranate or date polyphenols can directly interact with serum PON1, thus beneficially affecting PON1 structure and activity [53] and improving its stability [52], or it can also increases PON1 expression in the liver [54]. Similar antioxidant superior effects for the combination were observed in the mice peritoneal macrophages, in accordance with previous studies using PJ [8, 9], or with date seeds oil in in-vitro studies [55]. These effects could be related to the significant increment in macrophage PON2, or in serum PON1, as was previously shown for PJ effect in J774A.1 macrophages [56]. It should be noted however that only consumption of the combination significantly decreased MPM cholesterol content, and this could be

related to the observed reduction in the uptake of LDL by the cells. Finally, in the

mice aortas, only the combination of pomegranate and dates demonstrated significant

anti-atherogenic effects, as it decreased aortas' lipid peroxides content, as well as

triglyceride and cholesterol mass. These effects could be related directly to the date

and pomegranate constituents, and / or to the increased PON (PON1/PON2) activities.

In contrast to the results in MPM the date seeds treatment did not significantly affect

the aorta lipid peroxides content, although the increase in PON and the decrease in

cholesterol content. The aortas of the E^0 mice contain increased levels of lipid

peroxides more than the levels observed in MPM, thus the small increase in PON

activity is not enough to cause reduction in the lipid peroxides levels and probably a

higher date seeds concentration is needed to decrease aortas (vs. MPM) lipid

peroxides levels. We speculate that pomegranate or date polyphenols directly affect

cellular cholesterol biosynthesis rate by decreasing HMGCoA reductase mRNA

levels, or HMGCoA activity as was shown for resveratrol [57] or for the polyphenol

extract (Seapolynol(™) [58], respectively. These effects could be probably not related

to the reduction in oxidative stress. These results further point to the selective and

specific effects of date and pomegranate in various tissues and cells, resulting in from

tissue specific interactions with polyphenols which are related to CVD risk. The

biological relevance of direct anti-oxidative effects of polyphenols for CVD was not

established [59], but in the current study we have demonstrated, for the first time, in

vivo using the atherosclerotic E^0 mice that consumption of the combination of date

extracts and PJ decreased serum, macrophage and aortic oxidative stress, and these

effects could, at least in part, be anti-atherogenic.

In conclusion then , the current study clearly demonstrates the beneficial anti-

atherogenic effects of consuming the combination of pomegranate fruit   with date

fruit and seeds, on oxidative stress and lipids metabolism in atherosclerotic mice

serum, macrophages and aortas. These results are probably due to the unique and

different polyphenol's structure in pomegranate and in date which demonstrate an

added value to their anti – atherogenic properties.

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Notes

The authors declare no competing financial interest.

Abbreviations Used

DDW, double distilled water

DPPH, 1, 1-diphenyl-2-picryl-hydrazyl

FITC, fluorescein isothiocyanate

HDL, high density lipoprotein

LDL, low density lipoprotein

PON1, paraoxonase 1

PON2, paraoxonase 2

MPM, mouse peritoneal macrophages

E^0 mice, apolipoprotein E KO mice

PJ, pomegranate juice

GAE, gallic acid equivalent

TBARS, thiobarbituric acid reactive substances

AAPH, 2.2'-azobis, 2-amidinopropane hydrochloride

DCFH, 2', 7'-dichlorofluorescin diacetate

PBS, phosphate buffered saline

DMEM, dulbecco's modified eagle's medium

FCS, fetal calf serum

DHC, dihydrocumarin

References

1. Hayek T, Oikinine J, Brook JG, Aviram M. Increased plasma and lipoprotein lipid peroxidation in apoE deficient mice. *Biochem Biophys Res Commun*. **1994**, 201, 1567-1574.

2. Maor I, Kaplan M, Hayek T, Vaya Y, Hoffman A, Aviram M. Oxidized monocyte-derived macrophages inn aortic atherosclerotic lesion from E0 mice and from human carotid artery contain lipid peroxides and oxysterols. *Biochem Biophys Res Commun*. **2000**, 269, 775-780.

3. Lusis AJ. Atherosclerosis. *Nature*. **2000**, 404, 233-241.

4. Aviram M, Rosenblat M. Pomegranate protection against cardiovascular diseases. *Evid Based Complement Alternat Med*. **2012**, 2012, 382763.

5. Gómez-Caravaca AM1, Verardo V, Toselli M, Segura-Carretero A, Fernández-Gutiérrez A, Caboni MF. Determination of the major phenolic compounds in pomegranate juices by HPLC−DAD−ESI-MS. *J Agric Food Chem*. **2013**, 61, 5328-5333.

