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**Blueberry anthocyanins and pyruvic acid adducts: anti-cancer properties in breast cancer cell lines**

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|                               |  |



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4 **Blueberry anthocyanins and pyruvic acid adducts: anti-cancer properties in breast**  
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7 **cancer cell lines**

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**Abstract**

The purpose of this study was to investigate the anti-cancer properties of an anthocyanin-pyruvic acid adduct extract, which is being developed aiming to be further applied in the food industry. An anthocyanin extract from blueberry (extract I) and an anthocyanin-pyruvic acid adduct extract (extract II) were tested on two breast cancer cell lines (MDA-MB-231 and MCF7). Proliferation was assessed by SRB assay and <sup>3</sup>H-thymidine incorporation. Caspase-3 activity was determined in the presence of both extracts. Their capacity as chemoattractant and their invasive potential were also assayed.

In both cell lines, extracts I and II significantly reduced cell proliferation at 250 µg/ml, after 24h of cell incubation. Caspase-3 activity was not altered by the extracts (250µg/ml) in both cell lines, with exception for extract II in MCF-7, which increased its activity, probably explaining its effects on cell proliferation.

Both extracts (250 µg/ml) demonstrated significant anti-invasive potential in both cell lines. Furthermore, they did not demonstrate any capacity for chemotaxis.

In conclusion, blueberry anthocyanins and respective anthocyanin-pyruvic acid adducts demonstrated anti-cancer properties by inhibiting cancer cell proliferation and by acting as cell anti-invasive factors and chemoinhibitors. Anthocyanin-pyruvic acid adduct extract showed a more pronounced effect in MDA-MB-231, suggesting an effect independent of estrogen receptors.

**Keywords** – anthocyanins; anthocyanins derived pigments; breast cancer; invasion proliferation.

## Introduction

Breast cancer is one of the most commonly diagnosed cancers and the second leading cause of cancer deaths in women worldwide today (Lacey *et al.*, 2002). Several studies have established that estrogens are predominantly involved in the initiation and proliferation of breast cancer and efforts are now being devoted to develop agents that block estrogen formation and action. However, estrogen receptor negative breast cancer is generally unresponsive to these agents. It is therefore vital to developed drugs that demonstrate anticancer activity in both forms of breast cancer.

The progressing of a tumor from being *in situ* to invasive is a major prerequisite for cancer metastasis (Stetler-Stevenson *et al.*, 1993). Malignant tumors invade normal tissue, involving three independent processes: the degradation of the extracellular matrix (ECM) cell metastasis and proliferation. (Bohle and Kalthoff, 1999; Stetler-Stevenson *et al.*, 1993).

Dietary factors are widely believed to play an important role in determining the risk of many cancers, including those of breast. In addition, consumption of fruits and vegetables has been associated with a decreased risk of developing breast cancer (La Vecchia and Bosetti, 2006). Interest in anthocyanins, the flavonoid constituents that provide much of the flavor and color to these fruits and vegetables has increased immensely during the past decade as epidemiological evidence hints at their cancer preventive activity (Wang and Stoner, 2008).

In earlier studies, it has been shown that anthocyanin and other phenolic compounds may have potentially beneficial effects, including reducing the risk of cardiovascular diseases and cancers with antioxidant, anti-inflammatory, and chemoprotective properties (Mazza, 2007; Wang and Stoner, 2008). The exact related mechanisms are

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4 still uncertain, however, their ability to suppress the proliferation (Li *et al.*, 2009) and  
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6 angiogenesis (Matsubara *et al.*, 2005), in addition to the induction of cancer cell  
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8 apoptosis (Shin *et al.*, 2009) may have contributed to these beneficial effects. These  
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10 compounds have also been reported to be able to scavenge reactive oxygen species,  
11  
12 inhibit low-density lipoprotein oxidation in vitro, prevent platelet aggregation and  
13  
14 decrease serum lipids (Dell'Agli *et al.*, 2004; Han *et al.*, 2007; Liu *et al.*, 2008).  
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18 Efforts are being made in order to include natural compounds in common processed  
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20 food. Anthocyanins and anthocyanin-derived pigments, namely anthocyanin-pyruvic  
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22 acid adducts, might be of great importance if used in the food industry, not only because  
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24 they arise from natural precursors and present appreciably antioxidant properties (Faria  
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26 *et al.*, 2005), but specially due to their unusual colors in acidic conditions (more orange  
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28 hues). The present work aimed to study the effect of a blueberry (*Vaccinium myrtillus*)  
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30 extract and its anthocyanin-pyruvic acid adducts extract on several properties related to  
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32 cancer, in particular breast cancer. For this purpose, breast cancer cell lines MDA-MB-  
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34 231 (ER $\alpha$ (-)) and MCF7 (ER $\alpha$ (+)) were used. An anthocyanin extract from blueberry  
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36 (extract I) was used for comparison with an anthocyanin-pyruvic acid adduct extract  
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38 (extract II), prepared from the reaction of the same anthocyanins with pyruvic acid. Cell  
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40 viability was evaluated by MTS assay and proliferation was assessed by SRB assay and  
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42 methyl-[<sup>3</sup>H]-thymidine incorporation. In addition, caspase-3 activity was also  
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44 determined in the presence of both extracts. Cells invasive potential and the  
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46 chemoattractive capacity of these extracts were assayed using a commercial kit.  
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## Materials and methods

### Reagents

Toyopearl gel was purchased from Tosoh<sup>®</sup> (Tokyo, Japan); pyruvic acid, MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt), SRB (sulforhodamine B) from Sigma-Aldrich<sup>®</sup> (Madrid, Spain), trichloroacetic acid from Merck<sup>®</sup> (Darmstadt, Germany), and methyl-[<sup>3</sup>H]-thymidine (88.0 Ci/mmol) from Amersham (Arlington Heights, IL).

