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Rosemary extracts improve flow-mediated dilatation of the brachial artery and plasma PAI-1 activity in healthy young volunteers

Rosemary extracts and endothelial dysfunction

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Abstract

Polyphenol antioxidants decrease the risk of atherosclerosis. Our aim was to evaluate prospectively in healthy young participants the effect of oral rosemary extracts (RE), consisting of diphenols, upon endothelial dysfunction (ED), preceding structural atherosclerosis. We prospectively studied 19 healthy young volunteers, who received oral RE (77.7 mg) for 21 days, consisting of active substances carnosol (0.97 mg), carnosic (8.60 mg) and rosmarinic acid (10.30 mg). Before and after RE treatment, we evaluated fasting serum levels of plasminogen-activator-inhibitor-1 (PAI-1), vascular cell adhesion molecule 1 (VCAM-1), inter-cellular adhesion molecule 1 (ICAM-1), superoxide dismutase (SOD), glutathione peroxidase (GPX), fibrinogen, high-sensitivity capsular reactive protein (hs-CRP), tumor-necrosis factor α (TNF- α), the lipid profile and ED, characterised as flow-mediated dilatation (FMD) in the brachial artery of $< 4.5\%$, estimated by ultrasound measurements. After 21 days, any side effects were registered, mean FMD increased nonsignificantly ($6.51 \pm 5.96\%$ vs. $7.78 \pm 4.56\%$, $p = 0.546$) and ED decreased significantly (66.6% vs 16.6% , $p = 0.040$). Among serum markers, only mean PAI-1 level decreased significantly ($4.25 \pm 1.46\text{U/ml}$ vs $3.0 \pm 0.61\text{U/ml}$, $p = 0.012$) after 21-day RE supplementation. We conclude that oral RE supplementation has the potential to improve serum PAI-1 activity and ED in young and healthy individuals.

Key words: Rosemary extracts, endothelial dysfunction, flow-mediated dilatation, plasminogen-activator-inhibitor-1

Introduction

Moderate consumption of red wine and green tea polyphenols has been associated with decreased risk of cardiovascular morbidity and mortality [Sato et al., 2002; Grassi et al., 2008]. Recent clinical studies have demonstrated an improvement in flow-mediated dilatation (FMD) of the brachial artery in healthy young people after administration of polyphenols in green tea as well as in patients with documented atherosclerosis after administration of polyphenol isoflavone in soya beans [Nishioka et al., 2007; Alexopoulos et al., 2008; Chan et al., 2008].

In vitro and in vivo studies demonstrated strong antioxidant and anti-inflammatory activity of rosemary extracts (RE) attributed to their diphenolic constituents, carnosic and rosmarinic acid in particular [Aruoma et al., 1996; Zeng et al., 2001; Lin et al., 2008]. As antioxidants, RE are scavengers of free radicals, they decrease consumption of NO due to depletion of free radicals, decrease the injury of endothelial cells and LDL oxidation by free radicals [Petersen et al., 2003; Moreno et al., 2006; Lin et al., 2008].

In experimental animals, superoxide dismutase (SOD) and glutathione peroxidase (GPX), being the most important antioxidant defense systems, are most often increased after consumption of polyphenols as a consequence of diminished oxidative stress [Chan et al., 2008; He et al., 2009]. For the same reason, the serum lipid profile is improved, resulting in decreased total and LDL-cholesterol [Lecumberri et al., 2007].