6. Gil ML, Tomas-Barberan FA, Hess-Pierce B, Holcroft DM, Kader AA. Antioxidant activity of pomegranate juice and its relationship with phenolics composition and processing. *J Agric Food Chem*. **2000**, 48, 4581-4589.

7. Seeram NP1, Adams LS, Henning SM, Niu Y, Zhang Y, Nair MG, Heber D. In vitro antiproliferative, apoptotic and antioxidant activities of punicalagin, ellagic acid and a total pomegranate tannin extract are enhanced in combination with other polyphenols as found in pomegranate juice. *J Nutr Biochem*. **2005**, 16, 360-367.

8. Kaplan M, Hayek T, Raz A, Coleman R, Dornfeld L, Vaya J, Aviram M. Pomegranate juice supplementation to atherosclerotic mice reduces macrophage lipid

peroxidation, cellular cholesterol accumulation and development of atherosclerosis. *J Nutr*. **2001**, 131, 2082-2089.

9. Aviram M1, Dornfeld L, Rosenblat M, Volkova N, Kaplan M, Coleman R, Hayek T, Presser D, Fuhrman B. Pomegranate juice consumption reduces oxidative stress, atherogenic modifications to LDL, and platelet aggregation: studies in humans and in atherosclerotic apolipoprotein E-deficient mice. *Am J Clin Nutr*. **2000**, 71, 1062-1076.

10. Al-Jarallah A1, Igdoura F, Zhang Y, Tenedero CB, White EJ, MacDonald ME, Igdoura SA, Trigatti BL. The effect of pomegranate extract on coronary artery atherosclerosis in SR-BI/APOE double knockout mice. *Atherosclerosis.* **2013**, 228, 80-89.

11. Matthaiou CM, Goutzourelas N, Stagos D, Sarafoglou E, Jamurtas A, Koulocheri SD, Haroutounian SA, Tsatsakis AM, Kouretas D. Pomegranate juice consumption increases GSH levels and reduces lipid and protein oxidation in human blood. *Food Chem Toxicol.* **2014**, 73C, 1-6.

12. Aviram M, Rosenblat M, Gaitini D, Nitecki S, Hoffman A, Dornfeld L, Volkova N, Presser D, Attias J et al. Pomegranate juice consumption for 3 years by patients with carotid artery stenosis reduces common carotid intima-media thickness, blood pressure and LDL oxidation. *Clin Nutr*. **2004**, 23, 423-433.

13. Rosenblat M1, Volkova N, Aviram M. Pomegranate phytosterol (β-sitosterol) and polyphenolic antioxidant (punicalagin) addition to statin, significantly protected against macrophage foam cells formation. Atherosclerosis**. 2013**, 226, 110-117.

14. Hamoud S, Hayek T, Volkova N, Attias J, Moscoviz D, Rosenblat M, Aviram M. Pomegranate extract (POMx) decreases the atherogenicity of serum and of human monocyte-derived macrophages (HMDM) in simvastatin-treated

hypercholesterolemic patients: a double –blinded, placebo-controlled randomized

prospective pilot study. Atherosclerosis. 2014, 232, 204-210.

15. Espin JC, Larrosa m, Garcia-Conesa MT, Tomas-Barberan F. Biological

significance of urolithins, the gut microbial ellagic acid-derived metabolites: the

evidence so far. *Evid Based Complement Alternat Med*. **2013**, 2013, 270418.

16. Vayalil PK. Date fruits (Phoenix dactylifera Linn): an emerging medicinal food.

Crit Rev Food Sci Nutr. **2012**, 52, 249-271.

17. Rock W. Rosenblat M, Borochov-Neori H, Volkova N, Judeinstein S, Elias M,

Aviram M. Effects of date (Phoenix dactylifera L., Medjool or Hallawi variety)

consumption by healthy subjects on serum glucose and lipid levels and on serum

oxidative status; a pilot study. J Agric Food Chem. 2009, 57, 8010-8017.

18. Borochov-Neori H, Judeinstein S, Greenberg A, Volkova N, Rosenblat M, Aviram

M. Date (Phoenix dactylifera L.) fruit soluble phenolics composition and anti-

atherogenic properties in nine Israeli varieties. J Agric Food Chem. 2013, 61, 4278-

4286.

19. Habib HM1, Platat C, Meudec E, Cheynier V, Ibrahim WH. Polyphenolic

compounds in date fruit seed (Phoenix dactylifera): characterization and

quantification by using UPLC-DAD-ESI-MS. *J Sci Food Agric*. **2014**, 94, 1084-1089.