### Extract preparation

Two hundred grams of fresh blueberries (*Vaccinium myrtillus*) picked in field were subjected to extraction with 500 ml of 50% aqueous ethanol (pH 1.5) for 30 min at room temperature. The blueberry anthocyanin extract (I) was filtered in a 50 µm nylon membrane and then purified by Toyopearl gel column chromatography according to the procedure described previously (Pissarra *et al.*, 2003).

Anthocyanin-pyruvic acid adducts (Extract II) were prepared through reaction of the genuine anthocyanin extract with pyruvic acid in water (pH 2.6, 35 °C) at an approximate molar ratio pyruvic acid/anthocyanin of 50:1 during 5 days. The resulting extract was purified by Toyopearl gel column chromatography with the anthocyanin-pyruvic acid adducts fraction eluted with water/ethanol 20% (v/v) (Faria *et al.*, 2005).

The extract composition has been previously determined (Faria *et al.*, 2005): total phenolics in extract I was  $257.9 \pm 8.5$  mg/ L trolox equivalents and  $320.4 \pm 7.5$  mg/ L trolox equivalents for extract II; total flavonoids on the extracts were  $153.1 \pm 9.3$  mg/L

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4 quercetin equivalents and  $149.3 \pm 7.1$  mg/ L quercetin equivalents for extract I and II,  
5  
6 respectively; pigment composition for extract I was  $78.7 \pm 4.0$  mg/L malvidin-3-  
7  
8 glucoside and  $39.8 \pm 4.0$  mg/L malvidin-pyruvic acid adduct in extract II.  
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### 11 12 13 **HPLC conditions**

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15 All extracts were analysed by HPLC (Knauer K-1001) on a 250 x 4.6 mm i.d. reversed-  
16  
17 phase C18 column (Merck, Darmstadt); detection was carried out at 511 and 528 nm  
18  
19 using a diode array detector (Knauer K-2800). The solvents were A: H<sub>2</sub>O/HCOOH  
20  
21 (9:1), and B: H<sub>2</sub>O/CH<sub>3</sub>CN/HCOOH (6:3:1). The gradient consisted of 20-85% B for 70  
22  
23 min at a flow rate of 1.0 ml min<sup>-1</sup>. The column was washed with 100% B for 20 min and  
24  
25 then stabilized at the initial conditions for another 20 min.  
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### 32 **Cells and culture conditions**

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34 MCF-7 cells (poorly invasive; ER $\alpha$ (+)) were grown in Minimum Essential Medium  
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36 (MEM, Sigma, St. Louis, M.O., U.S.A.), supplemented with 10% foetal bovine serum,  
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38 100 units ml<sup>-1</sup> penicillin, 100  $\mu$ g ml<sup>-1</sup> streptomycin and 0.25  $\mu$ g ml<sup>-1</sup> amphotericin B (all  
39  
40 from Sigma). MDA-MB 231 cells (highly metastatic; ER $\alpha$ (-)) were grown in RPMI  
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42 1640 (Invitrogen, Carlsbad, CA ) supplemented with 2 mM glutamine, 15% foetal  
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44 bovine serum, 100 units ml<sup>-1</sup> penicillin, 100  $\mu$ g ml<sup>-1</sup> streptomycin and 0.25  $\mu$ g ml<sup>-1</sup>  
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46 amphotericin B (all from Sigma). Both cell lines were maintained in a humidified  
47  
48 atmosphere of 5% CO<sub>2</sub>-95% air. Culture medium was changed every 2 to 3 days and the  
49  
50 culture was split every 7 days. For subculturing, the cells were removed enzymatically  
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52 (0.25% trypsin-EDTA, 5 min, 37°C), split 1:4, and subcultured in plastic culture dishes  
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54 (21 cm<sup>2</sup>;  $\varnothing$  60 mm; Orange Scientific, Belgium).  
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### Cellular viability determined by the MTS reduction method

For the experiments, the MCF-7 and MDA-MB-231 cells were seeded into 96-well (0.37 cm<sup>2</sup>; Ø 6.9 mm; Orange Scientific, Belgium) plastic cell culture clusters. Cells were treated for 24 h and the cell viability was assessed by MTS assay. Briefly, culture medium was removed and cells were preincubated with the compounds to test in culture medium, at 37° for 24 h. This treatment was removed and the cells were incubated for 3 h with 100 µL of culture medium and 20 µL/well of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt). Absorbance was registered at 490 nm. Results were expressed in % of control.

