# Isoquercitrin isolated from *Hyptis fasciculata* reduces glioblastoma cell proliferation and changes β-catenin cellular localization

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**Isoquercitrin isolated from the aerial parts of *Hyptis fasciculata* was evaluated according to its capacity to interfere with glioblastoma (Gbm) cell growth. Gbm cells were incubated with isoquercitrin, quercetin, or rutin at concentrations of 25, 50, and 100 μmol/l for 24, 48, and 72 h. Quercetin and rutin affected Gbm cell proliferation after treatment times of longer than 24 h. However, increasing concentrations of isoquercitrin inhibited 50% of Gbm cell proliferation at 24 h and further reached nearly 90% inhibition at 72 h. This effect did not affect cell morphology, cell viability, or cleaved capase-3 levels, indicating that isoquercitrin did not induce Gbm cell death. A marked reduction in cyclin D1 levels and an increase in p27 levels were observed when 100 μmol/l of isoquercitrin was added to Gbm cells. Interestingly, nuclear β-catenin staining observed in a subpopulation of untreated Gbm cells was found in the cytoplasm after 100-μmol/l isoquercitrin treatment. Collectively, these data show that isoquercitrin reduces Gbm cell growth without inducing apoptosis, possibly by modulating the control of the cell cycle. Our data also suggest that β-catenin-mediated signaling may be involved on the antiproliferative activity of isoquercitrin. *Anti-Cancer Drugs* 20:543–552 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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## Introduction

Flavonoids are polyphenolic compounds found throughout the plant kingdom. Within individual plants they occur in every organ but are usually concentrated in the leaves and flowers [1]. Various epidemiological and dietary studies suggest that flavonoids may be efficient in preventing neurodegeneration and motor decline and may also protect against oxidative stress [2]. In addition, it has been shown that flavonoids and other polyphenolic compounds have the ability to influence processes that are deregulated during cancer development [3]. Various mechanisms by which flavonoids may affect tumorigenesis have been proposed, including antioxidant activities [4], the scavenging effect on activated mutagens and carcinogens [5,6], interaction with proteins that control cell cycle progression [7], and alteration of gene expression [8]. All these studies point to flavonoids as promising compounds for prevention as well as treatment of a wide range of human pathologies including cancer. However, as the biochemical activities of these compounds are dependent on the individual flavonoid structure, each compound needs to be studied systematically to assess its individual biological potency.

Quercetin (3,3′,4′,5,7-pentahydroxyflavone) is the major flavonoid found in the plant kingdom and it may be a powerful bioactive constituent of the human diet because of its excellent free radical scavenging activity [9]. The flavonoid isoquercitrin (quercetin 3-*O*-β-D-glucopyranoside), which is typically found in onions, is a quercetin moiety with a glucose molecule on C3 of the flavonoidic nucleus [10]. Several studies have shown that quercetin and its related compounds can induce growth inhibition and cell death in a variety of cancer cells [11–13]. For instance, dietary administration of quercetin and rutin prevents chemically induced carcinogenesis in rodents [14]. In addition, it has been recently reported that quercetin can trigger and/or stimulate glioma cells to undergo apoptosis using a mechanism that involves MAPK, Akt, and survivin [15,16]. Although quercetin represents a promising compound to be studied as a possible anticancer drug, the effects of isoquercitrin, particularly in brain tumors, remains poorly explored.

Glioblastoma (Gbm) is the most common subtype of primary brain tumors and is characterized by a high proliferative index, aggressive invasiveness, and short

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survival prognosis, being considered as one of the deadliest of human cancers [17]. Gbm is largely refractory to radiation and other adjuvant therapies in use today and accounts for poor survival rate in affected patients, which in many cases is lethal in less than 24 months of disease onset [18]. As current evidence from different studies suggest that brain tumors arise from a diverse set of molecular abnormalities, the control of apoptotic and cell cycle regulators in Gbm cells have gained new interest as a potential chemotherapy alternative.

This study investigates the effects of isoquercitrin, a flavonoid that has been isolated from the aerial parts of *Hyptis fasciculata*, in Gbm cell culture. Treatment of Gbm cells with increasing concentrations of isoquercitrin under varying culture times did not affect cell viability or morphology, but significantly reduced cell proliferation. Inhibition of proliferation induced by isoquercitrin was followed by the reduction of cyclin D1 levels and increasing p27 levels. In addition, isoquercitrin treatment affected β-catenin cellular localization in a subset of Gbm cells. The data presented here show that isoquercitrin presents antiproliferative properties and that these effects may be mediated through β-catenin signaling.

## Materials and methods

### Chemicals and reagents

All cell culture reagents were purchased from Invitrogen (Carlsbad, California, USA). Dimethyl sulfoxide (DMSO) was purchased from Sigma (St. Louis, Missouri, USA). Anti-cyclin D1, anti-β-catenin, anti-P27 Kip 1, and anticleaved caspase-3 were purchased from Cell Signaling (Danvers, Massachusetts, USA) and anti-ciclophylin-B was purchased from Affinity Bioreagents (Golden, Colorado, USA). All solvents and reagents used in this study were of analytical grade. Silica gel was purchased from Sigma and the deuterated solvents used for obtaining the nuclear magnetic resonance (NMR) spectra as well as the other solvents used to isolate and purify isoquercitrin were purchased from TEDIA (Fairfield, Ohio, USA).

### Plant material

*H. fasciculata* was collected in Campo Bom (Porto Alegre, Rio Grande do Sul, Brazil). After identification, a voucher sample 537 was deposited at the herbarium of the Botany department (UFRGS).

