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ORIGINAL PAPER

Antioxidant, anticancer and hepatoprotective activities of *Cotoneaster horizontalis* Decne extract as well as α-tocopherol and amygdalin production from in vitro culture

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Abstract The phytochemical analysis of the ethanolic extract of branches of *Cotoneaster horizontalis*, Decne revealed the presence of: β-carotene, ascorbic acid and less amounts of α-tocopherol and amygdalin (vitamin B_{17}) in proportions of: 2,500, 70, 0.093, 0.334 mg 100 g^{-1}, respectively. Acute oral toxicity test revealed its safety profile. In vitro study revealed its good 2, 2-diphenyl-1-picrylhydrazyl radical scavenging and anticancer activities. Invivo study, simultaneous administration of this extract at a dose of 100 or 200 mg kg^{-1} body weight for 4 weeks, exhibited a significant protection in a dose-dependant manner against hepatotoxicity induced by repeated dose of acetaminophen (1 g kg^{-1} body weight day^{-1}, p.o.) by preserving the liver function parameters, hepatic redox state and serum lipid profile near the healthy levels. Consequently, in vitro culture was carried out on full or half strength of Murashige and Skoog medium supplemented with different concentrations of benzyl amino purine or kinetin provided shootlets production; different concentrations of 2,4-dichlorophenoxy acetic acid and naphthalene acetic acid showed an increase of callus. Determination of α-tocopherol and amygdalin in different shootlets and callus extracts showed a pronounced increases up to 30.62 and 3.69 mg 100 g^{-1} in shootlet extract, respectively as well as 26.61 and 12.71 mg 100 g^{-1} in callus extract, respectively, as compared with those of the mother plant (0.76 and 0.11 mg 100 g^{-1} extract, respectively).

Keywords Cotoneaster horizontalis · Bioactive constituents · Tissue culture

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Introduction

Family Rosaceae is a well-known family for the presence of cyanogen glucosides like the natural chemotherapeutic agent amygdalin (Laetrile, vitamin B-17) (Worldwithoutcancer.org.uk). Genus *Cotoneaster* enjoy a considerable reputation in traditional medicine due to the use of their extracts as expectorant, anticancer, antioxidant, hepatoprotective, antispasmodic, cardiotonic, antiviral and diuretic (Palme et al. 1996; Cakilcioglu and Turkoglu 2010). Previously other constituents viz., flavonoids, phenolic acids

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(Palme et al. 1996; El-Mousallamy et al. 2000), cyanogen glycosides (Kapche et al. 2007) and tannins (Wang and Ji 2008) have been found in this genus. One of its species, *Cotoneaster horizontalis* Decne (Common Name: Rock-spray) is an ornamental shrub, with decorative fruits, cultivated in Egypt (Khalifa and Loutify 2006) but not of common occurrence and not previously phytochemically or biologically investigated. Its micropropagation as an alternative to conventional methods for vegetative propagation attracted much attention. The current study aimed at identifying a new drug rich in α-tocopherol (antioxidant) and amygdalin (anticancer), in addition to the investigation of other bioactive constituents in the ethanolic extract of *Cotoneaster horizontalis* and evaluation of its antioxidant and anticancer activities in vitro as well as its hepatoprotective potential in repeated dose model of acetaminophen hepatotoxicity in rats. In vitro culture study of the explant was carried out on MS media using different kinds and concentrations of cytokinins (for shootlets multiplication) and auxins (for callus production), with a strategy employed to increase accumulation of natural α-tocopherol and amygdalin as compared with those of the mother plant.

Materials and methods

Plant material

Branches of *C. horizontalis* were collected from El-Orman Botanical Garden, Giza, Egypt. A voucher specimen (C-432) was kept in the Department of Pharmacognosy, Faculty of Pharmacy, Cairo University.

Air-dried powdered branches (100 g) were macerated in ethanol (95 %) till exhaustion. The concentrated residue was used for antioxidant, cytotoxic and hepatoprotective effects.

