Free Radical Research, February 2007; 41(2):234–241

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The main components of St John's Wort inhibit low-density lipoprotein atherogenic modification: A beneficial "side effect" of an OTC antidepressant drug?[†]

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Accepted by Professor B. Halliwell

(Received 25 July 2006)

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Abstract

Hypericin and pseudohypericin are polycyclic–phenolic structurally related compounds found in *Hypericum perforatum L.* (St John's wort). As hypericin has been found to bind to LDL one may assume that it can act as antioxidant of LDL lipid oxidation, a property which is of prophylactic/therapeutic interest regarding atherogenesis as LDL oxidation may play a pivotal role in the onset of atherosclerosis. Therefore, in the present paper hypericin, pseudohypericin and hyperforin, an other structurally unrelated constituent in St John's wort were tested in their ability to inhibit LDL oxidation. LDL was isolated by ultracentrifugation and oxidation was initiated either by transition metal ions (copper), tyrosyl radical (myeloperoxidase/ hydrogen peroxide/tyrosine) or by endothelial cells (HUVEC). LDL modification was monitored by conjugated diene and malondialdehyde formation. The data show that all compounds (hypericin, pseudohypericin and hyperforin) at doses as low as 2.5 μmol/l are potent antioxidants in the LDL oxidation systems used. The results indicate that the derivatives found in *Hypericum perforatum* have possible antiatherogenic potential.

Keywords: Hypericum perforatum, *LDL oxidation, depression, atherosclerosis*

Introduction

St John's wort is frequently used as complementary and alternative medicine by patients suffering from depression [1–3]. The main active constituents of St John's wort are hyperforin and the phenolic compounds hypericin and pseudohypericin (Scheme 1).

There appeared to be a correlation between elevated serum lipid peroxidation and depressive symptoms [4,5]. Higher concentrations of lipid oxidation products [6], and lower serum antioxidant capacity due to altered nutritional behaviour and life style factors (exercise, smoking, alcohol consumption) [4] of depressive patients may play a role. Lower levels of vitamin E [7] have been found in plasma of patients with major depression. However, it should be mentioned that these data have to be interpreted cautiously as decreased levels of antioxidant vitamins can also be secondary phenomena due to malnutrition and sedentary life style which is common in depressive disorders.

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[†]Part of the data of this paper were presented at the XIIIth Biennial Congress of the International Society for Free Radical Research, Davos, Switzerland, August 15–19, 2006.

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2007 Informa UK Ltd.
DOI: 10.1080/10715760600978831

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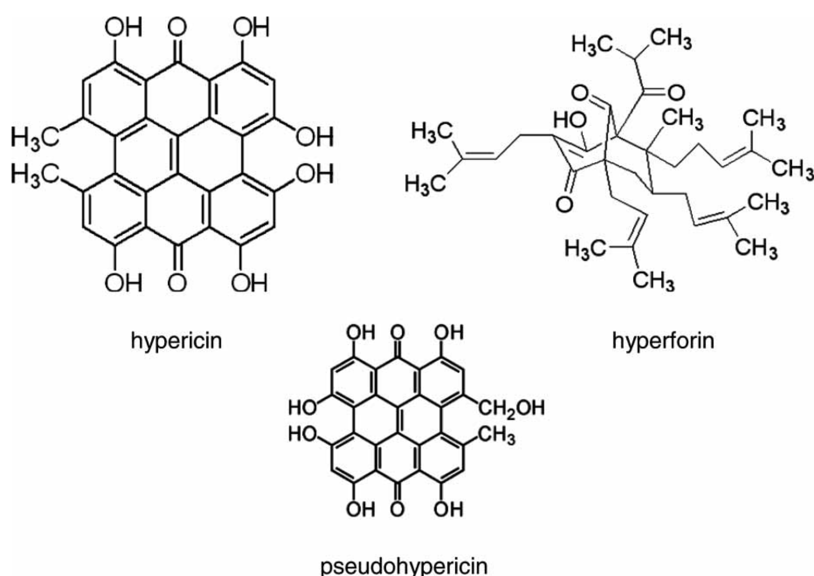

Scheme 1. Structures of St John's wort main antidepressant components.

