ANTI-PROLIFERATIVE EFFECTS OF A BLUEBERRY EXTRACT ON A PANEL OF TUMOR CELL LINES OF DIFFERENT ORIGIN

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Background: **Blueberries are among the fruits with the highest antioxidant activity and have been recognized by their health promoting properties.** *Aim:* **In vitro study of the anti-proliferative effects of a blueberry extract on a panel of cancer cells from different origin.** *Materials and Methods:* **A blueberry extract was produced using ethanol as extracting solvent. The anti-proliferative activity of the extract was evaluated against seven tumor cell lines. The properties of blueberry extract to decrease cell adhesion and migration were also investigated.** *Results:* **Blueberry extract showed a dose-dependent inhibitory effect on cell proliferation for all cell lines. Non-cytotoxic concentrations of the extract decreased cell adhesion in five of seven cell lines studied and inhibited the migration of MDA-MB-231 and PC-3 tumor cells.** *Conclusion:* **This work provides additional evidence regarding the ability of blueberry extract to inhibit the growth and decrease cell adhesion and migration of different cancer cell lines** *in vitro*.
Key Words: **anthocyanins, anti-proliferative, blueberry extract, cancer, polyphenols.**

Several studies have demonstrated the correlation between consumption of fresh fruits and vegetables with the prevention, delay or onset of chronic degenerative diseases including cancer. Fresh fruits and vegetables are rich sources of a large number of diverse nutrients, such as vitamins/antioxidants, trace minerals/micronutrients, phytosterols, novel enzymes, dietary fiber, and potent chemoprotectants [1].

Blueberry belongs to the *Ericaceous Vaccinium* group of deciduous shrub plants. American blueberries include the lowbush or wild blueberry (*Vaccinium angustifolium*, Aiton) and the highbush or cultivated blueberry (*Vaccinium corymbosum* L.), both of which have superior ranking among fruits and vegetables for their antioxidant capacity [2]. *In vitro* and *in vivo* studies have shown the chemopreventive effects of blueberries and their bioactive components on different types of cancer [3]. However, there is a growing interest in studying the potential of blueberry as an antitumor agent, specifically the cellular mechanisms related to the growth and dissemination of tumor cells.

The health promoting properties of blueberries have been attributed to the high antioxidant capacity of their polyphenolics compound. Polyphenols are capable of scavenging oxygenated free radicals, inhibiting or activating enzymes, or functioning as metal chelators, thus preventing damage to membrane lipids, proteins, and nucleic acids [4]. In addition, polyphenols have been reported to decrease leukocyte immobilization, induce apoptosis, inhibit cell proliferation and angiogenesis, modulate cell signaling and gene expression [5].

Uncontrolled proliferation is an important step in the initiation and progression of cancer [6]. On the other hand, tumor metastasis is a multistep process involving cell adhesion, degradation of the extracellular matrix, and cell migration [7]. Many *in vitro* studies have confirmed the anti-proliferative and proapoptotic effects of polyphenol- and anthocyanin-rich fractions purified from blueberry extract in cancer cell lines [8–13]. However, few studies have evaluated the properties of whole blueberry extract in key steps involved in cancer progression, such as cell adhesion and migration.

We have previously investigated the *in vitro* antitumor activity of two different vegetable extracts, specifically their effects on proliferation, adhesion and migration of melanoma and breast cancer cells [14, 15]. The aim of this work was to evaluate the *in vitro* anti-proliferative effects of a blueberry extract and its capacity to reduce cell adhesion and migration on a panel of tumor cells from different origin. The selected cell lines represent six human cancers: breast, colon, kidney, lung, cervix and prostate. Additionally, the murine colon carcinoma cell line CT26 was included in this study because it is commonly used in syngeneic mouse models of colon cancer. To the best of our knowledge, this is the first evaluation of the effects of whole blueberry extract on proliferation, adhesion and migration of human lung and kidney cancer cells.

MATERIAL AND METHODS

Reagents. 1,1-diphenyl-2-picrylhydrazyl (DPPH) and Folin Ciocalteu's phenol reagent were purchased from Sigma-Aldrich (USA). Gallic acid (GA) was supplied by Biopack (Argentina). Media and cell culture reagents were obtained from Gibco (USA).

Preparation of blueberry extract. Highbush blueberries (*Vaccinium corymbosum* L.) were supplied by Biomac SRL (Buenos Aires, Argentina). A 50/50 mixture of Misty and O'neal varieties of frozen blueberries (samples of 1 kg) were first homogenized in 0.5 l of 96% food grade ethanol (pH 4.2) using a domestic blender. Anthocyanins and other phenolic compounds in the homogenates were then extracted

at room temperature (RT, 22 °C) during 72 h, protected from light. The extract was filtered through a linen cloth squeeze bag and centrifuged at 2000 g for 10 min. The ethanol was evaporated at 35 °C using a laboratory rotary evaporator (Heidolph VV 2000, Germany). The aqueous extract thus obtained was sterile filtered using 0.22 µm membrane and stored at −20 °C for further characterization and cell-based experiments.