Preparation of standards

The standard stock solutions of: α-tocopherol (1 mg ml^{-1} absolute ethanol) and amygdalin (1 mg ml^{-1} 80 % ethanol) were stored in a refrigerator at −20 °C. Standard solutions were filtered through 0.45 μm filters before being injected to HPLC and diluted as necessary with ethanol. HPLC system consisted of two pumps (GBC LC 1110), UV/VIS detector (GBC LC 1210); an on-line degassing system; ODS column (Hypersil®, 5 μm, 250 mm × 4.6 mm, Beckman, Germany); mobile phases: methanol–water (96:4 v/v) for α-tocopherol and water–methanol–acetonitrile (70:25:5 v/v/v) for amygdalin; flow rate: 1 ml min^{-1}. Peaks were determined by means of retention time and UV spectra that were recorded for detection of α-tocopherol at 280 nm (Escriva and Esteve 2002) and amygdalin at 220 nm (Du et al. 2005). Quantification of α-tocopherol and amygdalin was achieved by measuring peak area against six concentrations of the standards (equivalent to 50, 100, 150, 200, 250 and 300 μg ml^{-1}) α-tocopherol and (100, 200, 500, 800, 1,000 and 1,500 μg ml^{-1}) amygdalin, plotting standard curves. The method was validated by determining: detection and determination limits, range of linearity and the precision using the standard solutions. The intra-day variability was determined by analyzing each sample at three times within the same day, and the inter-day reproducibility was performed on three consecutive days. Method accuracy was calculated by spiking the samples with two known concentrations of the standard compounds. The resultant samples were analyzed with the developed HPLC method. The spiked solutions were prepared in the concentration range of the calibration curve with two different concentration levels (low and high), and the experiments were carried out in duplicate at each level. The theoretical amount of the standards in the sample and their average percentage recovered in the spiked solutions was calculated to determine the accuracy of the method.

Quantitative estimation of ascorbic acid and β-carotene

Ascorbic acid content in the fresh branches of *C. horizontalis* was used to determine by 2,6-dichloro-indophenol titrimetric method (Neilsen 1998). The principle depends on the oxidation of L-ascorbic acid to L-dehydro ascorbic acid by the indicator dye 2, 6-dichloro-indophenol; vitamin C content was calculated (mg g^{-1} sample) according to the equation:

$$C \times V \times \mathrm{DF}/Wt,$$

where C is the ascorbic acid in mg ml^{-1} dye, V is the ml of the dye used for titration of diluted sample, DF is the dilution factor, and Wt is the sample weight (g). The results were the average of three determinations.

β-Carotene content in the air-dried powdered branches of *C. horizontalis* was determined by HPLC Hewlett Packered series 1050; sample was injected (25 μl) onto the Vydac polymer C18 column and chromatographed isocratically; mobile phase, methanol-THF (tetrahydrofuran) (95:5 v/v); flow rate, 1 ml min^{-1}. Detection of β-carotene content was carried out at 450 nm using a diode array detector (Konings and Roomans 1996).

In vitro antioxidant activity

The antioxidant activity was measured using the stable radical as 2,2-diphenyl-1-picryl-hydrazyl (DPPH) (Brand-Williams et al. 1995). Different concentrations of the extract of branches of *C. horizontalis* (0–250 μg ml^{-1} methanol) were added at an equal volume (2.5 ml) to methanolic solution of DPPH (0.3 mM, 1 ml) and

incubated in dark at room temperature for 30 min. Methanol was used as a control in place of the sample. Ascorbic acid (0–100 μg ml^{-1}) was used as a reference agent. The absorbance values were measured against methanol at 517 nm on spectrophotometer (Unicam UV–VIS spectrophotometer, Japan). The radical scavenged (%) was calculated using the formula:

Control absorbance
– Test absorbance/Control absorbance × 100

All tests were performed in triplicates and the graph was plotted with the average of three determinations. IC_{50}, the concentration of the tested sample in μg ml^{-1} that required scavenging 50 % of free radicals, was calculated.

Anticancer activity

The ethanolic extract of branches of *C. horizontalis* was tested against the following human tumor cell lines: hepatocellular carcinoma (HEPG2), breast adenocarcinoma (MCF7), colorectal carcinoma (HCT116), cervix adenocarcinoma (HELA), larynx carcinoma (HEP2) using the protein-staining Sulpho-Rhodamine-B assay (Skehan and Storeng 1990). The concentration of the extract which reduced survival to 50 % (IC_{50}) was mentioned.

