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Protective effects of *Peganum harmala* L. extract, harmine and harmaline against human low-density lipoprotein oxidation

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Abstract

Oxidative modification of low-density lipoprotein (LDL) particles has been implicated in the process of atherogenesis. Antioxidants that prevent LDL from oxidation may reduce atherosclerosis. We have investigated the protective effect of *Peganum harmala*-extract (P-extract) and the two major alkaloids (harmine and harmaline) from the seeds of *P. harmala* against $CuSO_4$-induced LDL oxidation. Through determination of the formation of malondialdehyde (MDA) and conjugated diene as well as the lag phase, the extract (P-extract) and compounds were found to possess an inhibitory effect. Moreover, harmaline and harmine reduced the rate of vitamin E disappearance and exhibited a significant free radical scavenging capacity ($DPPH^{\bullet}$). However, harmaline had a markedly higher antioxidant capacity than harmine in scavenging or preventive capacity against free radicals as well as inhibiting the aggregation of the LDL protein moiety (apolipoprotein B) induced by oxidation. The results suggested that *P. harmala* compounds could be a major source of compounds that inhibit LDL oxidative modification induced by copper.

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Introduction

A large body of evidence supports the key role of oxidized low-density lipoproteins (ox-LDLs) in the early inflammatory (De Lorgeil & Salen 2000) and more advanced stages of atherosclerosis lesions (Soledad et al 2002). LDL does not form atherosclerotic plaques in its native form, but oxidative modification of LDL is now recognised as a key event in the pathogenesis of atherosclerosis, leading to plaque build-up in arteries and consequently coronary heart disease (Stocker 1999; Stocker & Kaeney 2004). Since oxidative damage plays a key role in cardiovascular disease and other diseases, there has been considerable interest in the role of antioxidants to inhibit this process (Covas et al 2000). While endogenous lipophilic antioxidants such as vitamin E no doubt play a role in protecting cells and lipoproteins from oxidation, various studies using vitamin E supplements have not shown consistent results to improve protection against cardiovascular disease (O'Connor et al 1998). Dietary and natural antioxidants that inhibit LDL oxidation may therefore be important in protection against these diseases (Stocker 1999).

Dietary compounds, ubiquitous in vegetables and fruits and their juices, possess antioxidant activity that may have beneficial effects on human health (Pearson et al 1999). Recent epidemiological studies have shown that diets rich in plant-derived foods are associated with a protection against LDL oxidation (Sànchez-Moreno et al 2002) and reduced incidence of cardiovascular mortality (Hertog et al 1993). *Peganum harmala* is a herb native of the dry regions of the Mediterranean area. This plant is used in traditional medicine as an anthelmintic, lactogogue, antispasmodic, antipyretic, abortifacient, emetic (Kirtikar & Basu 1935; Chopra et al 1958), anticancerous (Bellakhdar 1997; Berrougui et al 2005), antiviral (Rashan et al 1989) and anti-hypertensive agent (Hmamouchi 1999). Hallucinogenesis (Grella et al 1998) and central monoamine oxidase inhibition effect (Fuller et al

1986), such as binding to various receptors including 5-HT receptors and the benzodiazepine-binding site of $GABA_A$ receptors (Rommelspacher et al 1980, 1985) have been reported also. The principal compounds of this plant are β-carboline alkaloids such as harmine, harmaline, harmalol, harman, vasicine and vasicinon (Kartal et al 2003; Monsef et al 2004). However, it has been reported that *P. harmala* contains some flavonoids (Sharaf et al 1997). *P. harmala* alkaloids have a wide spectrum of pharmacological action including platelet aggregation inhibition (Saeed et al 1993), monoamine oxidase inhibition (Adell et al 1996), anxiolytic and behavioural effects (Adell et al 1996; Berrougui et al 2003), and immunomodulatory effects (Wang et al 1996). There were some reports concerning the cardiovascular actions of harmala alkaloids such as harmine, harmaline and harmalol that reduced systemic arterial blood pressure and total peripheral vascular resistance (Aarons et al 1977).