Vitamin E may determine the oxidizability of lipids in LDL as it is a fat soluble vitamin which is transported mainly with the LDL fraction [8]. Several studies have shown depression and its associated symptoms to be a major risk factor for both the development of CVD and death after myocardial infarction [9–11]. Lipid oxidation especially in LDL may contribute to the pathogenesis of atherosclerotic plaque formation [12,13]. Several studies report on the beneficial effect of natural and synthetic phenolic compounds in cardiovascular disease [14]. It was therefore of interest if the lipophilic compounds in St John's Wort (hyperforin, hypericin and pseudohypericin) due to their chemical nature (Scheme 1) may have the potential to act as inhibitors of LDL atherogenic (oxidative) modification.

Materials and methods

Hypericin, pseudohypericin, hyperforin and myeloperoxidase (MPO) were from Calbiochem. Tyrosine (sodium salt) was from Sigma. Hypericin, pseudohypericin and hyperforin stock solutions were prepared in DMSO. Hyperforin supplied as a methanolic solution was evaporated by a stream of nitrogen and dissolved in DMSO. Control incubations received DMSO only (maximal 1%). All handling with the compounds was done under dimmed light.

Lipoprotein isolation

LDL preparations were isolated by ultracentrifugation as reported previously [15]. The final preparations were dialysed against 150 mmol/l NaCl containing 0.1 mmol/l EDTA or subjected to gel chromatography to get rid of KBr and filter sterilised. Protein was estimated by a modified Lowry method [16] using bovine serum albumin as a standard. All LDL concentrations are given as mg protein/ml.

Transition metal ion mediated LDL oxidation

Prior to LDL oxidation, the lipoprotein was equilibrated in phosphate buffered saline (PBS) pH 7.4 using Sephadex G-25 chromatography (PD-10 columns, Pharmacia). LDL (0.1 mg/ml) was incubated with 5 μmol/l Cu^{++} in the absence or presence of the respective compound at 37°C.

Metal ion independent LDL oxidation

For metal ion independent LDL oxidation LDL (0.1 mg/ml in 50 mmol/l phosphate buffer, pH 7.5, 100 μmol/l DTPA, 3 nmol/l MPO, 50 μmol/l tyrosine, 40 μmol/l H_2O_2) was incubated in the absence or presence of the respective compound (10 μmol/l) and conjugated diene (CD) formation was monitored.

Endothelial cell mediated LDL oxidation

Human umbilical vein endothelial cells (HUVEC) were isolated, cultured and used for cell mediated LDL oxidation as reported previously [17]. LDL concentration was 0.1 mg/ml RPMI-1640 medium and oxidation time was 18 h.

Estimation of LDL oxidation

LDL oxidation was monitored as the increase in CD formation by measuring $A_{234\,nm}$. Samples were