Determination of total phenolic content. Total phenolics were measured by colorimetric Folin-Ciocalteu method, as described [16]. Briefly, 50 µl of extract sample or GA solutions (used as standards) were mixed with 250 µl of Folin-Ciocalteu reagent, followed by the addition of 750 µl of 20% sodium carbonate solution. The volume was then made up to 5.0 ml with distilled water. After 2 h of incubation at RT, protected from light, the absorbance (Abs) was read at 760 nm with a spectrophotometer (Shimadzu, Japan). Total phenolic content was calculated from the standard curve and expressed as milligrams of GA equivalents per liter of aqueous extract (mg/l GAE). Data were reported as mean ± standard deviation (SD) of three independent assays.

Quantification of total anthocyanins. Total monomeric anthocyanins were determined by the differential pH method based on the property of anthocyanin pigments to change the color with pH [17]. Two dilutions of the same sample were prepared, the first one in potassium chloride buffer (0.025 M, pH 1.0) and the second one in sodium acetate buffer (0.4 M, pH 4.5). After equilibration at RT for 20 min, the Abs of two dilutions was read at 520 nm and 700 nm *versus* a blank cell filled with distilled water. Total monomeric anthocyanins were calculated as follows:

mg cyanidin 3-glucoside equivalent/ml of aqueous extract = (A · MW · DF · 10^3)/(ε · 1),

where A = (Abs 520 nm — Abs 700 nm) pH 1.0 — (Abs 520 nm — Abs 700 nm) pH 4.5; MW (molecular weight) = 449.2 g/mol for cyanidin-3-glucoside; DF = dilution factor; 1 = path length in cm; ε = 26 900 molar extinction coefficient, in L · mol^{-1} · cm^{-1}, for cyanidin-3-glucoside; and 10^3 = factor for conversion from g to mg.

Antioxidant activity analysis of blueberry extract. The antioxidant activity was determined using DPPH as a free radical based on the method described by Brand-Williams et al. with some modifications [18]. Briefly, 100 µl of extract sample was mixed with 3.9 mL of a 25 mg/l DPPH solution dissolved in absolute ethanol. For the control, 100 µl of ethanol was used instead of the extract solution. After incubation at RT in the dark for 30 min, the absorbance of samples and control was measured at 517 nm with a spectrophotometer (Shimadzu, Japan). The DPPH radical scavenging activity was calculated as follows:

DPPH scavenging activity (%) = 1 − (sample absorbance/control absorbance) · 100.

Cell culture. CT26 (American Type Culture Collection (ATCC): CRL-2638), HCT-116 (ATCC: CCL-247), MDA-MB-231 (ATCC: HTB-26), PC-3 (ATCC: CRL-1435), NCI-H125 (ATCC: CRL-5801) and HeLa (ATCC: CCL-2) cells were obtained from the ATCC. SN12C were kindly provided by Dr. Andrea Loaiza Perez (Institute of Oncology Angel H. Roffo, Buenos Aires, Argentina). CT26, PC-3, NCI-H125 and SN12C cells were cultured in RPMI 1640. MDA-MB-231 and HeLa cells were cultured in DMEM while HCT-116 was growth in MEM F12 50/50 medium. All media were supplemented with 10% fetal bovine serum and 50 µg/ml gentamicin (complete medium). Cells were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

Cell proliferation and cytotoxicity assays. Cells were seeded in complete medium at a density of 3 · 10^3 cells/well in 96-well plates for anti-proliferative activity analysis and 6 · 10^3 cells/well for cytotoxicity assay. After incubation at 37 °C for 24 h, cells were treated with blueberry extract at various concentrations ranging from 1.56 to 100 mg/l GAE or PBS (Control) in complete medium during 24 and 72 h for cytotoxicity and cell proliferation assay, respectively. After treatment, the medium was removed and attached cells were stained with a crystal violet solution 0.5% in methanol 20%. Cell viability was scored by dissolving the dye attached to cells in methanol/acetic acid (10%/5%) and reading the plates at 595 nm in a microtiter plate reader (Asyshitech GmbH, Austria). Cell growth in complete medium with PBS instead of blueberry extract was considered as 100%. All plates had control wells containing medium without cells to obtain a value for background, which was subtracted from the test sample readings. Data are expressed as mean ± SD. Six replicates per sample were used and each experiment was repeated three times independently. The concentration resulting in 50% of growth inhibition (IC_{50}) for each cell line was calculated from the growth curves for 72 h using GraphPad Prism version 6.00 (GraphPad Software, San Diego, California, USA).

