PHYTOTHERAPY RESEARCH
Phytother. Res. (2012)
Published online in Wiley Online Library
(wileyonlinelibrary.com) **DOI**: 10.1002/ptr.4688

Antiproliferative and Antiangiogenic Properties of Horse Chestnut Extract

Gabriela Mojžišová,[1]* Ján Mojžiš,[2] Martina Pilátová,[2] Lenka Varinská,[2] Lenka Ivanová,[2] Ladislav Strojný[1] and Ján Richnavský[3]

[1]Department of Experimental Medicine, University of P. J. Šafarik, Košice, Slovak Republic
[2]Department of Pharmacology, University of P. J. Šafarik Košice, Košice, Slovak Republic
[3]Gynecological and Obstetrical Department, 1st Private Hospital Košice-Šaca Inc., Košice-Šaca, Slovak Republic

This study was designed to examine the *in vitro* antiproliferative effect of the horse chestnut extract (HCE) on cancer cell lines. Furthermore, we have investigated the *in vitro* effect of HCE on some angiogenic events by using human umbilical vein endothelial cells. The cell proliferation was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and anchorage-independent growth by colony-forming assay. To understand the growth inhibitory effects, carcinoma cell lines (Jurkat, CEM, HeLa, and MCF-7) were treated with various concentrations of HCE. Incubation of Jurkat, CEM, HeLa, and MCF-7 cancer cells with HCE at 125 μg/mL for 72 h caused 93.7%, 32.3%, 20.4% and 40.4% reduction in cell survival. Colony-forming assay also confirmed growth-inhibitory effects of the compound studied. In HeLa HCE-treated cells, we found a significant increase in cells having sub-G_0/G_1 DNA content which is considered to be a marker of apoptotic cell death. Apoptosis was also further confirmed by DNA fragmentation analysis.Furthermore, HCE inhibited migration of human umbilical vein endothelial cells as well as decreased secretion of matrix metalloproteinase and vascular endothelial growth factor.In conclusion, the present study has assessed *the in vitro* antiproliferative/antiangiogenic potential of HCE. These results generate a rationale for *in vivo* efficacy studies with horse chestnut in preclinical cancer models. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: horse chestnut; antiproliferative; proapototic and antiangiogenic effects.

INTRODUCTION

Horse chestnut which is native to Europe have been used in the treatment of rheumatism and neuralgia as well as in conditions of venous congestion, particularly with dull, aching pain and fullness (Sirtori, 2001; Pittler and Ernst, 2006; Suter *et al.*, 2006).

The primary active constituent found in horse chestnut seed extract is aescin. Aescin is actually a mixture of triterpene saponins present in two forms, (α and β). A number of other products such as bioflavonoids (quercetin, kaempherol, and their diglycosyl derivatives), anti-oxidants (proanthocianidin A2), and the coumarins (aesculin and fraxin) have been isolated from the chestnut seeds (Bombardelli and Morazzoni, 1996; Sirtori, 2001). Aescin has been reported to act as an antiinflammatory, antiallergy, antioxidant, vasorelaxant compound (Carrasco and Vidrio, 2007; Wang *et al.*, 2009; Küçükkurt *et al.*, 2010; Lindner *et al.*, 2010).

On the other hand, antiproliferative effect of aescin was studied only marginally. Guo *et al.* (2003) found that beta(β)-aescin sodium can inhibit the growth of various human and mice tumor cell lines and their transplant tumors. Later, Niu and co-workers demonstrated that β-aescin is a potent natural inhibitor of cell proliferation and inducer of apoptosis either in HL-60 acute myeloid leukemia cells (2008a) or K562 chronic myeloid leukemia cells (2008b). Recently, aescin was found to exhibit significant antitumor effects in human hepatocellular carcinoma both *in vitro* and *in vivo* (Zhou *et al.*, 2009).

In the present work, we tested horse chestnut extract (HCE) for its antiproliferative and proapototic effects on cancer cell lines. Moreover, because of the critical role of angiogenesis in tumor growth and metastasis, we investigated whether HCE is able to modulate some critical steps in the process of neovascularization.

