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Antiproliferative action of water extracts of seeds or pulp of five different raspberry cultivars

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Abstract

In many recent publications the importance of various constituents of berries, anthocyanins and polyphenols, is shown *in vitro* and *in vivo* to be beneficial in protecting cells from different forms of cancer. As red raspberries are very rich in the in content of ellagic acid, the goal of this work was to study the cancer suppressive action of water raspberry extracts obtained by extracting water soluble constituents from pulp and from seeds, of five different raspberry cultivars: K81-6, Latham, Meeker, Tulameen and Willamette. The further aim was to compare their antiproliferative action to malignant human colon carcinoma LS174 cells and to normal immune competent cells, with the action of ellagic acid alone. Results from this study show that water extracts of raspberries seeds or pulp possess the potential for antiproliferative action against human colon carcinoma cells *in vitro*. The antiproliferative action of seeds extract was correlated with its content of ellagic acid. The cytotoxic activity of seeds extracts was not pronounced on normal human PBMC.

Keywords: Raspberry; Water extract; Colon carcinoma

1. Introduction

Recent publications dealing with cancer prevention frequently point to the importance of fruits and vegetables for diverse health benefits (Feldman, 2002; Liu, 2003). Among them, constituents of berries, anthocyanins and polyphenols such as ellagic acid, are shown *in vitro* and *in vivo* to be beneficial in protecting cells from various health injuries, such as ageing and different forms of cancer (Duthie, Gardner, & Kyle, 2003; Meyers, Watkins, Pritts, & Liu, 2003; Mullen et al., 2002; Whitley, Stoner, Darby, & Walle, 2003). Apart from that, the cancer preventive and cancer suppressive action of ellagic acid *in vivo* and *in vitro* has been reported in many papers too (Hirose, Nishikawa, Shibutani, Imai, & Shirai, 2002; Juranic, Zizak, Stanojkovic, & Radulovic, 2001; Khanduja, Gandhi, Pathania, & Syal, 1999; Thiem & Berge, 2003). As raspberries (Rubus idaeus L.) are very rich in ellagic acid (Mullen et al., 2002; Wada & Ou, 2002; Zafrilla, Ferreres, & Tomas-Barberan, 2001), the aim of this work was to study the cancer suppressive action of water raspberry extracts, obtained extracting water soluble constituents from pulp and from seeds, of five different raspberry cultivars and to assess the selectivity in the antitumor action. Their antiproliferative action to malignant and to normal immune competent cells was compared with the action of ellagic acid alone.

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2. Materials and methods

2.1. Plant extracts

Five different raspberry cultivars: K81-6, Latham, Meeker, Tulameen and Willamette were analyzed in this work. Weight percentages of seeds were determined after extraction, washing and air drying (Table 1). It was calculated that, if a person consumed 300 g of raspberries then the corresponding concentration of raspberry extract would be: water extract from 300 g of raspberries per 3 litres of blood plasma (i.e. it corresponded to extract from 100 mg of raspberries per ml plasma). To get the corresponding concentration of raspberry extract in our experiments, water extracts of pulp, or extracts of grinded seeds (corresponding to 150 mg of raspberries) were obtained by mixing $5 \times P$ mg of pulp, (P is quantity of pulp in 150 mg of raspberries), or $5 \times (150 - P)$ mg of seeds with 5 ml of complete nutrient medium for 24 h at 37 °C. Stock solutions were obtained after filtration of the extracts through Millipore filter pore size 0.22 μm before use.

2.2. Analisis of ellagic acid content

Identification, and determination of ellagic acid contents in these extracts were done by HPLC. A HP-1090 Liquid chromatograph with Diode-Array detector, and RP-18 column (LiChrospher® 250, 4 mm, 5 μm) were used. The flow rate of mobile phase using a binary mixture of rate of mobile phase was 1 ml/min, A: ACN; B: H20, and an elution gradient (0–5 min 5% A, 5–20 min 100% A). Aquisition was carried out at 255 nm. An external calibration method, using ellagic acid (Sigma Chemicals) as standard was used. Correlation was 0.999. Before injection, all samples were filtered using cellulose 0.45 μm filters.

Stock solution of ellagic acid was made at concentration of 10 mM in dimethylsulfoxide (DMSO), and this solution was diluted with nutrient medium and applied to target cells to various final concentrations ranging from 0 to 100 μM. All reagents were products of Sigma Chemicals.