Previously, we reported the vasorelaxant effect of a methanolic extract of *P. harmala* seeds, harmine and harmaline (Berrougui et al 2002). Tse et al (1991) reported that alkaloids contained in *P. harmala* showed an antioxidant activity when using harman in hepatic microsomal preparations. However, the antioxidant effect of the major compounds of this plant (harmine and harmaline) and related extracts have not been investigated thoroughly.

Therefore, this study has examined the antioxidant effect of the seeds of *P. harmala*, especially its extract (P-extract) and two major alkaloids (harmine and harmaline). The underlying mechanism of action was investigated using an in-vitro system (Cu^{2+}-oxidized human LDLs) and free radical scavenging test (DPPH).

Materials and Methods

Chemicals

Acetic acid, sulfuric acid, sodium phosphate, thiobarbituric acid, *n*-butanol, methanol, ethanol, *n*-isopropanol and hexane were purchased from Fisher (Montreal, Quebec). 1,1,3,3,-Tetraethoxypropane, D-α-tocopherol, butylated hydroxytoluene (BHT), cupric sulfate ($CuSO_4$), ethylenediaminetetraacetic acid (EDTA), lithium perchlorate, DPPH (1,1-diphenyl-2-picryl-hydrazyl), harmine and harmaline were obtained from Sigma (St Louis, MO, USA). Dialysis bags were purchased from Spectrum Medical Industries (Houston, TX, USA).

Subjects

Sera were obtained from 12 healthy volunteers (aged between 20 and 25 years). They were all in good health, without symptoms and signs of any arterial diseases established by a complete and negative clinical examination according to the WHO (World Health Organisation) criteria. Blood pressure profile, glycaemia, fibrinogen level, lipid and coagulation profiles were within normal ranges. The Ethics Committee of the Sherbrooke Geriatric University Institute approved the study, and all subjects gave written informed consent.

Plant extraction

Fresh seeds of *Peganum harmala* L. (Zygophyllaceae) were collected from the Atlas region of Morocco, in May 2000. They were identified by the botanical section of the Faculty of Medicine and Pharmacy (Rabat), where a specimen was preserved (number PH-00052). Fresh and powdered seeds were extracted by overnight maceration in MeOH/water (40:60, v/v). The extract obtained was concentrated by lyophilization to yield dry residues (6.8%, w/w). Phytochemical screening of this P-extract was investigated using a thin layer chromatography (TLC) system. Extraction, purification and identification of the alkaloids in P-extract were conducted using silica gel column chromatography followed by a mass spectra and NMR (nuclear magnetic resonance) analysis (Berrougui et al 2005).

Biochemical study

Lipoprotein isolation was performed according to the method of Sattler et al (1994). Briefly, human plasma was collected in EDTA ($0.4\,g\,L^{-1}$) and LDLs ($1.019 < d < 1.063$) were separated by ultra-centrifugation for 2 h at 15°C at 100 000 rev min^{-1} in a TLA 100.4 rotor. Isolated LDLs were dialysed overnight at 4°C with 10^{-2} M sodium phosphate buffer (pH 7) to remove the excess of buffer salt and EDTA. LDLs were then adjusted to a concentration of 100 μg protein mL^{-1} respectively by dilution in the same buffer. Proteins were measured by the Bradford method according to the recommended procedure (Biorad, Canada).

Copper-mediated LDL oxidation

LDLs ($100\,\mu g\,mL^{-1}$) were incubated in the absence or presence of increased concentrations of P-extract (0–$200\,\mu g\,mL^{-1}$), harmine or harmaline (10 μM), for the indicated periods at 37 °C in 10^{-2} mM sodium phosphate buffer (pH 7) containing 10 μM cupric sulphate. Oxidation reactions were stopped by simple cooling in an ice bath with EDTA addition and the lipid peroxides formed were measured immediately.

Biochemical markers of lipid peroxidation

Three markers were used to follow the copper-induced oxidation of LDL.