Cell adhesion assay. Exponentially growing cells were detached with trypsin/EDTA solution and seeded in 96-well plates at a density of 4 · 10^4 cells/well in complete medium containing increasing concentration of blueberry extract or PBS (control). After incubation at 37 °C for 2 h, the medium was removed and attached cells were stained as described above. Cell adhesion in complete medium with PBS instead of blueberry extract was considered as 100%. Data are expressed as mean ± SD. Six replicates per sample were used and each experiment was repeated three times independently.

Scratch assay. Cells (2 · 10^5 / well) were seeded in 12-well plates and cultured for 48 h in complete medium to form a confluent monolayer. Then, the monolayer was scratched with a pipette tip, washed with serum-free medium to remove floating cells, and photographed at 40× using an inverted microscope (Leica Microsystems CMS GmbH, Germany) with an attached Leica MC120 HD camera. Serial dilutions of blueberry extract or PBS (control) were added to the cells. After incubation at 37 °C for 24 h, cells were photographed again at three randomly selected sites per well. Cell migration was evaluated by measuring the cell-free surface at the beginning of the experiment (0 h) and the surface covered

by the cells at the end of the experiment (24 h) by using ImageJ Software (National Institute of Health, USA). Results are expressed as the percentage of covered surface in each condition. Cell migration in complete medium with PBS instead of blueberry extract was considered as 100%. Data are expressed as mean ± SD. The treatments were carried out in triplicate and each experiment was repeated three times independently.

Data analysis. Data are expressed as mean ± SD. Statistical differences between various concentrations of the extract were evaluated by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test *vs* control group. Differences were considered to be significant when $p < 0.05$.

RESULTS

Characterization of the blueberry extract. The values obtained for anthocyanins, total polyphenol content and radical scavenging capacity of the blueberry extract are shown in Table 1.

Effect of the blueberry extract on cell proliferation. The effect of the blueberry extract on growth of cancer cell lines was evaluated using the crystal violet staining assay. A range of concentration between 1.56 and 100 mg/l GAE was tested. For all tumor cell lines, the treatment for 72 h with the extract showed a dose-dependent decrease of cell proliferation (Fig. 1). The concentration resulting in 50% of growth inhibition calculated from the corresponding dose-response curve for each cell line is shown in Table 2. The IC_{50} values ranged from 19 to 53 mg/l GAE. After 24 h of treatment with the extract, the highest concentration tested (100 mg/l GAE) was cytotoxic in all cell lines, except for MDA-MB-231 (Fig. 2). Therefore, non-cytotoxic concentrations were selected for further experiments in order to evaluate the properties of the blueberry extract in key steps involved in cancer progression, such as cell adhesion and migration.

Effect of the blueberry extract on cell adhesion. Adhesion of tumor cells to a tissue culture plastic surface in the presence of increasing concentrations of the blueberry extract is shown in Fig. 3. Concentrations of 25 and 50 mg/l GAE significantly decreased cell adhesion in HCT-116 ($p < 0.05$), PC-3 ($p < 0.001$), NCI-H125 ($p < 0.05$) and SN12C ($p < 0.01$), whereas in CT26 and HeLa the extract did not have a significant effect at any of the concentrations assayed. In MDA-MB-231 tumor cells, an inhibitory effect on cell adhesion was only observed at 50 mg/l GAE.

Table 1. Total polyphenol, anthocyanin content and radical scavenging capacity of the blueberry extract

Sample	Total polyphenol[†] (mg/l GAE)	Total anthocyanin[‡] (mg/L cyanidin-3-glucoside equivalents)	Radical scavenging capacity[§] (%)
Blueberry extract	2109.76 ± 21.4	1027.82 ± 7.5	78.40 ± 0.5

Note: Data represent the means ± SD of three independent experiments. [†]Total polyphenol content was quantified by Folin-Ciocalteu assay as GAE. [‡]Anthocyanins were quantified by pH differential method as cyanidin-3 glucoside equivalents. [§]Radical scavenging capacity, representing the % of scavenged radicals from the total available, was measured by DPPH assay.

Effect of the blueberry extract on cell migration. We studied the effect of the blueberry extract on the invasive potential of cancer cell lines *in vitro*. Scratch assay was used to evaluate whether the blueberry extract could inhibit cell migration. Treatment with IC_{25} and IC_{50} of the extract significantly inhibited the migration ability of MDA-MB-231 ($p < 0.05$) and PC-3 ($p < 0.001$) tumor cells, while no significant effects were observed on CT26, HCT-116, HeLa, SN12C and NCI-H125 (Fig. 4).

DISCUSSION

Compared to other fruits and vegetables, a high antioxidant capacity has been reported for blueberries [19]. This antioxidant capacity, in addition to type and cultivar, can vary as a function of the climate and soil conditions in which they are grown, degree of ripeness, storage temperature and methods used for industrial processing [20].