2.3. Cell culture

Human colon carcinoma LS174 cells, were cultured as monolayers in the nutrient medium. The cells were grown at 37 °C in 5% CO_2 and humidified air atmosphere.

Table 1
The percentage of seeds and of pulp in different raspberry cultivars

Raspberry cultivars	Seed content (%)	Pulp content (%)
K81-6	5.14	94.9
Latham	3.28	96.7
Meeker	4.55	95.5
Tulameen	4.24	95.8
Willamette	4.49	95.5

2.4. Preparation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were separated from whole heparinized blood of two healthy volunteers by Lymphoprep™ (Oslo, Norway) gradient centrifugation. Interface cells, washed three times with Haemaccel (aqueous solution supplemented with 145 mM Na^+, 5.1 mM K^+, 6.2 mM Ca^{2+}, 145 mM Cl^- and 35 g/l gelatin polymers, pH = 7.4) were counted and resuspended in nutrient medium.

2.5. Treatment of LS 174 cells

Neoplastic LS174 cells were seeded (7000 cells per well) into 96-well microtiter plates, and 20 h later, after the cell adherence, five different, double diluted, concentrations of investigated extracts were added to the wells. Maximal final concentration of pulp or seed extracts applied to target cells was 67% i.e., stock extracts diluted to two third (corresponded to mentioned ratio of 100 mg of raspberries/ml plasma). Nutrient medium was RPMI 1640 medium, supplemented with l-glutamine (3 mM), streptomycin (100 μg/ml), and penicillin (100 IU/ml), 10% heat inactivated (56 °C) fetal bovine serum (FBS) and 25 mM Hepes, and was adjusted to pH 7.2 by bicarbonate solution. Only nutrient medium was added to the cells in the control wells.

2.6. Treatment of PBMC

PBMC were seeded (150,000 cells per well) into nutrient medium or in nutrient medium enriched with (5 μg/ml) phytohaemaglutinin (PHA) (Welcome) in 96-well microtiter plates. Two hours later, investigated extracts were added to the wells, in triplicate, to five final concentrations, except to the control wells where a nutrient medium only was added to the cells. Nutrient medium with corresponding concentrations of compounds, but void of cells was used as the blank.

2.7. Determination of cell survival

Cell survival was determined indirectly by measuring total cellular protein by the Kenacid Blue R (KBR) dye binding method (Clothier, 1995). Briefly, after 72 h of continuous extracts, or ellagic acid action, medium was discarded and target cells were washed twice with warm (37 °C) phosphate buffered saline (PBS). PBMC were always centrifuged 10 min at 2000 rpm and supernatant was aspirated leaving a small amount of medium in the aim to not disturb cells in the pellet. Then target cells were fixed for 20 min with 150 μl of a mixture of methanol and acetic acid (3:1) and stained 2–3 h with 0.04%

Coomassie Brilliant Blue R-250 in 25% ethanol and 12% glacial acetic acid, washed, and bound dye was dissolved in desorbing solution (1M Potassium acetate, 70% ethanol). Absorbance (A) at 570 nm was measured 2 h later. To get cell survival (%), A of a sample with cells grown in the presence of various concentrations of the investigated agent was divided with control optical density (the A of control cells grown only in nutrient medium), and multiplied by 100. It was implied that A of the blank was always subtracted from A of the corresponding sample with target cells. IC_{50} concentration was defined as the concentration of an agent inhibiting cell survival by 50%, compared with a vehicle-treated control.

3. Results and discussion

3.1. Ellagic acid content in seed and pulp extracts

The percentage of seeds and of pulp in different raspberry cultivars are given in Table 1. It is seen that the percentage of seeds in various sorts of raspberries increases from 3.28% found in Latham, to 5.14% found in K81-6. The concentrations of ellagic acid in mg%, or μM of different seeds or pulp raspberries extracts, are shown in Table 2.