Conjugated diene formation. LDLs ($100\,\mu g\,mL^{-1}$) oxidized alone or in the presence of various concentrations of P-extract (0–$200\,\mu g\,mL^{-1}$), harmine or harmaline were continuously monitored at 234 nm to detect the formation of conjugated dienes as described by Bennefont-Rousselot et al (1995).

Thiobarbituric acid-reactive substance (TBARS) formation. Mainly malondialdehyde (MDA) was assayed by high-performance liquid chromatography

(HPLC) (Agarwal & Chase 2002). The column was an HP hypersil 5 μm ODS 100 × 4.6 mm with a 5-μm ODS guard column and the mobile phase contained methanol–buffer (40:60, v/v). Fluorescence detector was set at an excitation wavelength of 515 nm and emission wavelength of 553 nm. Samples of LDLs were treated with butylated hydroxytoluene (BHT) and heat derivatized at 100°C for 1 h with thiobarbituric acid at an acidic pH. Samples were extracted with n-butanol and 10 μL volumes of the extract were injected.

Vitamin E (α-tocopherol) measurement. LDLs endogenous vitamin E was assayed as α-tocopherol, at different oxidation times, by reversed-phase-HPLC with electrochemical and UV detection (Khalil et al 2000). α-Tocopherol was assayed on a Sephasil peptide column (C_{18} 5 μm ST 4.6/250) (Pharmacia Biotech, Piscataway, NJ, USA), with methanol–ethanol–isopropanol (88:24:10, v/v/v) containing lithium perchlorate (20 μM) at a flow rate of 1 mL min^{-1}.

Apolipoprotein B (apo B) modification
Electrophoresis was run according to Laemmli (1970) on 7.5% SDS-polyacrylamide gel electrophoresis (PAGE) gels (non-reducing conditions; Mini Gel II from BioRad, Hercules, CA, USA). Gels were stained with Coomassie Brilliant Blue.

Free radical scavenging activity
Determination of the free radical scavenging activity of harmine and harmaline was conducted using the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) test (Mensor et al 2001). Briefly, DPPH solution dissolved in ethanol was added to the aqueous solution of P-extract at different concentrations and allowed to react at room temperature. After 30 min the absorbency (Abs) was measured at 518 nm. DPPH plus ethanol was used as a negative control and DPPH plus vitamin E was used as a positive control. Antioxidant activity was calculated using the following formula:

$$\text{Antioxidant activity}\% = 100 - \{[(\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}}) \times 100]/\text{Abs}_{\text{control}}]\}.$$

Statistical analysis

Values are expressed as the mean ± s.e.m. One-way analysis of variance followed by Tukey's multiple comparisons test was used for statistical analysis. Linear regression analysis was used to assess the association between two continuous variables. Statistical analyses were performed using Prism 2.0 version software.

Results

Phytochemical study

TLC analysis of P-extract in AcET/MeOH/NH_4OH 50% (9:1:1) eluent system, followed by a Dragendorff-reagent pulverization (potassium tetraiodobismuthate) of fluorescent spots at 365 nm, showed a high intensity of chestnut-orange bands indicating that the extract was rich in alkaloids. Principally, two intense bands were observed with retention factor (R_f) values of 0.62 and 0.36, suggesting that harmine and harmaline (R_f: 0.64 and 0.35, respectively (Munir et al 1995)) may have constituted the major alkaloids of P-extract. In addition, when TLC was sprayed by NEU reagent (2-aminoethyl-diphenylborate 1% in MeOH), yellow bands appeared indicating the presence of flavonoids in the extract. However, this extract was poor in triterpene and no tanins were observed under the $FeCl_3$ test.