### Cell proliferation determined by sulforhodamine B assay

The sulforhodamine B (SRB) assay, which measures whole-culture protein content as an index of tumour cell proliferation, was employed to determine the relative potencies of the anthocyanins and anthocyanin-pyruvic acid adducts extract in inhibiting the growth of MCF-7 and MDA-MB-231 cells, as described elsewhere (Miranda *et al.*, 1999). Briefly, cell cultures were plated on 96-well plates, and exposed to 200 µl of culture medium containing different concentrations of the tested compounds, solved in culture medium (controls were made in the presence of culture media). After 24 h of treatment, 50% TCA were added to the culture medium, for 1 h at 4°C in the dark, to fix cells. The plates were then washed with tap water to remove TCA, air-dried and stained for 15 min with 0.4% (w/v) SRB dissolved in 1% acetic acid. After incubation, cultures were rinsed four times with 1% acetic acid to remove residual dye, the plates were air-dried and the bound dye was solubilised with 200 µl of 10 mM Tris base solution (pH

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4 10.5). The absorbance of each well was determined at 492 nm in a microplate reader.  
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6 Results were expressed in % of control.  
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### 11 **Determination of DNA synthesis**

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13 MCF-7 and MDA-MB-231 cells were seeded into 24-well (1.65 cm<sup>2</sup>; Ø 14.5 mm;  
14  
15 Orange Scientific, Belgium) plastic cell culture clusters in a final volume of 0.5 ml  
16  
17 culture medium containing 10%FBS. After 24 h in culture, the cells were treated with  
18  
19 different concentration of anthocyanins and anthocyanin-pyruvic acid adducts in culture  
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21 medium (controls were made in the presence of culture media). After 24 h, the cells  
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23 were incubated with 0.2 ml of methyl-[<sup>3</sup>H]-thymidine (0.5 µCi/well) for 4 h. The  
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25 medium was removed and the cells were fixed by incubation in 10% TCA for 1 h at  
26  
27 4°C. The cells were then washed twice with 10% TCA to remove unbound radioactivity.  
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29 The plates were air-dried and the cells were lysed with 1 M NaOH (0.28 ml/well). A  
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31 0.25 ml aliquot of the lysate was neutralized with HCl prior to the addition of  
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33 scintillation fluid. The radioactivity of the samples was quantified by a liquid  
34  
35 scintillation counter. The counts (disintegrations per min) of each treatment were  
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37 averaged and expressed as percent of controls (adapted from (Miranda *et al.*, 1999)).  
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### 47 **Caspase 3 activity assay**

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49 Apoptosis was analysed by the caspase 3 activity of cell lysates following treatment  
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51 with anthocyanin and anthocyanin-pyruvic acid adducts for 6 h, using a caspase  
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53 colorimetric activity assay kit (Millipore<sup>®</sup>, Billerica, MA, APT131). Briefly, *p*-  
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55 nitroaniline (*p*-NA) moiety resulting from the hydrolysis of acetyl-Asp-Glu-Val-Asp *p*-  
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57 nitroaniline (Ac-DEVD-*p*NA) by caspase 3 activity was detected at 405 nm after  
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4 incubating the reaction mixture for 2 h at 37 °C. The concentration of the *p*-NA released  
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6 from the substrate was calculated from the absorbance values at 405 nm using a  
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8 calibration curve prepared with *p*-NA standards. Proteins from the lysate were  
9  
10 determined using Bradford's method (Bradford, 1976). Results are expressed in nmol *p*-  
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12 NA/mg protein.  
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### 18 **Effect on cell invasion capacity**

19  
20 The effect of the compounds on cells invasion potential was tested using Millipore®  
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22 QCM® 24-well assay kit, according to the manufacturer's instructions. Cells ( $7 \times 10^5$   
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24 cells/ml) were transferred to the rehydrated inserts and treated with the compounds to  
25  
26 test, solved in culture media. Also, control inserts (with cells and without compounds)  
27  
28 were used. After incubation for 24 h at 37°C, invaded cells were detached, lysed and  
29  
30 subsequently detected by CyQuant GR® dye. Detection was carried out at 480/520 nm  
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32 using a Perkin-Elmer LS 45 fluorometer.  
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### 40 **Chemoattractant potential**

41  
42 The chemoattractant potential of anthocyanins and anthocyanin-pyruvic acid adducts  
43  
44 was tested using the Millipore® QCM 24-well assay kit. Cells ( $7 \times 10^5$  cells/ml) were  
45  
46 transferred to the rehydrated inserts and the compounds were added to the basolateral  
47  
48 compartment, solved in culture media free of FBS. Control inserts without compounds  
49  
50 on the basolateral side were used. Cells were detached, lysed and subsequently detected  
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52 by CyQuant GR® dye. Detection was carried out at 480/520 nm using a Perkin-Elmer  
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54 LS 45 fluorometer.  
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### Statistical analysis

Values are expressed as the arithmetic mean  $\pm$  standard error mean (SEM). All the assays were performed in  $n \geq 3$ . Statistical significance of the difference between different compounds was evaluated by one-way analysis variance (ANOVA) followed by the Bonferroni test. Differences were considered to be significant when  $P < 0.05$ .

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## Results

An extract comprised of anthocyanins was prepared from blueberries (extract I) and other extract was prepared from these anthocyanins through reaction with pyruvic acid (extract II). The latter is comprised of anthocyanin-pyruvic acid adducts. The general structure of the compounds present in the different extracts is illustrated in Figure 1.

The effect of anthocyanins (extract I) and anthocyanin-pyruvic acid adducts (extract II) extract on cell viability was assayed by MTS method after 24 h of cell incubation with the extracts. A range of concentration between 50 and 500  $\mu\text{g/ml}$  was tested in both cell lines MCF7 and MDA-MB-231. Cellular viability was not significantly changed by the treatments with the extracts except for the highest concentration, 500  $\mu\text{g/ml}$  (table 1).

Two distinct methods were used to assay cellular proliferation after 24h incubation with the extracts to test: the SBR method, based on staining cellular proteins, and methyl- $[^3\text{H}]$ -thymidine incorporation in DNA.