### Extraction, isolation, and identification of isoquercitrin

Alcoholic extract was performed according to the method described by Matheus *et al.* [19]. Briefly, flowers and leaves were dried and ground (1010 g) and extracted using 96% ethanol, at room temperature, by static maceration. The ethanol extract was evaporated under reduced pressure and resuspended in water. Thereafter, a liquid–liquid extraction procedure was performed using hexane, dichloromethane, ethyl acetate, and *n*-butanol to obtain extracts of different polarities [20]. The ethyl acetate extract was submitted to a chromatography procedure on a silica column (F1, 90.0 × 2.5 cm) and elution was performed using dichloromethane/methanol gradient. One fraction from F1 column was submitted to a new chromatography procedure on silica column (F2, 60.0 × 2 cm; hexane/ethyl acetate and ethyl acetate/methanol gradients). The fractions obtained (total of 116) were grouped according to chromatographic similarities on thin-layer chromatography. The two thin-layer chromatography analyses, followed by spraying with 20% sulfuric acid in ethanol, showed a yellow color in the fractions 73 and 74 that is characteristic of the presence of a flavonoid in these fractions. These fractions (73 and 74) were dried and after total solvent evaporation, a yellow solid was obtained. This solid material, after analyses by $^{1}$H and $^{13}$C-NMR spectroscopy methodologies, was identified as isoquercitrin (Table 1).

### Flavonoid treatment of human glioblastoma cell culture

In this study, we used a human Gbm cell line Gbm 95 [22]. Low-passage cultures (below 50 passages) were cultured in Dulbecco's modified eagle medium/F12 supplemented with 10% fetal bovine serum on a 24-well plate or 25 cm$^2$ tissue culture flasks (Corning, São Paulo, Brazil). The medium was changed every 3 days until the culture was near confluence, in approximately 7 days.

**Table 1 Comparison of data from $^{1}$H-NMR and $^{13}$C-NMR obtained for the samples isoquercitrin with data from the literature [21]**

| | Reference [21] | | Sample | |
|---|---|---|---|---|
| C/H | $^{1}$H-NMR | $^{13}$C-NMR | $^{1}$H-NMR | $^{13}$C-NMR |
| 2 | | 155.8 | | 156.3 |
| 3 | | 132.5 | | 133.3 |
| 4 | | 177.3 | | 177.5 |
| 4a | | 103.5 | | 103.9 |
| 5 | | 160.7 | 12.9 (*sl*) | 161.3 |
| 6 | 6.18 (*d*) *J*1.9 Hz | 98.2 | 6.19 (*d*) *J*1.79 Hz | 98.7 |
| 7 | | 163.6 | | 164.2 |
| 8 | 6.45 (*d*) *J*1.9 Hz | 93.0 | 6.40 (*d*) *J*1.56 Hz | 93.5 |
| 8a | | 155.7 | | 156.2 |
| 1′ | | 121.1 | | 121.2 |
| 2′ | 7.58 (*d*) *J*2.0 Hz | 114.7 | 7.57 (*m*) | 115.3 |
| 3′ | | 144.3 | | 144.8 |
| 4′ | | 148.0 | | 148.5 |
| 5′ | 6.85 (*d*) *J*8.4 Hz | 115.7 | 6.96 (*d*) J9.10 Hz | 116.2 |
| 6′ | 7.65 (*dd*) *J*2.0 and 8.4 Hz | 120.7 | 7.57 (*dd*) J2.27 and 9.10 Hz | 121.6 |
| $1_{Glu}$ | 5.33 (*d*) *J*7.8 Hz | 102.0 | 5.45 (*m*) | 100.9 |
| $2_{Glu}$ | 3.10–3.73 (*m*) | 73.6 | 3.0–3.8 (group of signals) | 74.1 |
| $3_{Glu}$ | | 77.1 | | 77.6 |
| $4_{Glu}$ | | 70.7 | | 69.9 |
| $5_{Glu}$ | | 77.1 | | 76.5 |
| $6_{Glu}$ | | 60.0 | | 61.0 |

*d*, doublet; *dd*, doublet of doublets; *J*, coupling constant; *m*, multiplet; NMR, nuclear magnetic resonance; *sl*, singlet.

Then, cell cultures were either split or frozen in media containing 50% glycerol, 50% growth medium in cryotubes, and conserved in liquid nitrogen [22]. When 70% confluence was reached, Gbm cells were treated with flavonoids isoquercitrin, quercetin, and rutin for 24, 48, and 72 h at concentrations of 25, 50, and 100 µmol/l, respectively. DMSO used as a vehicle to solubilize the flavonoids was added to the control cultures at 1% v/v.

### [$^3$H]-thymidine incorporation assay

When 70% confluence was reached, Gbm cells were treated with flavonoids isoquercitrin, rutin, and quercetin (25, 50, and 100 µmol/l). After treatment, a [$^3$H]-thymidine 6 h-pulse was added. At the end of the 24, 48, or 72 h of incubation, the culture medium was collected and the reaction was stopped using 300 µl of ice-cold 10% trichloroacetic acid. [$^3$H]-thymidine incorporation was measured on a scintillation counter.

### Western blotting analysis

Cell lysate samples from Gbm-treated cells were harvested in a sample buffer (0.02 mmol/l dithiothreitol; 1.38 mmol/l sodium dodecyl sulfate; 125 mmol/l Tris-HCl, pH 6.8 and 20% glycerol). Protein quantification was performed using the Lowry method [23] and 30 µg of the total lysate was loaded in 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then electroblotted and transferred to a polyvinylidene fluoride membrane (HybondTM-P, Amersham Biosciences, São Paulo, Brazil). Membranes were preblocked in 5% non-fat dry milk in Tris-buffered saline 0.001% Tween 20 for 1 h and then incubated overnight with anti-cyclin D1 (1 : 2000), anti-p27 (1 : 2000), anticleaved caspase-3 (1 : 500), and anti-cyclophilin-B (1 : 1000) primary antibodies previously diluted in Tris-buffered saline 0.001% Tween 20 5% milk. Secondary antibodies conjugated with horseradish peroxidase were used to probe the membranes and the reaction was visualized using SuperSignal West Pico Chemiluminescent Substrate Pierce, (Rockford, Illinois, USA).