P. harmala inhibited conjugated diene and MDA formation upon Cu^{2+}-mediated oxidation in a dose-dependent manner

The effect of *P. harmala* on copper ion-induced LDL oxidation was investigated at increased concentration and oxidation time. LDL oxidation was monitored by the formation of conjugated dienes and TBARS as MDA equivalent. Incubation of LDL (100 μg mL^{-1}) with increasing concentration of *P. harmala* (0, 50, 100 and 200 μg mL^{-1}) inhibited the formation of conjugated dienes and MDA in a concentration-dependent manner. That inhibition is illustrated in Figure 1 by an increase of lag phase and reduction of maximal accumulation of oxidation products (OD_{max}) and maximal rate of oxidation (V_{max}) (Table 1) dependent on the concentration of P-extract. P-extract concentration was significantly correlated to the lag phase of conjugated diene formation and negatively correlated to the OD_{max}. P-extract at 50 and 100 μg mL^{-1} did not affect the LDL oxidation rate (V_{max}) while 200 μg mL^{-1} of P-extract significantly reduced the V_{max} of the conjugated dienes and MDA formation ($P < 0.001$ vs control) (Table 1).

Antioxidant activity of harmine and harmaline

To determine the compounds responsible for the antioxidant activity of the P-extract, the two main alkaloids contained in the extracts of P-extract (harmine and harmaline) were investigated for their antioxidant activity.

Representative oxidation kinetic curves (Figure 2) showed that harmine and harmaline inhibited LDL oxidation. Indeed, formation of conjugated dienes and MDA was significantly reduced in the presence of harmine or harmaline at 10 μM. This antioxidant effect was illustrated by a significant increase in the lag-phase time of conjugated dienes and MDA formation (Table 2). The effect of harmaline was more potent than that of harmine to protect LDL against oxidation. Both alkaloids significantly decreased the rate of LDL oxidation (V_{max}) and the maximal accumulation of oxidation products (OD_{max}) of conjugated dienes (Table 2).

To gain more insight into the effect of these alkaloids on LDL oxidation, we measured their action on the rate of disappearance of vitamin E (α-tocopherol). Oxidation of LDL alone resulted in a significant increase of the α-tocopherol disappearance rate (Figure 3), whereas in

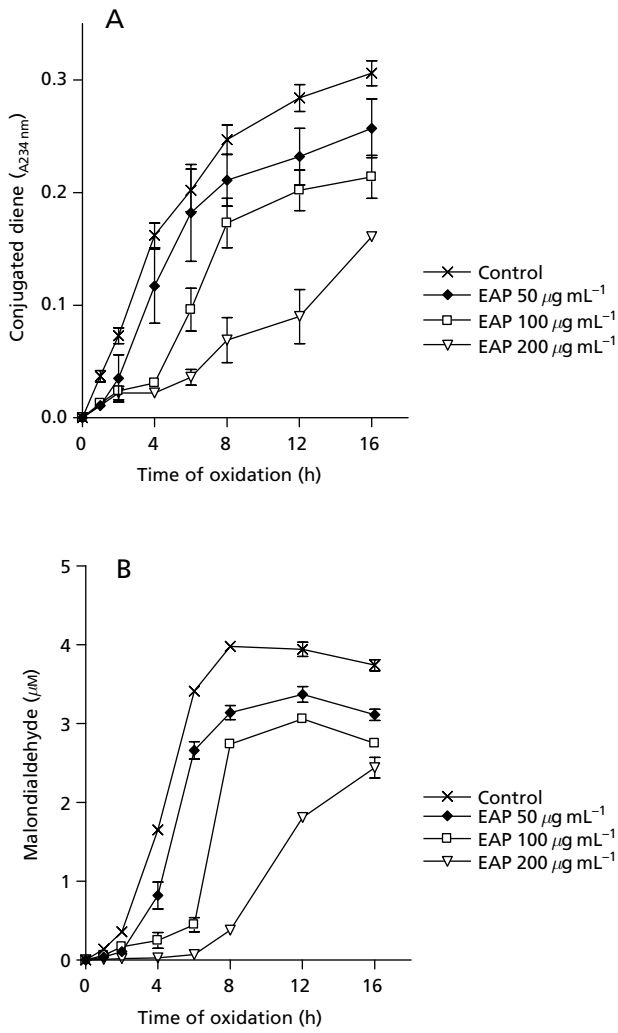

Figure 1 Effect of P-extract on conjugated diene (A) and malondialdehyde (MDA) production (B), upon incubation of human LDL (0.1 mg mL^{-1}) with 10 μM $CuSO_4$ in the presence of increasing concentrations of P-extract (EAP; 0–200 μg mL^{-1}). Results are expressed as mean ± s.e.m. of a minimum of three independent experiments.

the presence of harmine or harmaline, the depletion of α-tocopherol was significantly reduced after 1 and 6 h of oxidation.