As seen in figure 2, both extracts (250  $\mu\text{g/ml}$ ) were able to decrease MCF7 and MDA-MB-231 cell proliferation when evaluated by the SRB method. It is worth notice that extract II inhibited MDA-MB-231 cell proliferation in all concentrations tested.

The results obtained with methyl- $[^3\text{H}]$ -thymidine incorporation assay were much more pronounced (figure 3) than those obtained with the SRB method. In MCF7 cells, extract I inhibited proliferation only at 250  $\mu\text{g/ml}$ , but extract II showed inhibition in all concentrations. The results obtained with the MDA-MB-231 cells were more marked than with MCF7 cells. Extract I inhibited MDA-MB-231 cell proliferation around 65% and extract II inhibited cell proliferation around 70%.

Since caspase-3 is an enzyme involved in the early steps of apoptosis pathway, its activity was assessed in order to evaluate if this pathway could be responsible for the

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4 anti-proliferative effect of these compounds. Cellular incubation with the extracts did  
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6 not affect caspase-3 activity with the exception of extract II in MCF7, in which caspase-  
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8 3 activity was significantly increased (figure 4).  
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11 Concerning the invasion potential, matrigel inserts were used to evaluate this ability in  
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13 the presence of the tested compounds. The extracts were tested at 250 µg/ml since this  
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15 concentration showed the highest anti-proliferative potential without altering cell  
16  
17 viability. Extract I significantly inhibited cell invasion capacity in both cell lines, but  
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19 extract II was more efficient (table 2). Extract II inhibited the MDA-MB-231 invasion  
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21 capacity in 73% relatively to control (100%; cells in the absence of extracts). The  
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23 chemotaxis ability of these compounds was also tested, using the same matrigel inserts.  
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26 The extracts did not have the ability to attract cells. In fact, they inhibited migration of  
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28 both cell lines which indicates they were chemoinhibitors (table 2). Also, extract II had  
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30 a more noticeable effect than extract I, particularly in MDA-MB-231 cells.  
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## Discussion

Anthocyanins, a class of flavonoids, are reddish natural pigments extensively distributed in fruits and especially in berries. Epidemiological studies have positively correlated their consumption with reduced mortality by cardiovascular disease and some types of cancer (Rossi *et al.*, 2006; Tavani *et al.*, 2006). These compounds have been widely studied for their biological properties, namely antioxidant, anti-bacterial, anti-inflammatory, anti-cancer among others (Gerhauser, 2008; Heinonen, 2007; Wang and Stoner, 2008). Therefore, it could be of great importance if anthocyanins or anthocyanin-derived pigments could be used in the food industry. Efforts are being made in order to include these natural compounds in common processed food. The major interest of the putative use of these anthocyanin-derived pigments extract lies in its unusual colors in acidic conditions (more orange hues) even though arising from natural precursors.

The present work aimed to study the effect of a blueberry (*Vaccinium myrtillus*) extract and its anthocyanin-pyruvic acid adducts extract on several properties related to cancer, in particular breast cancer. In a previous study, the chemical characterization of these extracts has already been accomplished. Furthermore, the antioxidant properties of these extracts were investigated in several models, including the protection against oxidation of liposomes. The antioxidant capacity increased with the structural complexity of the anthocyanin-derived pigments of the extracts (Faria *et al.*, 2005).

For the present study, two human breast adenocarcinoma cell lines were used: MDA-MB-231 and MCF7. The major differences between these cell lines are the presence of the estrogen receptor (ER) and their invasive and metastatic potential: MDA-MB-231 is a hormone-independent cell line (ER $\alpha$ (-)) and have more invasive and metastatic

