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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, 0.5\(\mu\)mol 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 \(\beta\)-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™ 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**

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

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% Na₂CO₃ 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
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 x 10⁶ 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.** 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).

**Serum analyzes**

**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 10^9 cells/L in DMEM containing 5% FCS, 1x10^5 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 (2x10^6) 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 CaCl₂ 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.

**Statistics.** 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.
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<sup>0</sup> 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<sup>0</sup> 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).
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
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 E₀ 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 E₀ 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 
ellagittannins 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 is 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 electrophil 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<sup>0</sup> 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 E0 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 E⁰ 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 E⁰ 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.
Funding

We acknowledge the financial support from the Rappaport Family Research Institute in the Medical Sciences and from the Clinical Research Institute at Rambam Health Care Campus (CRIR).

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<sup>0</sup> 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
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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 E⁰ 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. E⁰ 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 E₀ mice of date seeds extract, or PJ, or Hallawi date extract, or their combination, on the mice peritoneal macrophages (MPM) atherogenic properties. E₀ 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 E₀ mice of: date seeds extract, PJ, Hallawi date extract, or their combination, on their aorta's atherogenic properties. E₀ 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<sup>0</sup> Mice on serum lipids and glucose levels

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

*p < 0.01 (vs. Placebo)
Dates - In vitro

A. Total Polyphenol Concentration

Polyphenol Concentration (µg GAE/ml)

Hallawi Extract Amari Extract Date Seeds Extract

B. Free Radical Scavenging Capacity

Free Radical Scavenging Capacity

Hallawi Extract Amari Extract Date Seeds Extract

C. TBARS

Copper Ion-induced LDL Oxidation

TBARS

Hallawi Extract Amari Extract Date Seeds Extract

D. Lipid Peroxides

Copper Ion-induced LDL Oxidation

Lipid Peroxides

Hallawi Extract Amari Extract Date Seeds Extract

E. HDL-mediated Macrophage Cholesterol Efflux

Control Cells +Amari Extract +Hallawi Extract +Date Seeds Extract

Figure 1

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Figure 2

A. Total Polyphenol Concentration

B. Free Radicals Scavenging Capacity

C. Lipid Peroxides

D. Copper Ion-induced LDL Oxidation

E. HDL-Mediated Macrophage Cholesterol Efflux
Figure 3

In Vivo \( E^2 \) Mice Serum

A. AAPH-Induced Serum Lipid Peroxidation

B. Serum PON1 Activity

* Significance level
In -Vivo: E0 Mice Peritoneal Macrophages (MPM)

Figure 4
In-Vivo: E0 Mice Aortas

A. Aorta’s Lipid Peroxides Content

B. Aorta’s PON Lactonase Activity

C. Aorta’s Cholesterol Content

D. Aorta’s Triglyceride Content

Figure 5