Harmine and harmaline inhibit apo B modification

The protection of harmine and harmaline against oxidation induced aggregation of the LDL protein moiety (apo B). SDS-PAGE analysis was carried out on oxidized LDLs either with or without alkaloids. Figure 4 depicts a profile of apoB modification after LDL oxidation. Following incubation with copper for 0, 1 and 4 h, the intensity of the apo B protein greatly decreased. Harmaline inhibited this effect but harmine did not.

Free radical scavenging properties of harmine and harmaline

In an attempt to gain more insight into the mechanisms at the origin of the antioxidant effect of harmine and harmaline, we investigated the radical scavenging potential of these two alkaloids. Thus, the reactivity towards the stable free radical DPPH was continuously monitored at 518 nm. Vitamin E (10 μM) was used as a positive control. Native absorption of these alkaloids did not interfere with the absorption maximum of DPPH at 518 nm (data not shown). As shown in Figure 5, harmine and harmaline (10 μM) exhibited a significant free radical scavenging capacity. Harmaline showed a higher free radical scavenging potency than harmine. However, when compared with vitamin E (α-tocopherol) at an equal concentration, harmaline presented less scavenging activity, approximately 50% lower than that of vitamin E (Figure 5).

Discussion

Modification of LDL particles induced by copper ion is related to free radical reaction, though the mechanism of the oxidant production has yet to be clearly elucidated.

Table 1 Effect of increased concentrations of antioxidant (P-extract) on the kinetics of LDL peroxidation induced by copper and represented by conjugated diene (CD) and MDA production

Kinetic parameters	Control	P-extract 50 μg mL^{-1}	P-extract 100 μg mL^{-1}	P-extract 200 μg mL^{-1}	Linear regression (r^2)	*P*
Lag phase (H):						
CD	0.59 ± 0.02	1.49 ± 0.30	4.4 ± 0.3***	6.68 ± 0.62***	0.964	0.018
MDA	1.97 ± 0.05	2.92 ± 0.17***	5.46 ± 0.05***	7.21 ± 0.05***	0.953	0.023
OD_{max}:						
CD (234 nm)	0.306 ± 0.011	0.257 ± 0.026	0.214 ± 0.019**	0.161 ± 0.001**	0.975	0.012
MDA (μM h^{-1})	3.74 ± 0.027	3.37 ± 0.1*	3.06 ± 0.005***	2.44 ± 0.13***	0.998	< 0.001
V_{max} (μM h^{-1}):						
CD (234 nm)	0.043 ± 0.002	0.048 ± 0.009	0.035 ± 0.004	0.022 ± 0.004**	0.831	0.088
MDA (μM h^{-1})	0.76 ± 0.02	0.71 ± 0.06	0.7 ± 0.02	0.28 ± 0.01***	0.840	0.083

Data are expressed as means ± s.e.m. of three to five separate experiments. *P < 0.05, **P < 0.01 and ***P < 0.001, compared with controls (analysed using one-way analysis of variance followed by a Dunnet post-test).