### MTT assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was used to assay mitochondrial activity in viable cells. Cells were plated at a concentration of $1.0 \times 10^4$ cells/well in 96-well tissue culture plates in a medium containing 10% fetal bovine serum and cultured for 24 h before treatment with flavonoids (25, 50, and 100 µmol/l) for 24, 48, and 72 h. MTT was added to each well at a final concentration of 150 µg/ml 2 h before cell harvesting. The formazan reaction product was dissolved with DMSO and quantified spectrophotometrically at 570 nm using a UV–Vis system.

### Immunocytochemistry

Immunostaining was performed as described by Garcia-Abreu *et al.* [24,25]. Briefly, cells were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) of pH 7.6, washed with PBS, and permeabilized with 0.1% Triton X-100 in PBS for 5 min. Samples were then blocked for 1 h with PBS containing 5% bovine serum albumin. A rabbit anti-β-catenin (1 : 200) primary antibody was incubated for 1 h at room temperature. Specific secondary antibodies conjugated with Cy3 fluorochrome (1 : 10 000) were incubated for 1 h at room temperature. After PBS washes, slides were mounted and observed in a Nikon TE 2000 inverted microscope (Melville, New York, USA). Images were captured using a CoolSNAP-Pro (Media Cybernetics, Bethesda, Maryland, USA) digital camera.

### Statistical analysis

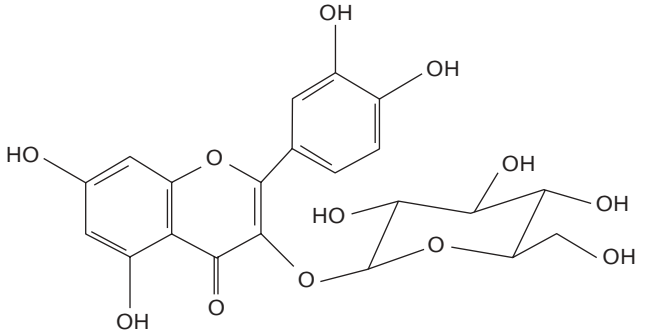
Each experiment was performed at least three times and triplicates were performed in [$^3$H]-thymidine and MTT assay. Cell staining quantification was performed by counting the number of DAPI and the number of non-nuclear and nuclear β-catenin staining in randomly chosen microscope fields, and then a percentage proportion of non-nuclear and nuclear over total cells was applied. Statistical analysis of the data was performed using the Mann–Whitney *U* test (GraphPad Prism version 4.00, Graphpad software Inc., 2003, La Jolla, California, USA). Statistical significance set at $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ was compared with vehicle treatment.

## Results

### Nuclear magnetic resonance identifies the isoquercitrin flavonoid

Despite its use in popular medicine in Brazil for the treatment of different diseases [26], there are only a few studies about the chemistry or pharmacology of *H. fasciculata* species. Thus, an ethanolic extract from the aerial parts of this plant was prepared and, after being dried and resuspended in water, a liquid–liquid extraction procedure was performed to obtain four new extracts with different polarities (hexane, dichloromethane, ethyl acetate, and *n*-butanol). The fractions 73 and 74 of the ethyl acetate extract of the aerial parts of *H. fasciculata* were analyzed through NMR (Table 1). The $^1$H-NMR spectrum shows two hydrogens at δ 6.19 and δ 6.40 with a coupling constant around 1.8 Hz indicative of a meta coupling and suggestive of hydrogens in the positions 5 and 7 of ring A (Table 1). Three other groups of signals at δ 6.96, 7.57, and 7.58 seem to belong to ring B of the flavonoid suggesting a substitution pattern with hydroxyl groups in the positions 3′ and 4′. It is also possible to see a group of signals referring to the hydrogens of glucose. This is confirmed by the signal at 5.45 with a coupling constant of 7 Hz, which represents the anomeric hydrogen of glucose in β-orientation. A comparison of both the $^1$H-NMR and $^{13}$C-NMR profiles for this molecule with previously published data [21] strongly suggests this compound to be isoquercitrin (Fig. 1).