20. Juhaimi FA, Ghafoor K, Ozcan MM. Physical and chemical properties,

antioxidant activity, total phenol and mineral profile of seeds of seven different date

fruit (Phoenix dactylifera L.) varieties. *Int J Food Sci Nutr*. **2012**, 63, 84-89.

21. Habib HM, Ibrahim WH. Effect of date seeds on oxidative damage and

antioxidant status in vivo. *J Sci Food Agric*. **2011**, 91, 1674-1679.

22. Takaeidi MR, Jahangiri A, Khodayar MJ, Siahpoosh A, Yaghooti H, Rezaei S, Salecheh M, Mansourzadeh Z. The effect of date seed (Phoenix dactylifera) extract on paraoxonase and arylesterase activities in hypercholesterolemic rats. *Jundishapur J Nat Pharm Prod*. **2014**, 9, 30-34.

23. Singleton, V. L., Rossi, J. A. Colorimetry of total phenolics with phosphomolybdic   phosphotungstic acid reagents. *Am J Enol Viti* **1965**, 16, 144-158.

24. Blois MS. Antioxidant determination by the use of a stable free radical. *Nature*. **1958**, 181, 1199-1200.

25. Aviram M. Plasma lipoprotein separation by discontinuous density gradient ultracentrifugation in hyperlipoproteinemic patients. *Biochem Med*. **1983**, 30, 111-118.

26. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem*. **1951**, 193, 265-275.

27. Buege JA, DS Aust. Microsomal lipid peroxidation. *Methods. Enzymol*. **1978**, 52, 302-310.

28. El-Saadani M, Esterbauer N, El-Sayed M, Goher M, Nassar AY, Jurgens G. Spectrophotometric assay for lipid peroxides in serum lipoproteins using commercially available reagent. *J Lipid Res*. **1989**, 30, 627-630.

29. Gu X, Kozarsky K, Krieger M. Scavenger receptor class B, type I-mediated [3H] cholesterol efflux to high and low density lipoproteins is dependent on lipoprotein binding to the receptor. *J Biol Chem*. **2000**, 275, 29939–30001.

30. Gaidukov L, Tawfik DS. High affinity, stability, and lactonase activity of serum paraoxonase PON1 anchored on HDL with ApoA-I. *Biochemistry*. **44** (2005) 11842-11854.

31. Frei B, Stocker R, Ames BN. Antioxidant defenses and lipid peroxidation in human blood plasma. *Proc Natl Acad Sci USA*. **1988,** 85, 9748-9752.

32. Bass DA, Parce JW, Dechatelet LR, Szejda P, Seeds MC, Thomas M. Flow cytometric studies of oxidative product formation by neutrophils: a graded response to membrane stimulation. *J. Immunol.* **1983**, 130, 1910–1917.

33. LeBel CP, Ischiropoulos H, Bondy SC. Evaluation of the probe 2',7'-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. *Chem Res Toxicol*. **1992**, 5, 227-231.

34. Liu Y1, Mackness B, Mackness M. Comparison of the ability of paraoxonases 1 and 3 to attenuate the in vitro oxidation of low-density lipoprotein and reduce macrophage oxidative stress. *Free Radic Biol Med*. **2008**, 45, 743-748.

35. Horke S, Witte I, Wilgenbus P, Krüger M, Strand D, Förstermann U. Paraoxonase-2 reduces oxidative stress in vascular cells and decreases endoplasmic reticulum stress-induced caspase activation. *Circulation*. **2007**, 115, 2055-2056.

36. Seeram NP, Henning SM, Zhang Y, Suchard M, Li Z, Heber D. Pomegranate juice ellagitannin metabolites are present in human plasma and some persist in urine for up to 48 h. *J Nutr*. **2006**, 136, 2481-2485.

37. Seeram NP, Zhang Y, McKeever R, Henning SM, Lee Ru-po, Suchard MA, Li Z, Chen S, Thames G, Zerlin A, Nguyen M, Wang D, Dreher M, Heber D. Pomegranate juice and extracts provide similar levels of plasma and urinary ellagitannin metabolites in human subjects. *J Med Food.* **2008,** 11, 390-394.

38. Bialonska D, Kasimsetty SG, Khan SI, Ferreira D. Urolithins, intestinal microbial metabolites of Pomegranate ellagitannins, exhibit potent antioxidant activity in a cell-based assay. *J Agric Food Chem*. **2009**, 57, 10181-10186.