MATERIALS AND METHODS

Horse chestnut dry extract (botanical name is *Aesculus hippocastanum* L, semen) was a gift from Calendula a. s. (Nová Ľubovňa, Slovak Republic). The standardized extract of horse chestnut contains 18%–22% of aescin. 3-(Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was from Sigma-Aldrich Chemie (Steinheim, Germany); Cycle TEST™ PLUS DNA reagent kit and propidium iodide were purchased from Becton Dickinson Biosciences (BDB, San Jose, CA, USA). Quantikine® Human VEGF Immunoassay for the quantitative determination of human vascular endothelial growth factor (VEGF) concentrations in cell culture supernates, serum, and plasma was from R&D systems, Inc. Minneapolis, MN, USA. Collagenase II was purchased from Invitrogen (Carlsbad, CA, USA).

* Correspondence to: Gabriela Mojžišová, Department of Experimental Medicine, University of P. J. Šafarik, Tr. SNP 1, 040 66 Košice, Slovak Republic.
E-mail: gabriela.mojzisova@upjs.sk

Received 04 March 2011
Accepted 07 March 2012

Cell lines and culture

The following human cancer cell lines were used for this study: Jurkat (human acute T-lymphoblastic leukemia), CEM (acute T-lymphoblastic leukemia), HeLa (cervical carcinoma cells), and MCF-7 (breast cancer cells). All cell lines used were kindly provided by Dr Hajduch (Olomouc, Czech Republic). Jurkat, CEM, and HeLa cells were cultured in RPMI 1640 medium (PAA Laboratories, Pasching, Austria). MCF-7 were cultured in growth medium consisting of high glucose Dulbecco's Modified Eagle Medium. Both media were with Glutamax-supplemented with 10% fetal calf serum, penicilin (100 IU/mL), and streptomycin (100 μg/mL) (all from Invitrogen, Carlsbad, CA USA), in the atmosphere of 5% CO_2 in humified air at 37 °C. Cell viability, estimated by trypan blue exclusion, was greater than 95% before each experiment.

Cytotoxicity assay

The cytotoxic effect of HCE was studied by using colorimetric microculture assay with the MTT end point (Mosman, 1983). The method is based on the conversion of a tetrazolium salt MTT in cells to insoluble formazan.

Briefly, 8×10^3 cells were plated per well in 96 well polystyrene microplates (SARSTEDT, Nümbrecht, Germany) in the culture medium containing the tested HCE at the doses of 500–15.6 μg/mL. After 72 h of incubation, 10 μL of MTT (5 mg/mL) was added in each well. After an additional 4 h, during which insoluble formazan was produced, 100 μL of 10% sodium dodecyl sulfate was added in each well, and another 12 h was allowed for the formazan to be dissolved. The absorbance was measured at 540 nm with the use of the automated MRX microplate reader (Dynatech Laboratories, Billinghurst, West Sussex, UK). The absorbance of the control wells was taken as 100%, and the results were expressed as a percentage of the control.

Colony formation analysis

For colony formation assay, HeLa cells were seeded in six-well plates at a density of 1000 cells per well and allowed to adhere for 10 h before treatment. Culture medium containing variable concentrations of HCE was added to cells and incubated for 14 days. The cells were then fixed in buffered formalin (pH 7.2) and stained with 0.01% crystal violet. The crystal violet stain was then extracted with 10% acetic acid for 60 min and read at 540 nm. Cell survival at each drug concentration was expressed as a percentage of survival of controls (no drug added).

Cell cycle analysis

The cell cycle distribution in cells treated with HCE was analyzed by propidium jodide DNA staining using Cycle TEST™ PLUS DNA reagent kit. Briefly, 5×10^5 HeLa cells were treated with HCE for 24, 48, and 72 h. After treatment, cells were harvested, washed three times in citrate buffer, and stained according to the manufacturer's instructions. Data acquisition was performed within 1 h after staining on a FACS Vantage SE flow cytometer using CellQuest Pro software (both from BDB), information being stored for 5×10^4 events per sample. The data were analyzed using Win MDI software. Percentages of cells corresponding to G_0/G_1, S, and G_2/M cell cycle phases were calculated. The sub-G_0/G_1 fraction of cells was identified as the apoptotic population.

