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# Flavonoids Inhibit Angiogenic Cytokine Production by Human Glioma Cells

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**VEGF and TGF-β1 are cytokines that stimulate tissue invasion and angiogenesis. These factors are considered as molecular targets for the therapy of glioblastoma. Bevacizumab, a recombinant humanized monoclonal antibody developed against VEGF, inhibits endothelial cell proliferation and vessel formation. Flavonoids obtained from *Dimorphandra mollis* and *Croton betulaster* have been described as proliferation inhibitors of a human glioblastoma derived cell line. VEGF and TGF-β1 levels were dosed by ELISA in a GL-15 cell line treated with bevacizumab and also with the flavonoids rutin, 5-hydroxy-7,4′-dimethoxyflavone, casticin, apigenin and penduletin. Rutin reduced the VEGF and TGF-β1 levels after 24 h but not after 72 h. The other flavonoids significantly reduced TGF-β1 production. Bevacizumab reduced only the VEGF levels in the supernatant from GL-15 cultures. These results suggest that the flavonoids studied, and commonly used in popular medicine, present an interesting subject of study due to their potential effect as angiogenic factor inhibitors. Copyright © 2010 John Wiley & Sons, Ltd.**

*Keywords:* angiogenesis; angiogenic factor inhibitors; flavonoids; VEGF; TGF-β1; glioblastoma.

## INTRODUCTION

Tumors must generate a new vascular supply to grow beyond a critical size and for metastasis. They do so by secreting proangiogenic cytokines that promote the new formation of blood vessels (Folkman, 1985). Angiogenesis, the formation of new blood vessels from existing vasculature, is fundamental for a variety of physiological and pathological processes including tumor growth and metastasis (Folkman, 1995; Ferrara, 2001). Malignant gliomas are typically angiogenic tumors and express great amounts of angiogenic factors (Hanahan and Folkman, 1996).

Among these factors, vascular endothelial growth factor (VEGF) and transforming growth factor-β1 (TGF-β1) are preeminent glioblastoma-associated multifunctional cytokines that stimulate migration, tissue invasion and angiogenesis. VEGF is a secreted heparin-binding glycoprotein and one of the most potent endothelial cell-specific mitogens. It is known to play a key role in tumor angiogenesis (Ferrara and Henzel, 1989; Ferrara and Davis-Smyth, 1997). It is not only a potent and specific mitogen to endothelial cells but also increases vascular permeability and promotes extravasation of proteins from tumor vessels, thus playing a pivotal role in effusion formation (Senger *et al*., 1983; Dvorak *et al*., 1995; Mesiano *et al*., 1998). Several studies have found a strong association between high tumor VEGF expression and advanced tumor stage or poor survival (Salven *et al*., 1997; Tanigawa *et al*., 1997; Chow *et al*., 1999). Bevacizumab (Avastin®, Roche), a recombinant humanized monoclonal antibody developed against VEGF, binds to soluble VEGF, preventing receptor binding and inhibiting endothelial cell proliferation and vessel formation (Ranieri *et al*., 2006). TGF-β1 overexpression has been associated with several cancers and correlates with tumor progression, angiogenesis and poor prognosis (Bierie and Moses, 2006). It has featured prominently amongst the cytokines studied for its capacity to regulate new blood vessel formation *in vitro* and *in vivo*. The role of TGF-β1 in carcinogenesis is complex with the suppressor activities dominating in normal tissue, but inducing cell proliferation in tumorigenesis, due to changes in the TGF-β1 signaling pathways (Oft *et al*., 1998; Gordinier *et al*., 1999). TGF-β1 increased expression in cancer has been reported (Gordinier *et al*., 1999; Ivanović *et al*., 2003) as well as its role in angiogenesis and the relationship between its expression and VEGF up-regulation in cancer (Cheng *et al*., 2000; Vinals and Pouyssegur, 2001; Xiong *et al*., 2002). Therefore, these cytokines are considered to be molecular targets for the therapy of glioblastoma (Farhadi *et al*., 2005; Wick *et al*., 2006).