39. Mertens-Talcott SU, Jilma-Stohlawetz P, Rios J, Hingorani L, Derendorf H. Absorption, metabolism, and antioxidant effects of pomegranate (*Punica granatum L.)* polyphenols after ingestion of a standardized extract in healthy human volunteers. J Agric Food Chem. 2006, 54, 8956-8961.

40. Cerda B, Espı´n JC, Parra A, Martı´nez P, Toma´s-Barbera´n FA. The potent in Vitro antioxidant ellagitannins from pomegranate juice are metabolized into bioavailable but poor antioxidant hydroxy-6H-dibenzopyran-6-one derivatives by the colonic microflora of healthy humans. *Eur J Nutr*. **2004**, 43, 205-220.

41. Seeram NP, Aronson WJ, Zhang Y, Henning SM, Moro A, Lee R-P, Sartippour M, Harris DM, Retting M, Suchard MA, Pantuck AJ, Belldegrun A, Heber D. Pomegranate ellagitannins derived metabolites inhibit prostate cancer growth and localize to mouse prostate gland. *J Agric Food Chem. 2007, 55, 7732-7737.*

42. Brown JE, Khodr H, Hider RC, Rice-Evance CA. Structural dependence of flavonoid interactions with Cu2+ ions: implications for their antioxidant properties. *Biochem J*. **1998**, 330, 1173-1178.

43. Ishimoto H, Shibata M, Myojin Y, Ito H, Sugimoto Y, Tai A, Hatano T. In vivo anti-inflammatory and antioxidant properties of ellagitannin metabolite urolithin A. *Bioorg Med Chem Lett*. **2011**, 21, 5901-5904.

44. O'Connor, P. M., Zysow BR, Schoenhaus SA, et al. Prebeta-1 HDL in plasma of normolipidemic individuals: influences of plasma, age, and gender. *J Lipid Res*. **1998**, 39, 670–678.

45. Patel PJ1, Khera AV, Wilensky RL, Rader DJ. Anti-oxidative and cholesterol efflux capacities of high-density lipoprotein are reduced in ischaemic cardiomyopathy. *Eur J Heart Fail*. **2013,** 15, 1215-1219.

46. Hafiane A1, Jabor B1, Ruel I1, Ling J1, Genest J2. High-density lipoprotein mediated cellular cholesterol efflux in acute coronary syndromes. *Am J Cardiol*. **2014**, 113, 249-255.

47. Chen JH1, Wang CJ, Wang CP, Sheu JY, Lin CL, Lin HH. Hibiscus sabdariffa leaf polyphenolic extract inhibits LDL oxidation and foam cell formation involving up-regulation of LXRα/ABCA1 pathway. *Food Chem*. **2013**, 141, 397-406.

48. Helal O1, Berrougui H, Loued S, Khalil A. Extra-virgin olive oil consumption improves the capacity of HDL to mediate cholesterol efflux and increases ABCA1 and ABCG1 expression in human macrophages. *Br J Nutr*. **2013**, 109, 1844-1855.

49. Hendrich AB. Flavonoid-membrane interactions: possible consequences for biological effects of some polyphenolic compounds. *Acta Pharmacol Sin*. **2006**, 27, 27-40.

50. Forman HJ, Davies KJA, Ursini F. How do nutritional antioxidants really work: nucleophilic tone and para-hormesis versus free radical scavenging in vivo. *Free Radic Biol Med.* **2014**, 66, 24-35.

51. Abuelgassim AO. Effect of Flax Seeds and Date Palm Leaves Extracts on Serum Concentrations of Glucose and Lipids in Alloxan Diabetic Rats. *Pakistan Journal of Biological Sciences. **2010**, 13: 1141-1145*.

52. Aviram M, M Rosenblat, S Billecke, et al. Human serum paraoxonase (PON 1) is inactivated by oxidized low density lipoprotein and preserved by antioxidants. *Free Radic Biol Med*. **1999**, 26, 892-904.

53. Atrahimovich D, Vaya J, Khatib S. The effects and mechanism of flavonoid-rePON1 interactions. Structure-activity relationship study. *Bioorg Med Chem.* **2013,** 21**,** 3348-3355.

54. Khateeb J1, Gantman A, Kreitenberg AJ, Aviram M, Fuhrman B. Paraoxonase 1 (PON1) expression in hepatocytes is upregulated by pomegranate polyphenols: a role for PPAR-gamma pathway. Atherosclerosis. **2010** , 208, 119-125.

55. Ines D, Sonia B, Fatma BA, Souhali B, Hamadi A, Hamida T, Basma H. Date seed oil inhibits hydrogen-peroxide-induced oxidative stress in human epidermal keratinocytes. *Int J Dermatol.* **2010**, 49, 262-268.