The antioxidant capacity of blueberries has been attributed to the high concentration of phenolic compounds, particularly anthocyanins [21]. Therefore, extraction protocols to obtain anthocyanin-rich blueberry extracts are desirable. However, the production of blueberry extracts with high content of anthocyanins and other phenolic compounds using relatively simple, low cost and easy to scale up processes while remain food safe has some limitations. Therefore, there is a growing interest to find new extraction methods to obtain berry extracts with high yields and low cost, while preserving the biological activity of phenolic compounds.

Comparison of different solvents for extraction of anthocyanins and other phenolic compounds from blueberries have been performed [22]. The most common solvents are methanol, ethanol, acetone and even water acidified with several organic and inorganic acids. From these extraction methods, acidified methanol is the most efficient [23]. However, due to the toxicity of methanol, for use in food industry ethanol is preferred.

In this work, anthocyanins and other phenolic compounds from highbush blueberries (*Vaccinium corymbosum* L.) were extracted at RT using ethanol as extracting solvent due to its low cost and easy recovery by evaporation. Since anthocyanins, along with other flavonoids, are localized in the peel and seeds of the berries [24], we introduced an additional homogenization step to achieve a more efficient extraction of these phytochemicals.

Bioactive compounds in blueberries have been shown to inhibit cancer cell proliferation *in vitro* [8–13]. Most investigations of this inhibitory effect on cell proliferation are focused on individual compounds, such as anthocyanins and phenolic acids. However, a single anthocyanin or a few anthocyanins may lose their bioactivity or not behave in the same way as the compounds in natural fruits and vegetables [13]. Also, the protective effect of the blueberries observed might be due to synergistic or additive actions of several bioactive compounds more than a particular compound of extract. For example, the treatment of a human leukemia cell line with quercetin and ellagic acid in combination enhanced apoptosis and inhibited cell proliferation compared with the single
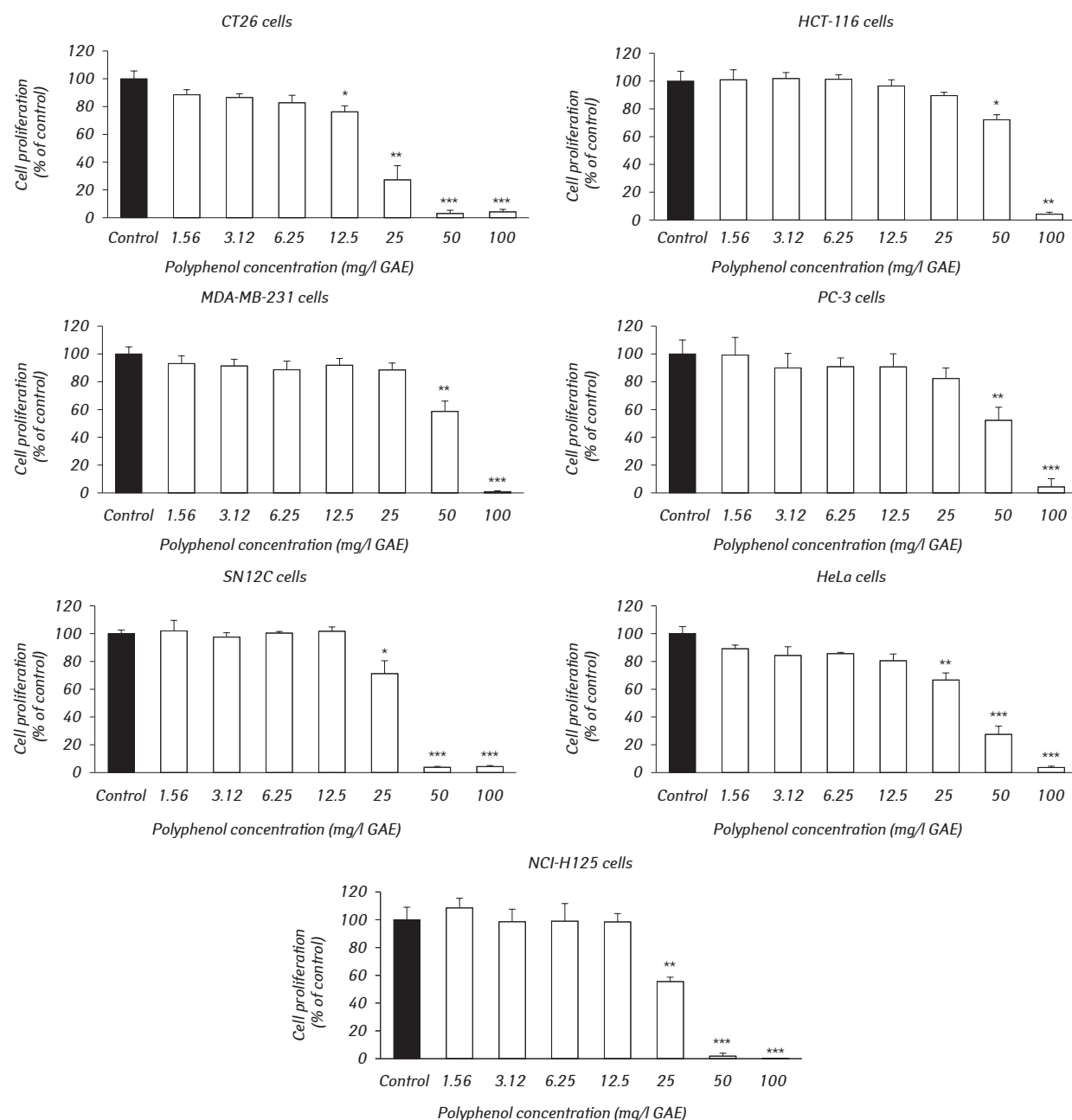
Fig. 1. Effect of the blueberry extract on cell proliferation. Cells were seeded in 96-well plates 24 h before treatment. Increasing concentrations of blueberry extract, expressed as mg/l of GAE, were added to the wells in complete medium. After 72 h, the cells were stained with crystal violet and absorbance at 595 nm was read. Proliferation percent for each concentration is expressed as mean ± SD (n=6) of three independent experiments, referred to PBS-treated cells as control (100% cell proliferation). Statistical analysis was done using One-way ANOVA and Dunnett's post test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ *vs* control