DNA fragmentation assay

Treated and untreated cells (1×10^6) were washed twice in phosphate-buffered saline w/o calcium and magnesium. Cell lysis was performed in a buffer containing 10 mmol/L tris (hydroxymethyl)aminomethane, 10 mmol/L EDTA, and 0.5% Triton X-100. Afterwards, proteinase K (1 mg/mL) was added, and lysate was incubated for 60 min at 37 °C. Then, it was heated at 70 °C for 10 min followed by addition of RNAase (200 μg/mL) and another 1 hr incubation at 37 °C. Samples were subsequently transferred to 2% agarose gel and run with 40 V at 3 h. DNA fragments were visualized by UV transluminator by ethidium bromide staining.

Vascular endothelial growth factor quantification

Vascular endothelial growth factor protein released into the conditioned medium was measured by using a commercial enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems) according to the manufacturer's instructions. HeLa cells (1×10^5) were seeded in six-well plates in 2 mL of complete growth medium. After 24 h, the cells were washed with phosphate-buffered saline and preconditioned for 1 h at 37 °C in 1 mL of RPMI containing 2% fetal bovine serum. The preconditioned medium was replaced with 1 mL RPMI containing 2% fetal bovine serum alone or with 125 μM $CoCl_2$ (as an *in vitro* model of hypoxia) in the absence or presence of HCE (125, 62.5 and 31.2 μg/mL). After 24-h incubation to allow VEGF protein secretion under the various culture conditions, medium was collected and 1 mM of phenyl methyl sulfonyl fluoride was added. The supernatant was clarified by centrifugation for 5 min at 15000 *g* and stored at −70 °C until quantification for VEGF. The assay was repeated three times with similar results. Data from three independent experiments were pooled for statistical analysis.

Endothelial cell isolation and cell cultures

Human umbilical vein endothelial cells (HUVECs) were isolated from freshly collected human umbilical cords by collagenase digestion of the umbilical vein interior according to the methods described by Marin *et al.* (2001). Cells were plated in 100 × 20 mm tissue culture dishes (SARSTEDT, Nümbrecht, Germany) coated with 1.5% gelatin. Cells were grown to confluence in cultivation medium. Primary cultures were harvested at confluence with 0.05% trypsin–0.02% EDTA (Invitrogen) and plated at a split ratio of 1:3 in tissue culture dishes. The cells were fed with fresh medium a day before each individual experiment. The endothelial identity of the cells was confirmed by their "cobblestone" morphology and CD31 expression. The cells were stained with a

combination of CD45-FITC (BDB)/CD31-PE (Caltag, Burlingame, CA, USA) monoclonal antibodies and analyzed by flow cytometry. Endothelial cells were identified as CD31+CD45− cells and represented almost 100% of all cells in every primary culture.

Endothelial cell migration assay

The motility of HUVECs was assayed using wound healing assay (Martínez-Poveda *et al.*, 2005). Briefly, HUVECs were cultured on the 24-well plate in the cM199 medium to reach confluence. A 2 mm tip of a pipette was used to wound the monolayer of cells. Afterwards, the medium was replaced with fresh endothelial cell growth factor and heparin-free medium containing the studied compound at different concentrations in the presence of 25 ng/mL of recombinant VEGF. The wounded area was photographed at the start ($t = 0$ h) and at time point $t = 17$ h. Quantitation of cell migration was performed as described by Cheung and Li (2002). The experiments were performed in duplicate wells and repeated three times.

Gelatinase zymography

Gelatinolytic activities of secreted matrix metalloproteinases (MMPs) were analyzed by zymography on gelatin-containing polyacrylamide gels as described by Newcomb *et al.* (2005). With the use of this technique, both active and latent species can be visualized.