56. Shiner M, Fuhrman B, Aviram M. Macrophage paraoxonase 2 (PON2) expression is up-regulated by pomegranate juice phenolic anti-oxidants via PPAR gamma and AP-1 pathway activation. *Atherosclerosis*. **2007**, 195, 313-321.

57. Il Jin Choa, Ji Yun Ahna, Suna Kima, Myung Sook Choib, Tae Youl Haa, Resveratrol attenuates the expression of HMG-CoA reductase mRNA in hamsters. *Biochemical Biophysical Research Communications*. **2008,** 367, 190–194.

58. Yeo AR1, Lee J1, Tae IH1, Park SR1, Cho YH2, Lee BH3, Shin HC4, Kim SH4, Yoo YC1. Anti-hyperlipidemic effect of polyphenol extract (Seapolynol(™) and Dieckol isolated from Ecklonia cava in in vivo and in vitro models. *Prev Nutr Food Sci.* **2012**, 17, 1-7.

59. Hollman PC1, Cassidy A, Comte B, et al.The biological relevance of direct antioxidant effects of polyphenols for cardiovascular health in humans is not established. *J Nutr.* **2011**, 141, 989S-1009S.

Legend to figures

Figure 1: Anti-oxidative properties of Amari date extract, Hallawi date extract, or date seeds extract, and their ability to affect macrophage cholesterol efflux. (A) Total polyphenol concentration in Hallawi date extract, or in Amari date extract, or in date seeds extract was determined as described under the Methods section, and is expressed as μg of GAE. (B) DPPH (100μmoL/L) was incubated with increasing polyphenols concentrations (0-5μg GAE/mL) of Hallawi date extract, or Amari date extract or of date seeds extract, and the DPPH absorbance was monitored at time 0 and after 5 minutes of incubation. (C&D) LDL (100μg protein /mL) was pre-incubated with increasing polyphenols concentrations (0-5μg GAE/mL) of Hallawi date extract, or of Amari date extract, or of date seeds extract, followed by the addition of copper ions. The extent of LDL oxidation was determined by the TBARS assay (C) or by the lipid peroxides assay (D). (E) J774A.1 cell line macrophages were pre-incubated for 20h with 2 or 5 μg GAE/mL of Hallawi date extract, or Amari date extract or of date seeds extract. Control cells were incubated with similar volume of 50% ethanol. After cell wash, the extent of HDL-mediated cholesterol efflux from the cells was determined as described under the Methods section. Results are the mean ±SD of three different experiments, *p‹ 0.01 vs. Control Cells.

Figure 2: Anti-oxidative properties and ability to affect macrophage cholesterol efflux of: PJ, its major polyphenols (Punicalain, Punicalagin, Gallic Acid), or their metabolites (Urolithin A, Urolithin B). (A) Total polyphenol concentrations in PJ, in gallic acid, in urolithin A, in urolithin B, in punicalain or in punicalagin were determined as described under the Methods section, and is expressed as μg of GAE. (B) DPPH (100μmol/L) was incubated with increasing polyphenols concentrations

(0-5μg GAE/mL) of PJ, or of gallic acid, or of urolithin A, or of urolithin B, or of punicalain or of punicalagin, and the DPPH absorbance was determined at time 0 and after 5 minutes of incubation. (C&D) LDL (100μg protein /mL) was pre-incubated with increasing polyphenols concentrations (0-5μg GAE/mL) of PJ, or of gallic acid, or of urolithin A, or of urolithin B, or of punicalain, or of punicalagin, followed by the addition of copper ions. The extent of LDL oxidation was determined by the TBARS assay (C) or by the lipid peroxides assay (D). (E) J774A.1 macrophages were pre-incubated for 20h with 2 or 5 μg GAE/mL of PJ, or of gallic acid, or of urolithin A, or of urolithin B, or of punicalain or of punicalagin. Control cells were incubated with similar volume of 50% ethanol. After cell wash, the extent of HDL-mediated cholesterol efflux from the cells was determined as described under the Methods section. Results are the mean ±SD of three different experiments, *p‹ 0.01 vs. Control Cells.