Flavonoids are polyphenolic compounds largely found in plants. Microbicidal and antineoplastic

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properties have been attributed to these substances (Boudet, 2007). Rutin is a glycosylated flavonoid, also known as quercetin 3-*O*-rutinoside (Martinez-Flores *et al*., 2002). In Brazil, it is extracted from the seeds of *Dimorphandra mollis* (Leguminosae-Mimosoidae), a typical 'cerrado' region tree (Lorenzi, 1949; Schultz, 1984), and commercialized as a phytotherapeutic drug for the treatment of vascular disorders and hypertension. The flavonoids 5-hydroxy-7,4′-dimethoxyflavone, casticin, apigenin and penduletin were extracted from *Croton betulaster* Müll. Arg., a shrub from the Euphorbiaceae family species found in the 'cerrado' region of Bahia-Brazil (Cordero, 1995) but also found in a large variety of plants around the world (Hajdú *et al*., 2007; Han *et al*., 2007).

The present study evaluated the *in vitro* effects of those flavonoids and of bevacizumab, on the production of angiogenic cytokines by a human glioblastoma derived cell line (GL-15).

## MATERIAL AND METHODS

**Cell culture.** GL-15, a human glioblastoma derived cell line, was maintained in a humidified atmosphere composed of 95% air and 5% $CO_2$ at 37°C in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, 100 units/mL penicillin G, 100 μg/mL streptomycin, 7 mM glucose, 2 mM glutamine and 0.011 g/L pyruvic acid.

**Flavonoids extracts.** The flavonoid rutin was extracted from *Dimorphandra mollis* seeds by recrystallization in tetrahydrofuran (THF) according to Merck SA procedures (98% purity). The air-dried leaves (600.0 g) of *C. betulaster* were extracted with hexane three times and the solutions were concentrated under vacuum. This procedure was repeated with dichloromethane and with methanol. The repeated silica gel column chromatography of the dichloromethane extract from the leaves of *C. betulaster* with a gradient of hexane–EtOAc successfully gave acacetin (10 mg), genkwanin e 7-methyl ether acacetin (40 mg), casticin (449 mg), penduletin (81 mg), salvigenin and 5-hydroxy-3,6,7,4′-tetramethoxyflavone (7 mg), 5-hydroxy-3,6,7,4′-tetramethoxyflavone (9 mg) pectolinarigenin and santin (3 mg). The methanol extract was dissolved in MeOH–$H_2O$ (9:1) and partitioned against hexane, dichloromethane, AcOEt and *n*-butanol. The AcOEt-soluble portion from the methanol extract was chromatographed on a silica gel column eluted with gradient of hexane–EtOAc and gave apigenin (5 mg) and apigenin-8-C-*β*-D-glucopyranoside (30 mg) (Barbosa *et al*., 2000).

The *Croton betulaster* flavonoids 5-hydroxy-7,4′-dimethoxyflavone, casticin (3′,5-dihydroxy-3,4′,6,7-tetramethoxyflavone), apigenin (5,7,4′-trihydroxyflavone) and penduletin (4′,5-dihydroxy-3,6,7-trimethoxyflavone) obtained as described above were dissolved in dimethyl sulfoxide (DMSO) and used to treat the GL-15 cells.

**Flavonoids cells treatment.** The cell cultures were grown to confluence in 35 mm polystyrene plates and treated with rutin, 5-hydroxy-7,4′-dimethoxyflavone, casticin, apigenin and penduletin at concentrations of 50 μM and 100 μM for 24 and 72 h. At the end of the incubation periods, the supernatants were collected and stored at −20°C until required. Cultures without treatment or treated with DMSO were used as a control. All assays were performed at least three times.

**Anti-VEGF treatment.** In order to observe whether VEGF inhibition could affect TGF-β1 secretion, the GL-15 cultures were treated with bevacizumab (Avastin®, Roche), an anti-VEGF humanized monoclonal antibody, diluted in culture medium at final concentrations of 0.1,1.0 and 10 μg/mL and incubated for 24 and 72 h.

**Cytokine dosages.** The VEGF and TGF-β1 levels were measured in GL-15 cultures supernatants using ELISA (Quantikine, R&D Systems, Minneapolis, MN), according to the manufacturer's instructions.

Briefly, the samples of supernatants were placed in 96-well plates coated with monoclonal detective antibodies and were incubated for 2 h. To determine TGF-β1 levels the samples were previously submitted to acidic treatment in order to activate the latent form of this growth factor. After washing with PBS, conjugated horseradish peroxidase antibody was added to bind to the cytokines. After incubation and washing a chromogenic substrate was added and the absorbance of each well was measured at 450 nm. The concentrations of VEGF and TGF-β1 were determined by interpolating from standard curves obtained with known concentrations of standard protein.