3.2. Cytotoxic effects of extracts and ellagic acid on LS174 cells

Microphotographs of human colon carcinoma LS174 cells obtained 72 h after continuous pulp or seed extracts action (presented in Fig. 1), roughly show that both types of extracts induce the decrease in the number of survived cells in relation to controls, i.e. the cells grown in the presence of nutrient medium only. Decrease in L174 cell survival induced by pulp or seed extracts is shown in Fig. 2 and Fig. 3, respectively. The applied concentration of pulp extracts, led to a pronounced decrease in number of surviving cells than corresponding concentrations (to its content in raspberries) of seed extracts. There were no changes in the pH of seed extracts, but some degree of enhanced acidity was pronounced in the pulp extracts. This can be seen in Table 3.

Table 2
The concentrations of ellagic acid in extracts of seeds or pulps, obtained from different raspberry cultivars

Raspberry cultivars	Seed		Pulp	
	mg%	μM	mg%	μM
K81-6	0.330	10.0	0.062	1.8
Latham	0.145	4.0	0.119	3.5
Meeker	0.181	5.0	0.114	3.4
Tulameen	0.303	9.0	0.136	4.0
Willamette	0.482	14.0	0.093	2.7

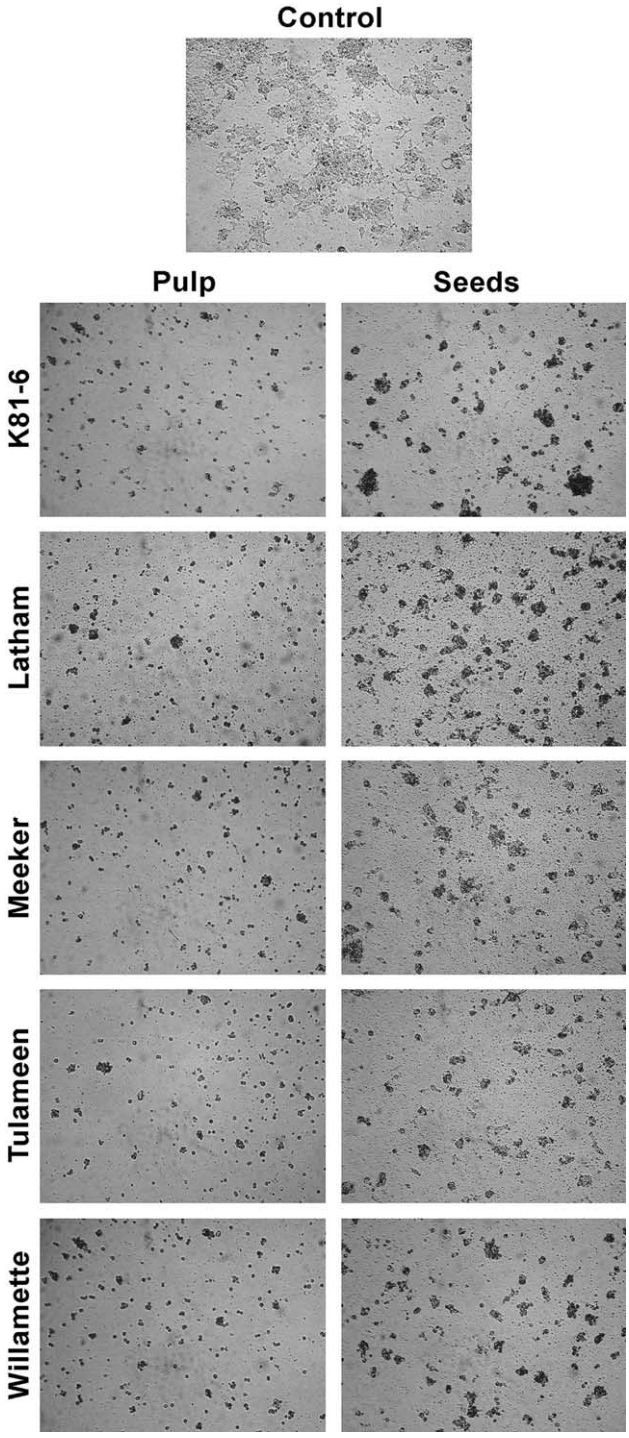

Fig. 1. Microphotographs of human colon carcinoma LS174 cells obtained 72 h after continuous pulp or seed extract action.