Conditioned media were obtained by incubating cells in 10 cm^2 dishes for 48 h with 1 mL of serum-free cultivation medium to which appropriate concentration of the HCE was added. Phorbol 12-myristate 13-acetate was used as a positive control. The conditioned media were centrifuged for 20 min at 1000 *g* in a microfuge to remove cells and cellular debris, and samples were frozen at −80 °C until use. Samples were put into the buffer containing 3% (w/v) sodium dodecyl sulfate and 10% (w/v) glycerol and then applied to 10% (w/v) polyacrylamide gels copolymerised with 2 mg/mL gelatin. After electrophoresis gels were renatured in 2.5% Triton X-100 (2 × 15 min), then incubated overnight at 37 °C in development buffer (50 mM Tris–HCl, pH 7.6; 10 mM CaCl2; 50 mM NaCl; 0.05% Brij35 [MP Biomedicals, Santa Ana, CA, USA]). Gels were stained with 0.5% Coomassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid for 40 min at room temperature and then distained for 1 h in 50% methanol and 10% acetic acid. Data from three independent experiments were pooled for statistical analysis.

Proteolytic activity for MMP-9 in the gel was visualized as clear white bands at 92 kDa against a dark background. The gelatinase standard (Chemicon) was used as a positive control for gelatin zymography.

Statistical analysis

For all experiments, mean values and standard deviations (from five experiments) were calculated using the ArcusQuickstat software package. To evaluate the statistical significance of observed differences between groups, Student's *t*-test was employed. The statistical significance was considered to be present if $p < 0.05$.

RESULTS AND DISCUSSION

Cytotoxicity assay

The sensitivity of cancer cells to HCE treatment differed among cell types. Survival of Jurkat as well as CEM, HeLa, and MCF-7 cells exposed to various HCE doses is shown in Table 1. HCE at 125 μg/mL for 72 h caused 93.7%, 32.3%, 20.4%, and 40.4% reduction in cell survival. Moreover, HCE in dose 31.2 μg/mL significantly decreased Jurkat survival ($p < 0.05$). In HUVECs, significant cytotoxicity was observed after HUVECs were incubated with HCE at two highest doses used (500 and 250 μg/mL) ($p < 0.001$).

Colony formation assay

To determine whether HCE can inhibit tumorigenesis of HeLa cells, we did colony formation assay. Compared with the control, HCE significantly inhibited the capacity of producing colonies in HeLa cell lines in all tested doses (125–7.8 μg/mL $p < 0.001$, $p < 0.01$, and $p < 0.01$). These results thus suggest that HCE treatment is highly effective in suppressing the colony-forming ability of human cancer cells (Table 2).

Cell cycle analysis

The distribution of HeLa cells in different phases of the cell cycle is shown in Table 3. After treatment with HCE at a dose of 62.5 μg/mL for 24 h, we found an increase in cells having sub-G_0/G_1 DNA content that was accompanied by a slight decrease in G_2/M phase cells. After 72-h treatment, nearly 40% of cells exposed to HCE were found to have sub-G_0/G_1 DNA content. These results indicate that HCE causes the appearance of the fraction of cells with sub-G0/G1 DNA content, which is suggestive of apoptosis (Table 3).

DNA fragmentation assay

Analysis of DNA fragmentation by agarose gel electrophoresis is one of the most widely used biochemical markers for cell death. The detection of internucleosomal DNA cleavage (DNA laddering) is considered to be an indicator of apoptosis.

As shown in Figure 1A, treatment of Jurkat cells with HCE resulted in the formation of definite fragments that could be seen via electrophoretic examination as a characteristic ladder pattern (62.5 μg/mL for 48 and 72 h). In HeLa cells (Fig. 1B), HCE produced similar effects.

Suppression of vascular endothelial growth factor secretion by horse chestnut extract

As shown in Figure 2, chemical hypoxia with $CoCl_2$ enhanced VEGF secretion. Twenty-four hours of treatment with HCE in doses 125–31.2 μg/mL significantly inhibited $CoCl_2$-induced VEGF secretion ($p < 0.001$; $p < 0.01$). Under normoxic conditions, no significant

Table 1. Effect of HCE on the viability (%) of different cancer cell lines and HUVECs