**Statistical analysis.** Statistical significance between two groups (defined as $p = 0.05$) was evaluated using the Mann-Whitney *U*-test.

## RESULTS

### Flavonoid effects

In the presence of the rutin, GL-15 glioma cells produced lower levels of VEGF at 50 μM and 100 μM, at 24 h (median 484.5 pg/mL and 586.6 pg/mL, respectively) when compared with non-treated controls (median 1823.6 pg/mL). TGF-β1 secretion was also inhibited by the two concentrations of rutin in this period (median 1038.4 pg/mL and 877.7 pg/mL). However, at 72 h, the inhibition was reversed and the VEGF and TGF-β1 levels were similar in the supernatants from treated and non-treated cultures (Fig. 1).

The other flavonoids analysed did not inhibit significantly VEGF secretion by GL-15 cells at 24 and 72 h at 50 μM or 100 μM concentrations. Casticin, apigenin and penduletin, but not 5-hydroxy-7,4′-dimethoxyflavone, significantly reduced TGF-β1 levels in both periods studied (Figs 2 and 3).

### Bevacizumab

It was observed that the three adopted concentrations of bevacizumab (0.1, 1.0 and 10 μg/mL) significantly

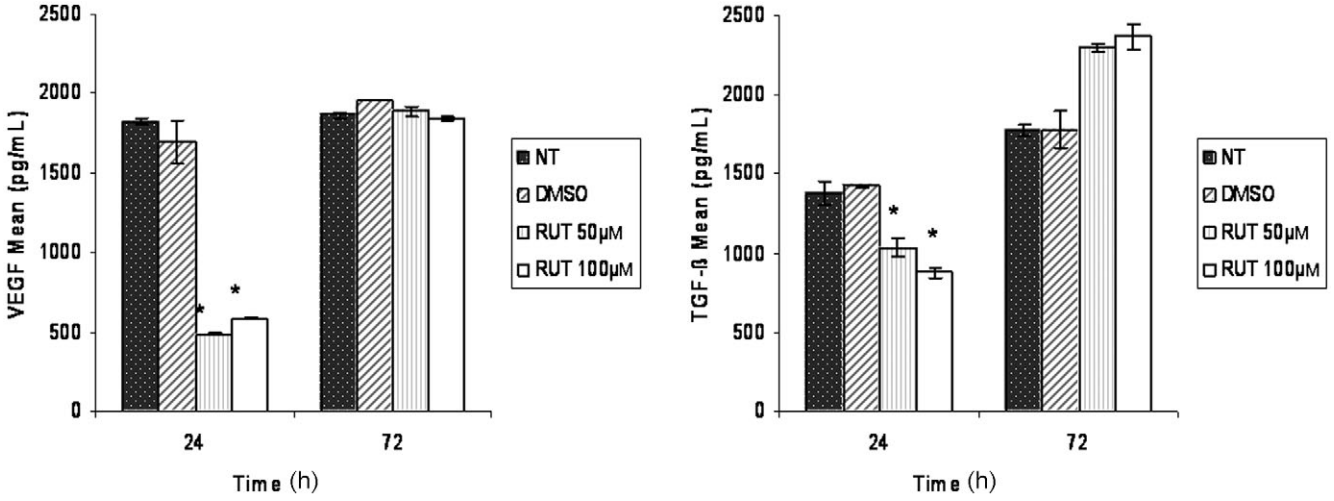


**Figure 1.** GL-15 cells treated with rutin (RUT) showed reduced VEGF and TGF-β1 production after 24 h but not after 72 h. NT, no treatment; DMSO, dimethyl sulfoxide; $*p \leq 0.05$.