The decrease in the survival of LS174 cells determined after 72 h of continuous action of pure ellagic acid is shown in Fig. 4. Concentrations (%) of seed or pulp extracts which induced a 50% decrease in LS174 cell survival, as well as concentrations of ellagic acid (μM) in these extracts, or concentration of pure ellagic acid compound, which led to 50% decrease in malignant

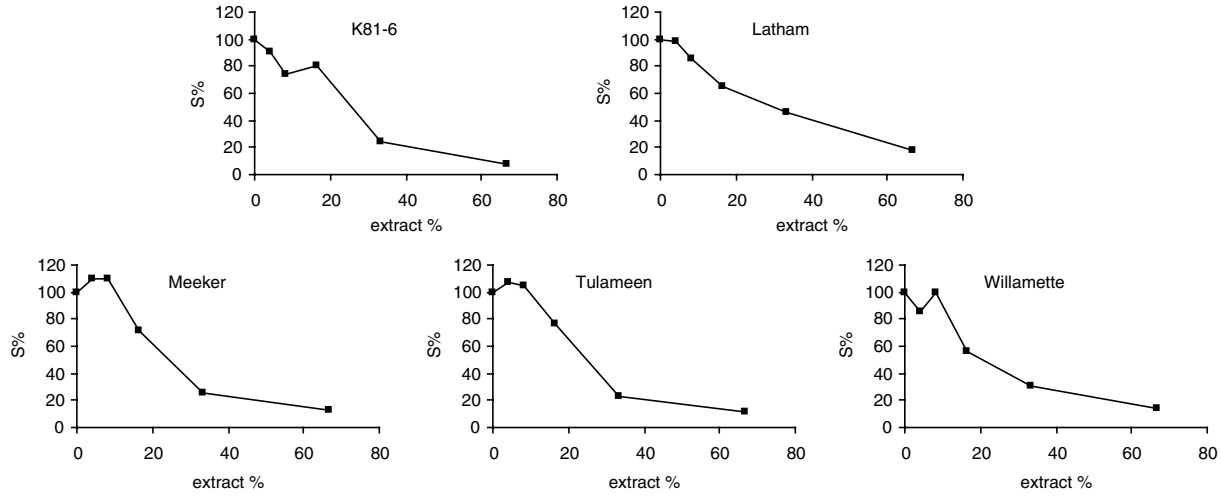

Fig. 2. Survival of L174 cells determined by KBR test, 72 h after continuous action of water extracts of raspberry pulp. It is given as a function of different concentrations of extracts of five different raspberry cultivars.

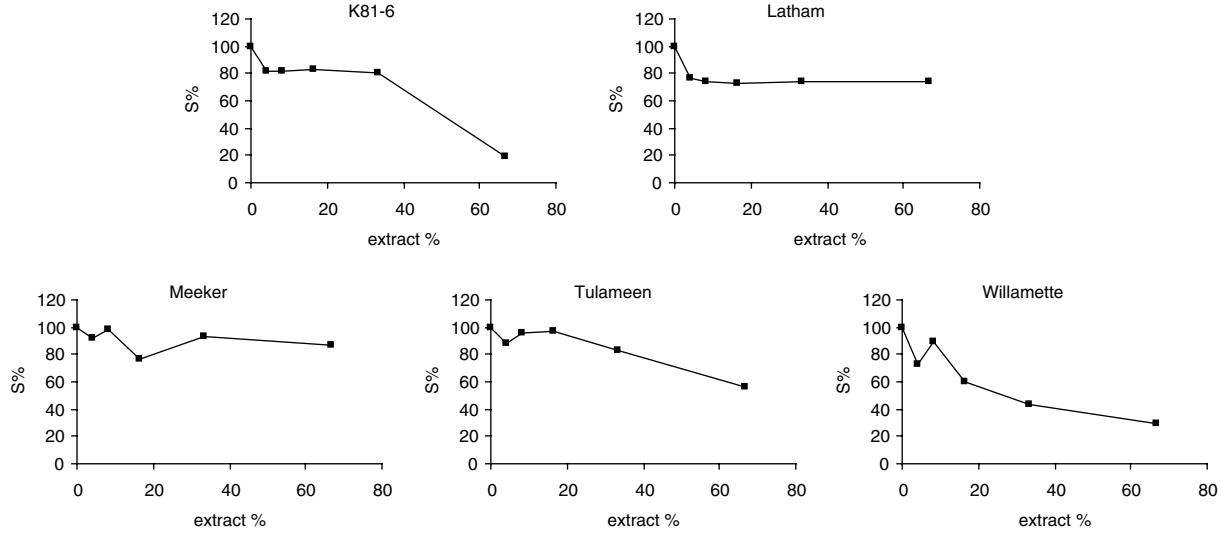

Fig. 3. Survival of L174 cells determined by KBR test, 72 h after continuous action of water extracts of raspberry seeds. It is given as a function of different concentrations of extracts of five different raspberry cultivars.