Figure 3: The effect of consumption by the atherosclerotic E^0 mice of date seeds extract, or PJ, or Hallawi date extract, or their combination, on the mice serum oxidative stress and on serum PON1 activity. E^0 mice, at the age of 6 weeks, consumed, for 3 weeks, 0.5μmoL GAE/mouse/day, of: PJ, Hallawi extract, date seeds extract, or of the combination (PJ +Hallawi date extract +date seeds extract). The placebo mice received similar volume of 50% ethanol. At the end of the study, blood samples were collected. (A) The susceptibility of the mice sera to AAPH-induced oxidation was determined by the TBARS assay. (B) Serum PON1 arylesterase activity was measured as described under the Methods section. Results are the mean ±SD (n=4) in each mouse group, *p‹ 0.01 vs. Placebo.

Figure 4: The effect of consumption by E^0 mice of date seeds extract, or PJ, or

Hallawi date extract, or their combination, on the mice peritoneal macrophages

(MPM) atherogenic properties. E^0 mice, at the age of 6 weeks, consumed, for 3

weeks, 0.5µmoL GAE/mouse/day, of PJ, or of Hallawi extract, or of date seeds

extract, or of the combination (PJ +Hallawi date extract +date seeds extract). The

placebo mice received similar volume of 50% ethanol. At the end of the study, MPM

were harvested and the following analyses were performed: (A) MPM oxidative stress

was determined by the DCFH assay, (B) MPM PON2 lactonase activity, (C) MPM

cholesterol content, and (D) MPM uptake of LDL (labeled with FITC). Results are the

mean ±SD (n=4) in each mouse group, *p‹ 0.01 vs. Placebo.

Figure 5: The effect of consumption by E^0 mice of: date seeds extract, PJ, Hallawi

date extract, or their combination, on their aorta's atherogenic properties.

E^0 mice, at the age of 6 weeks, consumed for 3 weeks, 0.5µmoL GAE/mouse/day, of

PJ, or of Hallawi extract, or of date seeds extract, or of the combination (PJ +Hallawi

date extract +date seeds extract). The placebo mice received similar volume of 50%

ethanol. At the end of the study the mice aortas were collected and the following

analyzes were performed: (A) Aorta's lipid peroxides content, (B) Aorta's PON

lactonase activity, (C) Aorta's cholesterol content, and (D) Aorta's triglyceride

content. Results are the mean ±SD (n=4) in each mouse group, *p‹ 0.01 vs. Placebo.

Table 2: Effect of consumption of Hallawi Date extract, or Date Seeds extract, or Pomegranate juice (PJ), or their combination by E^0 Mice on serum lipids and glucose levels

	Cholesterol (mg/dL)	Triglyceride (mg/dL)	Glucose (mg/dL
Placebo Mice	393±40	283±16	99±19
+ Hallawi Extract	413±67	312±57	116±10
+Date Seeds Extract	242±11 *	142±14 *	100±8
+PJ	389±53	288±8	135±25
+Hallawi Extract +Date Seeds Extract + PJ	300 ±11 *	168±14*	122±12

*p< 0.01 (vs. Placebo)