compound. These results suggest a synergistic rather than an additive effect [25]. It has been reported that both whole cranberry juice and blueberry extract had higher anti-proliferative activity than isolated anthocyanin and proanthocyanidin fractions despite the increased

Table 2. IC$_{50}$ values of blueberry extract for the panel of tumor cell lines calculated from the proliferation assays after 72 h of treatment

Cell line	Tumor type	IC$_{50}$ (mg/L GAE) ± SD
CT26	Mouse colon carcinoma	19.1 ± 1.3
HCT-116	Human colorectal carcinoma	51.2 ± 5.7
MDA-MB-231	Human breast carcinoma	53.2 ± 0.2
PC-3	Human prostate carcinoma	49.2 ± 2.3
HeLa	Human cervical adenocarcinoma	36.5 ± 0.4
SN12C	Human renal cell carcinoma	26.1 ± 0.6
NCI-H125	Human lung adenocarcinoma	25.8 ± 1.2

Note: IC$_{50}$ values are expressed as mean ± SD of three independent experiments.

polyphenolic content of the different fractions [26, 27]. Even, two-by-two and a combination of all three fractions of these extract did not improve the anti-proliferative activity. Also, these compounds are phenolic in nature and could be degraded or/and oxidized or lost during fractionation process affecting its bioactive properties. Together, these results showed that whole cranberry juice and blueberry extract were more active than isolated fractions. Therefore, it is more valuable to investigate the anti-cancer activities of blueberry phytochemicals mixtures than pure compounds.