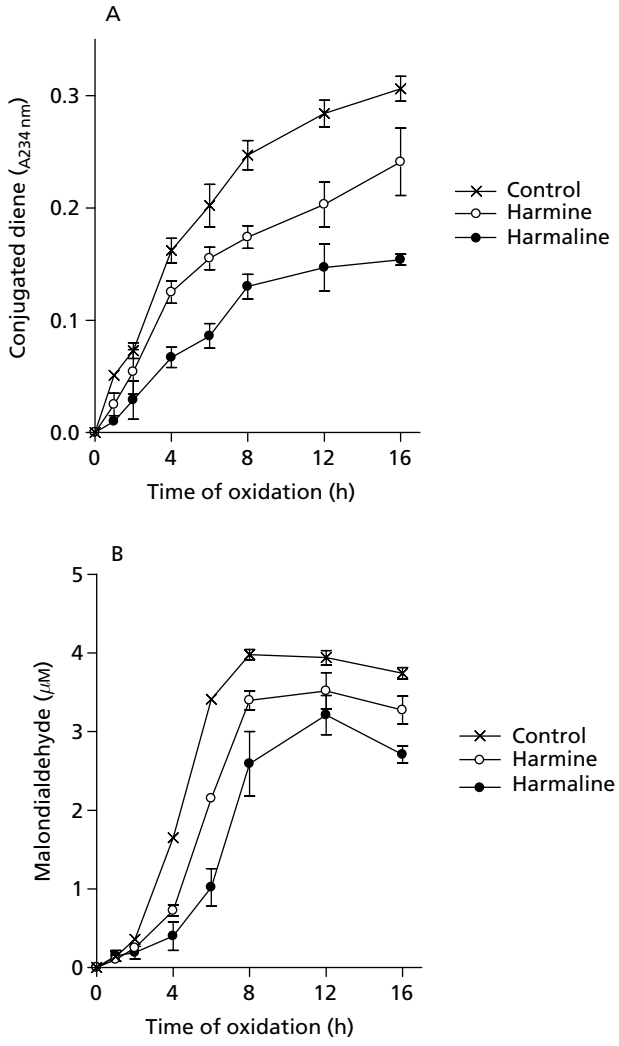

Figure 2 Effect of harmine and harmaline (10 μM) on conjugated diene (A) and malondialdehyde (MDA) production (B), upon incubation of human LDL (0.1 mg mL^{-1}) with 10 μM $CuSO_4$ with or without the presence of harmine or harmaline. Results are expressed as mean ± s.e.m. of a minimum of three independent experiments.

LDL oxidation may require the generation of hydroxyl radicals produced by the Fenton reaction (Zhang 1999). Copper ions induce oxidation of polyunsaturated fatty acid (PUFA) of LDLs, which results in an elevation of lipid peroxides, and depletion of natural antioxidants such as α-tocopherol. Formation of fatty acid-hydroperoxides from PUFA is followed by fragmentation of fatty acids to short-chain aldehydes, mainly MDA, that modify apoprotein B chemically and structurally, and alter the binding specificity of LDL molecules (Esterbauer et al 1991). Oxidative modification of LDLs plays a crucial role in the pathogenesis of atherosclerosis and high levels of oxidized LDLs are found in various acute coronary syndromes, indicating that oxidized LDLs might be a marker for atherosclerosis (Marrugat et al 2004).

P. harmala is a plant rich in β-carboline alkaloids. These compounds are naturally present in the human food chain and are supposed to occur endogenously in normal body constituents. They are found in the blood plasma, heart, kidney, liver and in brain tissue, where they have been proposed to be the endogenous ligands for benzodiazepine and imidazoline receptors (Rommelspacher et al 1980; May et al 1994; Hudson et al 1999). β-Carboline alkaloids also act as inhibitors of monoamine oxidase (MAO) and activators of 5-HT receptors. Previously, we demonstrated that harmala alkaloids exerted a sedative-like effect when administered to mice (Berrougui et al 2003). Kim et al (2001) have investigated the protective effects of these alkaloids against the damage induced by oxidative stress. Those authors showed that β-carbolines depressed the loss of viability in PC-12 cells through a scavenging action on reactive oxygen species and inhibition of the oxidation of thiols. The vasorelaxant effect of *P. harmala* extract and pure compounds from seeds of *P. harmala* have been reported (Shi et al 2001; Berrougui et al 2002). The toxicity and secondary effects of these alkaloids present a big challenge with respect to their pharmacological use. Previously, we conducted a toxicological study of *P. harmala* in mice and our results showed that high oral administration of P-extract (10 mg kg^{-1}) did not induce mortality and only a little secondary effect was noted, such as partial tremor. However, an in-vivo study of *P. harmala* alkaloid toxicity may provide more evidence or information for the biological active doses.