Dose HCE (μg/mL)	Jurkat	CEM	HeLa	MCF-7	HUVEC
500.0	0.0	0.0	0.0	$1.3 \pm 0.1^{***}$	$4.1 \pm 0.2^{***}$
250.0	0.0^{***}	$8.7 \pm 0.9^{***}$	$48.6 \pm 7.5^{***}$	$54.7 \pm 3.6^{***}$	$56.6 \pm 9.3^{**}$
125.0	$6.3 \pm 0.8^{***}$	$67.7 \pm 11.3^{**}$	$79.6 \pm 6.9^{*}$	$59.6 \pm 8.9^{**}$	100
62.5	$56.1 \pm 8.1^{**}$	100	82.7 ± 8.7	$70.5 \pm 5.4^{*}$	100
31.2	$77.3 \pm 6.6^{*}$	100	92.2 ± 12.1	96.1 ± 10.2	100
15.6	80.8 ± 11.0	100	90.4 ± 10.5	100	100

HCE, horse chestnut extract; HUVEC, human umbilical vein endothelial cell.
$^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ versus control. Cells were incubated with different doses of HCE in triplicate.

Table 2. Effect of HCE on HeLa cells survival studied by colony-forming assay

Dose HCE (μg/mL)	% of cell survival
125.0	$16.5 \pm 2.8^{***}$
62.5	$19.5 \pm 3.3^{***}$
31.2	$45.3 \pm 4.1^{***}$
15.6	$62.1 \pm 3.9^{**}$
7.8	$76.9 \pm 4.7^{*}$

HCE, horse chestnut extract.
$^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ versus control.

Table 3. Effect of horse chestnut extract on cell cycle in HeLa cells incubated for 24, 48, and 72 h

	sub-G0/G1	G0/G1	S	G2/M
K	1.5	62.1	16.3	19.7
24 h	7.8	59.1	14.3	16.4
48 h	15.8	52.3	12.7	14.4
72 h	37.1	39.5	9.2	10.8

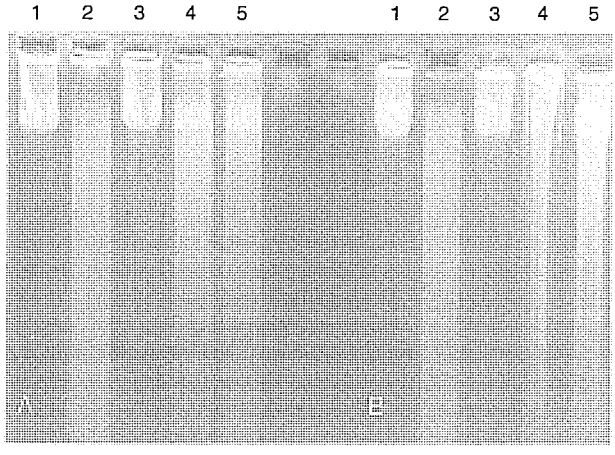

Figure 1. DNA fragmentation of Jurkat (A) and HeLa (B) cells after 24, 48, and 72 h incubation with horse chestnut extract in a dose of 62.5 μg/mL. Apoptotic DNA fragmentation was qualitatively analyzed by DNA gel electrophoresis. The extracted DNA was loaded on a 2% agarose gel and stained with ethidium bromide. Lanes indicate different treatments: lane 1–negative control, lane 2–positive control (etoposide 50 μL/mL), lane 3–DNA fragmentation after 24 h of incubation, lane 4–DNA fragmentation after 48 h of incubation, and lane 5–DNA fragmentation after 72 h of incubation with horse chestnut extract.

impact of HCE on the levels of VEGF protein in HeLa cells was observed.

Endothelial cell migration

Migration of endothelial cells in response to angiogenic factors plays an important role in angiogenesis. In this assay, we studied the ability of examined compound to inhibit endothelial cell migration in a wound closure assay. After wounding with a pipet tip, solvent controls reformed a confluent monolayer within 24 h of incubation. HCE was added in doses 125–16.6 μg/mL. In the presence of this extract, a potent dose-dependent inhibition of endothelial cell migration was observed (Fig. 3).

Gelatinase zymography

To examine whether the studied compound modulate the secretion of MMP-9 from HUVECs, we determined the MMP-9 level in the conditioned medium by gelatin zymography.

