

## Research Article

# Evaluation of the anti-angiogenic effect of aloe-emodin

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**Abstract.** The present study identified aloe-emodin (AE, a hydroxyanthraquinone from *Aloe vera* and other plants) as a new anti-angiogenic compound with inhibitory effects in an *in vivo* angiogenesis assay and evaluates its effects on specific key steps of the angiogenic process. AE inhibits endothelial cell proliferation, but this effect is not cell specific, since AE also inhibits tumor cell proliferation. Cell migration and invasion are not remarkably affected by AE. On the other hand, AE has different ef-

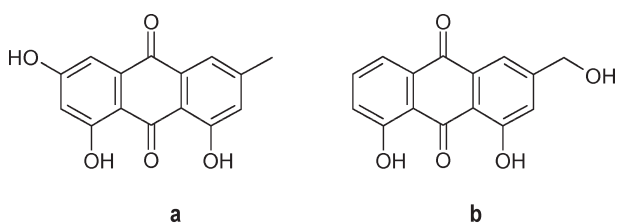
fects on endothelial and tumor cell gelatinases. Two main targets of the pharmacological action of AE as an anti-angiogenic compound seem to be urokinase secretion and tubule formation of endothelial cells. Finally, AE produces a remarkable photocytotoxic effect on tumor cells. Taken together, our data indicate that AE can behave both as an anti-tumor and an anti-angiogenic compound and suggest that AE could be a candidate drug for photodynamic therapy.

**Keywords.** Aloe-emodin, emodin, angiogenesis, cancer, photodynamic therapy.

## Introduction

Emodin is a natural anthraquinone present in some species of *Penicillium* and in plants of common use in traditional medicine that has been shown to have a potent anti-tumor effect [1–7]. Aloe-emodin (AE) is a hydroxyanthraquinone found in *Aloe vera*, as well as in leaves and roots of other plants, usually in combination with its glycosides or in an anthrone reduced form [8]. AE is structurally very similar to emodin (Fig. 1) and has also shown anti-tumor effects [9–13].

Persistent, deregulated angiogenesis is associated with some pathological conditions including cancer as well as ophthalmic, skin and inflammatory diseases, among others [14]. Furthermore, angiogenesis is one of the six hallmarks of cancer and it is required for both cancer progression and metastasis [15]. Therefore, inhibition of pathological angiogenesis has become an attractive therapeutic alternative for the treatment of angiogenesis-dependent diseases [16]. Mechanistically, angiogenesis is



**Figure 1.** Chemical structures of emodin (a) and aloe-emodin (AE) (b).

a very complex process in which several key steps are involved. When quiescent endothelial cells are activated by some pro-angiogenic signal, they change their phenotype to become highly proliferative and able to migrate, remodel the surrounding extracellular matrix (ECM) and finally to differentiate to form new vessels. Any of these key steps can be a potential pharmacological target to inhibit angiogenesis and, hence, to treat angiogenesis-dependent diseases [17, 18].

Our group is actively involved in the identification of new natural modulators of angiogenesis and in the investigation of their specific effects on the different steps of the

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angiogenic process [19–23]. In the present study, AE was evaluated as a potential new natural anti-angiogenic compound both with an *in vivo* assay and different *in vitro* assays to study its effects on endothelial cell proliferation, ECM remodeling potential, migration, invasion and differentiation to form tubule-like structures. Furthermore, its potential photobiological activity to modulate tumor cell survival was also studied.

## Material and methods

**Material and reagents.** Cell culture media were purchased from Gibco (Grand Island, NY, USA) and Bio-Whittaker (Walkersville, MD, USA). Fetal bovine serum (FBS) was a product of Harlan-Seralab (Belton, UK). Matrigel was purchased from Becton Dickinson (Bedford, MA, USA), and Calcein-AM was from Molecular Probes (Eugene, OR, USA). AE was supplied by Sigma-Aldrich (St. Louis, MO, USA). Stock solution (10 mg/mL) was prepared in DMSO and stored in aliquots at  $-20^{\circ}\text{C}$ . In all the assays, the vehicle (DMSO) was present at less than 1% (v/v) and controls with the vehicle alone were carried out in parallel. Supplements and other chemicals not listed in this section were obtained from Sigma-Aldrich. Plastic ware for cell culture was supplied by NUNC (Roskilde, Denmark).