No study had been conducted to investigate the antioxidant effect of these compounds towards lipid peroxidation. In this study, we have investigated, for the first time, the protective effect of P-extract and pure compounds from *P. harmala* towards the oxidation of human LDLs.

The results showed that P-extract protected LDLs against lipid peroxidation by decreasing the conjugated diene and MDA formation, in a dose-dependent manner. The elongation of the lag phase and the reduction of the OD_{max} and V_{max}, mainly at 200 μg mL^{-1} P-extract, supported these protective effects. Lag phase corresponds to the period during which endogenous LDL antioxidants (e.g. α-tocopherol and β-carotene) are depleted (Esterbauer & Ramos 1996).

In the phytochemical assay, TLC analysis showed that P-extract contained a large series of alkaloids, of which harmaline and harmine were the dominant products (results not shown). Our results showed that harmaline as well as harmine inhibited lipid peroxidation of LDLs. Harmaline was more efficient than harmine in protecting LDLs against oxidation. This was illustrated in three ways. Firstly, the extended lag phase and reduction of maximal accumulation of oxidation products. Secondly, by the decrease in the rate of disappearance of α-tocopherol in the presence of harmaline more than with harmine. Thirdly, by the decline in the oxidation rate during the propagation phase.

We explored the inhibitory effect of alkaloids on the copper-induced alteration of apo B. Harmaline, but not harmine, abolished the Cu^{2+}-induced aggregation of LDLs and protected the modification of amino-acid-apo

Table 2 Effect of harmine and harmaline (10 μM) on the kinetics of LDL peroxidation induced by copper and represented by conjugated diene (CD) and MDA production

Kinetics parameters	Control	Harmine (10 μM)	Harmaline (10 μM)
Lag phase (H):			
CD	0.59 ± 0.02	1.7 ± 0.23*	4 ± 0.5***
MDA	1.97 ± 0.05	3.65 ± 0.12*	4.56 ± 0.36***
OD_{max}:			
CD (234 nm)	0.306 ± 0.011	0.24 ± 0.03	0.154 ± 0.005**
MDA (μM h^{-1})	3.74 ± 0.027	3.517 ± 0.233	3.21 ± 0.25
V_{max}:			
CD (234 nm)	0.043 ± 0.002	0.035 ± 0.0027	0.026 ± 0.0003***
MDA (μM h^{-1})	0.76 ± 0.02	0.698 ± 0.0093	0.563 ± 0.144

Data are expressed as means ± s.e.m. of three to five separate experiments. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ compared with controls (analysed using one-way analysis of variance followed by a Dunnet post test).

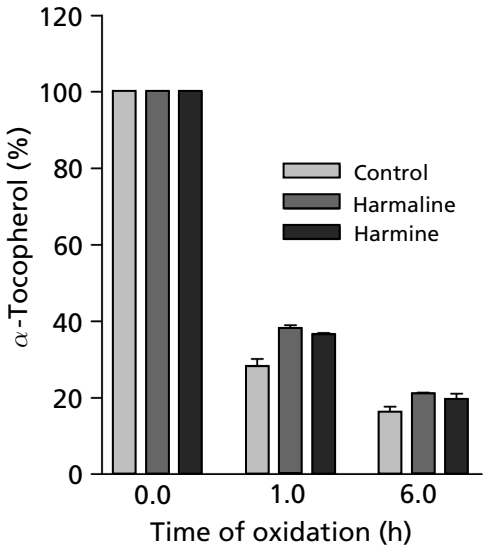

Figure 3 Effect of harmine or harmaline (10 μM) on endogenous α-tocopherol (%) disappearance during oxidation of LDL. Oxidation was induced by incubation of human LDL (0.1 mg mL^{-1}) with copper (10 μM). Results present mean ± s.e.m. of a minimum of three independent experiments.