In comparison to other non-phenolic antioxidants, the advantages of RE are their more extensive effect upon the nuclear factor kappa B (NF- κ B) pathway, resulting in more pronounced decrease in inflammation [Aggarwal et al., 2004; Lin et al., 2008]. Their activity involving the NF- κ B pathway is important in the control of the immune and inflammatory response, cellular proliferation and survival [Aggarwal et al., 2004; Lin et

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4 al., 2008]. Therefore, RE may prevent several chronic and progressive inflammatory
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6 and autoimmune diseases, particularly atherosclerosis, Alzheimer's disease and aging in
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8 general [Lin et al., 2008]. Inflammatory activity of the NF- κ B pathway, exerting its
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10 effect by expressing cytokines, chemokines, cell adhesion molecules, immunoreceptors,
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12 etc., was studied by evaluating serum markers such as high-sensitivity C-reactive
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14 protein (hs-CRP), fibrinogen, tumor necrosis factor- α (TNF- α) and serum
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16 plasminogen-activator-inhibitor type 1 (PAI-1) activity [Pasten et al. 2007; Chan et al.,
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18 2008; Lin et al., 2008]. Studies even demonstrated that diphenols in RE decrease PAI-1
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20 activity by several pathways, in addition to NF- κ B transcription, TNF- α reduction and
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22 human peroxisome proliferator-activated receptor gamma activation [Rau et al. 2006].
23
24 Arterial endothelial dysfunction (ED) is an early event in atherogenesis, preceding
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26 structural atherosclerotic changes by years. It can be evaluated by ultrasound
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28 measurement of the flow-mediated dilatation (FMD) in the brachial artery and by
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30 measuring serum markers such as vascular cell adhesion molecule 1 (VCAM-1) and
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32 inter-cellular adhesion molecule 1 (ICAM-1) [Celermajer et al., 1992; Hwang et al.,
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34 1997; Jadhav et al., 2003].

35
36 Our aim was to evaluate prospectively the effect of 21-day oral RE supplementation
37
38 with active substances carnosol, carnosic and rosmarinic acid, upon FMD and serum
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40 markers ICAM-1, VCAM-1, PAI-1, hs-CRP, fibrinogen, SOD, GPX, TNF- α , total
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42 serum cholesterol, triglycerides, LDH-cholesterol and HDL-cholesterol level in healthy
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44 young volunteers.
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54 55 56 57 Materials and methods

58 59 *Subjects studied* 60

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4 We studied 19 healthy young volunteers, 7 men and 12 women, mean age 34.3 ± 7.7
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6 years. 8 (42.1%) were smokers and 3 (15.8%) with the family history of symptomatic
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8 atherosclerosis. Inclusion criteria: more than 18 years old, negative urine- β -HCG test in
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10 young women, written informed consent. A week before the start and during the study
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12 we recommended discontinuation of all previously used antioxidants, vitamins and
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14 alcohol intake. We also recommended the use of a well-balanced diet of 1500 – 2000
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16 kcal/day with approximately 30% proteins, 60% carbohydrates and 10% fat with at least
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18 one daily ration of meat, but only up to 2 apples per day [Chun et al., 2008].
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23 Exclusion criteria: any known chronic, malignant disease and/or the use of any
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25 medication, pregnancy, breast-feeding and simultaneous participation in another clinical
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27 study.
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30 *Study design.*

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33 Observational prospective monocenter study performed at the University Clinical
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35 Centre, Maribor, approved by the national ethics committee (Number 58/06/06). Written
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37 informed consents were obtained before the start of the study from each participant. The
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39 study protocol conformed to the ethical guidelines of the Declaration of Helsinki. The
40
41 study was also approved by the National Agency for Medical Products and Medical
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43 Devices (Number 297-2006-001894-12) and registered at EMEA (EudraCT number
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45 2006-001894-12; Protocol Code Number ROS-001).
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50 *Study protocol*