Gelatin zymography revealed that HCE reduced MMP-9 activity in HUVECs in a concentration-dependent manner. The active bands of MMP-9 gradually diminished when HUVECs were treated with different doses of HCE (from 15.6 to 250 μg/mL) (Fig. 4).

The bands of the standard for MMP-9 reflect inactive as well as active forms of the studied enzyme. We determined only the pro-enzyme of MMP-9 in our samples, not an active form of this enzyme. Therefore, we can claim that HCE treatment decreased the secretion of MMP-9 but not inhibited MMP activity.

DISCUSSION

Cancer is a major disease at a worldwide level accounting for more than 7 million deaths per year. Progress made in cancer therapy has not been sufficient to significantly lower annual death rates from most epithelial tumor types resulting in an urgent need for new strategies in cancer control.

Living organisms, including plants, microbes, and marines organisms, provide rich sources of chemically diverse bioactive compounds (Schwartsmann *et al.*, 2002). The possibility that intake of natural substances might reduce risk of cancer has attracted attention as eventual chemopreventive or chemotherapeutic agent.

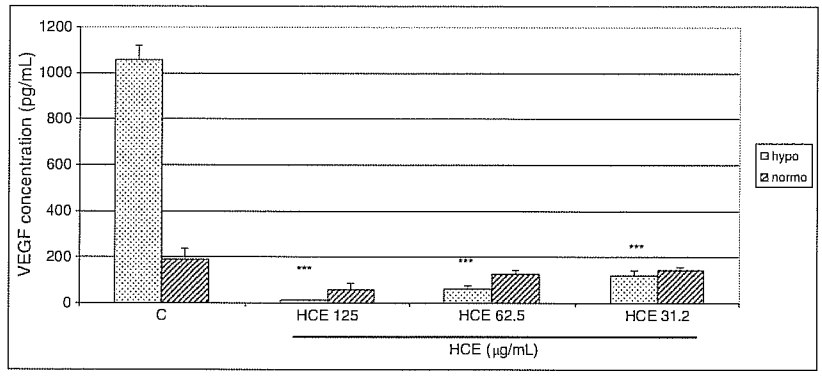

Figure 2. Horse chestnut extract (HCE) decreased vascular endothelial growth factor (VEGF) protein in cultured cells treated with cobalt chloride ($CoCl_2$). Cells were exposed to HCE for 24 h in the absence or presence of 125 µM $CoCl_2$. VEGF protein concentrations in the culture media were determined by enzyme-linked immunosorbent assay. Data shown are representative of three independent experiments. *** $p < 0.001$.

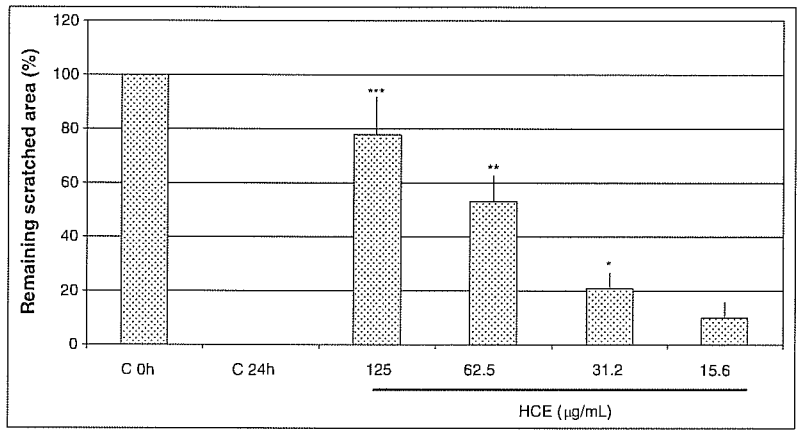

Figure 3. Inhibitory effect of horse chestnut extract (HCE) on human umbilical vein endothelial cells migration in wound migration assays. Percentage of remaining scratched area was calculated after being marked and quantified by the histogram function of the Adobe Photoshop 5.5 program. C 0h and C 24h–controls after 0 and 24 h of incubation. Experiments were performed in triplicate. *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$ versus C24h.