B protein residue of the protein moiety of LDLs, indicating that there was insignificant oxidative modification of LDLs. As mentioned above, harmaline inhibited the maximal production of oxidation product and therefore protected the apolipoprotein moiety against alteration. These results were in agreement with Pari et al (2000) who showed that β-carbolines could quench singlet oxygen, superoxide and hydroxyl radicals and inhibit the oxidative formation of higher molecular weight aggregates of the test protein.

We evaluated the radical scavenging activity of the compounds tested using a stable free radical, DPPH, in an homogenous ethanolic solution. DPPH, as a weak hydrogen atom abstractor, was considered a good generator for peroxyl $ROO^{\bullet}$ radicals (Blois 1958; Sànchez-Moreno et al 2002). Our results showed that the two alkaloids, harmaline and harmine, were able to scavenge free radicals generated by DPPH. However, harmaline was threefold more potent than harmine but less potent than vitamin E, used as a positive control.

In terms of the structure relationship, the results indicated that the change of β-carboline to dihydro-β-carboline (i.e. harmine vs harmaline) increased the potency of the antioxidant activity of the alkaloids.

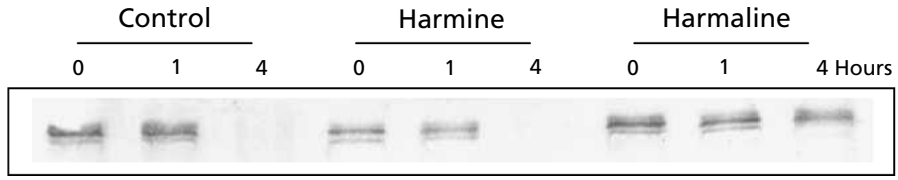

Figure 4 Harmaline, but not harmine, inhibited LDL apoB aggregation induced by copper-initiated peroxidation. Human LDL (0.1 mg mL^{-1}) was incubated with 10 μM $CuSO_4$ (0, 1 and 4 h) in the presence of harmine or harmaline. SDS-PAGE profile of apoB. Pool 1 represents control LDL apoB oxidized in the same experimental conditions. Pools 2 and 3 represent LDL apo B oxidized in the same experimental conditions in the presence of harmine or harmaline, respectively.

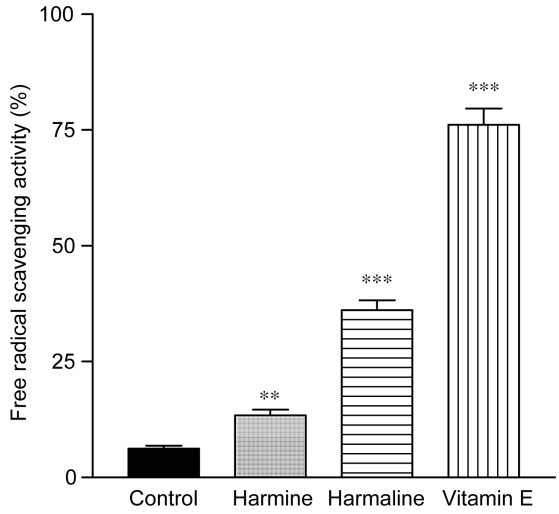

Figure 5 Radical scavenging activity of harmine or harmaline (10 μM) as measured by the DPPH system. Percentage of inhibition of DPPH radical was evaluated by a decrease in the absorbance at 517 nm after 30-min incubation. Values are the mean ± s.e.m. of a minimum of three independent experiments.

Furthermore, this difference in activity related to the structure has been widely reported in the literature. Shi et al (2001) showed the same change between harmine and harmaline induced a change in the potency of the vasorelaxation effect of these alkaloids.

In conclusion, the present findings suggested that alkaloids from seeds of *P. harmala* exerted an antioxidant effect towards in-vitro oxidation of LDLs. The results lend support to the theory that one of the roles of the β-carbolines is to offer protection against oxidative stress. The active ingredient in this plant is mostly harmaline and its activity might be mediated by chelating Cu^{2+}, scavenging free radicals and thus inhibiting the oxidation of human LDLs.

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