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4 potential than MCF7. These two cell lines are frequently used in this type of studies  
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6 (Castillo-Pichardo *et al.*, 2009; Davis *et al.*, 2008; Shankar *et al.*, 2009).  
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9 Cellular viability was assayed by MTS method after 24h of incubation with the extracts  
10  
11 and it was decreased with the highest concentration (500 µg/ml). Since the  
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13 concentration 500 µg/ml of both the extracts was cytotoxic, the following experiments  
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15 were performed only with noncytotoxic concentrations.  
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18 Cell proliferation was assessed by SBR method which consists of staining cellular  
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20 proteins with SRB and measuring the dye spectrophotometrically. This method is  
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22 considered to provide information about cellular proliferation, since protein synthesis  
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24 and cellular proliferation are tightly connected. A decrease of protein synthesis, an  
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26 index of cellular proliferation, was observed. DNA synthesis was evaluated through  
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28 methyl-<sup>3</sup>H-thymidine incorporation. A concentration-dependent inhibition of cell  
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30 proliferation was also observed.  
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34  
35 Altogether, these results show that anthocyanins, as can be confirmed in the literature  
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37 (Jing *et al.*, 2008; Li *et al.*, 2009; Syed *et al.*, 2008), but also the new anthocyanin  
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39 derived-pigment were capable of inhibiting cellular protein synthesis and reduced DNA  
40  
41 incorporation in cancer cell lines. The polyphenols mode of action on cell growth is not  
42  
43 well-established. However, it is now well accepted that some of these compounds may  
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45 interact with steroid receptors, such as phytoestrogens (Damianaki *et al.*, 2000), which  
46  
47 did not seem the case, since the results were different in estrogen -dependent and -  
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49 independent cancer cell lines. Steroid receptor-independent pathways such as blocking  
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51 cell cycle progression, increased apoptosis and reduced cell survival signaling are also  
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53 accepted mechanisms of the polyphenols inhibitory effect on cell proliferation.  
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4 Caspases are a family of cysteine proteases which are sequentially activated and  
5 therefore used as a measure of caspase-mediated apoptosis induction. Caspase-3 activity  
6 was significantly increased only for extract II in MCF7. This indicates that extract II  
7 could interfere with apoptosis pathway in MCF7 cell line, but did not explain the results  
8 of proliferation inhibition. Neither extracts induced caspase activation in MDA-MB-  
9 231, possibly because the antiproliferative effect did not involve an apoptotic process or  
10 DNA fragmentation and apoptosis did not always require caspase activation. Induction  
11 of apoptosis may be due to oxidative stress or through apoptosis inducing factors where  
12 caspase activation is not essential (caspase-independent pathways). A previous study  
13 has shown that anthocyanins activate caspase-independent apoptosis in prostate cancer  
14 cells (Reddivari *et al.*, 2007).  
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30 It has been shown that interactions between cells and extracellular matrix (ECM) can be  
31 critical for invasion, migration and metastasis of tumors (Liotta *et al.*, 1980). Therefore,  
32 invasion potential is an essential parameter to assess if these compounds could have the  
33 ability to suppress cancer cell metastasis, within a concentration showing no toxicity  
34 (250 µg/ml).  
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42 Both extracts inhibited cell invasion capacity in both cell lines. ECM has to be  
43 proteolytically degraded to allow cell migration and invasion. The activation of these  
44 matrix-degrading proteinases is essential for the tumor cells to cross the basement  
45 membrane. Some studies revealed that proteinases related to degradation of matrix were  
46 required for cancer cell metastasis and enhanced production of metalloproteinases  
47 (MMPs) and urokinase-type plasminogen activator (u-PA), a serine proteinase,  
48 correlates with migration, invasion and angiogenesis of the tumors (Kim, 2003; Mackay  
49 *et al.*, 1990). In fact, some studies showed that anthocyanins decrease the expressions of  
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4 MMP-2 (matrix metalloproteinase-2) and u-PA (Chen *et al.*, 2006; Huang *et al.*, 2008)  
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6 which could explain their ability to inhibit cell invasion. It is most likely that extract II,  
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8 which decreased cell invasion in a more pronounced way, interfere with MMP-2 and  
9  
10 with other MMPs, enhancing, thus, the effect.  
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14 Additionally, anthocyanins and anthocyanin-pyruvic acid adduct extracts potential as  
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16 chemotactic factors were tested. The extracts not only did not have the ability to attract  
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18 cells but also inhibited cell migration. Cells did not migrate in direction of the tested  
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20 compounds, which make them chemoinhibitors. The cells responded to the compounds  
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22 as a negative chemotactic signal, which could be interesting in the perspective of  
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24 inhibiting cell migration and invasion, implicated in metastatic efficiency.  
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28 Studies on anthocyanins uptake after their consumption suggest that blood levels are far  
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30 below the levels required to exhibit anti-carcinogenic effects *in vitro*. Thus, it is unclear  
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32 whether the concentrations *in vivo* are sufficient to exert anti-carcinogenic effects.  
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34 Nevertheless, the knowledge that the ingestion of these compounds could be an  
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36 advantage and a target to future pharmacologic research is valuable.  
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40 In summary, both anthocyanin and anthocyanin-pyruvic acid adduct extracts were  
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42 efficient in inhibiting breast cancer cell lines proliferation in noncytotoxic  
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44 concentrations. Anthocyanin-pyruvic acid adduct extract was able to activate caspase-3  
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46 in MCF7 cells, demonstrating an interference with apoptosis pathway. Both extracts  
47  
48 were capable of inhibiting cell invasion and revealed no chemotactic properties. The  
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50 results suggested an effect independent of estrogen receptors. Altogether, this study  
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52 demonstrated promising chemopreventing effects, particularly for the new anthocyanin-  
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54 pyruvic acid adducts.  
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### Acknowledgements

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### Figure and Table Captions

**Table 1** – Effects of prepared blueberry (*Vaccinium myrtillus*) extract I and II on cell viability evaluated by MTS assay in (A) MCF7 cells and in (b) MDA-MB-231 cells. Cells, seeded in 24 well plates, were treated with each compound for 24 h. Results are means  $\pm$  S.E.M.. \*Significantly different from control (no compounds),  $P < 0.05$ .

**Table 2** – Effects of prepared blueberry (*Vaccinium myrtillus*) extract I and II on cell (A) invasion and (B) chemotaxis. Cells were treated with 250  $\mu\text{g/ml}$  of each compound for 24 h. Results are presented as % of control (control 100%) and are means  $\pm$  S.E.M.. \*Significantly different from control (no compounds),  $P < 0.05$ .