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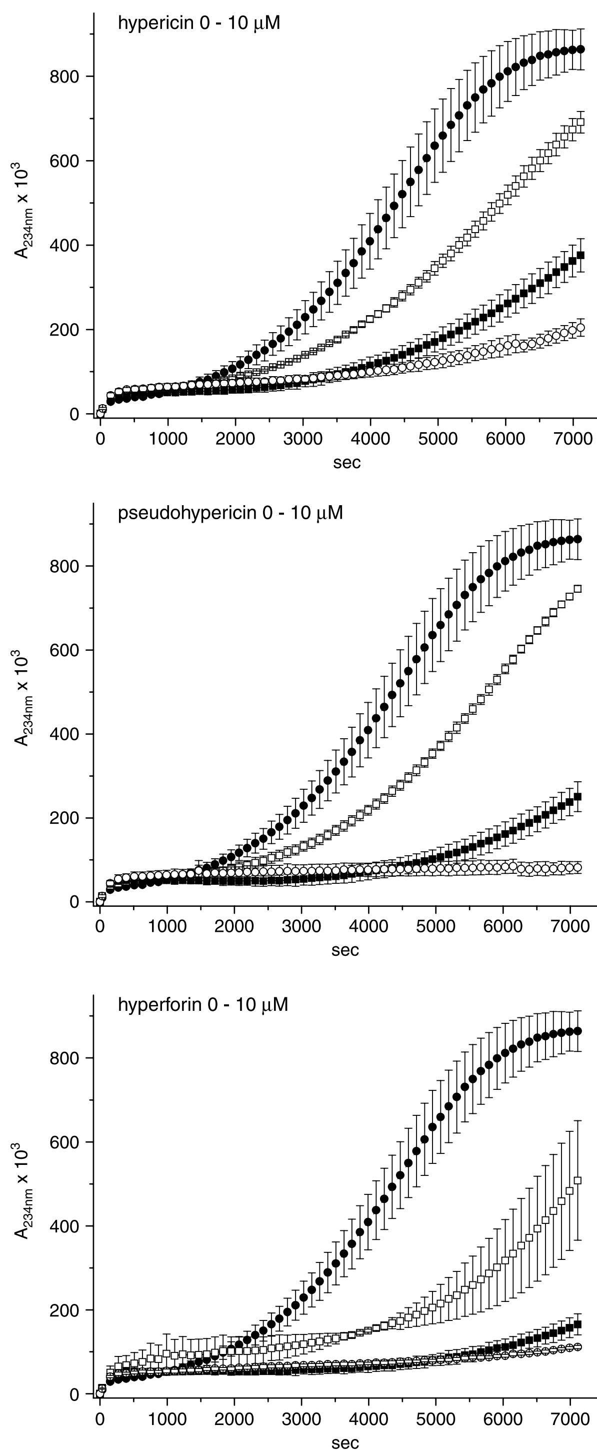

Figure 1. Influence of hypericin, pseudohypericin or hyperforin on LDL lipid oxidation induced by transition metal ions. LDL (0.1 mg/ml) was incubated with the respective compound at 37°C in the absence or presence of 5 μmol/l Cu^{++} and oxidation was monitored as the increase in CD formation as given in methods ($n = 3$). Control, ●; 2.5 μmol/l, □; 5 μmol/l, ■; 10 μmol/l, ○.

measured using a Hitachi UV/VIS 2000 spectrophotometer equipped with a thermostated (37°C) 6-position cuvette sampler. Lag time calculation was done according to Esterbauer et al. [8].

TBARS formation in LDL was measured after oxidation and was stopped by the addition of 50 μmol/l EDTA and 20 μmol/l BHT as reported and expressed as malondialdehyde equivalents [18].

Binding of hypericin, pseudohypericin and hyperforin to isolated LDL

LDL (1 mg/ml PBS, 50 μmol/l EDTA) was incubated in the absence or presence of hypericin, pseudohypericin or hyperforin (all 20 μmol/l) for 1 h at room temperature and subsequently samples were applied to gel chromatography to remove EDTA and unbound compounds. The protein fractions were subjected to lipoprotein oxidation after dilution to 0.1 mg/ml PBS as indicated in the figure legends.