**Fig. 1**



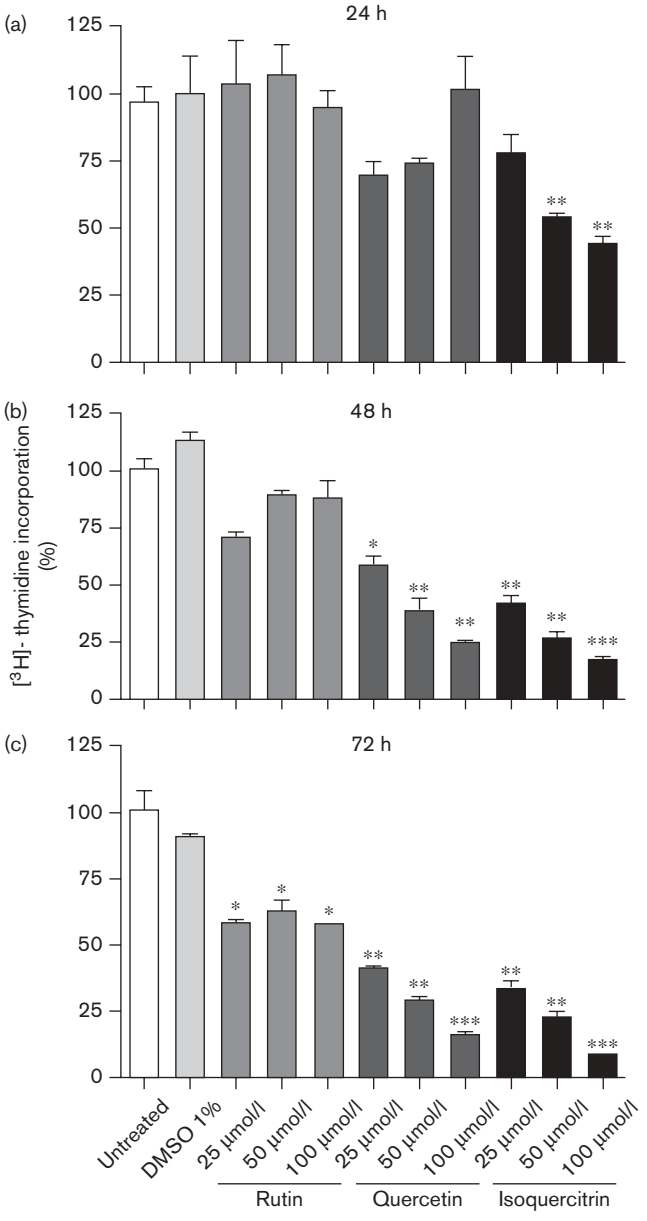

Structure of isoquercitrin.

Furthermore, the multiplicity shown in $^{13}$C-NMR using the attached proton test technique is the same as that seen for the isoquercitrin spectrum [27]. Taken together, the data indicate this molecule to be isoquercitrin with the predicted chemical structure shown in Fig. 1.

### Effects of isoquercitrin on glioblastoma cell proliferation

To investigate whether isoquercitrin isolated in this study had an effect on Gbm cell growth, we treated Gbm cultures with 25, 50, and 100 µmol/l of this flavonoid for 24, 48, and 72 h. To compare specific activities of isoquercitrin, commercial flavonoids rutin and quercetin were also tested in Gbm cells at the same concentrations of isoquercitrin. Different concentrations of isoquercitrin reduced [$^3$H]-thymidine incorporation in Gbm cell culture and this reduction increased with culture time (Fig. 2a). After 24 h of 50 and 100 µmol/l of isoquercitrin treatment, we observed a significant reduction in Gbm [$^3$H]-thymidine incorporation of 40 and 53%, respectively, when compared with cells incubated with DMSO 1% used as a vehicle control (Fig. 2a). A major effect in Gbm proliferation was observed at 48 h when increasing concentrations of isoquercitrin reduced [$^3$H]-thymidine incorporation by 60, 72, and 83%, respectively, when compared with untreated or DMSO-treated cells (Fig. 2b). Likewise, after 72 h of isoquercitrin treatment, the inhibition of Gbm proliferation reached its maximum effect. Increasing concentrations of isoquercitrin reduced [$^3$H]-thymidine incorporation by 67, 77, and 91% in Gbm cells (Fig. 2c). Our analysis did not reveal significant alteration in Gbm proliferation when both rutin and quercetin were added to the culture for 24 h at the same concentration used for isoquercitrin (Fig. 2a). However, we noticed that increasing concentrations of quercetin induced similar inhibition on [$^3$H]-thymidine incorporation in Gbm cells in treatment periods longer than 24 h (Fig. 2b and c). Interestingly, increasing concentrations of rutin only resulted in significant inhibition of Gbm cell proliferation after 72 h of treatment (Fig. 2c).