Acute toxicity study

Acute oral toxicity test was conducted for ethanolic extract of branches of *C. horizontalis* according to OECD (1996) guidelines 423, for acute toxic classic method using female albino rats.

Hepatoprotective activity

Hepatoprotective activity of the ethanolic extract (100 or 200 mg kg^{-1} body weight) or silymarin (50 $mgkg^{-1}$ body weight) was measured according to Kulkarni et al. (2002); liver damage in female Swiss strain albino rats (150 ± 20 g) was induced by simultaneous daily administration of acetaminophen (1 g kg^{-1} body weight, p.o.).

Biochemical analysis

Serum alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), total bilirubin (T.B), total protein (T.P), albumin (Alb), triglycerides (TG), total cholesterol (TC) were assayed by colorimetric methods using commercial kits supplied by Spectrum Diagnostics (Hannover, Germany), and Stanbio Laboratory LiquiColor (Boerne, TX, USA) for the determination of high density lipoprotein-cholesterol (HDL). γ-glutamyl transferase (GGT) was assayed by kinetic photometric method using kits supplied by Greiner Diagnostic GmbH (Bahlingen, Germany). While low density lipoprotein (LDL) and very low density lipoprotein (VLDL) were calculated by Friedewald's equation (1972) and atherogenic index (A.I) = (TC–HDL/HDL). Oxidative stress markers in liver tissue as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST), reduced glutathione (GSH) and malondialdehyde (MDA) were assayed using commercial kits supplied by Biodiagnostics (Giza, Egypt).

In vitro culture of explant

The explant of *C. horizontalis* was sterilized with aqueous ethanol (70 %) for 1 min, immersed in a solution of commercial Chlorex (10 %) with a few drops of tween 20 for 10 min, rinsed exhaustively in sterile distilled water and immersed in 0.1 % mercuric chloride solution with a few drops of tween-20 for 10 min, then rinsed in sterile distilled water. Explant cuttings (1–1.5 cm) were aseptically sectioned and cultured on half strength MS medium (Murashige and Skoog 1962) supplemented with 25 g l^{-1} sucrose and 7 g l^{-1} agar, supplemented with concentrations: 0, 2, 4 and 6 mg l^{-1} of BAP or kin, adjusted to pH 5.7 ± 0.1 and incubated at 24 ± 1 °C under 16-h photoperiod (day light fluorescent tube at 1500 lux.). After 4 weeks of incubation, the produced shootlets were subcultured every 4 weeks under the same conditions. For callus induction, leaf discs from shootlet multiplications were cultured on full strength MS supplemented with concentrations: 0, 2, 4 and 6 mg l^{-1} of 2,4-dichlorophenoxy acetic acid (2,4-D) and naphthalene acetic acid (NAA). After 4 weeks of incubation, the produced callus was subcultured 4 times (every 4 weeks) under the same conditions.

Preparation of samples for HPLC analysis

Air-dried powders (DP) of: in vitro shootlets (14 samples), callus (6 samples) and branches of *C. horizontalis* (5 g, each) were separately extracted with *n*-hexane (50 ml × 4) then with methanol (95 %) (50 ml × 4) by sonication at room temperature (15 min, each).

a. Each of the accurately weighed concentrated *n*-hexane extracts was prepared and saponified (Escriva and Esteve 2002). The remaining residues (unsaponifiable matters) were separately dissolved in 5 ml of the mobile phase: methanol–water (96:4 v/v).
b. Each of the accurately weighed concentrated methanolic residues was redissolved in 5 ml methanol (HPLC-grade) prior to HPLC analysis. A volume of 10 μl was injected in the HPLC system.

Data analysis

The biochemical results were statistically analysed using the Statistical Package for Social Sciences (SPSS, version 17; SPSS Inc, Chicago) for Windows followed by Dennett's Multiple Comparison Test (DMCT). $P < 0.05$ was considered as significant.

Results and discussion

Phytochemical analysis

Vitamins analysis of the plant under investigation revealed the presence of valuable amounts of β-carotene (2,500 mg 100 g^{-1}) and ascorbic acid (70 mg 100 g^{-1}) and small amounts of α-tocopherol (0.093 mg 100 g^{-1}) and amygdalin (0.334 mg 100 g^{-1}).