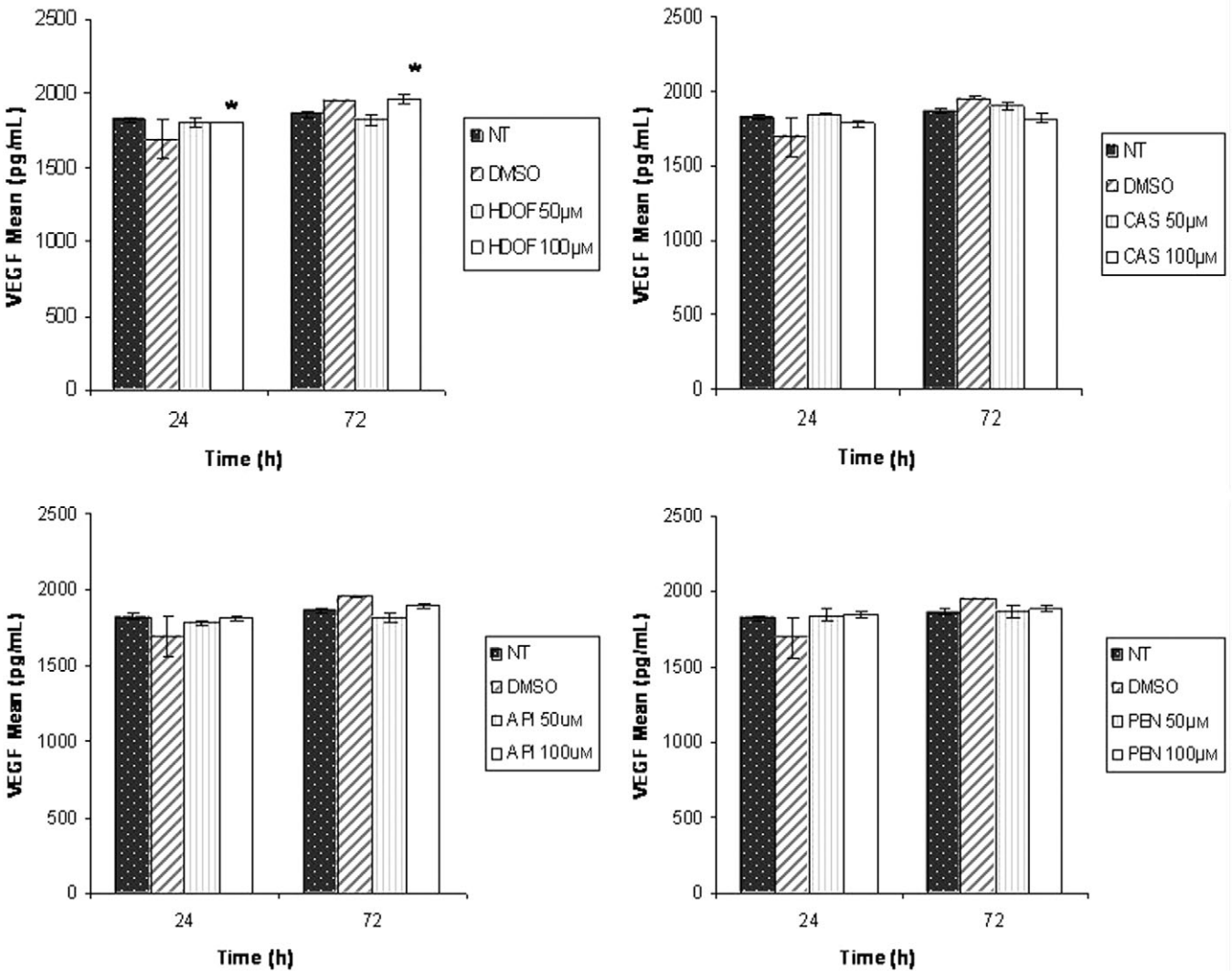


**Figure 2.** VEGF levels in GL-15 supernatant were not altered by treatment of cells with the *C. betulaster* flavonoids 5-hydroxy-7,4′-dimethoxyflavone (HDOF), casticin (CAS), apigenin (API) and penduletin (PEN).