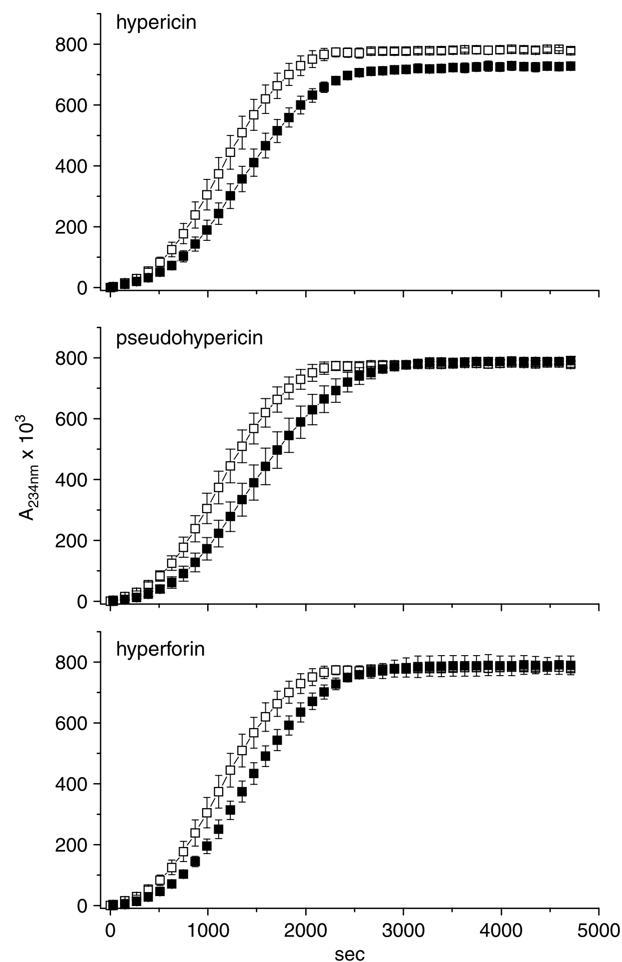

Figure 2. Binding of hypericin, pseudohypericin and hyperforin to isolated LDL. LDL was incubated for 60 min at room temperature with 20 μmol/l of the respective compound and further treated as described in methods. 0.1 mg/ml LDL protein was subjected to metal ion dependent oxidation. The extent of oxidation was monitored as the increase in CD formation as given in methods ($n = 3$). Control, □; pre-treatment, ■.

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Binding of hypericin, pseudohypericin and hyperforin to LDL in plasma

Plasma was incubated in the absence or presence of hypericin, pseudohypericin and hyperforin (10 μmol/l) for 30 min at room temperature and subsequently subjected to ultracentrifugation to isolate the LDL fraction. The LDL fraction was re-centrifuged and subsequently analyzed for bound compounds by HPLC/MSMS.

Sample preparation for HPLC/MSMS

Hundred microliter of isolated LDL (5.7–6.4 mg/ml LDL protein) was extracted twice with 1 ml ethyl-acetate/*n*-hexane (70:30). After centrifugation at 2000*g* for 10 min, the organic layer was separated and evaporated by a gentle stream of nitrogen. The extract was reconstituted with 200 μl acetonitrile.

HPLC/MSMS

The HPLC system consisted of a quarternary LC pump (Waters 2695, Waters Corp., Milford, MA, USA) with degasser and a sample cooling (5°C) autosampler (Waters, 2777C Sample Manager) with integrated column heater. Injection volume was 15 μl. For chromatographic separation at 25°C a Restek C18 column 5 μm, 50 × 2.1 mm i.d. with integrated guard column was used. The HPLC eluents were 20 mmol ammonium formate (A) and acetonitrile (B). Gradient elution was started with 60% A and 40% B and was linearly changed to 100%B within 10 min. After analysis the column was washed for 5 min with acetonitrile and equilibrated for 5 min with initial gradient conditions. The flow rate was 0.25 ml/min and was introduced without splitting into the electrospray ion source (ESI) of a triple-stage quadrupole mass spectrometer (Micromass, Quatro Micro, Altrincham, UK). The mass spectrometer was optimized for selected reaction monitoring in the negative ion mode for the following transitions: m/z 503–m/z 405 for hypericin, m/z 535–m/z 383 for hyperforin, and m/z 519–m/z 487 for pseudo-hypericin [19].

Tissue factor activity assay

HUVEC were incubated for 12 h in the presence of 0.1 mg/ml LDL with or without the respective compound (10 μmol/l). Tissue factor assay for the quantification of the procoagulant activity of the endothelial cells was performed as previously described [20].