**Cell cultures.** Bovine aortic arches were isolated from calves immediately after death at the local slaughterhouse Famadesa (Málaga), transported to the lab immersed in PBS containing penicillin-streptomycin and amphotericin at standard cell culture concentrations, and used immediately upon arrival for isolation of primary bovine aortic endothelial cells (BAEC) by a collagenase treatment, as first described by Gospodarowicz et al. [24]. BAEC were cultured and maintained as described elsewhere [22]. Human umbilical vein endothelial cells (HUVEC) were isolated by a modified collagenase treatment [25], and maintained as described [19]. Transformed human HT-1080 fibrosarcoma, U2-OS osteosarcoma and MDA-MB231 breast carcinoma cells were supplied by ATCC and maintained in culture [26].

***In vivo* angiogenesis assay.** The *in vivo* chicken chorioallantoic membrane (CAM) assay was carried out as described elsewhere [21], using fertilized chick eggs provided by Granja Santa Isabel (Córdoba, Spain). Briefly, eggs were incubated horizontally at  $38^{\circ}\text{C}$  in a humidified incubator, windowed by day 3 of incubation and processed by day 8. The tested compound stock solution was added to a 0.7% solution of methylcellulose in water, and 10- $\mu\text{L}$  drops of this solution were allowed to dry on a teflon-coated surface in a laminar flow hood. Then, the methylcellulose discs were implanted on the CAM, the eggs were sealed with ad-

hesive tape and returned to the incubator for 48 h. Negative controls were always made with DMSO mixed with the methylcellulose. Five eggs were used for each tested dose of AE. After the reincubation, CAMs were examined under a stereomicroscope. The assay was scored as positive when two independent observers reported a significant reduction of vessels in the treated area. In our hands, this procedure yields no false positive.

***In vitro* angiogenesis assays.** A number of *in vitro* assays were carried out to test the specific effects of AE treatment on several key steps of the angiogenic process in both endothelial and tumor cells. These assays included MTT cell proliferation assay, tube formation by endothelial cells on Matrigel, zymographic assays for the detection of gelatinases and urokinase in conditioned media of control and AE-treated cells, and fluorescent cell migration and invasion assays. We have described all of these assays extensively elsewhere [19, 23]. In these *in vitro* assays, AE treatments were carried out under conditions (AE concentration and time of treatment) that did not produce any cytotoxic effect on cells.

**Photocytotoxicity assay.** Human U2-OS osteosarcoma and HT-1080 fibrosarcoma cells were seeded in 96-well plates at  $4 \times 10^3$  cells per well. After 24 h of incubation, culture medium was removed and serial dilutions of AE (each concentration in quadruplicate) in culture medium were tested at a final volume of 0.1 mL per well. After another 24-h incubation, medium was removed, wells were gently washed with PBS, and new culture medium (now without AE) was added. Plates were exposed for 1 h to a photoactivation treatment with two sources of white light (over and below the plates), Sylvania GTE standard F20W/154-RS daylight, with a light fluence rate of  $23.9 \text{ W/m}^2$ , maintaining a constant temperature of  $25^{\circ}\text{C}$  during the complete exposure period. Light fluence rate was measured with a spectroradiometer Sphereoptics SMS-500 (Sphereoptics-Hoffman, LLC, Contoocook, NH, USA). Control plates were not irradiated. Subsequently, a last 24-h incubation was carried out and the MTT assay was carried out.

**Statistics and image analysis.** All quantitative data are expressed as means  $\pm$  standard deviation (SD). Two-tailed Student's *t*-test was used for evaluations of pair of means, to establish which groups differed from the control group. Quantitative analysis of images was performed with the NIH Image 1.6 Program.

## Results and discussion

**AE inhibits *in vivo* angiogenesis in the CAM assay in a dose-dependent manner.** The CAM assay was used

to determine the ability of AE to inhibit angiogenesis *in vivo*. Table 1 shows that 50 nmol AE per CAM produced *in vivo* inhibition of angiogenesis in 60% of treated eggs, 30 nmol AE per CAM produced *in vivo* inhibition of angiogenesis in 50% of treated eggs, and 10 nmol AE did not produce any inhibition of angiogenesis in the CAM assay. These inhibitory doses are similar to those of other anti-angiogenic compounds found by us to inhibit angiogenesis in the CAM assay [19, 23], and are much lower (by three orders of magnitude) than the doses of the anti-angiogenic compound ursolic acid needed to inhibit angiogenesis in the CAM assay [21].