Table 3
The pH of 66.7% pulp or seeds extracts of various red raspberries cultivars

Raspberry cultivars	Extract pH	
	Seed	Pulp
K81-6	7.2	5.5
Latham	7.1	6.3
Meeker	7.2	5.6
Tulameen	7.1	5.4
Willamette	7.1	5.6

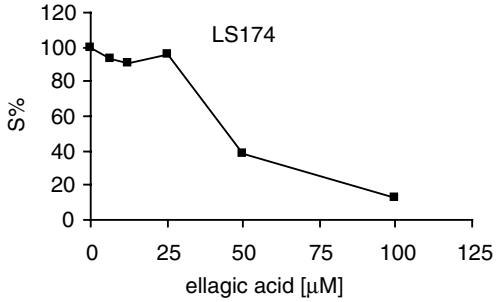

Fig. 4. Survival of L174 cells determined by KBR test is given as a function of different concentrations of ellagic acid, at the end of 72 h of its continuous action.

LS174 cells survival given in Table 4, showed that the antiproliferative activity of pulp extracts was more pronounced than the activity of the corresponding seeds extracts. The antiproliferative activity of pulp extracts of different raspberry cultivars towards LS174 cells decrease in the order: Willamette > Latham > Meeker > K81-6 > Tulameen. The cytotoxic activity of seed extracts was the highest for Willamette and decreased for

Table 4
Concentrations of seed or pulp extracts, and corresponding quantity of raspberry fruits of which extract induced 50% decrease in LS174 cell survival, as well as the corresponding concentrations of ellagic acid in these extracts

Raspberry cultivars	IC_{50} (extract%)	Raspberries fruits (g)	Ellagic acid (μM) in IC_{50} extract
K81-6			
Pulp	32.2	144	0.58
Seed	49.9	223	4.99
Latham			
Pulp	29.9	134	1.04
Seed	>67	>300	>2.67
Meeker			
Pulp	31.0	139	1.05
Seed	>67	>300	>3.33
Tulameen			
Pulp	33.3	149	1.33
Seed	>67	>300	>6.00
Willamette			
Pulp	29.7	13.3	0.80
Seed	27.1	121	3.80
Ellagic acid			58.67[a]

[a] Concentration of pure ellagic acid (μM) which induced 50% decrease in cell survival.

K81-6, and was not as pronounced for other raspberry cultivars.

3.3. Cytotoxic effects of extracts and ellagic acid on PBMC

The absence of a dose dependent decrease in PBMC survival is found when concentrations up to 67% of water seeds or pulp raspberry extracts or 50 μM ellagic acid alone were applied on stimulated PBMC (Table 5 and in Fig. 5). It is interesting to note that pulp extracts of Meeker, and of K81-6 raspberries showed very strong cytotoxic activity to non-stimulated PBMC isolated from only one person; IC_{50} of extracts were 7.32% and 13.2%, respectively. The importance of nutrition in cancer prevention has become well recognized in the recent times, and in order to decrease the incidence of aging health diseases, or cancer, the World Health Organization (WHO) recommends a daily intake of at least 400 g of vegetables and fruit (Hoffmann, Boeing, Volatier, & Becker, 2003). On the basis of epidemiological data (Block, Patterson, & Subar, 1992) it was estimated that the overall occurrence of cancer, could be reduced at least 20–30% by healthy nutrition (Steinmetz & Potter, 1996). In relation to this, an increasing number of publications have studied the cancer preventive, or cancer suppressive action of many nutrition constituents; among them ellagic acid was frequently mentioned (Hirose et al., 2002; Juranic et al., 2001; Khanduja et al., 1999; Mertens-Talcott, Talcott, & Percival, 2003; Thiem & Berge, 2003).