**Figure 1** – **A)** HPLC chromatogram of the blueberry (*Vaccinium myrtillus*) extract I recorded at 528 nm: (1) Delphinidin-3-Galactose, (2) Delphinidin-3-Glucose, (3) Cyanidin-3-Galactose, (4) Delphinidin-3-Arabinose, (5) Cyanidin-3-Glucose, (6) Petunidin-3-Galactose, (7) Cyanidin-3-Arabinose, (8) Petunidin-3-Glucose, (9) Peonidin-3-Galactose, (10) Petunidin-3-Arabinose, (11) Peonidin-3-Glucose, (12) Malvidin-3-Galactose, (13) Peonidin-3-Arabinose, (14) Malvidin-3-Glucose, (15) Malvidin-3-Arabinose; General structure of blueberry (*Vaccinium myrtillus*) anthocyanidins. R1 and R2, independently of each other, are H, OH or OMe. R3 is glucose, galactose or arabinose. **B)** HPLC chromatogram of blueberry extract II after 5 days of reaction with pyruvic acid recorded at 511 nm: (1) Delphinidin-3-Galactose(or Glucose)-Pyruvic acid adduct, (2) Delphinidin-3-Arabinose-Pyruvic acid adduct, (3) Cyanidin-3-Galactose(or Glucose)-Pyruvic acid adduct, (4) Cyanidin-3-Arabinose-

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Pyruvic acid adduct, (5) Petunidin-3-Galactose(or Glucose)-Pyruvic acid adduct, (6) Petunidin-3-Arabinose-Pyruvic acid adduct, (7) Peonidin-3-Galactose(or Glucose)-Pyruvic acid adduct, (8) Peonidin-3-Arabinose-Pyruvic acid adduct, (9) Malvidin-3-Galactose-Pyruvic acid adduct, (10) Malvidin-3-Glucose-Pyruvic acid adduct, (11) Malvidin-3-Arabinose-Pyruvic acid adduct; C, HPLC chromatogram of the blueberry extract C recorded at 570 nm: (1) Vinylpyrano-Delphinidin-3-Galactose(or Glucose)-Catechin, (2) Vinylpyrano-Delphinidin-3-Glucose(or Galactose)-Catechin, (3) Vinylpyrano-Cyanidin-3-Galactose(or Glucose)-Catechin, (4) Vinylpyrano-Cyanidin-3-Arabinose-Catechin, (5) Vinylpyrano-Petunidin-3-Glucose(or Galactose)-Catechin, (6) Vinylpyrano-Petunidin-3-Arabinose-Catechin, (7) Vinylpyrano-Peonidin-3-Galactose(or Glucose)-Catechin, (8) Vinylpyrano-Malvidin-3-Arabinose-Catechin, (9) Vinylpyrano-Malvidin-3-Glucose(or Galactose)-Catechin. General structure of blueberry (*Vaccinium myrtillus*) anthocyanidin-pyruvic acid adducts. R1 and R2, independently of each other, are H, OH or OMe. R3 is glucose, galactose or arabinose.

**Figure 2** - Effects of prepared blueberry (*Vaccinium myrtillus*) extract I and II on cellular growth. A – MCF7; B – MDA-MB-231. Cells were treated with the compounds or the culture medium for 24 h. Cellular growth was measured after staining cellular protein with sulforhodamine B. Columns represent mean values  $\pm$  S.E.M.. \* Significantly different from control (no compounds),  $P < 0.05$ .

**Figure 3** - Effects of prepared blueberry (*Vaccinium myrtillus*) extract I and II on cellular growth. A – MCF7; B – MDA-MB-231. Cells were treated with the compounds or the culture medium for 24 h. Cellular growth was measured after incubation for 4 h

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4 with 0.5  $\mu\text{Ci}$  methyl- $[\text{}^3\text{H}]$ -thymidine per well, cell lysis and liquid scintillation counting.  
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7 Columns represent mean values  $\pm$  S.E.M.. \*Significantly different from control (no  
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9 compounds),  $P < 0.05$ .

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14 **Figure 4** - Effects of prepared blueberry (*Vaccinium myrtillus*) extract I and II on  
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16 caspase-3-activity. Cells were treated with 250  $\mu\text{g/ml}$  of each extract or culture medium  
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18 for 6 h. Caspase-3 activity was measured as *p*-nitroaniline (p-NA) formation and  
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20 detected at 405 nm. Columns represent mean values  $\pm$  S.E.M.. \*Significantly different  
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22 from control (no compounds),  $P < 0.05$ .  
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**Table 1****A – MCF-7**

| Concentration $\mu\text{g/ml}$ | 50              | 100             | 250             | 500              |
|--------------------------------|-----------------|-----------------|-----------------|------------------|
| Extract I                      |                 |                 |                 |                  |
| (% control)                    | 103.4 $\pm$ 0.7 | 107.2 $\pm$ 0.0 | 108.5 $\pm$ 2.1 | 24.7 $\pm$ 2.9 * |
| Extract II                     |                 |                 |                 |                  |
| (% control)                    | 99.4 $\pm$ 0.8  | 100.7 $\pm$ 2.9 | 91.3 $\pm$ 7.2  | 20.8 $\pm$ 1.2*  |

**B – MDA-MB-231**

| Concentration $\mu\text{g/ml}$ | 50              | 100             | 250             | 500              |
|--------------------------------|-----------------|-----------------|-----------------|------------------|
| Extract I                      |                 |                 |                 |                  |
| (% control)                    | 103.6 $\pm$ 1.3 | 105.7 $\pm$ 2.0 | 111.9 $\pm$ 6.5 | 81.0 $\pm$ 7.7 * |
| Extract II                     |                 |                 |                 |                  |
| (% control)                    | 103.5 $\pm$ 0.5 | 110.0 $\pm$ 1.6 | 108.0 $\pm$ 4.5 | 77.5 $\pm$ 1.7 * |

**Table 2****A - Invasion**

|             | MCF-7      | MDA-MB-231 |
|-------------|------------|------------|
| Extract I   |            |            |
| (% control) | 65.2±5.2 * | 45.6±9.1 * |
| Extract II  |            |            |
| (% control) | 57.5±8.1 * | 27.0±0.9 * |