It have been shown that cell lines of different origins respond with varying degrees of sensitivity regarding their growth toward berry extracts [9, 26, 28]. We evaluated
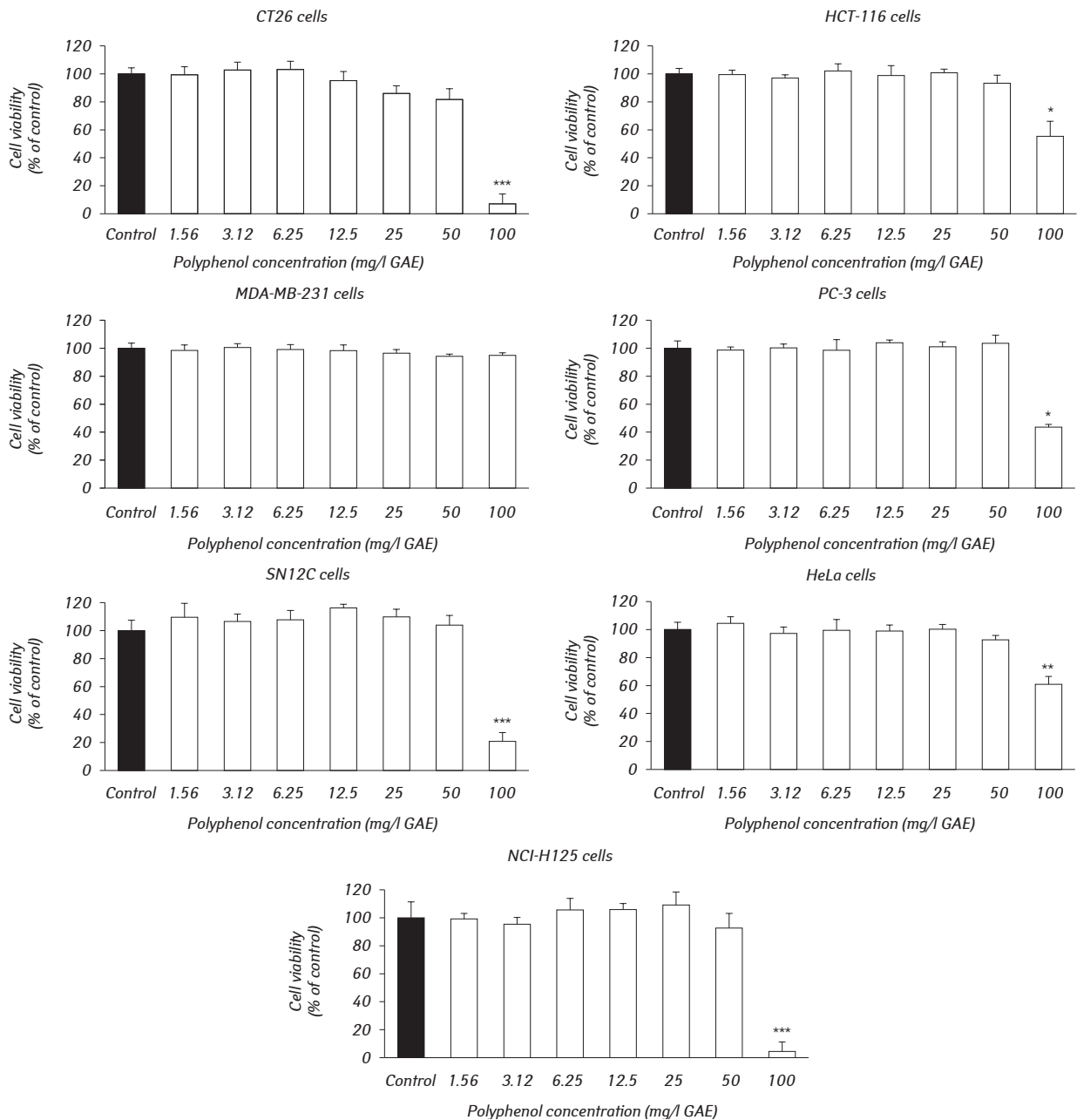
Fig. 2. Effect of the blueberry extract on cell viability. Cells were seeded in 96-well plates 24 h before treatment. Increasing concentrations of blueberry extract, expressed as mg/l of GAE, were added to the wells in complete medium. After 24 h, the cells were stained with crystal violet and absorbance at 595 nm was read. Viability percent for each concentration is expressed as mean ± SD (n = 6) of three independent experiments, referred to PBS-treated cells as control (100% cell viability). Statistical analysis was done using One-way ANOVA and Dunnett's post test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ *vs* control

the effects of whole blueberry extract on the proliferation of a panel of cancer cell lines (six human cell lines from different tumor types and one murine colon carcinoma cell line). The properties of the extract on key steps involved in cancer progression, such as cell adhesion and migration, were also investigated. A concentration-dependent inhibition of cell proliferation was observed in all cell lines treated with the extract for 72 h. Our results are in agreement with previous studies that reported the anti-proliferative activity of whole blueberry extracts in breast and colon cancer cell lines [27, 29]. Similarly, extracts from strawberry cultivars have been shown to reduce the proliferation of human colon (HT29) and breast (MCF-7) cancer cells in a dose-dependent manner [30]. Also, an anti-proliferative study conducted

by Seeram et al. [9] on human oral (KB, CAL-27, breast (MCF-7), colon (HT29, HCT-116) and prostate (LN-CaP) cancer cell lines revealed that methanol extracts of blueberry, blackberry, black raspberry, cranberry, red raspberry and strawberry inhibited the growth of all cancer cells but with a marked variation for both the type of berry and the specific cell line studied.