Statistical analysis

Data were calculated as means ± standard deviation (SD) of 3–7 experiments. Specific effects were evaluated by one-way analysis of variance (ANOVA) plus Tukey–Kramer Multiple Comparisons Test. $p < 0.05$ was regarded statistically significant.

Results

Effect of hypericin, pseudohypericin and hyperforin on metal ion induced LDL oxidation

Figure 1 presents the effect of the main components contributing to the antidepressant effect of St John's wort on transition metal ion dependent LDL oxidation monitored by formation of CDs. As low as 2.5 μmol/l of hypericin, pseudohypericin or hyperforin showed significant inhibition of lipid oxidation. At the highest concentration used (10 μmol/l) complete inhibition of LDL oxidation was seen over the time course investigated.

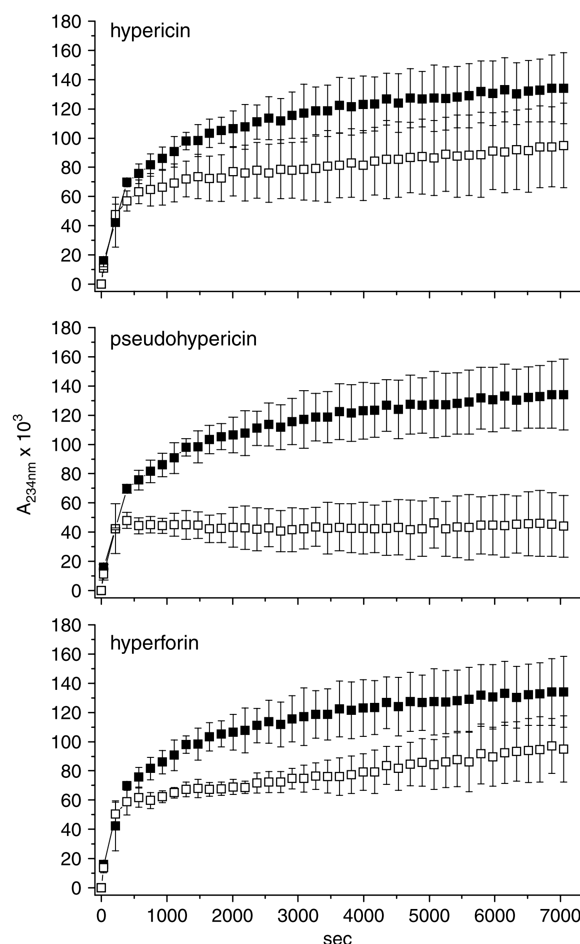

Figure 3. Influence of hypericin, pseudohypericin or hyperforin on LDL lipid oxidation induced by tyrosyl radicals. LDL (0.1 mg/ml) was incubated with the respective compound as described for metal ion independent LDL oxidation in methods and oxidation was monitored as the increase in CD formation ($n = 3$). Control, ■; 10 μmol/l compound, □.

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Binding of hypericin, pseudohypericin and hyperforin to isolated LDL

Isolated LDL pre-treated with hypericin, pseudohypericin or hyperforin and subsequently purified by gel chromatography to get rid of unbound compound showed significantly less oxidation compared to control LDL (Figure 2).

Effect of hypericin, pseudohypericin and hyperforin on tyrosyl radical induced LDL oxidation

Figure 3 presents the effect of hypericin, pseudohypericin and hyperforin on metal ion independent (tyrosyl radical induced) LDL oxidation. All compounds showed significant inhibition of lipid oxidation in this LDL oxidizing system.

Effect of hypericin, pseudohypericin and hyperforin on endothelial cell mediated LDL oxidation and endothelial cell morphology

Hypericin, pseudohypericin and hyperforin antagonized endothelial cell mediated LDL oxidation as seen in Figure 4. At 10 μmol/l all compounds significantly inhibited lipid oxidation as estimated by formation of TBARS in the culture medium. In parallel to the LDL antioxidant effect, the cytotoxic effect of oxidized LDL on HUVEC as indicated by morphology alteration (rounding of EC) was prevented (Figure 4).