In vitro antioxidant potential

The antioxidant effect (Fig. 1) showed the dose–response curve of DPPH scavenging activity of ethanolic extract of branches of *C. horizontalis* with IC_{50} values of 19.3 µg ml^{-1}.

Cytotoxicity activity

The ethanolic extract of branches of *C. horizontalis* showed dose-dependent cytotoxicity against different human cancer cell lines (HEPG2, MCF7, HCT116 and HELA) (Table 1) which mainly due to anti-tumor potential of amygdalin (Brent 2005), antioxidant potential of α-tocopherol, β-carotene and ascorbic acid contents and other phenolics detected during preliminary phytochemical screening (Rajneesh et al. 2008). The plant extract failed to exert a considerable effect on larynx carcinoma cell line.

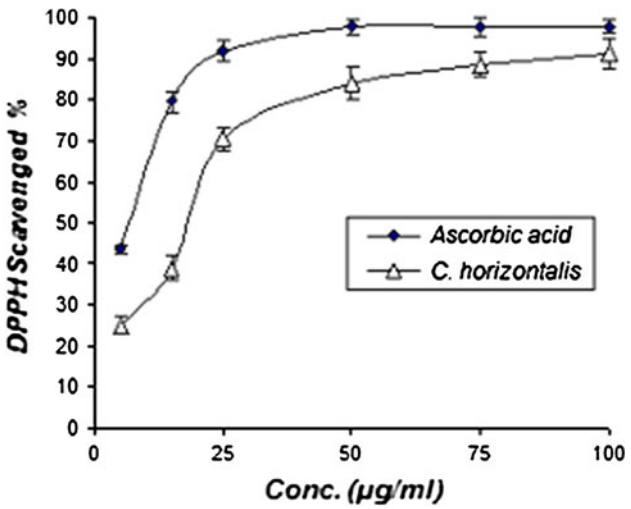

Fig. 1 Dose–response curve of DPPH scavenging activity of ethanolic extract of Cotoneaster horizontalis Decne and ascorbic acid

Acute toxicity test

The acute toxicity test of the ethanolic extract of *C. horizontalis* showed no signs of toxicity up to 2 g kg^{-1} b.wt. and was considered as safe (OECD Guidelines. 1996). One-tenth and one-twentieth of the maximum dose (100 and 200 mg kg^{-1} body weight) of the extract were selected for evaluation of hepatoprotective potential.

Effect on liver function

Results of acetaminophen intoxication (Table 2) recorded a significant elevation of serum marker enzymes: ALT, AST, ALP, GGT, and T.B with a decrease in T.P as compared to the normal group (Mohit et al. 2011). Meanwhile, the simultaneous oral administration of the extract of *C. horizontalis* or silymarin with acetaminophen for 4 weeks seemed to preserve the structural integrity of the hepatocellular membrane in a dose-dependant manner as evidenced from significant reduction in acetaminophen-induced rise in the serum enzymes and the increase of the serum T.P level. The increase in serum proteins is a clear demonstration of detoxification of acetaminophen reactive metabolite (Davern et al. 2006).

Effect on serum lipid profile

Acetaminophen intoxication associated with hyperlipidemia (Raghavendran et al. 2005) as evidenced from a

Table 1 Cytotoxic activity of the ethanolic extract of branches of *Cotoneaster horizontalis* on different human cell lines

Conc (µg ml^{-1})	HEPG2	MCF7	HCT116	HELA	HEP2
	Survival %				
0.00	100	100	100	100	100
0.81[a]	–	50	–	–	–
0.87[a]	50	–	–	–	–
1.00	48	44	74	74	97
2.50	58	45	51	51	74
2.89[a]	–	–	50	–	–
5.00	53	36	44	80	55
8.19[a]	–	–	–	50	–
10.00	44	31	23	28	64

[a] Doses gave IC_{50} of HEPG2, MCF7, HCT116 and HELA, respectively. HEPG2, hepatic carcinoma cell line; MCF7, breast carcinoma cell line; HCT116, colon carcinoma cell line; HELA, cervix carcinoma cell line; HEP2, larynx carcinoma cell line

significant increase in the serum levels of triglycerides (TG), total cholesterol (TC), LDL, VLDL and atherogenic index (A.I) and a decrease in the level of high density lipoprotein (HDL) as compared with normal control rats (Table 3). The hypercholesterolemia could be attributed to decreased secretion of bile, bile salts and total biliary fatty acids, while the increase in the TG could be due to the inhibition of hepatic cells to produce lipoproteins and lipases which were essential for triglyceride metabolism (Bhaumik and Sharma 2002). Consequently, a significant change in the level of serum lipid profile toward the normal values after treatment with *C. horizontalis* extract or silymarin was a clear manifestation of their protection against acetaminophen intoxication.