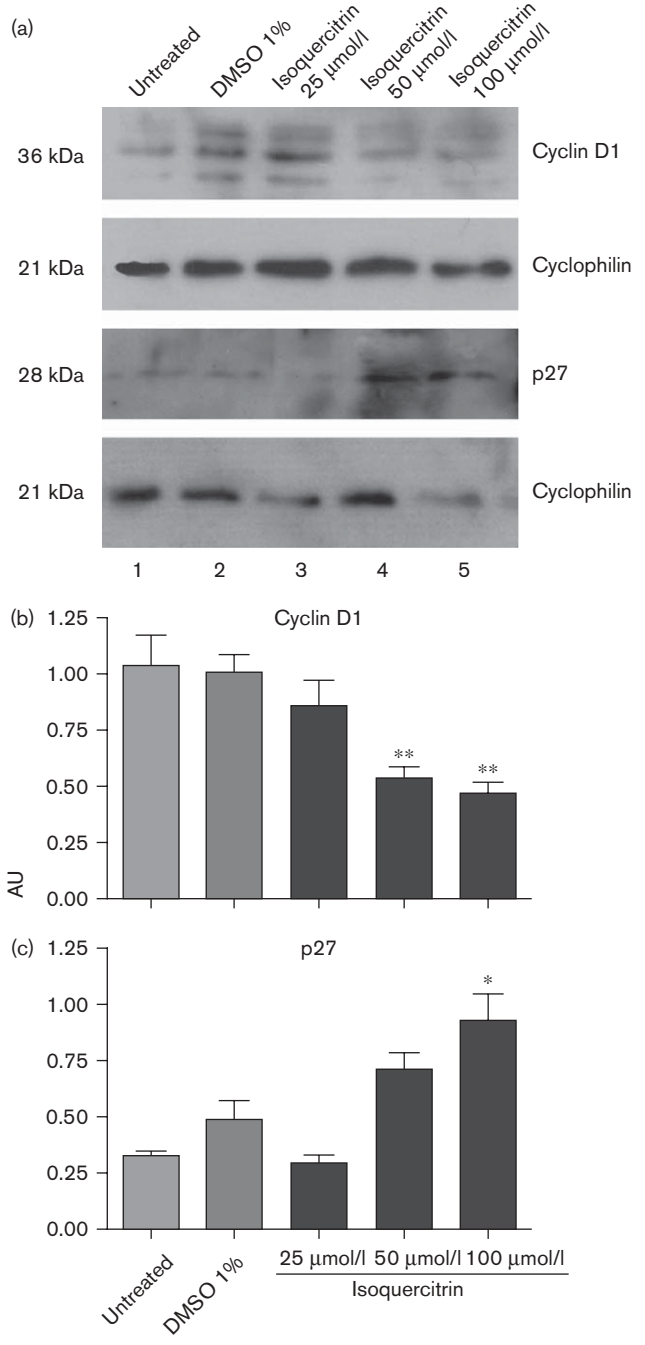
**Fig. 2**




Isoquercitrin inhibits glioblastoma (Gbm) cell proliferation. (a) Gbm cells were treated with 25, 50, and 100 µmol/l of rutin, quercetin, and isoquercitrin for 24 h, (b) 48 h, and (c) 72 h. [$^3$H]-thymidine incorporation was decreased significantly after treatment with isoquercitrin at nearly all times tested. No significant effect was observed after 24 h treatment with quercetin and rutin; however, [$^3$H]-thymidine incorporation was decreased significantly after treatment with quercetin for 48 and 72 h and decreased significantly after treatment with rutin for 72 h. Every bar represents triplicates of four independent experiments. DMSO, dimethyl sulfoxide. *$P<0.05$; **$P<0.01$; ***$P<0.001$.

To analyze whether the effects on cell proliferation promoted by isoquercitrin in Gbm cells could involve cell cycle control, we investigated the levels of cyclin D1 and p27 proteins that positively and negatively control the cell cycle, respectively. Therefore, we analyzed

**Fig. 3**




Cyclin D1 and p27 levels are altered after isoquercitrin treatment. (a) Western blot of total lysate from glioblastoma (Gbm) cells were treated with 25, 50, and 100 µmol/l of isoquercitrin for 24 h. Lanes 1 and 2 are control conditions of either untreated or vehicle treatment with 1% dimethyl sulfoxide (DMSO). Cell homogenates were assayed by western blot with anti-cyclin D1, anti-p27 and anti-cyclophilin B was used as a loading control. (b,c) Histograms showing an index of expression in arbitrary units (AU), calculated by dividing the densitometry band value of cyclin D1 or p27 of each condition by cyclophilin. Notice that 100 µmol/l of isoquercitrin (lane 5) decreased cyclin D1 level and increased p27, but lower concentrations did not affect cyclin D1 or p27 in Gbms cells. Every bar represents three independent experiments. *$P<0.05$; **$P<0.01$.

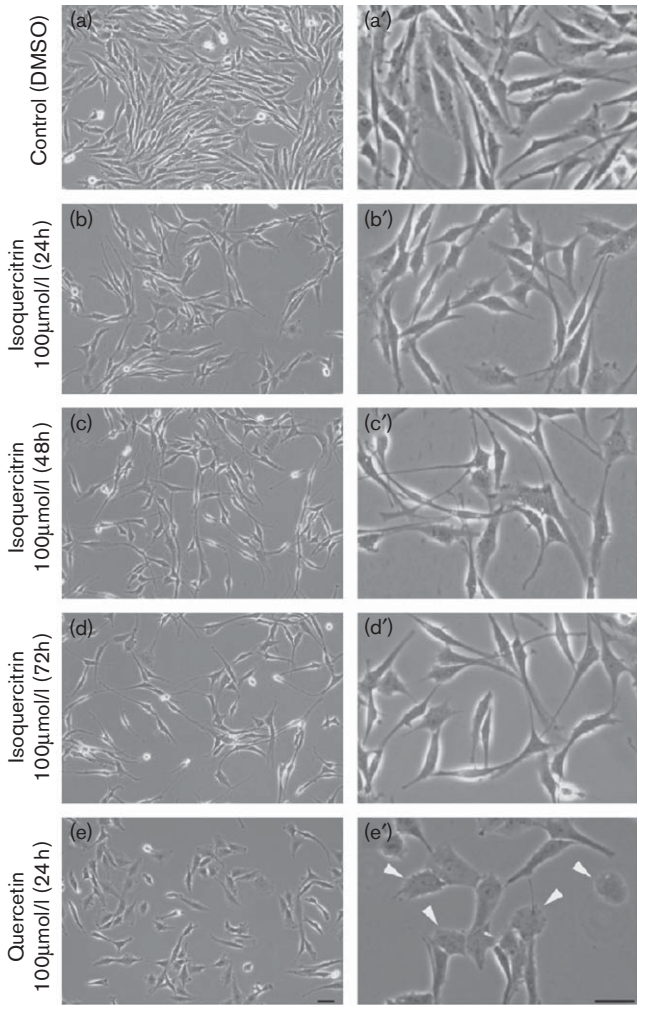
isoquercitrin-treated Gbm cell lysate by western blotting and probed with anti-cyclin D1 and anti-p27 antibodies (Fig. 3a). Gbm cells treated with 100 µmol/l of isoquercitrin revealed a marked reduction in the levels of cyclin D1 (Fig. 3a, lane 5). Conversely, we observed that 100 µmol/l of isoquercitrin increased levels of p27 protein (Fig. 3a, lane 5). Densitometry analysis of the western blot bands revealed that reduction of cyclin D1 and induction of p27 levels were significant when 100 µmol/l of isoquercitrin was used (Fig. 3b and c). These results show that isoquercitrin negatively regulates Gbm cell proliferation in a concentration and time-dependent manner.

### Effects of isoquercitrin on morphology, cell viability, and death in glioblastoma cells

Although our results suggested an effect on cell cycle, it is possible that isoquercitrin promotes Gbm cell death as previously reported for other flavonoids [28,29]. First, we analyzed Gbm cells under conventional light microscopy after 24, 48, and 72 h of isoquercitrin treatment. We did not observe any major change in isoquercetrin-treated Gbm cell morphology when compared with DMSO-treated cells at all times approached in this study (Fig. 4a–d). Cells maintained a healthy appearance, its fibrous-like morphology that is typical of this type of tumor in culture (Fig. 4a and b). Isoquercitrin-treated cell did not show signs of detachment during all treatments time. In addition, isoquercitrin-treated cultures often exhibited lower cell densities compared with control cultures, consistent with the proposed antiproliferative effect of this flavonoid (Fig. 4b and d′). In contrast, we noticed that 100-µmol/l quercetin treatment of Gbm cells altered the morphology to a rounded shape (Fig. 4e and e′).