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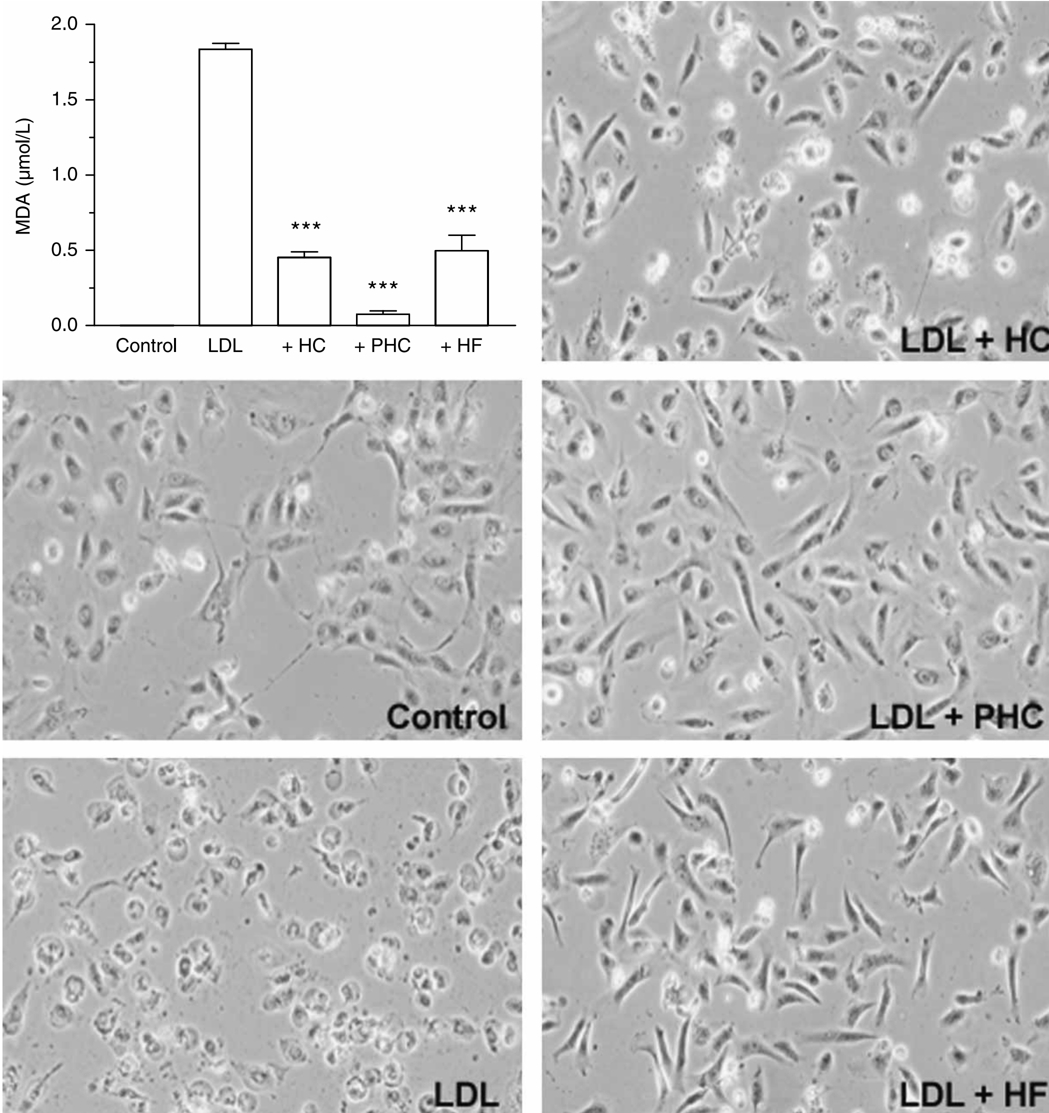

Figure 4. Influence of hypericin, pseudohypericin or hyperforin on endothelial cell mediated LDL lipid oxidation. LDL (0.1 mg/ml) was incubated with HUVEC for 18 h in the absence or presence of the respective compound (10 μmol/l) as described in methods and oxidation was monitored as the increase in TBARS formation ($n = 3$). Photomicrographs of endothelial cells exposed to LDL with or without the respective compound after 18 h of oxidation.