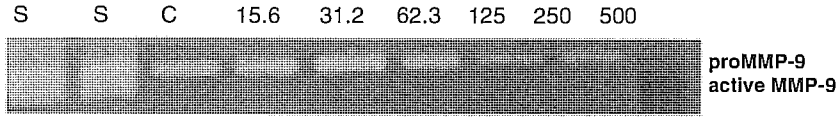

Figure 4. Matrix metalloproteinase (MMP)-9 protein secreted from human umbilical vein endothelial cells. Results show that horse chestnut extract downregulates secretion of MMP-9 in a dose-dependent manner. Cells were incubated in the absence (control; lane 3) or presence (lanes 4–9) of different doses of horse chestnut extract (µg/mL), and the level of MMP-9 protein secreted into the medium was measured by gelatin zymography. This is a representative gel picture of one of three separate experiments with similar results. S–MMP-9 standard.

In the present study, we have investigated the inhibitory effect of HCE on cancer as well as endothelial cell functions. To our knowledge, this is the first report about the pro-apoptotic and antiproliferative effects of HCE in human cancer cells.

Cell viability and colony formation assays demonstrated that HCE had potent antiproliferative effects in cancer cells that were in a dose-dependent and time-dependent manner. In an attempt to determine the mechanism responsible for the antiproliferative effects of the compound tested, we assessed apoptosis DNA fragments assay and DNA content analysis. We have shown that the decrease in cell viability by HCE was associated with increase in the fraction of cells with sub-G_0/G_1 DNA content which is considered a marker of apoptotic cell death. In addition, DNA fragmentation was also clearly present as determined by agarose gel electrophoresis. Although the precise mechanism of HCE pro-apoptotic effect has not been elucidated yet, we suggest that β-aescin, a principal active constituent in horse chestnut may be responsible for apoptosis-inducing activity of HCE. Ability of β-aescin to induce apoptosis was documented by Niu and co-workers (2008a, b). The mechanisms by which β-aescin inhibits cancer cell growth remain poorly understood. It can exert its growth-inhibitory effects through inhibition of signal transducer and activator of transcription 3. It was found that suppression was mediated through the inhibition of activation of upstream

kinases c-Src, Janus-activated kinase 1, and Janus-activated kinase 2. Moreover, β-aescin also downregulated the expression of signal transducer and activator of transcription 3-regulated gene products, such as cyclin D1, Bcl-2, Bcl-xL, survivin, and Mcl-1 (Tan *et al.*, 2010). Furthermore, expression of cellular inhibitor of apoptosis protein-2, cyclin D1, and cyclooxygenase-2 in cancer cells treated with β-aescin was also downregulated (Harikumar *et al.*, 2010).

It is well known that the growth and spread of some cancers absolutely depends on the development of a tumor-associated vasculature by a process known as angiogenesis. Thus, there is considerable interest in finding ways to inhibit angiogenesis as an effective means for preventing tumor progression and metastasis (Rosen, 2001; Folkman, 2006). It has been documented that many natural compounds inhibit angiogenesis (Mojzis *et al.*, 2008a; Keshavarz *et al.*, 2010; Jeong *et al.*, 2011).

Few years ago, Baronikova and co-workers (2004) found no effect of HCE in their *ex vivo* model of angiogenesis. Here, in a series of complementary assay systems, we investigated the effects of HCE on endothelial cell viability, migration, MMP activity as well as on VEGF secretion in *in vitro* conditions. Contrary to aforementioned study, we found that HCE significantly suppressed an important basic step in angiogenesis.

In our study, HCE treatment showed an inhibition in the migration of HUVECs. Although this effect might be due to reduced proliferation of endothelial cells, we found that migration of endothelial cell was inhibited also in doses of HCE which did not interfere with cell viability. Like in cancer cells, this effect may also be mediated by β-aescin. Wang and co-workers (2008) described the ability of β-aescin to block migration of endothelial cells. Simultaneously, they found that β-aescin increased thrombospondin-1 (TSP-1) expression in HUVECs. Thrombospondin-1 is a potent angiogenesis inhibitor, and several lines of evidence indicate that the antiangiogenic activity of TSP-1 is mediated through the inhibition of endothelial cell migration (Ren *et al.*, 2006).