The mode of action of polyphenols on cell proliferation is not well-known. However, it is now well accepted that some of these compounds may interact with steroid receptors, such as phytoestrogens [31]. Steroid receptor-independent pathways such as blocking cell cycle progression, increased apoptosis and reduced cell survival signaling are also accepted mechanisms of the polyphenol inhibitory effect on cell proliferation [10].
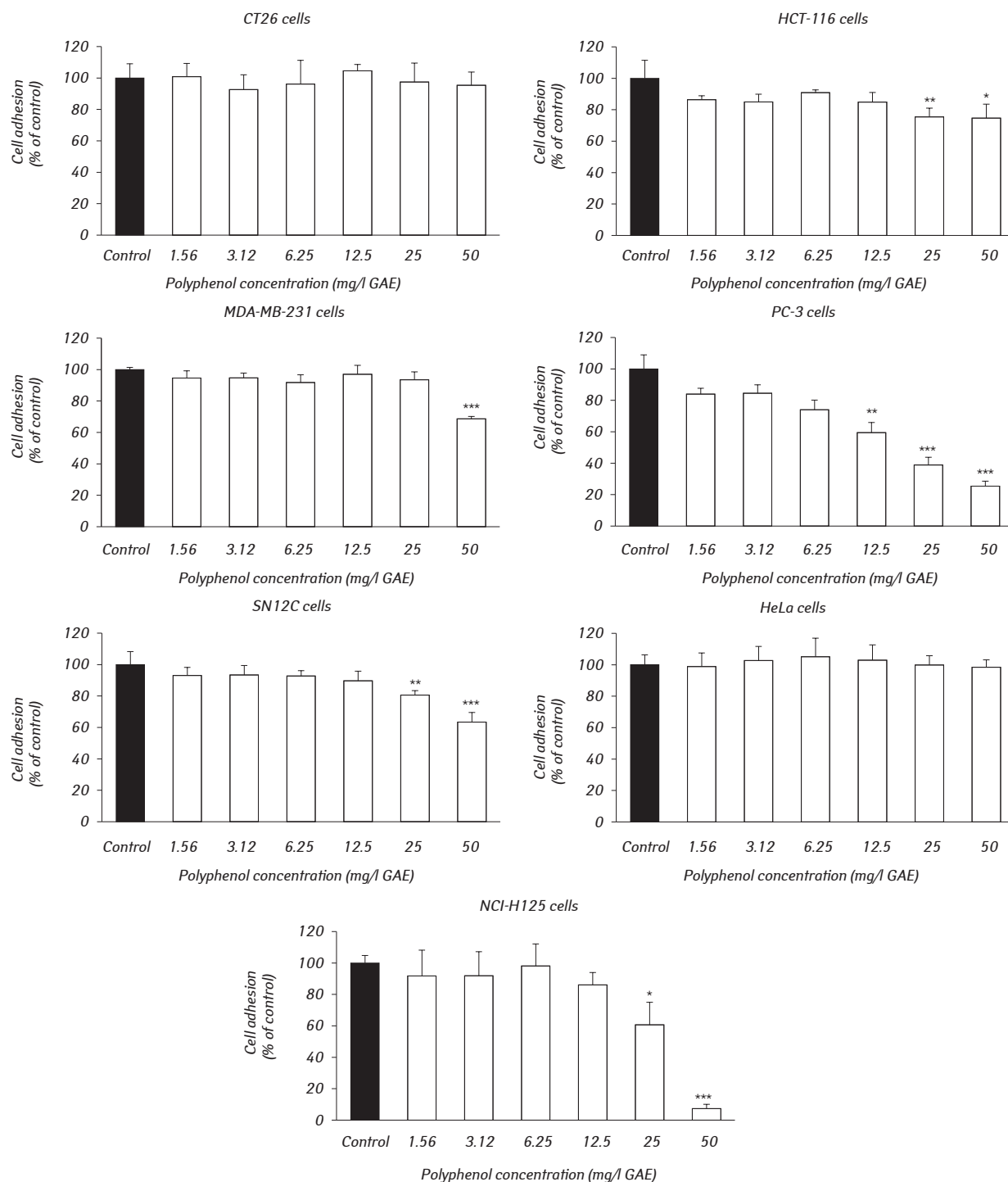
Fig. 3. Effect of the blueberry extract on cell adhesion. Cells were seeded in 96-well plates and treated with increasing concentrations of blueberry extract, expressed as mg/L of GAE. After 2 h, the cells were stained with crystal violet and the absorbance at 595 nm was read. Cell adhesion percent for each concentration is expressed as mean ± SD (n = 6) of three independent experiments, referred to PBS-treated cells as control (100% cell adhesion). Statistical analysis was done using One-way ANOVA and Dunnett's post test. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ *vs* control