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53 On day 0, volunteers were clinically examined and screened for baseline demographic
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55 data, family history and history of prior diseases. Blood pressure and pulse were
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57 measured and standard ECG was recorded. When all the inclusion criteria were fulfilled
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59 and contraindications were excluded, first written informed consent was obtained. For
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4 the next four weeks the volunteers discontinued the use of any vitamin and antioxidant
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6 supplementation and started the recommended diet [Chun et al., 2008].
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9 On day 7, inclusion and exclusion criteria were rechecked and second written informed
10
11 consent was obtained. Blood pressure and pulse were measured. Fasting blood samples
12
13 were drawn from the antecubital vein to estimate serum levels of PAI-1, SOD, ICAM-1,
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15 VCAM-1, fibrinogen, total serum cholesterol, triglycerides, GPX, LDH-cholesterol and
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17 HDL-cholesterol levels between 8:00 and 9:00 a.m. Vascular ultrasound examination
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19 was performed to estimate FMD of the brachial artery between 9:00 and 11:00 a.m.
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23 Starting on day 7 the volunteers took oral supplementation (tablets) of RE each day for
24
25 the next next 21 days. The tablets consisted of 77.7 mg of RE with the active substances
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27 carnosol (0.97 mg), carnosic acid (8.60 mg) and rosmarinic acid (10.30 mg), gained by
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29 high performance liquid chromatography (HPLC) and supplied by Vitiva d.o.o.,
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31 Slovenia [Škrinjar et al., 2007]. The specific dosage was chosen on the basis of previous
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33 studies, showing good tolerance without side effects [Samman et al., 2001].
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37 Each week (day 14, day 21 and day 28) the volunteers were interviewed for any side
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39 effects.
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42 On day 28, between 8:00 and 9:00 a.m., blood samples were redrawn. Between 9:00 and
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44 11:00 a.m. FMD was re-evaluated by ultrasound measurements of the brachial artery.
45

46 *Vascular ultrasound examination*

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48 FMD was studied using a high-resolution B mode Phillips ultrasound system with a 7
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50 MHz linear array transducer. The subjects rested in the supine position for ten minutes
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52 before haemodynamic measurements were performed. The right brachial artery was
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54 scanned in the longitudinal section 2 to 15 cm above the elbow to find the clearest
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56 images of the anterior and posterior wall layers. The mean arterial diameter was
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4 measured at the end of diastole. At least three cardiac cycles were analysed for each
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6 scan and the measurements averaged. The flow velocity was measured at a fixed
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8 incident angle of 68° to the vessel with the range gate of 1.3 mm located in the centre of
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10 the artery. The baseline blood flow was estimated by multiplying the velocity time
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12 integral of the Doppler flow signal (corrected for incident angle) with the vessel cross-
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14 sectional area. Hyperaemic flow increase was induced by inflation of a blood pressure
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16 tourniquet placed around the forearm to a pressure of 200 mmHg for 4 minutes.
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Hyperaemic flow was recorded for the first 15 seconds and diameter measurements were taken 45-60 seconds after cuff deflation. In this way FMD was defined as the endothelium-dependent dilatation, expressed as the percentage change of the diameter after reactive hyperaemia, relative to the baseline scan. FMD, expressed in percentages, was calculated from the brachial artery baseline diameter (D_1) and 60-second postdeflation diameter (D_2), measured as described previously. The formula to calculate is following: $FMD (\%) = (D_2 - D_1) / D_1$. The same investigator, who was blind to the subjects' characteristics, carried out all measurements [Celermajer et al., 1992]. The FMD levels of less than 4.5% were defined as ED as demonstrated by clinical studies [Jadhaw et al., 2003].

Blood samples and laboratory methods

Total serum cholesterol, HDL-cholesterol and triglycerides were estimated by the colorimetric method (Ektachem 250 Analyzer, Eastman Kodak Company, Rochester, USA). LDL-cholesterol level was calculated by means of the Friedewald's equation ($LDL\text{-cholesterol} = \text{Total cholesterol} - HDL\text{-cholesterol} - 4.5 \cdot \text{Triglycerides}$). The lipid profile was estimated at the start and 3 weeks after treatment.

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4 Blood samples to estimate PAI-1, SOD, ICAM-1, VCAM-1, GPX, hs-CRP, TNF- α and
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7 fibrinogen were drawn in fasting state between 8:00 and 10:00 a.m. to avoid circadian
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9 variations. Blood samples were centrifugated and plasma was frozen and stored at -
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12 70°C. Estimations were performed a few weeks later and were blinded to other
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14 investigators.