Dates -In vitro
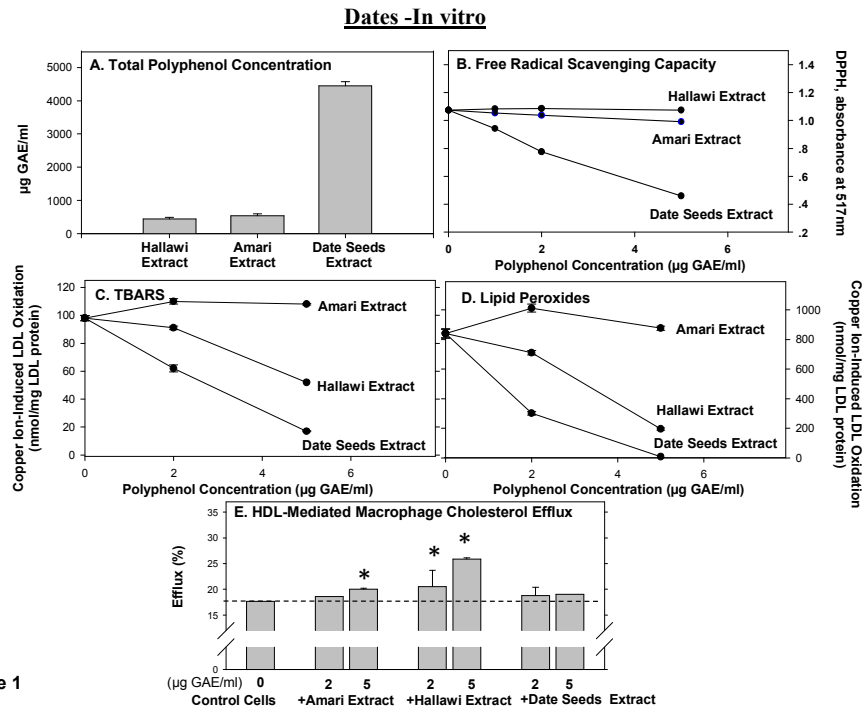
Figure 1

Pomegranate –In Vitro

Figure 2

In Vivo : E⁰ Mice Serum

A. AAPH-Induced Serum Lipid Peroxidation

A. Serum PON1 Activity

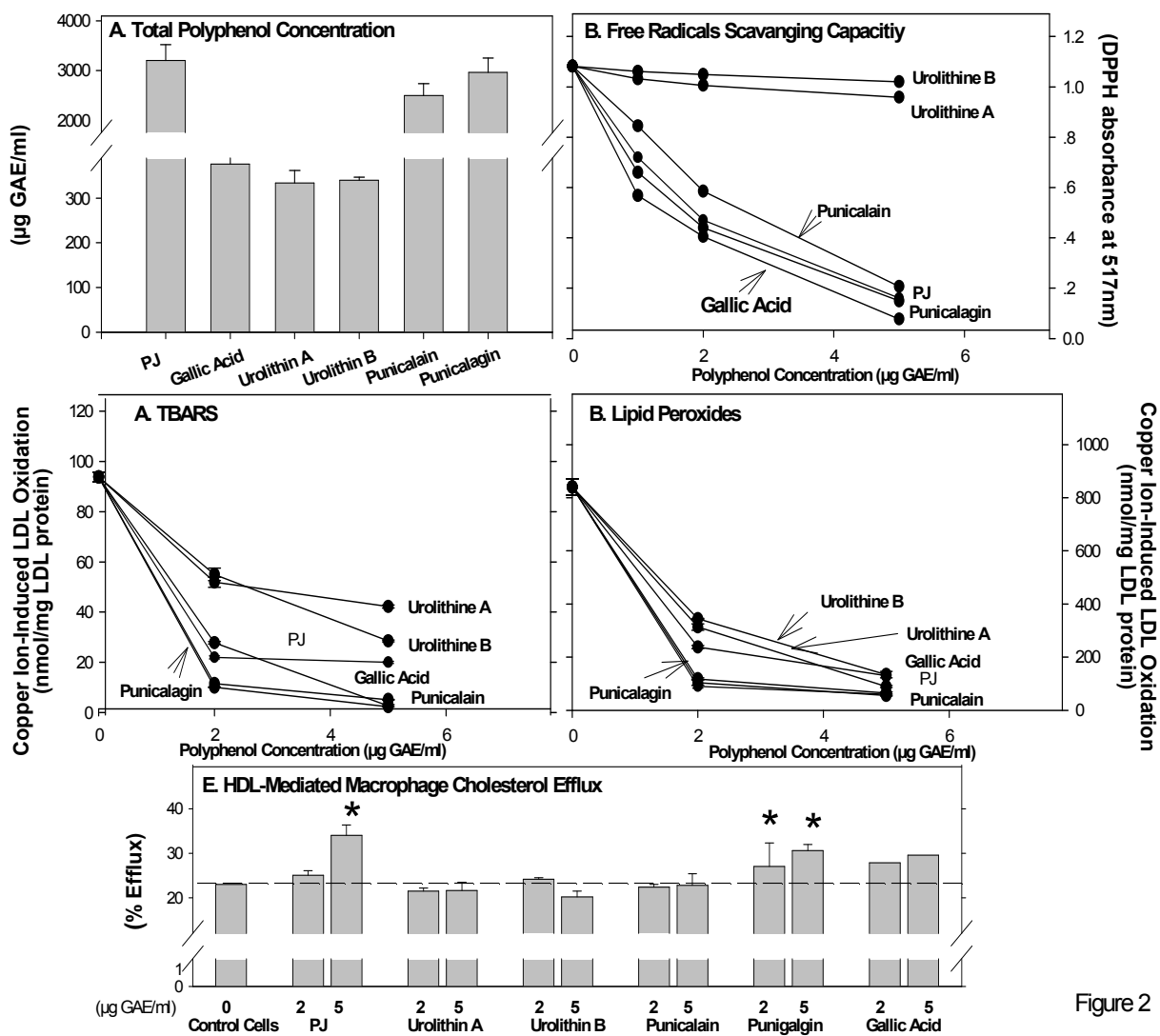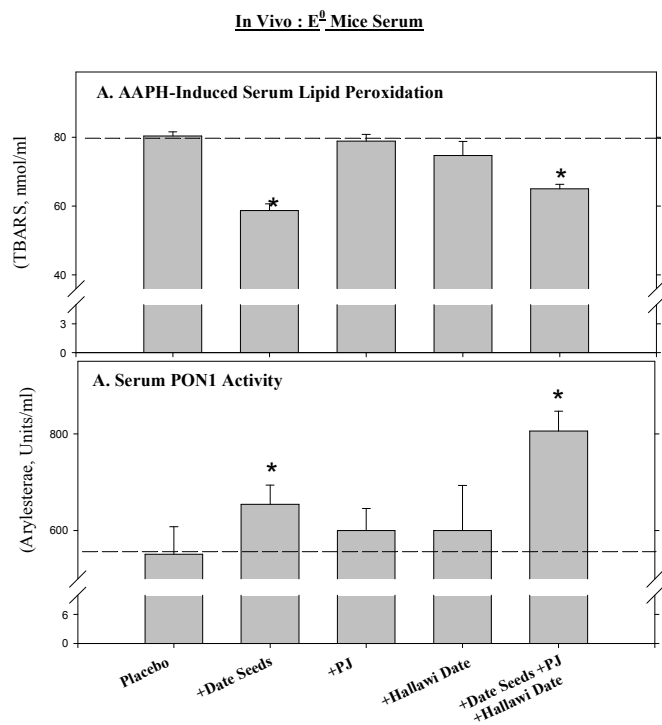Placebo   +Date Seeds   +PJ   +Hallawi Date   +Date Seeds +PJ +Hallawi Date