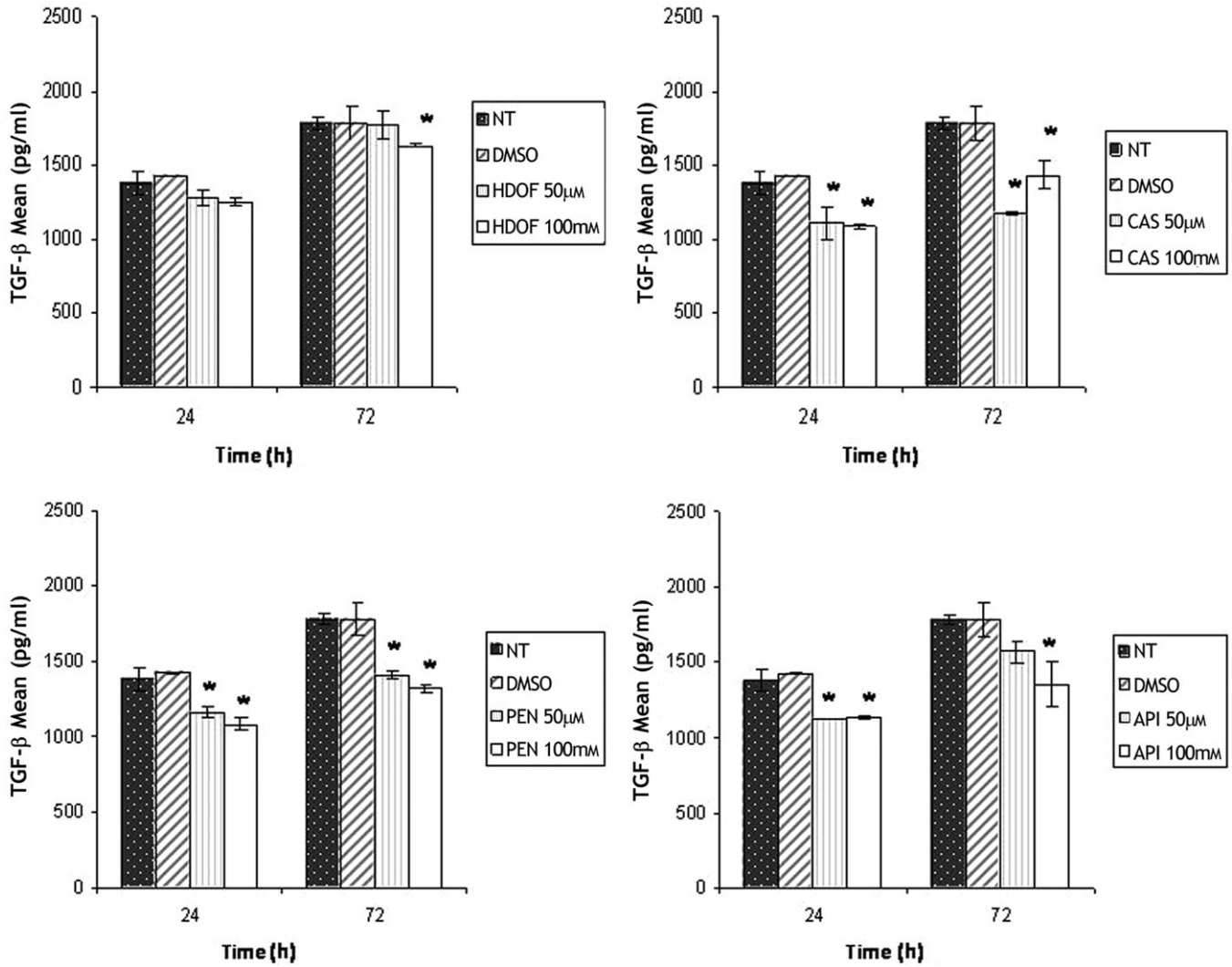


**Figure 3.** The treatment with 5-hydroxy-7,4′-dimethoxyflavone (HDOF) had no effect on TGF-β1 levels. The other flavonoids extracted from *C. betulaster* casticin (CAS), apigenin (API) and penduletin (PEN) reduced TGF-β1 production by GL-15.

reduced the levels of VEGF ($p < 0.01$) in the supernatant of GL-15 cultures in a dose-dependent manner at 24 and 72 h but had no effect on the TGF-β1 production (Fig. 4).

## DISCUSSION

Malignant glioblastoma multiforme (GBM) is one of the most lethal forms of adult human cancers. The median survival rate of one year has remained essentially unchanged for a large number of years despite aggressive treatment regimens that include surgery, radiation and chemotherapy.

The anticancer effects of some nutrients or traditional medicinal plants have generated much investigation in order to discover new therapeutic agents (Barbosa *et al*., 2003). Apigenin, a common dietary flavonoid, widely distributed in many fruits and vegetables possesses antitumor properties against, especially, prostate cancer (Cordeiro, 1995). Casticin, another flavonoid, inhibits mouse lymphocyte growth and proliferation of human tumor cells (Scheck *et al*., 2006) and penduletin presents an inhibitory effect on mouse tsFT210 cancer cells (Fang *et al*., 2007).