Effect on liver oxidative markers

Results in Table 4 showed that the elevated level of MDA was due to excessive formation of free radicals and activation of lipid peroxidation system that altered membrane fluidity and permeability, led to a loss of membrane integrity. At the same time, there was a depletion in the intracellular pool of GSH content that affected the removal of the free radicals through GPx and GST activities (Chin et al. 2009), associated with a decrease in the activity of the antioxidant enzymes (SOD, CAT, GPx, GR and GST) resulted in inadequate detoxification mechanism to prevent the formation of excessive reactive radicals (Mohit et al. 2011). However, treatment with *C. horizontalis* extract or silymarin along with acetaminophen significantly increased the activities of antioxidant enzymes and cellular content of GSH, provided hepato-protection by eliminating the deleterious effects of the excessive free radicals and toxic metabolites of acetaminophen and inhibiting lipid peroxidation.

Validation data

The detection limit values of α-tocopherol and amygdalin were calculated as the concentrations corresponding to three times the height of the background noise: 0.12 and 0.11 μg ml^{-1}, respectively, and determination limits were defined as three times the detection limit: 0.36 and 0.33 μg ml^{-1}, respectively. The measurements of intra- and inter-day variability were utilized to determine the repeatability of the HPLC method. The relative standard deviation (RSD) was considered as a measure of precision. The results show that the overall intra- and inter-day variations indicated that RSD was less than 1.94 and 1 % for α-tocopherol and amygdalin, respectively. Their recovery tests ranged from 100.78 to 98.34 %, respectively, with RSD <2 %. Linear regression analysis for each standard curve was performed, the regression equations: $Y = .52682X - 13.227$, $r^2 = 0.9998$ (α-tocopherol), $Y = 4.3097X + 157.8744$, $r^2 = 0.9843$ (amygdalin), X referred to the concentration of the standard compounds, Y the peak area, and r the correlation coefficient of the equation.

Table 2 Effect of concomitant daily administration of the ethanolic extract of branches of *Cotoneaster horizontalis* or silymarin with acetaminophen for 4 weeks on serum liver function in albino rats

Parameters		Control	Acetaminophen (1 g/kg b.wt)	Ethanolic extract of *C. horizontalis* (mg/kg body weight)		Silymarin (mg kg^{-1} body weight) 50
				100 +Acetaminophen (1 g kg^{-1} b.wt)	200	
L.W	(g/100 g b.wt)	2.99 ± 0.08	3.63 ± 0.05[b]	3.36 ± 0.05[d]	3.17 ± 0.06[e]	3.12 ± 0.05[e]
ALT	(U/l)	22.9 ± 1.94	45.4 ± 2.31[b]	38.0 ± 1.40[d]	36.3 ± 1.82[d]	30.3 ± 1.16[e]
AST		51.7 ± 2.01	86.1 ± 2.98[b]	72.8 ± 2.54[d]	70.4 ± 3.28[d]	62.6 ± 3.63[e]
ALP		121.8 ± 5.18	242.3 ± 9.02[b]	201.1 ± 6.60[e]	169.0 ± 5.10[e]	152.5 ± 5.38[e]
GGT		3.55 ± 0.17	8.04 ± 0.28[b]	5.82 ± 0.23[e]	5.13 ± 0.13[e]	5.00 ± 0.15[e]
T.B	(mmole/l)	12.8 ± 5.12	18.8 ± 0.68[b]	15.6 ± 1.03[d]	14.9 ± 0.51[c]	14.5 ± 0.68[e]
T.P	g/l	5.68 ± 0.20	4.91 ± 0.13[a]	5.60 ± 0.16[d]	5.81 ± 0.13[e]	5.85 ± 0.16[e]
Alb.		3.09 ± 0.09	2.52 ± 0.07[b]	2.86 ± 0.03[c]	2.91 ± 0.08[d]	2.98 ± 0.12[d]