**B - Chemotaxis**

|             | MCF-7      | MDA-MB-231 |
|-------------|------------|------------|
| Extract I   |            |            |
| (% control) | 38.5±8.2 * | 78.4±0.4 * |
| Extract II  |            |            |
| (% control) | 27.5±9.4 * | 32.5±8.9*# |

Figure 1

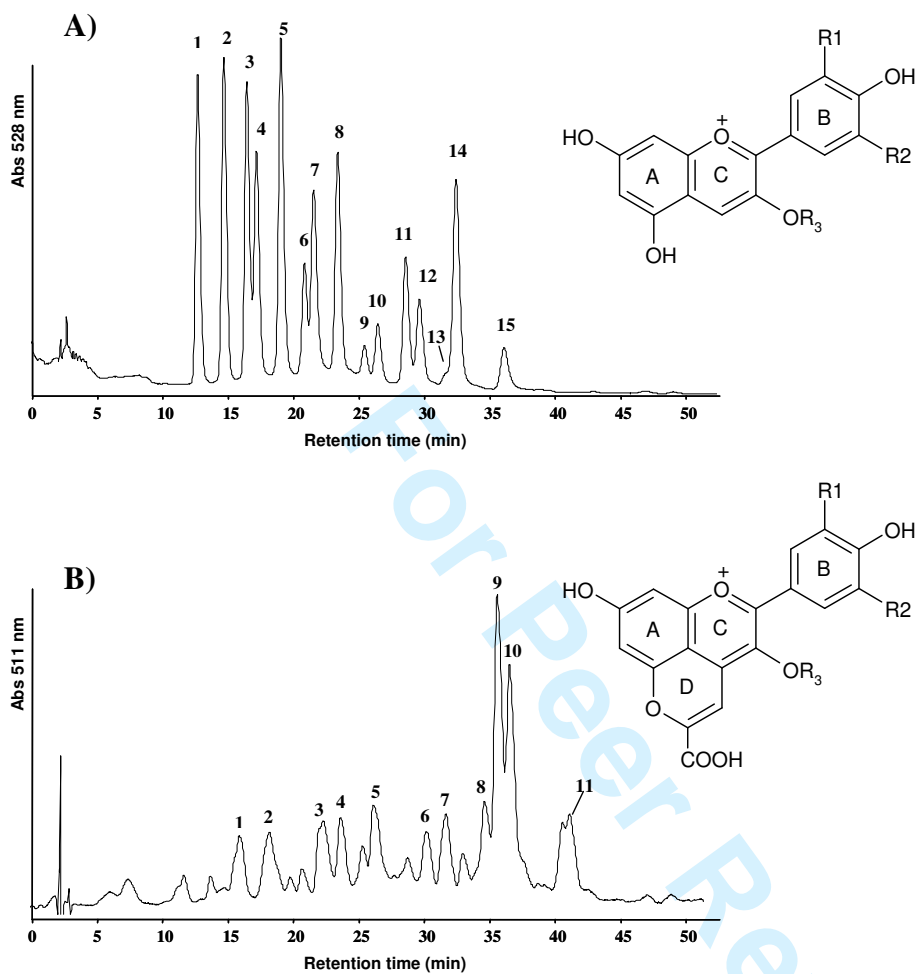
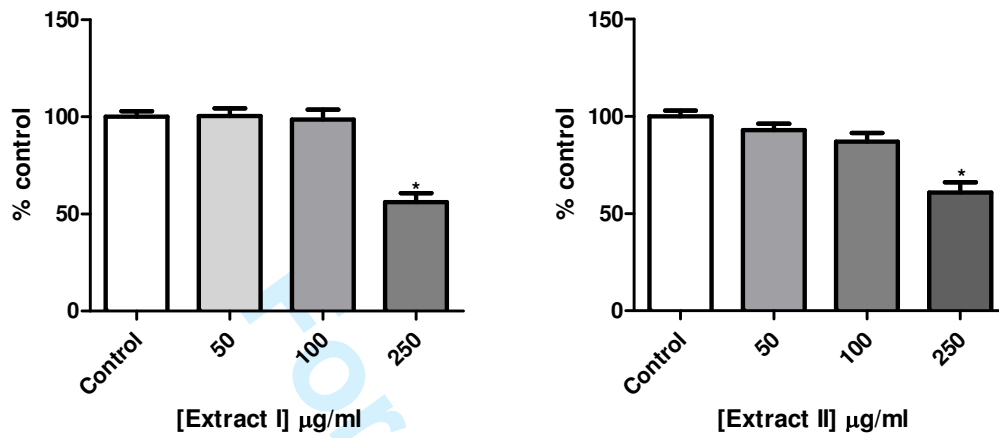