Metastasis is the leading reason for the resultant mortality of cancer patients. The process is composed of a number of sequential events called metastatic cascade, in which occur changes in cell-cell and cell-matrix adhesion, as well as in cell migration [32]. It has been shown that a large number of natural products, including berries, inhibit metastatic potential in several cancer cell lines. These products can act on key proteins as some metalloproteases and also can regulate the AKT, NFκB, AP-1, c-Jun and ERK signaling pathways, which are important in control of metastasis-related proteins [24, 33]. Our *in vitro* experiments showed that non-cytotoxic concentrations of the blueberry extract decreased cell adhesion in five of seven cell lines studied (HCT-116, PC-3, NCI-H125, MDA-MB-231 and SN12C) and inhibited the migration of MDA-MB-231 and PC-3 cells. Since cell adhesion and migration play important roles in tumor progression and metastasis, the
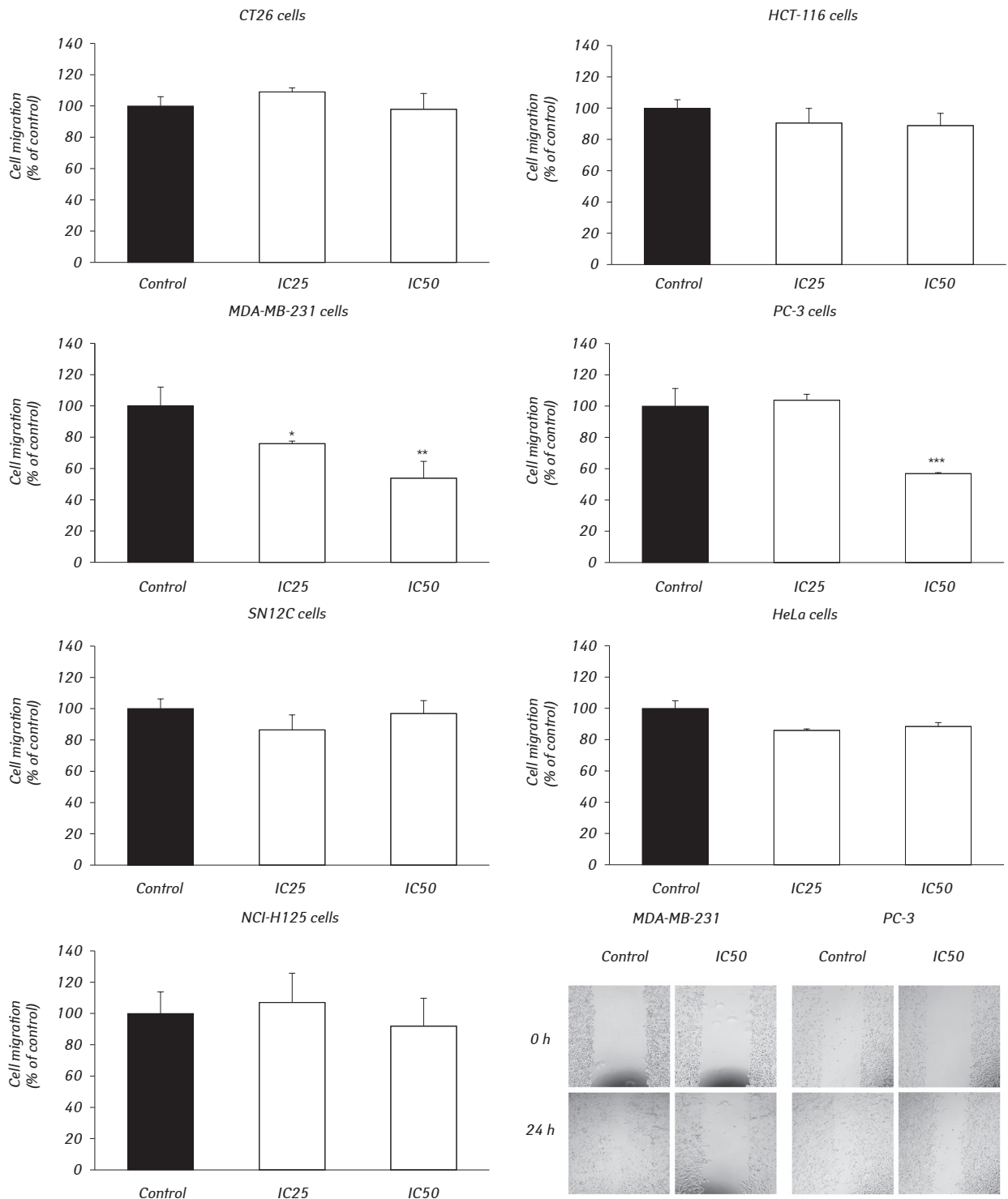
Fig. 4. Effect of the blueberry extract on cell migration in the scratch assay. Confluent monolayers of cells in 12-well plates were scratched with a pipette tip and then treated with IC_{25} and IC_{50} of the blueberry extract or PBS as a control in complete medium. Images were taken using an inverted microscope at the beginning of the assay and after 24 h of treatment. Cell migration was evaluated by measuring the cell-free surface at the beginning of the experiment (0 h) and the surface covered by the cells at the end of the experiment (24 h) by using ImageJ Software. Results are expressed as the percentage of covered surface in each condition, referred to PBS-treated cells as control (100% cell migration). The means ± SD (n = 3) of three independent experiments are illustrated in the graphs. Statistical analysis was done using One-way ANOVA and Dunnett's post test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ *vs* control. Images of representative assays for MDA-MB-231 and PC-3 cell lines are shown

inhibition of these events in various cancer cells by the blueberry extract suggest its use as a potential anticancer agent. However, further *in vitro* experiments are necessary to elucidate the molecular mechanisms underlying the inhibitory effects of the extract on cell proliferation, adhesion and migration.

In conclusion, this work provides additional evidence regarding the ability of blueberry extract to inhibit the growth and decrease cell adhesion and migration of different cancer cell lines *in vitro*. Further studies, such as tumor progression and chemically-induced carcinogenesis assays in BALB/c mice, are currently undergo-

ing in order to investigate the potential of the blueberry extract for the prevention and treatment of cancer.

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