Furthermore, we found that HCE suppressed the secretion of MMP-9. Endothelial cell invasion is an essential event during angiogenesis, and endothelial cells have to degrade the extracellular matrix to permit the cell movement. Matrix metalloproteinases, particularly MMP-2 and MMP-9, play a crucial role in tumor invasion and angiogenesis. They are enzymes that break down extracellular matrix proteins to allow further differentiation and spread of endothelial cells during angiogenesis. Several reports indicate that MMPs play important role in the initiation of angiogenesis (Folkman, 2006). Moreover, it is suggested that most cancer-associated deaths are due to invasion of tumors into vital organs, which in turn is due to the expression of MMP-9 (Stahtea *et al.*, 2008). How HCE inhibit secretion of MMP-9 is not known. However, as documented by Harikumar *et al.* (2010) β-aescin, a principal component of HCE, blocked expression of MMP-9 in *in vitro* conditions. Besides this, β-aescin may also influence MMP activity via TSP-1 up-regulation. Overexpression of TSP-1 decreases active MMP-9 levels in mammary tumors (Ren *et al.*, 2006). Moreover, suppression of MMP-9 activity is not only associated with inability of endothelial cells to degrade the extracellular matrix. Ten years ago, it has been demonstrated that MMP-9 mediates release of VEGF from extracellular stores (Bergers *et al.*, 2000). Vascular endothelial growth factor is a potent angiogenic factor and is a prime regulator of endothelial cell proliferation. Through its own receptors (primarily VEGFR2), it also regulates multiple facets of tumor angiogenesis (Ferrara *et al.*, 2003). Activation of oncogenes as well as inactivation of tumor suppressor genes during malignant transformation is often associated with overexpression of VEGF. Additionally, its production is enhanced by hypoxia, which often occurs in tumors (Rosmorduc and Housset, 2010). Here, we have demonstrated that HCE significantly suppressed hypoxia-induced secretion of VEGF in HeLa cells. We suggested that this effect may be associated with pleiotropic pharmacological effects of aescin. Besides modulation of VEGF release via MMP-9 inhibition, it was found that β-aescin downregulated VEGF expression because of the inhibition of signal transducer and activator of transcription 3 and NF-κB signaling pathway or by increasing expression of TSP-1 (Wang *et al.*, 2008; Harikumar *et al.*, 2010; Tan *et al.*, 2010)

In the present study, HCE exhibited antiproliferative and antiangiogenic effects. Which active compound(s) in HCE is responsible for its antiproliferative/antiangiogenic effects cannot be deduced from this study. We suggest that effects of HCE presented in this study are largely due to its β-aescin content. However, it seems that the horse chestnut plant extract is more potent than pure β-aescin.

Like in all plant-derived extracts, there are many different components with various pharmacological mechanisms in the HCE. Horse chestnut is known to contain other constituents such as flavonoids and coumarins (Sirtori, 2001; Kapusta *et al.*, 2007). We and others have shown that the flavonoids may inhibit proliferation of cancer cells as well as inhibit crucial steps in angiogenesis (Mojzis *et al.*, 2008a; Mojzis *et al.*, 2008b; Oh *et al.*, 2010; Pilátová *et al.*, 2010). Moreover, some coumarins were also found to inhibit above-mentioned processes (Huang *et al.*, 1994; Lee *et al.*, 2006). We suggest that there is a potential and likelihood for interaction between these compounds, which could act synergistically to enhance the bioactivity of HCE.

From these experiments, we conclude that HCE significantly reduces cancer cell growth in a dose-dependent manner. Furthermore, HCE is also a potent angiogenesis inhibitor. Taken together, antiangiogenic and apoptosis-inducing potential of HCE generate a rationale for *in vivo* efficacy studies with this seed extract in preclinical cancer models.

Acknowledgements

This work was supported by the Slovak Research and Development Agency under the contract no. APVV-0325-07. This work was also partially supported by the Agency of the Slovak Ministry of Education for the Structural Funds of the EU, under projects ITMS: 26220120058 (20%) and ITMS: 26220120039 (20%).

Conflict of Interest

The authors have declared that there is no conflict of interest.

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