This *in vivo* assay identifies AE as a new anti-angiogenic compound, but gives no information on which specific steps of angiogenesis are targeted by AE. Very recently, it has been reported that emodin behaves as an anti-angiogenic compound by targeting vascular endothelial growth factor (VEGF)-induced endothelial cell proliferation, migration, invasion and tubule formation [27]. To get new, additional insights on the features of AE as an anti-angiogenic compound, and to compare them with those exhibited by emodin, we carried out a complete set of *in vitro* assays that we have previously used to characterize the anti-angiogenic effects of other compounds from natural sources, including aeroplysinin-1, homocysteine, ursolic acid, puerpene, hypericin and hyperforin [19–23, 28].

**AE inhibits both endothelial and tumor cell proliferation.** To characterize an anti-angiogenic compound, it is advisable to study its effects on the different steps involved in angiogenesis. Angiogenesis involves local proliferation of endothelial cells in response to an angiogenic stimulus. Viability assays are easily adapted to carry out fast, sensitive and efficient determinations of the effects of drugs on cell survival and/or proliferation. Therefore, these assays are often employed for primary screening of new drugs with potential pharmacological use. In fact, several of the best characterized compounds described unambiguously as anti-angiogenic were initially detected and selected for their capability to interfere with endothelial cell growth. This is the case for the extremely selec-

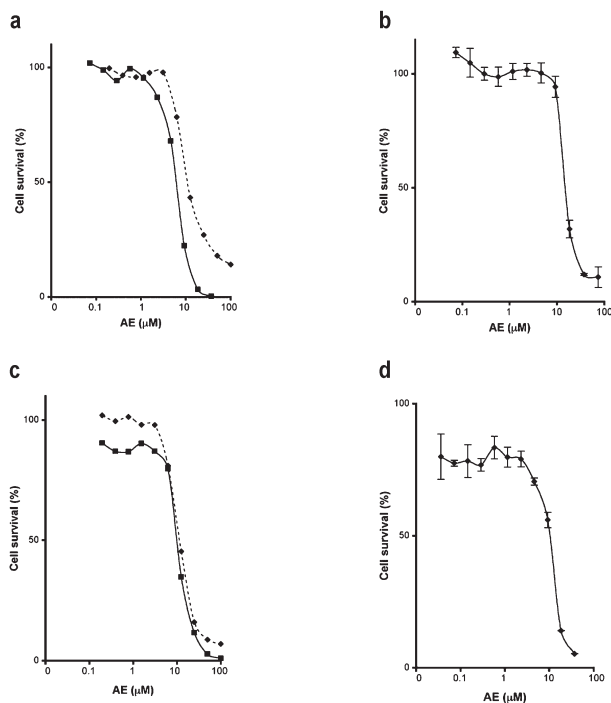
tive inhibitor of endothelial cell proliferation TNP-470, a synthetic analog of fumagillin with enhanced anti-angiogenic properties [29, 30]. Many other natural compounds with anti-angiogenic effects do, indeed, inhibit endothelial cell proliferation [16, 19, 31, 32]. However, although desirable, specificity for just endothelial cells is not common among the described anti-angiogenic compounds that inhibit cell proliferation [20].

We investigated the ability of AE treatment to inhibit the growth of endothelial and tumor cells. Figure 2 shows the mean survival curves obtained with the MTT assay for two endothelial cell primary cultures (BAEC and HUVEC, Fig. 2a and b) and two human tumor cell lines (HT-1080 fibrosarcoma and MDA-MB231 breast carcinoma, Fig. 2c and d), under both conditions of high and low proliferation rates (Fig. 2a and c). The estimated  $IC_{50}$  values are summarized in Table 2. All of them are in the micromolar range and there are not remarkable differences related to cell type and proliferation rate. These data point to a nonspecific cytotoxic effect of long-term (3 days) treatments with micromolar concentrations of AE, similar to those previously shown to exhibit cytotoxic effects on

**Table 1.** Effects of aloe-emodin (AE) on *in vivo* angiogenesis as determined by the chorioallantoic membrane (CAM) assay<sup>a</sup>.

Doses (nmol/egg)	Inhibition		
	Negative	Slight	Clear
10	2/2	0/2	0/2
30	2/4	1/4	1/4
50	2/5	1/5	2/5

<sup>a</sup> Data are the number of CAM eggs exhibiting no inhibition (Negative), slight inhibition (Slight) or clear inhibition (Clear) of angiogenesis.