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16 Serum PAI-1 activity was estimated by the chromogenic method (Berichrom PAI by
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18 Dade Behring, Marburg, Germany). 1 unit (U) of PAI-1 activity is defined as the
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20 amount of PAI-1 that inhibits one international unit of human single tissue-plasminogen
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22 activator. Normal levels are 0.3-3.5 U/ml
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26 Serum SOD was estimated by an in-house method with xanthine, INT / 2-(4-
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28 iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride and xanthine oxidase
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30 (Randox, Crumlin-UK). Serum SOD activity determines indirectly the grade of
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32 inhibition of generation of superoxide radicals by xanthine and xanthine oxydase.
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34 Normal values were 164-240 U/ml.
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38 Serum ICAM-1 was estimated by ELISA H uman sVCAM-1 Immunoassay (R&D
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40 Systems, Inc., Minneapolis, USA, normal levels 115-306 ng/ml).
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43 Serum VCAM-1 was estimated by ELISA Human sICAM-1 Immunoassay (R&D
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45 Systems, Inc., Minneapolis, USA, normal levels 395 -714 ng/ml).
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48 Serum GPX was estimated by an in-house method with glutathione (GSH), cumene
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50 hydroperoxide and glutathione reductase (GR) (Randox, Crumlin-UK). GPX determines
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52 indirectly the rate of formation of oxidized glutathione; normal values were 4171-10881
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54 U/l.
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57 Serum TNF- α was estimated by quantitative immunometric chemoluminescence
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59 IMMULITE[®] TNF- α test, performed by automatic analyzer IMMULITE[®], DPC
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(Diagnostic Products Corporation, Los Angeles, United States of America, normal levels up to 8.1 pg/ml).

Serum hs-CRP was estimated by immunoturbidimetric method with latex particles (PETIA) (Siemens Healthcare Diagnostics, former Dade Behring, Newark, USA); normal levels were < 3.0 mg/l.

Serum fibrinogen was estimated by the modified Clauss method (automatic analyser Dade Behring, Marburg GmbH, Germany); normal levels were 2.2 g/l – 4.2 g/l.

Statistical analysis

Statistics was performed by IBM PC, SPSS for Windows. The values were expressed as means \pm standard deviations or proportions where necessary. The differences among the groups were tested by the two-sided Student's t-test for means \pm standard deviations and by the chi-square test for proportions. The P value < 0.05 was statistically significant.

Results

During 21 days of oral RE supplementation, any side effects were registered.

Comparisons of mean levels of tested variables before and after 21 days of RE supplementation are displayed in Table 1. After 21 days, mean FMD of the brachial artery increased nonsignificantly ($5.1 \pm 5.3\%$ vs. $7.1 \pm 3.8\%$, $P = 0.189$). However, the rate of ED decreased significantly (68.4% vs. 15.9% , RR 4.3, 95% CI 1.468 to 12.791, $P = 0.0038$). Mean PAI-1 level decreased significantly (4.4 ± 1.3 U/ml vs. 3.3 ± 0.7 U/ml, $P = 0.0025$) as well. Median levels of FMD increased from 3.76% to 6.7% (Figure 1) and median PAI-1 levels decreased from 4.9 U/ml to 3.3 U/ml (Figure 2). 21 days of RE supplementation did not have any significant effect upon serum lipid profile, mean serum SOD, ICAM-1, VCAM-1, GPX, hs-CRP, TNF- α or fibrinogen level (Table 1).

Discussion

Three weeks of oral RE supplementation with the active substances carnosol, carnosic and rosmarinic acid resulted in significant decrease in mean serum PAI-1 activity, significant improvement of ED, characterised as FMD < 4.5% and in nonsignificant increase in mean FMD. To our knowledge, this was the first clinical study, evaluating the effect of oral RE supplementation on endothelial vascular function.