Table 5
Concentrations (%) of seed or pulp raspberries extracts, of which extract induced 50% decrease in PBMC survival

	IC_{50} (extract%)			
	PBMC #1		PBMC #2	
	−PHA	+PHA	−PHA	+PHA
Pulp				
K81-6	13.11	>67	>67	>67
Latham	>67	>67	>67	>67
Meeker	7.32	>67	>67	>67
Tulameen	>67	>67	>67	>67
Willamette	>67	>67	>67	>67
Seed				
K81-6	>67	>67	>67	>67
Latham	>67	>67	>67	>67
Meeker	>67	>67	>67	>67
Tulameen	>67	>67	>67	>67
Willamette	>67	>67	>67	>67
Ellagic acid			>50 μM[a]	>50 μM [a]

[a] Concentration of pure ellagic acid (μM) which induced 50% decrease in PBMC survival.

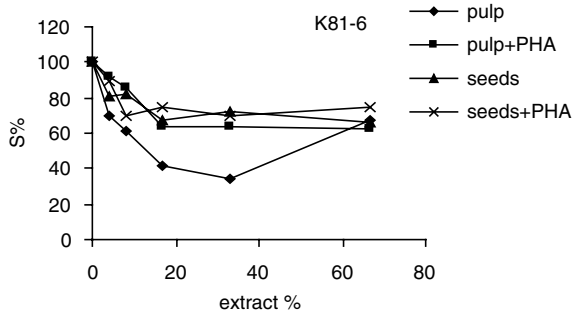

Fig. 5. Survival of PBMC (non-stimulated, or stimulated to proliferation by PHA) determined by KBR test, 72 h after continuous action of seed or pulp K81-6 raspberry water extracts. It is given as a function of different concentrations of seed or pulp berry extracts. Cultivars are given in legend. Representative experiment.

Results from this work show that ellagic acid alone exerts its cytotoxic action towards colon carcinoma LS174 cells. Apart from that, extracts of red raspberry pulp or seeds express their antiproliferative action towards investigated colon carcinoma LS174 cells, too. The action of pulp extracts was more pronounced than the action of the corresponding seed extracts, and it was not related to their contents of ellagic acid, but could, at least partially, be connected with the lowered pH of the nutrition medium. The antiproliferative action of the most strong seed extracts was correlated with their ellagic acid content, although it was higher than the action of the same concentrations of ellagic acid alone. This is in accordance with reports that combinations of ellagic acid with some other berry constituents

in berry extracts (Bagchi, Sen, Bagchi, & Atalay, 2004; Huang et al., 2002; Liu et al., 2002; Meyers et al., 2003; Seeram, Adams, Hardy, & Heber, 2004), could lead to the greater cytotoxicity of investigated extracts than ellagic acid alone. Results from this work support data on possible synergistic or additive antiproliferative, anti-angiogenic, antioxidant, and anti-carcinogenic potential, of the anthocyanins, proanthocyanidins, and flavonol glycosides within berry extracts (Bagchi et al., 2004; Huang et al., 2002; Liu et al., 2002; Seeram et al., 2004). Regarding the activity of seed extracts, data from this work indicate that daily consumption of a minimum of 121 g of raspberries of Willamette, or 233 g of K81-6 cultivars, could provide enough antiproliferative potential to suppress the growth of neoplastic colon carcinoma cells.

Although the cytotoxic action of pulp extracts of two different cultivars was pronounced on only one person's PBMC, this may be due to higher acidity, i.e. lower pH of the nutrition medium (with 10% FBS), which is rather difficult to be achieved in 99% human sera.

The biochemical basis of the antiproliferative action of extracts was not studied in this work because the mechanism of ellagic acid or of anthocyanins antiproliferative action is already published (Bagchi et al., 2004; Huang et al., 2002; Juranic et al., 2001; Liu et al., 2002; Mertens-Talcott et al., 2003; Meyers et al., 2003; Seeram et al., 2004).

In conclusion, results from this study show that water extracts of raspberry seeds or pulp possess the potential for antiproliferative action against human colon carcinoma cells *in vitro*. The action of pulp extracts was higher than that of seed extracts and was not directly connected with its content of ellagic acid, but could at least partially be connected with the enhanced acidity of the nutrient medium. In contrast, the antiproliferative action of seeds extracts was correlated with its content of ellagic acid. The cytotoxic activity of seeds extracts was not as pronounced on normal human PBMC.

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