Each value represents the mean of 8 rats ± SE

L.W Liver weight, *ALT* alanine transaminase, *AST* aspartate transaminase, *ALP* alkaline phosphatase, *GGT* γ-glutamyl transferase, *T.B* total bilirubin, *T.P* total protein, *Alb* albumin

Significant at [a] $P < 0.01$; [b] $P < 0.001$ versus control. Significant at [c] $P < 0.05$; [d] $P < 0.01$; [e] $P < 0.001$ versus acetaminophen

Table 3 Effect of concomitant daily administration of the ethanolic extract of branches of *Cotoneaster horizontalis* or silymarin with acetaminophen for 4 weeks on serum lipid profile in albino rats

Parameters		Control	Acetaminophen (1 g/kg b.wt.)	Ethanolic extract of *C. horizontalis* (mg/kg body weight)		Silymarin (mg/kg body weight) 50
				100 +Acetaminophen (1 g/kg b.wt.)	200	
TG	mmole/l	0.74 ± 0.03	1.12 ± 0.04[a]	0.93 ± 0.02[b]	0.81 ± 0.02[b]	0.79 ± 0.02[b]
TC		2.29 ± 0.03	3.73 ± 0.08[a]	3.00 ± 0.07[b]	2.64 ± 0.06[b]	2.56 ± 0.08[b]
HDL		1.14 ± 0.02	0.87 ± 0.03[a]	1.06 ± 0.02[b]	1.16 ± 0.02[b]	1.16 ± 0.03[b]
LDL		0.82 ± 0.05	2.35 ± 0.07[a]	1.52 ± 0.08[b]	1.11 ± 0.07[b]	1.04 ± 0.09[b]
VLDL		2.60 ± 0.11	3.96 ± 0.13[a]	3.30 ± 0.06[b]	2.88 ± 0.05[b]	2.68 ± 0.08[b]
A.I		1.02 ± 0.05	3.33 ± 0.13[a]	1.85 ± 0.09[b]	1.29 ± 0.07[b]	1.23 ± 0.10[b]

Each value represents the mean of 8 rats ± SE

TG Triglycerides, *TC* total cholesterol, *HDL* high density lipoprotein, *LDL* low density lipoprotein, VLDL very low density lipoprotein, A.I atherogenic index

Significant at [a] $P < 0.001$ versus control. Significant at [b] $P < 0.001$ versus acetaminophen

Table 4 Effect of concomitant daily administration of the ethanolic extract of branches of *Cotoneaster horizontalis* or silymarin with acetaminophen for 4 weeks on liver antioxidant activities in albino rats

Parameters		Control	Acetaminophen (1 g/kg b.wt)	Ethanolic extract of *C. horizontalis* (mg/kg body weight)		Silymarin (mg/kg body weight) 50
				100 +Acetaminophen (1 g/kg b.wt)	200	
SOD	U/mg protein	3.72 ± 0.07	2.43 ± 0.14[a]	3.08 ± 0.09[b]	3.83 ± 0.17[d]	3.77 ± 0.19[d]
CAT		6.42 ± 0.18	3.39 ± 0.21[a]	4.57 ± 0.14[d]	5.39 ± 0.19[d]	6.02 ± 0.19[d]
GPx		5.52 ± 0.14	4.17 ± 0.03[a]	4.43 ± 0.09[b]	4.96 ± 0.08[d]	5.25 ± 0.04[d]
GR		4.75 ± 0.17	3.94 ± 0.08[a]	4.25 ± 0.03[c]	4.34 ± 0.06[d]	4.45 ± 0.08[d]
GST		5.07 ± 0.10	3.59 ± 0.11[a]	4.11 ± 0.04[d]	4.76 ± 0.06[d]	4.97 ± 0.08[d]
GSH	μmole/g liver	50.2 ± 0.99	31.0 ± 0.66[a]	36.3 ± 0.65[d]	42.9 ± 0.99[d]	47.5 ± 0.89[d]
MDA	nmole/mg protein	0.48 ± 0.02	0.85 ± 0.03[a]	0.62 ± 0.03[d]	0.52 ± 0.02[d]	0.51 ± 0.03[d]