Next, we analyzed Gbm cell viability by an MTT assay after 24, 48, and 72 h treatment with different concentrations of isoquercitrin, quercetin, or rutin. No significant changes were observed in the presence of different concentrations of rutin and isoquercitrin at all times tested (Fig. 5a–c). However, in quercetin-treated Gbm cultures, we noticed a marked reduction in viability that was time dependent and correlates with previous reports [15,30] (Fig. 5a–c). We finally analyzed whether isoquercitrin treatment affects apoptosis in Gbm cells. Therefore, we checked the levels of cleaved caspase-3, which plays a key role in cell apoptosis linking elements of different stimuli that lead to cell death [31]. The three isoquercitrin concentrations used in this study were not able to induce α-cleaved caspase-3 in Gbm cells (Fig. 5d, lanes 3–5) as compared with mitomycin C, an inducer of this product (Fig. 5b, lane 6). These results show that isoquercitrin isolated in this study did not alter cell morphology, cell viability and did not induce caspase-3-dependent apoptosis when added to Gbm cells for 24 h.

**Fig. 4**




Isoquercitrin does not affect cell morphology. (a) Phase contrast photomicrography of untreated glioblastoma (Gbm) cultured cells and (b–d) isoquercitrin-treated Gbm culturered cells for 24, 48, or 72 h. (e) Gbm cells treated with 100 μmol/l of quercetin. No morphological changes were induced by the treatment with isoquercitrin, but Gbm cells treated with quercetin displayed a round morphological shape (arrowhead). Scale bar 50 μm. DMSO, dimethyl sulphoxide.

### Effects of isoquercitrin on beta-catenin distribution in glioblastoma cells

As the Wnt pathway regulates cell proliferation, which is misregulated in many cancers, and has been reported to be modulated by polyphenols [32,33], a possible effect of isoquercitrin was investigated by examining β-catenin cellular distribution, an important indicator of Wnt/β-catenin activity. Therefore, we performed β-catenin immunostaining in Gbm cells and quantified its cellular localization (Fig. 6). We observed that 23% of β-catenin staining in untreated Gbm cells was found in the nuclei, whereas 45% appeared to be non-nuclear staining and 31% of the cells did not stain for β-catenin (Fig. 6a–c), suggesting an activation of Wnt/β-catenin signaling in a subpopulation of Gbm cells in our cultures (Fig. 6a–c). When 100 μmol/l of isoquercitrin was added to Gbm cells, nuclear β-catenin staining dramatically decreased to 4%, while non-nuclear staining increased to 77% (Fig. 6a–e). Quantification revealed that these changes promoted by isoquercitrin are statistically significant and that the decrease in β-catenin-negative cells from 31% (untreated) to 18% (isoquercitrin-treated) was not significant (Fig. 6a). These results suggest that isoquercitrin treatment alters the distribution of β-catenin in Gbm cells. The decrease in nuclear β-catenin is consistent with a decrease in Wnt/β-catenin signaling activity.