Figure 2

A – MCF-7



B – MDA-MB-231

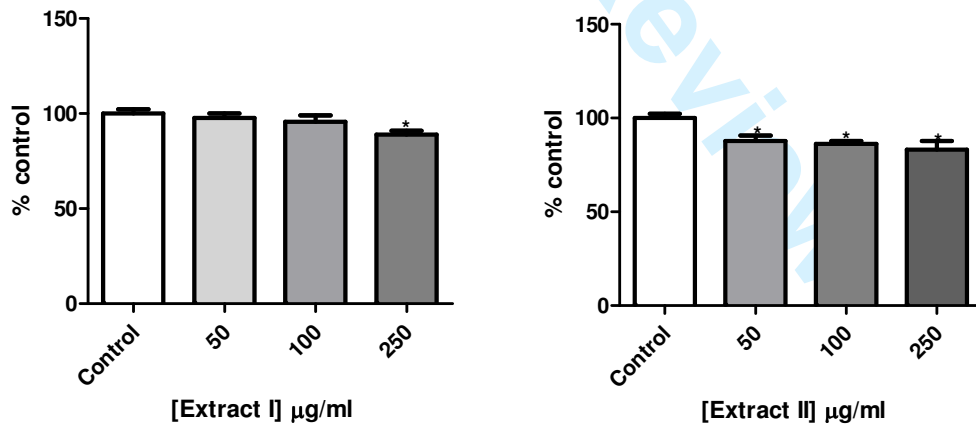
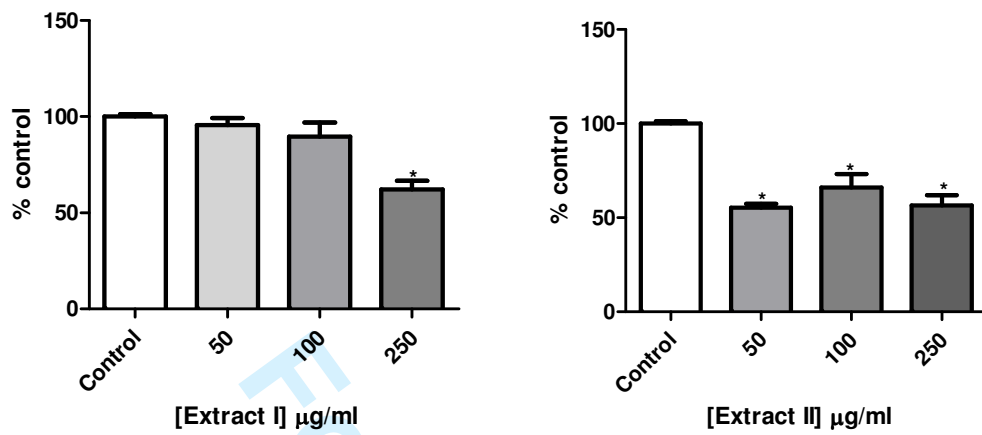
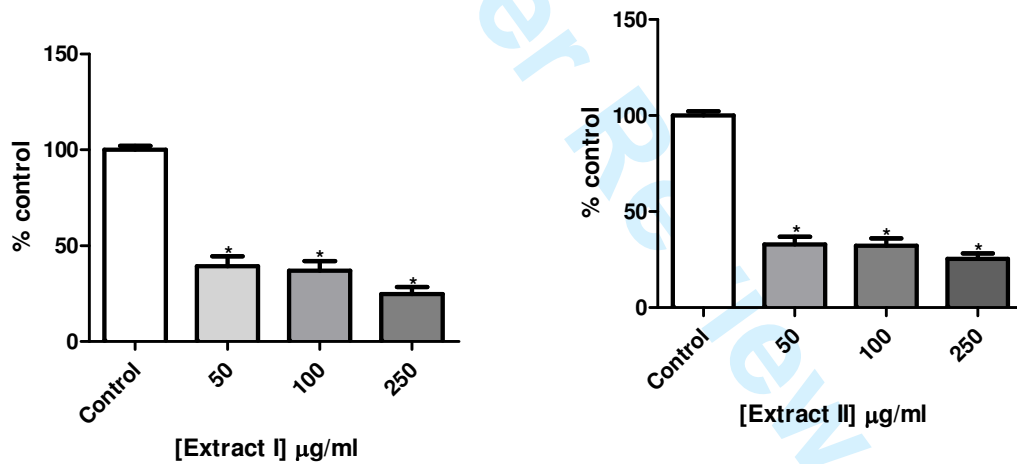


Figure 3

## A – MCF-7



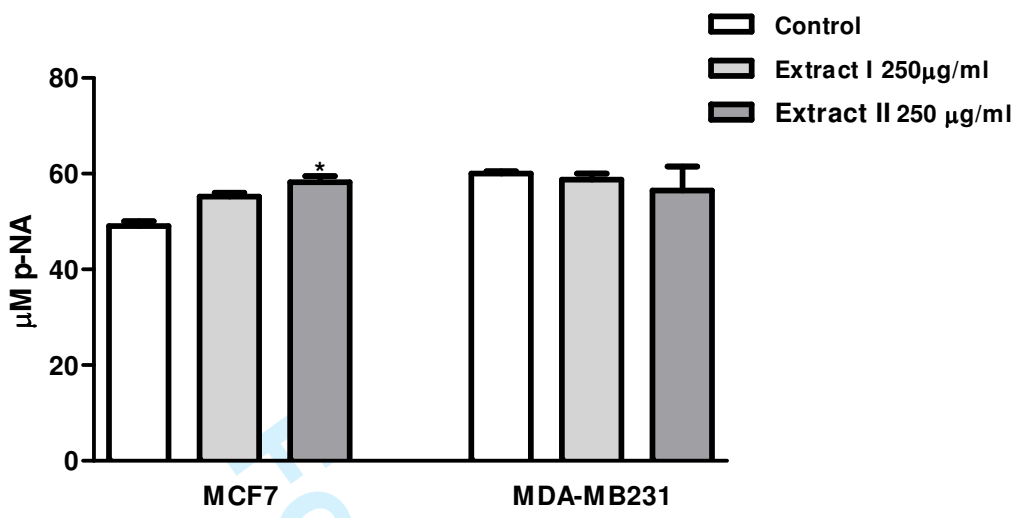
## B – MDA-MB-231





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Figure 4



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