Each value represents the mean of 8 rats ± SE

Significant at [a] $P < 0.001$ versus control. Significant at [b] $P < 0.05$; [c] $P < 0.01$; [d] $P < 0.001$ versus APAP

SOD Superoxide dismutase, *CAT* catalase, *GPx* glutathione peroxidase, *GR* glutathione reductase, *GST* glutathione-S-transferase, *GSH* reduced glutathione, *MDA* malondialdehyde

Estimation of α-tocopherol and amygdalin (Table 5)

Analysis of α-tocopherol in *n*-hexane extracts of different shootlets cultures of *C. horizontalis* relative to the concentration of the reference sample found that full MS exhibited a good increase in α-tocopherol (0.238 mg 100 g^{-1} DP) which was significantly increased after the addition of 2 and 6 mg l^{-1} BAP (1.746 and 0.796 mg 100 g^{-1} DP, respectively) followed by half MS with 2, 4 or 6 mg l^{-1} BAP. Kin treatments were generally non-significant as compared with results of the mother plant (0.093 mg 100 g^{-1} DP) (Fig. 2). Callus cultured on MS treated with 6 mg l^{-1} NAA significantly increased its content to 0.286 mg 100 g^{-1} DP, followed by 2 mg l^{-1} NAA (0.266 mg 100 g^{-1} DP). Quantitative analysis of amygdalin content in methanolic extracts of the same cultures relative to reference amygdalin revealed that full MS supplemented with 4 mg l^{-1} kin exhibited a significant result (9.33 mg 100 g^{-1} DP); pronounced results were recorded for callus cultured on MS supplemented with 2 mg l^{-1} 2,4-D (13.64 mg 100 g^{-1} DP) followed by 4 and 6 mg l^{-1} 2,4-D (9.755 and 6.106 mg 100 g^{-1} DP, respectively) as compared with that of the mother plant (0.334 mg 100 g^{-1} DP) (Fig. 3).

In conclusion, the ethanolic extract of branches of *C. horizontalis* could be considered as a potential source of a natural antioxidant with hepatoprotective and

Table 5 α-Tocopherol and amygdalin contents in extracts of different in vitro shootlet cultures and callus of *Cotoneaster horizontalis* resulted from different treatments

Treatment (mg/L hormones)	α-Tocopherol (mg 100 g^{-1} dry powder ± SD)	Amygdalin (mg 100 g^{-1} dry powder ± SD)
Plant aerial part	0.093 ± 0.002	0.334 ± 0.002
Shootlets		
F (control)	0.238 ± 0.003	0.707 ± 0.001
2 (BAP)	1.746 ± 0.010	1.713 ± 0.023
4 (BAP)	0.187 ± 0.002	4.559 ± 0.100
6 (BAP)	0.796 ± 0.001	1.780 ± 0.023
2 (Kin)	0.059 ± 0.000	1.564 ± 0.034
4 (Kin)	0.089 ± 0.000	9.330 ± 0.410
6 (Kin)	0.095 ± 0.000	3.033 ± 0.012
H (control)	0.236 ± 0.002	2.459 ± 0.041
2 (BAP)	0.244 ± 0.000	0.430 ± 0.003
4 (BAP)	0.318 ± 0.000	2.775 ± 0.014
6 (BAP)	0.299 ± 0.003	0.712 ± 0.004
2 (Kin)	0.044 ± 0.001	0.998 ± 0.002
4 (Kin)	0.049 ± 0.003	1.291 ± 0.003
6 (Kin)	0.137 ± 0.003	2.519 ± 0.013
Callus		
2 (2,4-D)	0.122 ± 0.000	13.64 ± 0.124
4 (2,4-D)	0.153 ± 0.001	9.755 ± 0.133
6 (2,4-D)	0.247 ± 0.000	6.106 ± 0.062
2 (NAA)	0.266 ± 0.002	0.982 ± 0.002
4 (NAA)	0.199 ± 0.003	1.883 ± 0.025
6 (NAA)	0.286 ± 0.004	2.347 ± 0.014