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Effect of hypericin, pseudohypericin and hyperforin on endothelial cell tissue factor activity

The endothelial cell oxidized LDL induced tissue factor activity in HUVEC as indicated by shortening of coagulation times in an one-stage clotting assay (see Methods). Cell oxidized LDL reduced the coagulation time from 123.7 ± 3.7 (control) to 81.4 ± 3.3 s, $p < 0.05$. Hypericin, pseudohypericin and hyperforin antagonized oxLDL induced tissue factor activity (oxLDL + hypericin: 203.5 ± 24.7 s, $p < 0.001$; oxLDL + pseudohypericin: 189.6 ± 5.4 s, $p < 0.001$; oxLDL + hyperforin: 146.2 ± 7.9 s, $p < 0.01$).

Detection of hypericin, pseudohypericin and hyperforin in LDL isolated from plasma preincubated with the respective compound

Plasma was incubated in the absence or presence of hypericin, pseudohypericin and hyperforin (10 μmol/l) for 30 min at room temperature and subsequently subjected to ultracentrifugation to isolate the LDL fraction. The LDL fraction was further purified by re-centrifugation and subsequently analyzed for bound compounds by HPLC/MSMS as described in methods. All compounds were found to bind to LDL in plasma as shown in the chromatograms depicted in Figure 5. The following concentrations were measured in isolated LDL: hypericin, 0.54 μg/mg LDL; pseudohypericin, 1.02 μg/mg LDL; and hyperforin, 1.96 μg/mg LDL.

Discussion

In the present study the main constituents of the commonly used natural antidepressant St John's Wort (*Hypericum perforatum*) hypericin, pseudohypericin and hyperforin were found to inhibit lipoprotein oxidation as tested in different LDL oxidizing systems. All substances bind to LDL in plasma and may therefore protect LDL also *in vivo* from oxidative insults. The total antioxidant capacity of these compounds may be based on the binding of transition metal ions and/or chain breaking activity. Based on their structure as polyphenols [21], hypericin and pseudohypericin may act in both ways as the phenolic hydroxy groups (position 1, 6, 8 and 13 in the ring system [22]) in the vicinity of the oxo function (position 7 and 14) may be capable of binding transition metal ions [23]. Hyperforin may possess chain-breaking activity. Major depression has been found to be accompanied by lower serum vitamin E—one of the principal protective factors against LDL oxidation *in vivo* [8]—resulting in lower antioxidant defence [7]. A finding which is in line with the notion that depression is correlated with increased lipid peroxidation [6].

An interrelationship exists between depression and cardiovascular disease. On the one hand, depression