**Figure 2.** Survival curves of endothelial and tumor cells treated with AE. (a) Bovine aortic endothelial cells (BAEC); (b) human umbilical vein endothelial cells (HUVEC); (c) HT-1080; (d) MDA-MB231. AE concentrations are represented on logarithmic scale. Experiments with controlled culture conditions to warrant low control proliferation rates were carried out with BAEC and HT-1080 cells (curves in a and c with experimental points represented as diamonds). Depicted data are means of values (with coefficient of deviation values among independent experiments lower than 20%) of four (two in the cases of low control proliferation rates) independent experiments with quadruplicate samples each.

**Table 2.** Effects of AE on endothelial and tumor cell growth<sup>a</sup>.

Cell line	IC <sub>50</sub> (μM)
BAEC	6 (13*)
HUVEC	14
HT-1080	10 (12*)
MDA-MB231	11

<sup>a</sup>IC<sub>50</sub> values were calculated from dose-response survival curves (Fig. 2) as the concentrations of AE yielding a 50% of control cell survival. \*Values in parenthesis correspond to experiments with controlled culture conditions to warrant low control proliferation rates. IC<sub>50</sub> values are expressed as means values (with coefficient of deviation values among independent experiments lower than 20%) of four (two in the cases of low control proliferation rates) independent experiments with quadruplicate samples each.

other tumor cell cultures and to induce apoptosis and autophagy in the C6 glioma cell line [13, 33]. This cytotoxic effect seems very different to the described inhibition by emodin of VEGF-induced endothelial cell proliferation, which leads to endothelial cell arrest in G<sub>0</sub>/G<sub>1</sub> phase of cell cycle [27].

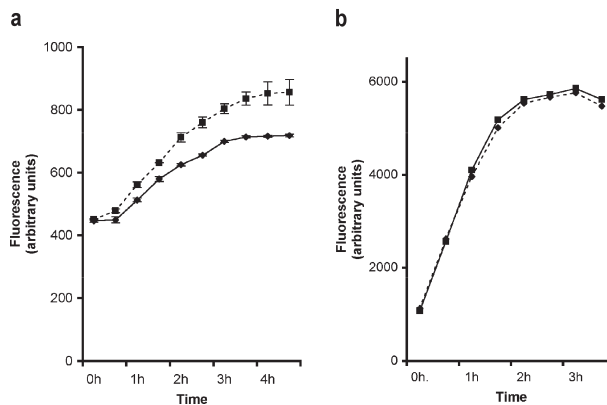
#### AE is a weak inhibitor of endothelial cell migration.

Cell migration is a key step shared by both angiogenesis and tumor progression [34, 35]. Figure 3 shows the effects of AE on both endothelial cell (BAEC, Fig. 3a) and tumor cell (HT-1080, Fig. 3b) migration, as determined by a continuous fluorescence assay. These results clearly show that 25 μM AE is only a weak inhibitor of endothelial cell migration. However, it must be underscored that this effect does seem to be endothelial cell specific, since no effect was found on tumor cell migration. On the other hand, these effects are different to those previously reported for emodin, in which only VEGF-induced migration was tested in a wound healing repair assay, showing that 5 μM emodin was able to completely suppress this VEGF-induced migration of human umbilical endothelial cells [27].

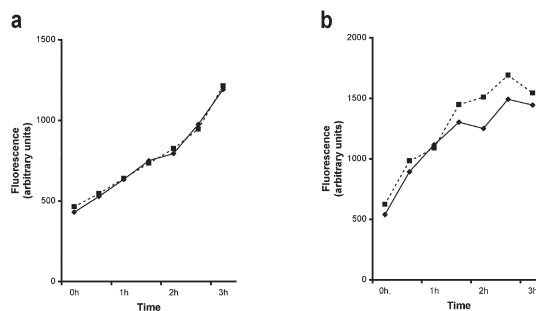
#### AE does not inhibit endothelial and tumor cell invasion.

Cell invasion is another key step shared by both angiogenesis and tumor progression. Figure 4 shows the effects of AE on both endothelial cell (BAEC, Fig. 4a) and tumor cell (HT-1080, Fig. 4b) invasion, as determined by a continuous fluorescence assay. These data clearly show that AE has no relevant anti-invasive effect, in contrast to other anti-angiogenic compounds previously described [19, 26, 28, 31, 36, 37].

**AE has different effects on endothelial and tumor cell gelatinases.** Matrix metalloproteinases 2 and 9 (MMP-2, MMP-9), commonly named gelatinases, are two key extracellular enzymes involved in ECM remodeling, an essential step required for both angiogenesis and metastasis [38–40].