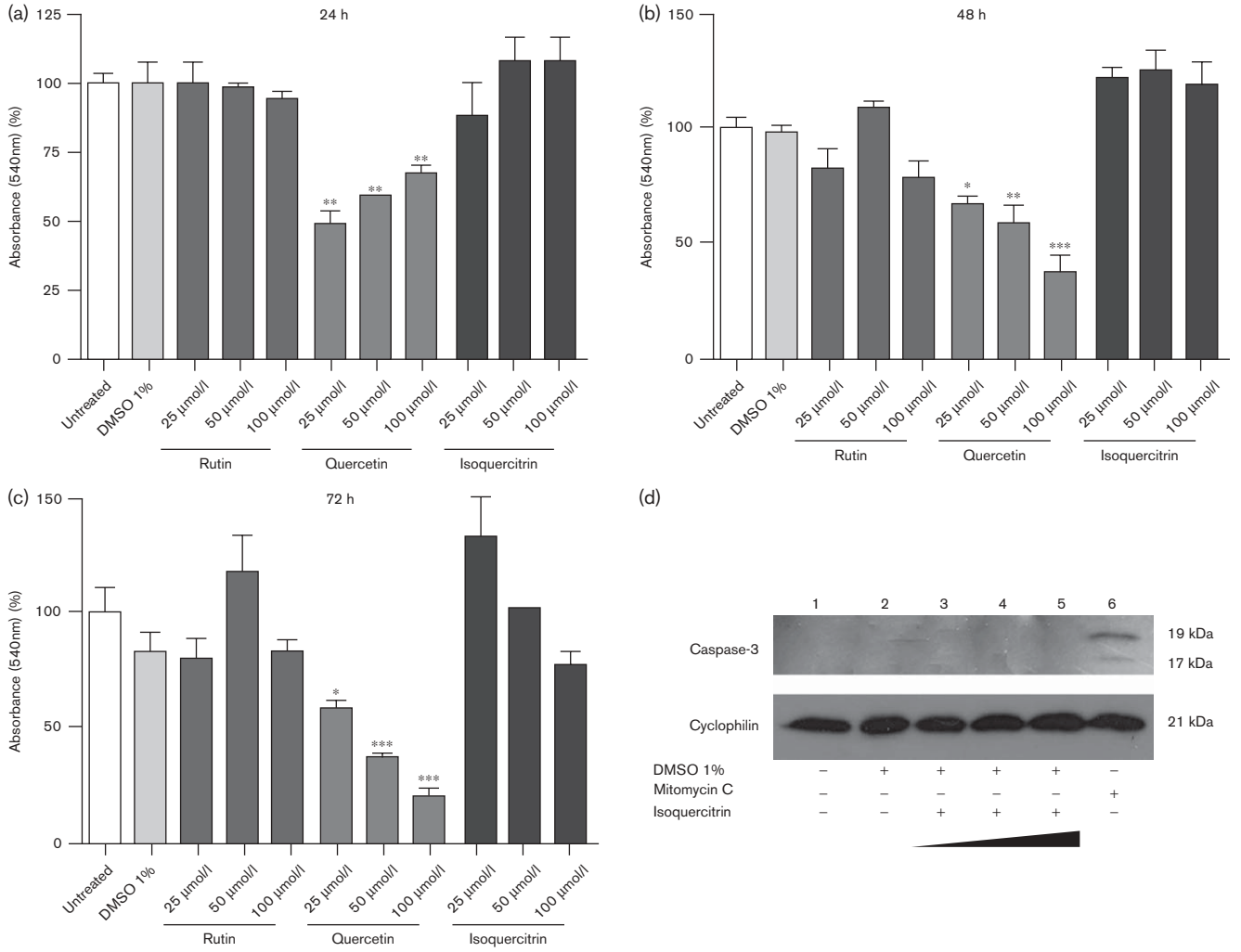
## Discussion

In this study, we identified a flavonoid molecule isolated from *H. fasciculata* which resembles the chemical properties of isoquercitrin and inhibits proliferation in Gbm cells without the induction of capase-3-dependent apoptosis or alteration of cell morphology. In addition, this molecule changes the β-catenin distribution in a subpopulation of Gbm cells from nuclear to cytoplasmic, suggesting that isoquercitrin could inhibit the canonical Wnt pathway.

The anticancer properties of flavonoids have been widely documented, particularly for quercetin and rutin [14,34,35]. One proposed mechanism is that quercetin inhibits the growth of malignant cells by interfering with threonine and serine/threonine kinases [36,37], thereby arresting the growth of malignant cells in the late G1 phase [38]. However, it remains poorly explored whether isoquercitrin, a glycosilated flavonoid derived from quercetin, is able to control the growth of cancer cells. Therefore, we attempted to isolate, identify, and initiate functional studies characterizing the biological effects of isoquercitrin. We used a standard protocol for flavonoid isolation in small amounts and determined the chemical structure by spectral methods, including UV [39], $^{1}$H and $^{13}$C-NMR [40], and mass spectroscopy [41,42]. Our analysis by $^{1}$H-NMR and $^{13}$C-NMR found considerable similarities between the *H. fascilulata* compound isolated in this study and those reported elsewhere (Table 1), [21] which allowing us to determine that the purified molecule in our study was isoquercitrin.

Our results show that only 50–100 μmol/l of isoquercitrin is sufficient to inhibit approximately 50% of [$^{3}$H]-thymidine incorporation in Gbm cells during 24 h of treatment. However, longer isoquercitrin treatments induced a more robust inhibition on [$^{3}$H]-thymidine incorporation. By comparing isoquercitrin treatments with rutin and quercetin treatments, we only observed such reductions in [$^{3}$H]-thymidine incorporation after 48 h for quercetin and after 72 h for rutin indicating that these effects are time dependent and compound specific.