Impaired endothelial vascular function precedes atherosclerosis and its improvement by pharmacological agents would delay the onset of symptomatic atherosclerosis such as acute myocardial infarction or stroke, which are among the most prevalent causes of cardiovascular morbidity and mortality [Celermajer et al., 1992; Jadhav et al., 2003].

In vivo and in vitro studies demonstrated that the chronic and progressive nature of atherosclerosis is driven by several oxidative and inflammatory mechanisms such as inactivation of NO, oxidative alteration of DNA, proteins and lipids, activation of redox-sensitive genes, etc. [Moreno et al., 2006; Chang et al., 2008; Lin et al., 2008]. In vitro, animal and human studies suggest that cellular antioxidant enzymes such as SOD and GPX may protect against atherosclerosis [Blankenberg et al., 2003]. Nevertheless, meta-analysis of studies with antioxidant vitamin supplements and Selenium does not support a possible role of their supplementation in reducing the risk of cardiovascular disease. However, in this meta-analysis studies with phenolic antioxidants were not included and, at present, no definite conclusion can be drawn to justify the use of antioxidants for the prevention of atherosclerotic events [Bjelakovic et al., 2008; Katsiki et al., 2009].

The key event in the development and progression of atherosclerosis is migration of smooth muscle cells from media to the subendothelial region. In rosemary, carnosic

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4 acid is the major phenolic constituent. It is a typical diphenol with potent chain-
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6 breaking antioxidant activity [Petersen et al., 2003; Lin et al., 2008]. It inhibits the
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8 activation and expression of matrix metalloproteinase-9 through down-regulation of
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10 NF- κ B, preventing the migration of human vascular smooth muscle cells [Lin et al.,
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12 2008]. Major active components in our oral RE were carnosic and rosmarinic acid,
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14 which both possess significant antioxidant and anti-inflammatory activity. Both have
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16 been successfully used as antioxidants in the food industry, but their anti-inflammatory
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18 and antimicrobial effects were also demonstrated in human studies [Martinez-Tome et
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20 al., 2001; Samman et al., 2001; Osakabe et al., 2004; Lukaczer et al., 2005].

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26 In our study serum PAI-1 activity was most significantly affected. Our findings are
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28 consistent with a study on the cultured human coronary artery cells, demonstrating
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30 downregulation of PAI-1 gene expression in arterial endothelial cells [Pasten et al.,
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32 2007]. Decreasing serum PAI-1 activity may reflect molecular contribution to
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34 cardiovascular protection by the use of polyphenols [Pasten et al., 2007]. Increased PAI-
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36 1 levels are associated with impaired endogenous fibrinolysis and increased risk of
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38 thrombotic events and progression of atherosclerosis. Decreasing PAI-1 by RE could
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40 prevent atherosclerosis and its complications [Pasten et al., 2007]. However, further
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42 randomised studies with RE in established atherosclerosis would be necessary to
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44 demonstrate prevention of further thrombotic complications.

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49 In our healthy volunteers, mean baseline levels of serum SOD, ICAM-1, VCAM-1,
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51 GPX, TNF- α , hs-CRP and fibrinogen were within normal limits and therefore, not
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53 surprisingly, RE supplementation had only a nonsignificant impact on their mean levels
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55 as estimated after 21 days.
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4 In our volunteers, mean FMD changed nonsignificantly after RE supplementation.
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6 However, after 21 days, ED rate improved significantly (from 68.4% to 15.9%, $p =$
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8 0.0038), suggesting an improvement of the balance between endothelial
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10 vasoconstriction and vasodilatation. This seems to correlate with improved endogenous
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12 fibrinolysis, characterised by decreased PAI-1 activity and with nonsignificant increase
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14 in SOD and nonsignificant decrease in VCAM-1, ICAM-1, fibrinogen, hs-CRP and
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16 TNF- α [Nishioka et al., 2007; Chan et al., 2008; He et al., 2009; Nieuwdorp et al.,
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18 2009].
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24 Our results suggest that oral RE supplementation in young and apparently healthy
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26 individuals is safe and has the potential to improve endothelial function, impaired
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28 endogenous fibrinolysis, characterised by a decrease in serum PAI-1 activity and
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30 resulting atherosclerosis. However, further randomised clinical studies are needed,
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32 particularly in patients with symptomatic atherosclerosis, arterial hypertension or
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34 diabetes, dyslipidemia and smoking to confirm their beneficial antioxidant and anti-
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36 inflammatory effect upon arterial endothelial function and symptomatic atherosclerosis.
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46 and the European Regional Development Fund.
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For Peer Review