**Figure 3.** Effects of AE on endothelial and tumor cell migration. Migrating controls and 25 μM AE-treated BAEC (a) and HT-1080 (b) cell values are represented by squares and diamonds, respectively. Data are given as arbitrary units of fluorescence and they are means ± S.D. of three different samples.

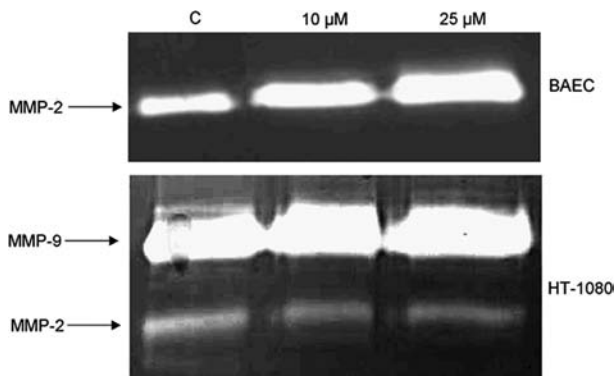


**Figure 4.** Effects of AE on endothelial and tumor cell invasion. Invading controls and 25 μM AE-treated BAEC (a) and HT-1080 (b) cell values are represented by squares and diamonds, respectively. Data are given as arbitrary units of fluorescence and they are means ± S.D. of three different samples.

Figure 5 shows that AE has very different effects on the secretion of gelatinases to conditioned media from endothelial (BAEC) and tumor (HT-1080) cells. The quantification of the bands is reported in Table 3. BAEC cells only express MMP-2. As shown in Figure 5 and Table 3, AE treatment induces a dose-dependent increase in the levels of MMP-2 in BAEC conditioned media. On the other hand, human HT-1080 fibrosarcoma cells do express both MMP-9 and MMP-2 activities. Figure 5 and Table 3 show that AE treatment has no significant effect on HT-1080 cell MMP-9 levels. HT-1080 cell MMP-2 levels are decreased, in contrast with the inducing effect observed in BAEC conditioned media.

It is noteworthy that the effects of AE treatment on endothelial cell MMP-2 levels here shown are very different to the total inhibitory effect produced by 5 μM emodin [27].

**AE is a potent inhibitor of urokinase secretion.** Urokinase (a serine protease) is another key extracellular enzyme involved in ECM remodeling related to angiogenesis



**Figure 5.** Effect of AE on endothelial and tumor cell gelatinase production. Controls and samples from 10 and 25  $\mu\text{M}$  AE-treated BAEC and HT-1080 cells after 24 h of treatment are shown.

**Table 3.** Quantification of the bands of gelatinase activity in conditioned media of endothelial and tumor cells treated with AE<sup>a</sup>.

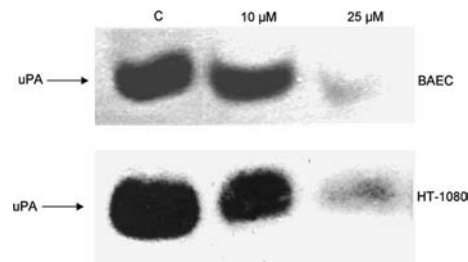
Cell line	Gelatinase	Activity (%)	
		10 $\mu\text{M}$ AE	25 $\mu\text{M}$ AE
BAEC	MMP-2	155	206
HT-1080	MMP-9	119	114
	MMP-2	55	71

<sup>a</sup> Bands of activity in the gels shown in Figure 5 were quantified using image analysis and their values were expressed as the percentage of gelatinase activity in conditioned media from control, untreated cells.

and metastasis [41–43]. However, very recently it has been shown that urokinase is not a target for emodin, since up to 25  $\mu\text{M}$  emodin treatments produce no decrease of urokinase proteolytic activity levels [27]. This is not the case for AE treatment. Figure 6 and Table 4 show that AE treatment induces a dose-dependent decrease in the levels of urokinase in both BAEC and HT-1080 cell conditioned media, with an almost complete inhibition at 25  $\mu\text{M}$  AE.

These data strongly suggest that urokinase is one of the main key targets of the pharmacological action of AE.