Figure 3
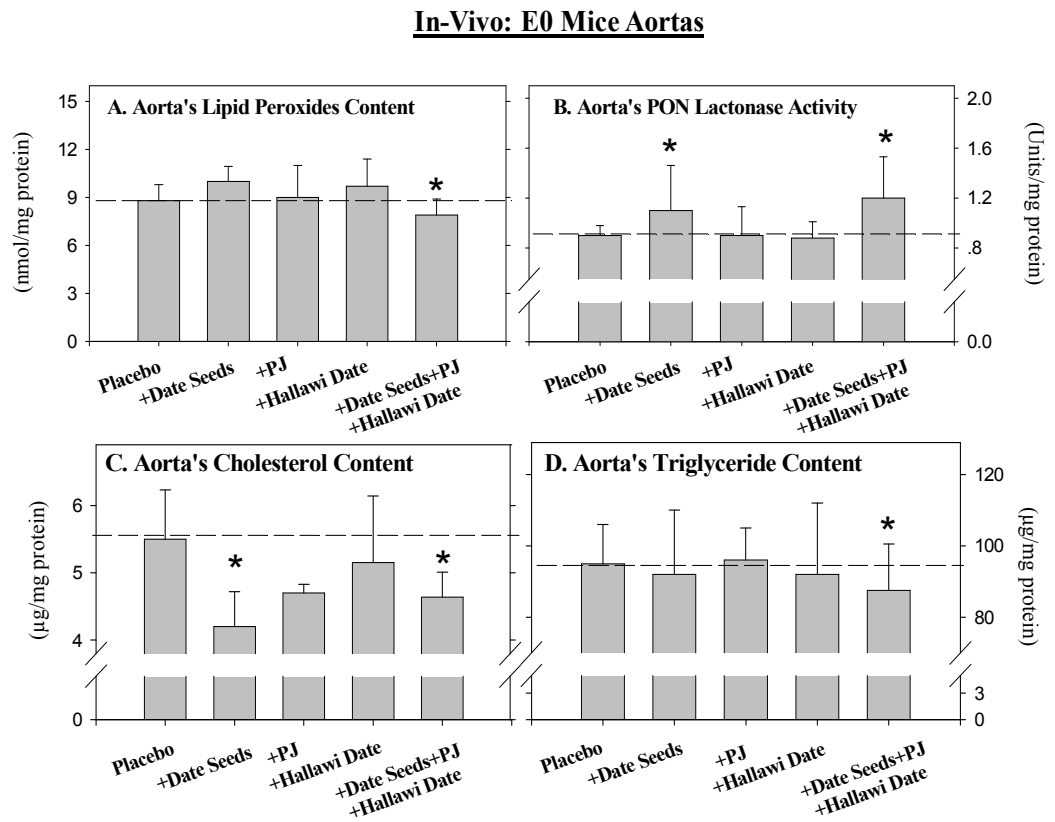
In -Vivo: E0 Mice Peritoneal Macrophages (MPM)

Figure 4

In-Vivo: E0 Mice Aortas

Figure 5