Table 1: Comparisons of mean levels of tested variables before and after treatment with oral formulation of rosemary extracts for 21 days

Variables	Before treatment	After treatment	P
Mean FMD \pm SD (%)	5.1 \pm 5.3	7.1 \pm 3.8	0.1897
Mean serum cholesterol \pm SD (mmol/l)	4.8 \pm 0.9	4.7 \pm 0.6	0.689
Mean serum triglycerides \pm SD (mmol/l)	1.0 \pm 0.7	1.3 \pm 1.2	0.3528
Mean serum LDL-cholesterol \pm SD (mmol/l)	2.9 \pm 0.9	2.8 \pm 0.7	0.7045
Mean serum HDL-cholesterol \pm SD (mmol/l)	1.6 \pm 0.4	1.6 \pm 0.3	1.0000
Mean PAI-1 \pm SD (U/ml)	4.4 \pm 1.3	3.3 \pm 0.7	0.0025
Mean serum SOD \pm SD (U/ml)	184.5 \pm 5.8	187.0 \pm 7.4	0.2541
Mean serum VCAM-1 \pm SD (ng/ml)	634.8 \pm 188.6	622.3 \pm 153.8	0.824
Mean ICAM-1 \pm SD (ng/ml)	292.2 \pm 91.6	280.6 \pm 99.3	0.710
Mean GPX \pm SD (U/l)	8513.3 \pm 1796.0	7931.6 \pm 1662.2	0.3070
Mean serum fibrinogen \pm SD (mg/l)	3.5 \pm 0.8	3.2 \pm 1.3	0.3973
Mean hs-CRP \pm SD (mg/l)	1.6 \pm 0.4	1.6 \pm 0.9	0.459
ED (%)	13 (68.4)	3 (15.9)	0.0038
Mean red blood cell count \pm SD ($10^{12}/l$)	4.5 \pm 0.5	4.5 \pm 0.5	1.000
Mean platelet count \pm SD ($10^9/l$)	247.3 \pm 55.9	237.5 \pm 48.6	0.567
Mean white blood cell count \pm SD ($10^9/l$)	6.7 \pm 2.1	6.8 \pm 1.9	0.878
TNF- α \pm SD (pg/ml)	9.3 \pm 2.7	8.0 \pm 2.3	0.118

Legend:

SD, standard deviation; LDL, low density lipoproteins; HDL, high-density lipoproteins;

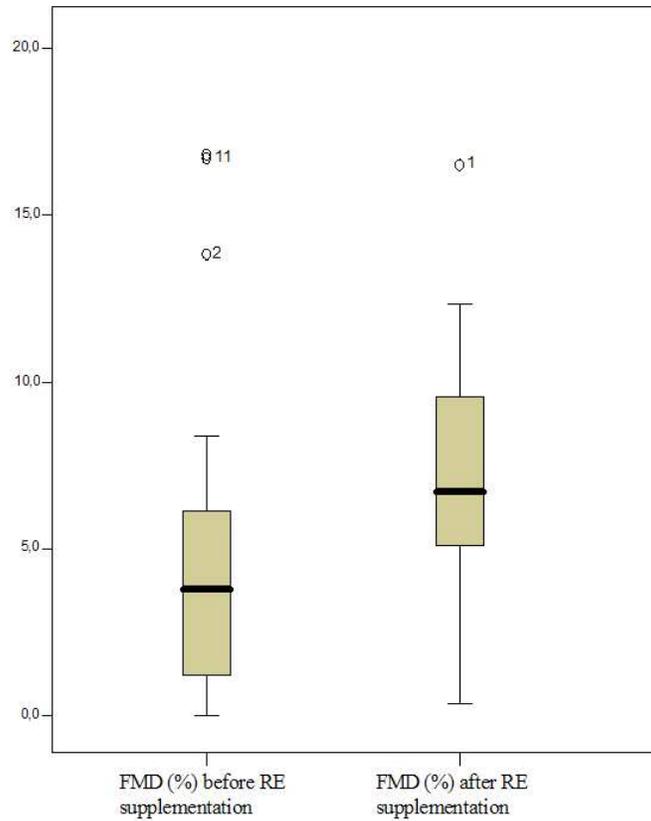
PAI-1, plasminogen activator inhibitor type 1; U, unit; SOD, sodium dysmutase;