**AE inhibits tubule formation of endothelial cells on Matrigel.** The final event during angiogenesis is the organization of endothelial cells in a three-dimensional network of tubes. *In vitro*, endothelial cells plated on Matrigel align themselves forming cords. In our hands, these tubule-like structures are much more stylized with HUVEC than with BAEC [19]. Figure 7 shows that treatment with 25  $\mu\text{M}$  AE for 24 h completely inhibits tubule formation of HUVEC on Matrigel. A quantification of the density of tubules also shows that a treatment with 10  $\mu\text{M}$  AE produced a 30% decrease as compared to control, untreated HUVEC (result not shown). The AE concentrations required to inhibit the differentiation of HUVEC cells did not affect their viability after 24 h (results not

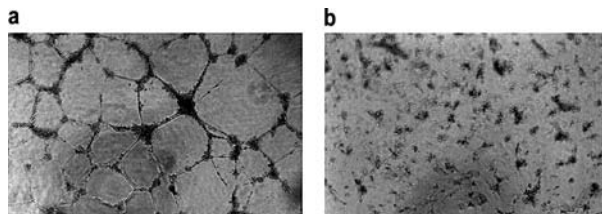


**Figure 6.** Effect of AE on endothelial and tumor cell urokinase production. Controls and samples from 10 and 25  $\mu\text{M}$  AE-treated BAEC and HT-1080 cells after 24 h of treatment are shown. uPA: urokinase.

**Table 4.** Quantification of the bands of urokinase activity in conditioned media of endothelial and tumor cells treated with AE<sup>a</sup>.

Cell line	Urokinase activity (%)	
	10 $\mu\text{M}$ AE	25 $\mu\text{M}$ AE
BAEC	94	18
HT-1080	66	15

<sup>a</sup> Bands of activity in the gels shown in Figure 6 were quantified using image analysis and their values were expressed as the percentage of urokinase activity in conditioned media from control, untreated cells.



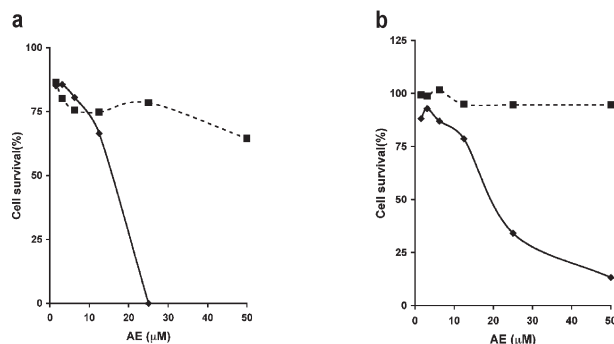
**Figure 7.** Inhibition by AE of endothelial tubule formation on Matrigel. (a) Control, untreated HUVEC cells 24 h after seeding over Matrigel. (b) HUVEC seeded over Matrigel and treated with 25  $\mu\text{M}$  AE for 24 h.

shown). Therefore, AE treatment has another key target in this essential step of the angiogenesis process, in addition to its potent inhibitory effect on urokinase.

#### **AE produces a photocytotoxic effect on tumor cells.**

Due to its anthraquinone chemical structure, AE photoexcitation could result in cytotoxicity. It has been shown that photoexcitation of AE does induce cytotoxicity and photo-oxidative damage of RNA and DNA in human skin fibroblasts, through the formation of singlet oxygen [44, 45]. Figure 8 shows that photoexcited AE is much more cytotoxic than unexcited AE for human cancer cells. This remarkable result suggests that AE could be a candidate for photodynamic therapy for some kinds of cancer [46].

**Concluding remarks.** Our studies clearly show that AE is another natural anti-angiogenic compound with a nar-



**Figure 8.** Photocytotoxicity of photoexcited AE on tumor cells. Survival curves are depicted for human HT-1080 fibrosarcoma (a) and U2-OS osteosarcoma (b) cells both in the presence of unexcited AE (squares) and photoexcited AE (diamonds). Data are means of four different determinations, and they are shown as percentage of the values obtained with their respective controls of cells in the absence of AE.

rower spectrum of targets than many others [19, 20, 23, 28]. Its effects on tumor cells agree with the previous suggestion that AE also behaves as an anti-tumor compound [9–13]. The differences observed between the effects of AE on endothelial *versus* tumor cells open a window for its potential therapeutical application as either an anti-angiogenic or an anti-tumor drug. On the other hand, AE photoexcitability and its demonstrated photocytotoxic effects on tumor cells point to AE as a potential candidate drug for photodynamic therapy. Finally, in spite of the structural similarity of AE and emodin, their respective anti-angiogenic effects are very different, which suggests that they target different steps of the angiogenic process.

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