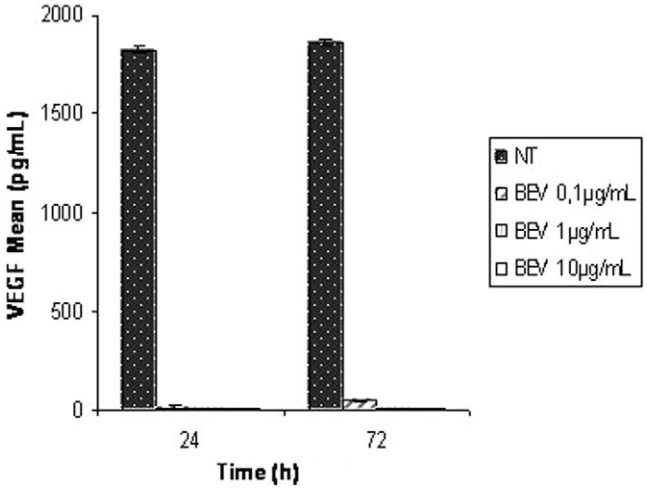


**Figure 4.** VEGF was not detected in the supernatant from GL-15 culture treated with 0, 1 μg, 1 μg and 10 μg/mL of bevacizumab (BEV). NT, no treatment.

Angiogenesis appears as a key target due to its involvement in solid tumor growth and dissemination. The prevention of neovascularization might be achieved by reducing the level of expression of tumor derived angiogenic factors (Haïdara *et al*., 2006).

Molecules such as flavonoids, antioxidants and retinoids have been shown to act in the tumor microenvironment (Li *et al*., 2005). Flavonoids are a large group of aromatic plant secondary metabolites that are produced by the plant for the purpose of protection against photosynthetic stress, reactive oxygen species and wounds. They have been shown to inhibit cancer development in various animal models, exhibiting antioxidant activities and have produced the most compelling data for the antitumor activities of plant secondary metabolites in various types of cancers (Noonan *et al*., 2007). Flavonoids extracted from *Dimorphandra mollis* and *Croton betulaster*, native medicinal plants from Bahia-Brazil, have been described as inhibitors of proliferation of a human glioblastoma derived cell line (Kuo *et al*., 1997; Lahiri-Chatterjee *et al*., 1999).

In the present study, it was shown that rutin reduced the level of expression of VEGF and TGF-β1 production in the GL-15 GBM human cell line, after 24 h of treatment. These results are partly in accord with those of Schindler and Mentlein (Costa *et al*., 2008) that showed an inhibitory effect of rutin on VEGF production by MDA human breast cancer cells and by glioma cells. In our study, this effect disappeared after 72 h of contact, underlining either the instability of the molecule during the time course of the culture, or the development of a resistance to the drug or even a capacity of the cells to metabolize the drug.

The 5-hydroxy-7,4-dimethoxyflavone had no effects on VEGF or TGF-β1 expression by GL-15 cells. However, the other flavonoids analysed – casticin, apigenin and penduletin – significantly reduced TGF-β1 release, without modifying VEGF expression. The effect on TGF-β1 release was maintained during the whole experimental time period. These results sustain the hypothesis of Schindler and Mentlein who suggested a relationship between the sugar moiety of the flavonoids, present in rutin, but not in the other studied flavonoids, and their suppression of VEGF secretion (Schindler and Mentlein, 2006).

It has been proposed that TGF-β1 increases VEGF synthesis and a relationship between the effects of TGF-β1and VEGF had also been demonstrated on endothelial cells, through a cascade of autocrine or paracrine reactions (Ferrari *et al*., 2006). Because of this modulation, TGF-β1 levels were also dosed in bevacizumab treated cells. The results obtained with bevacizumab, which completely blocked VEGF but did not have any effect on TGF-β1 levels, did not confirm such a relationship in the GL-15 glioma cell line.

Several reports show the anticancer action of those flavonoids as antiproliferation or apoptosis induction (Li *et al*., 2005; Csupor-Löffler *et al*., 2008) but, for the first time, VEGF and TGF-β1 production and the effect of flavonoids on TGF-β1 levels expressed by this glioma cell line has been shown. These results suggest that flavonoids present are of interest for glioma treatment due their potential inhibitory effects on angiogenic factors.

## Conflict of Interest

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

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