VCAM-1, Vascular cell adhesion molecule 1; ICAM-1, Inter-Cellular Adhesion

Molecule 1; GPX, Glutathione peroxidase 1; hs-CRP, high-sensitivity C-reactive

protein; ED, endothelial dysfunction; TNF- α , tumor necrosis factor α

Figure 1: Box-plots for median levels, 25th and 75th percentiles of flow-mediated dilatation (FMD) of the right brachial artery before and after rosemary extracts (RE) supplement

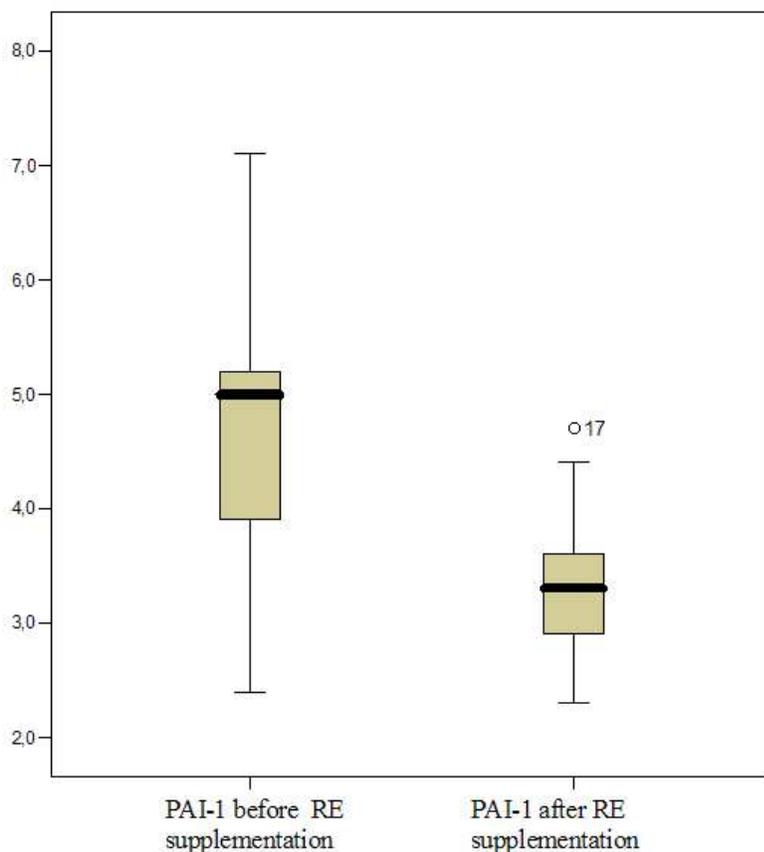


o11, o2, o1, outliers (values between 1.5 and 3.0 box lengths from the upper or lower edge of the box)

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Figure 2: Box-plots for median levels, 25th and 75th percentiles of plasminogen-activator-inhibitor type 1 (PAI-1) before and after rosemary extracts (RE) supplement



o17, outlier (value between 1.5 and 3.0 box lengths from the upper or lower edge of the box)

169x210mm (96 x 96 DPI)