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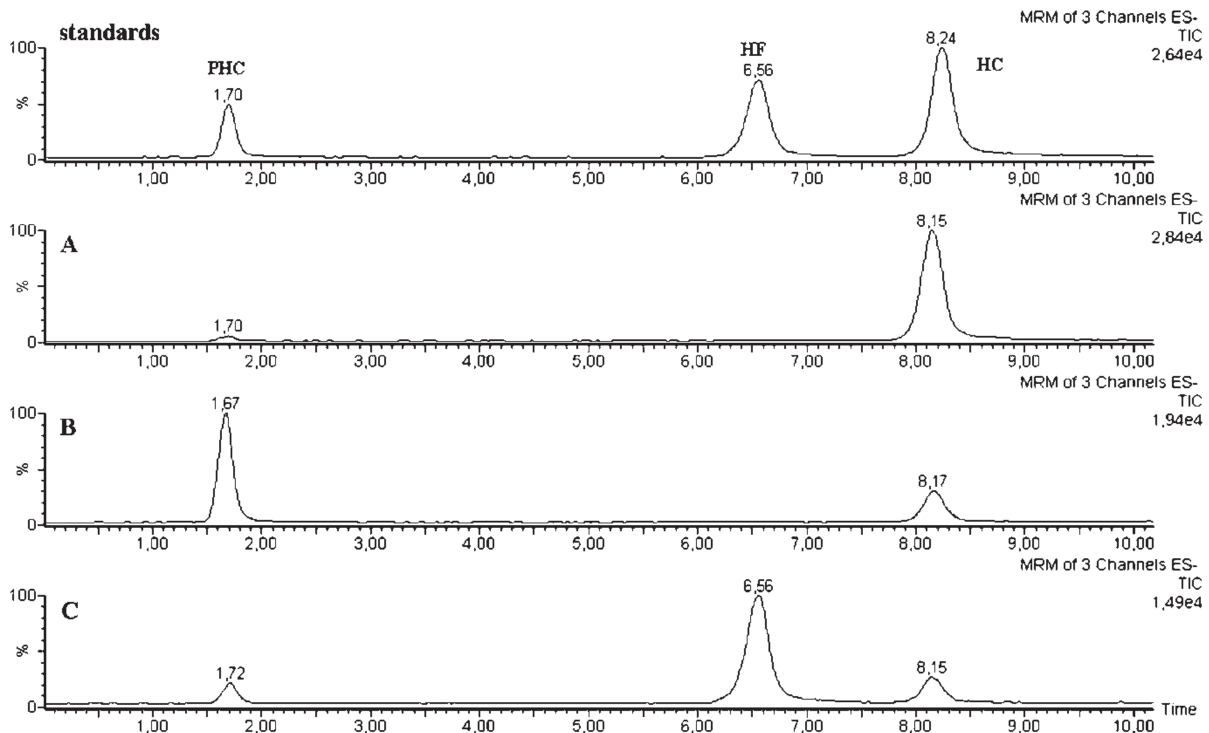

Figure 5. HPLC/MSMS-chromatograms of organic extracts obtained from purified LDL isolated from plasma pretreated with: hypericin(A); pseudohypericin(B); and hyperforin(C). Samples were prepared for HPLC/MSMS as given in methods.

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is a risk factor for coronary heart disease [9], on the other hand it is known that clinical sequelae of atherosclerosis, such as myocardial infarction lead to depression and this is associated with higher mortality [24].

Atherogenesis is a chronic process, which may—beside other pathogenic mechanisms—be also caused by increased lipid oxidation of LDL [12,13]. The products of LDL oxidation have been found to initiate plaque formation in the vessel wall [25]. Thus attempts have been made to slow or reverse this process of atherogenesis by antioxidative compounds [8]. One of the main problems in the development of an effective antioxidant is that many of these compounds do not associate with the target molecule (i.e. LDL). The active agents in St John's Wort fulfil these requirements as they bind to LDL in plasma which holds true for the main natural antioxidant vitamin E.

From pharmacokinetic studies in humans it is known that after multiple dose administration of a Hypericum extract typical maximal plasma levels of hypericin are 15 nmol/l, of pseudohypericin are 28 nmol/l and of hyperforin are 355 nmol/l [26]. Delaey et al. found that hypericin binds extensively to LDL [27]. Thus one may speculate that these compounds may reach relevant concentrations to protect LDL. Thus, based on our results, one may speculate that a combined antioxidative/antidepressive effect is achieved during therapy with St John's Wort. To test this hypothesis, randomised prospective clinical studies focusing on cardiovascular endpoints in patients with depression under therapy with *Hypericum perforatum* extract vs. established antidepressant drugs are mandatory.

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Acknowledgement

Sabine Schreier was supported by the OENB Jubiläumsfonds #10537.

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