**Fig. 5**



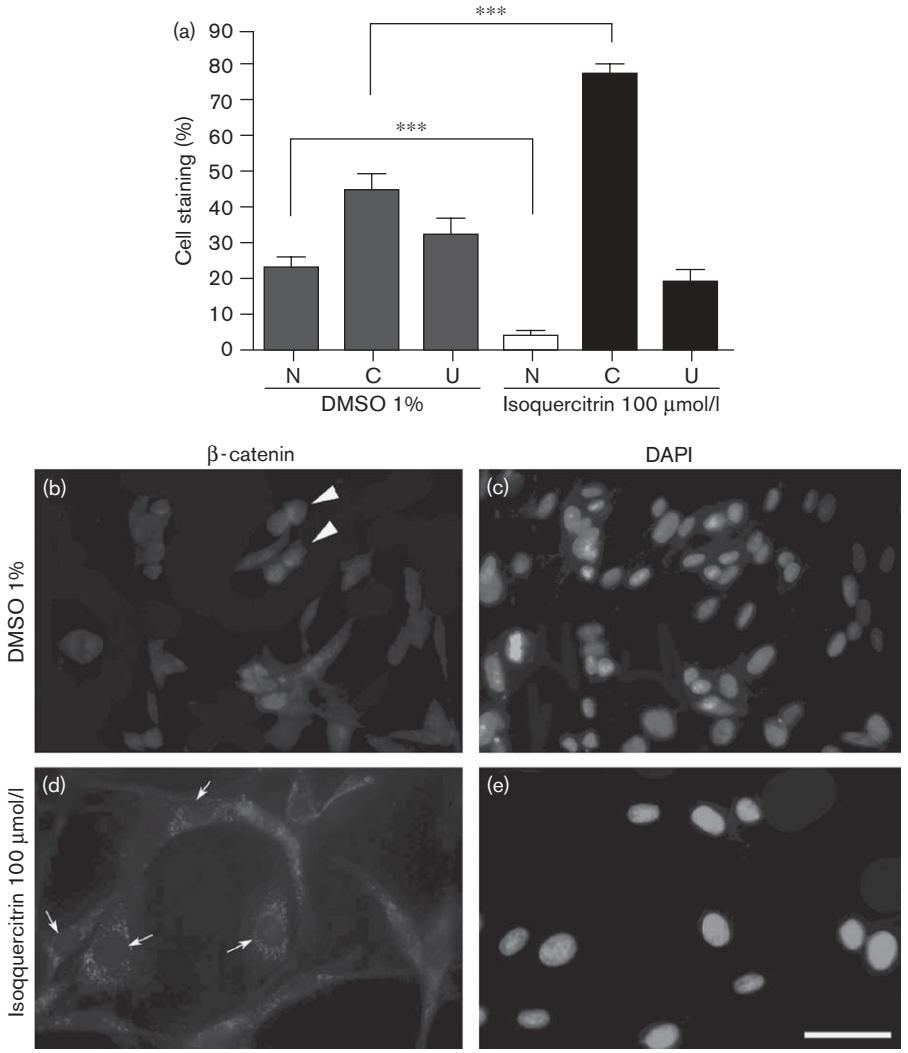

Isoquercitrin does not affect viability and does not induce apoptosis in glioblastoma (Gbm) cells. (a) Gbm cells were treated with 25, 50, and 100 µmol/l of rutin, quercetin, and isoquercitrin for 24 h, (b) 48 h, (c) and 72 h, respectively. Histograms showing the absorbance of the MTT assay in different treatment conditions. Notice that quercetin treatment decreases cell viability at all tested time points. (d) Cell homogenates were assayed by western blot with anti-cleaved caspase-3 and anti-cyclophilin-B was used as a loading control. Lanes 1 and 2 were control condition either untreated or vehicle treatment with 1% dimethyl sulfoxide (DMSO); lanes 3–5 were Gbm cells treated with isoquercitrin 25, 50, and 100 µmol/l; and lane 6 cells treated with mitomycin C (5 µg/ml). Notice that only cells treated with mitomycin C displayed high levels of cleaved caspase-3. $*P<0.05$; $**P<0.01$; $***P<0.001$.

In addition, the isoquercitrin concentrations used in this study were not toxic to Gbm cells in treatments as long as 72 h, but quercetin, at the same concentrations, significantly decreased Gbm cell viability within the first 24 h of treatment. In accordance, we also found that isoquercitrin affected two major components involved in cell cycle control. Isoquercitrin added to Gbm cell induced an increase in p27 and a decrease in cyclin D1 protein levels.

Several cyclins were described controlling the transition phases of cell cycle, and overexpression of cyclins has been linked to the development and progression of cancer [43,44]. In combination, these findings show that isoquercitrin and quercetin act through different mechanisms, although they are structurally related. In addition, these results indicate that isoquercitrin effects Gbm cell proliferation through the regulation of cell cycle rather than cell death. Given that quercetin has been reported as an inducer of apoptosis in glioma cells [15] and that the isoquercitrin used in this study did not induce apoptosis, rather it affected cell proliferation, it may be possible that structural differences such as the presence of glucose replacing a hydroxyl group in isoquercitrin are responsible for the discrepant biological effects.

The Wnt pathway plays many important roles in controlling cell fate, proliferation, migration, tissue

**Fig. 6**




Isoquercitrin affects β-catenin localization. β-catenin immunostaining of Gbm cells treated with 100 µmol/l isoquercitrin or a vehicle control of dimethyl sulfoxide (DMSO) 1% (control). (a) Quantification of nuclear or cytoplasmic β-catenin immunostaining. Cell staining quantification was performed by counting the number of DAPI-stained nuclei and the number of non-nuclear and nuclear β-catenin staining in randomly chosen microscope fields to calculate percentage proportion of nuclear (N), cytoplasmic (C), or unstained (U). (b) In control conditions, a subpopulation of Gbm cells display nuclear β-catenin localization (arrowhead). (d) In cells treated with isoquercitrin, β-catenin staining is primarily localized in the cytoplasm (arrow) and nuclear β-catenin localization has decreased. Panels (c) and (e) show blue nuclei DAPI staining. Scale bar 50 µm; Each bar represents triplicates of three independent experiments. ***$P<0.001$.

architecture, and organogenesis during embryonic development and throughout the lifetime of metazoan organisms [45]. In the absence of Wnt, β-catenin is phosphorylated by a complex of proteins including axin, adenomatous polyposis coli, glycogen synthase kinase (GSK)3β, and casein kinase. Phosphorylated β-catenin is recognized by β-TrCP, an F-box component of the E3 ubiquitin ligase complex, which promotes β-catenin ubiquitination and degradation by the ubiquitin–proteasome system [46]. Binding of Wnt ligands to their receptors, LRP6/5 and frizzled, leads to the activation of the adaptor protein, disheveled, and the inhibition of GSK-3β activity, reducing β-catenin phosphorylation and subsequent degradation. Stabilized cytoplasmic β-catenin is able to translocate to the nucleus where it binds to members of the T-cell factor/lymphoid-enhancing factor family of transcription factors [47]. Deregulation of this pathway is associated with many diseases including cancer [48], as increased nuclear β-catenin levels promote the transcription of T-cell factor/lymphoid-enhancing factor target genes, including c-jun, c-myc, fibronectin, cyclin D1, and fra-1 [49–51]. We found two pieces of evidence that could support a modulation of Wnt signaling by isoquercitrin isolated in this study. First,

we detected a decrease in the levels of cyclin D1 protein when Gbm cells were treated with isoquercitrin (Fig. 3). Second, the amount of nuclear β-catenin staining was reduced in isoquercitrin-treated Gbm cells compared with the untreated Gbm cells (Fig. 6b, c and e). Although further investigation is required to confirm our results, the presented data favor an interpretation that the flavonoid isolated in this study is capable of inhibiting the canonical Wnt/β-catenin pathway. It is worth mentioning that recent studies did not find a correlation between growth stimulatory effects of apple polyphenols on HT29 human colon tumor cells through the Wnt pathway [33]. However, other reports have shown that quercetin presents a strong inhibitory effect on Wnt/β-catenin pathway, particularly through GSK3β [32].

In summary, we presented the isolation of a flavonoid from *H. fasciculata*, characterized as isoquercitrin, which is capable of inhibiting Gbm cell proliferation in a time and concentration-dependent manner without promoting cytotoxicity or caspase-3 dependent apoptosis at concentrations up to 100 µmol/l. Further investigation is needed to validate the effectiveness of isoquercitrin isolated from *H. fasciculata* as an anticancer therapy.

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