F full MS, *H* half MS media

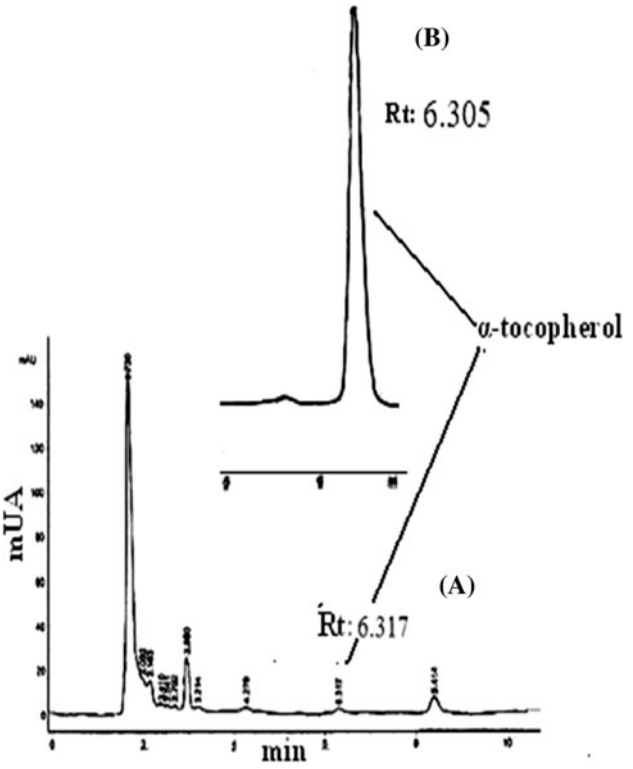

Fig. 2 HPLC chromatogram of **a** *n*-hexane extract of *Cotoneaster horizontalis*, **b** standard α-tocopherol

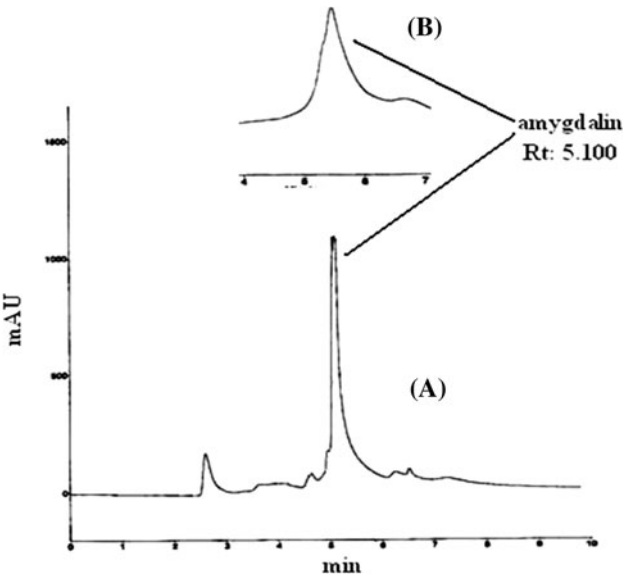

Fig. 3 HPLC chromatogram of **a** alcoholic extract of *Cotoneaster horizontalis*, **b** standard amygdalin

hypolipidemic properties for its content of natural vitamin E (0.76 mg 100 g^{-1} extract) and amygdalin (0.11 mg 100 g^{-1} extract). In vitro cultures showed that the use of full MS with 2 mg l^{-1} BAP or 6 mg l^{-1} of NAA induced vitamin E production and full MS supplied with 4 mg l^{-1} kin or 2 mg l^{-1} 2,4 D induced amygdalin.

To our knowledge, all results on *C. horizontalis* are presented here for the first time; as a trial to introduce a valuable medicinal plant which may have a beneficial value in the improvement of anticancer and hepatoprotection against toxicity induced by over doses of acetaminophen.

Author contribution Nadia Sokkar designed the experiments, conceptual advice and contributed to the preparation of the manuscript. Omyma El-Gindi gave technical support. Shaza Mohamed prepared the manuscript. Sawsan sayed supervised the tissue culture experiments. Zeinab Ali supervised the biochemical work. Iman Alfishawy performed the experiments.

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Conflict of interest The authors declare that they have no conflict of interest.

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