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New approach for treatment of primary liver tumors: The role of quercetin

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ABSTRACT

Hepatocellular carcinoma (HCC) is the most common primary liver tumor (PLT), with cholangiocarcinoma (CC) being the second most frequent. Glucose transporter 1 (GLUT-1) expression is increased in PLTs and therefore it is suggested as a therapeutic target. Flavonoids, like quercetin, are GLUT-1 competitive inhibitors and may be considered as potential therapeutic agents for PLTs. The objective of this study was evaluation of quercetin anticancer activity in three human HCC cell lines (HepG2, HuH7, and Hep3B2.1–7) and in a human CC cell line (TFK-1). The possible synergistic effect between quercetin and sorafenib, a nonspecific multikinase inhibitor used in clinical practice in patients with advanced HCC, was also evaluated. It was found that in all the cell lines, quercetin induced inhibition of the metabolic activity and cell death by apoptosis, followed by increase in BAX/BCL-2 ratio. Treatment with quercetin caused DNA damage in HepG2, Hep3B2.1–7, and TFK-1 cell lines. The effect of quercetin appears to be independent of P53. Incubation with quercetin induced an increase in GLUT-1 membrane expression and a consequent reduction in the cytoplasmic fraction, observed as a decrease in ^{18}F-FDG uptake, indicating a GLUT-1 competitive inhibition. The occurrence of synergy when sorafenib and quercetin were added simultaneously to HCC cell lines was noticed. Thus, the use of quercetin seems to be a promising approach for PLTs through GLUT-1 competitive inhibition.

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Introduction

The hepatocellular carcinoma (HCC) is the most frequent primary liver tumor (PLT) (approximately 80%), with cholangiocarcinoma (CC) being the second most frequent (approximately 10%) (1,2). HCC is currently the second leading cause of death by cancer worldwide (3–6). This type of tumor does not have a specific treatment, with the prognosis being very poor and the survival diminished (4,7,8). Among the therapeutic options available, liver transplantation and surgical resection are the only curative therapies. However, majority of the patients are diagnosed in advanced stages and thus are not suitable for surgical therapies (9–11). As an alternative to surgery, there are other therapies such as transcatheter arterial chemoembolization that, in most of the cases, has a merely palliative role, in part due to its high chemoresistance (12–17). More recently, sorafenib, a nonspecific multikinase inhibitor, has come to be used in clinical practice in patients with advanced HCC. However, it only increases the survival time of patients with this type of tumor, from 7.9 to 10.7 months (9).

CC is a malignant tumor that originates in epithelial cells of the biliary tree (18). With regard to prognosis, at an advanced stage of the disease, CC is taken as a devastating type of cancer and the only curative treatment possible is surgical resection. Nevertheless, when diagnosed, majority of the patients are at an advanced stage of the disease, making it impossible to perform this therapy. Under these conditions patients are, most of the time, conducted to palliative treatments (19,20). In this context, it is imperative to find new therapies and new therapeutic targets for these types of tumor.

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In recent years, several studies indicate that glucose transporter 1 (GLUT-1) is overexpressed in HCC, promoting tumorigenesis. Furthermore, in 2009, Amann and colleagues revealed that when suppressing GLUT-1 expression using siRNA, tumorigenesis of HCC cell cultures was significantly reduced. These results suggest that GLUT-1 plays a direct role in HCC carcinogenesis and may be a promising therapeutic target for this type of tumor (21–23).With regard to the relationship between CC and GLUT-1, a recent study showed that GLUT-1 expression in CC is correlated with poor prognosis and aggressive behavior and, according to the authors, GLUT-1 might be a therapeutic target for CC (24).

GLUT-1 has three ATP binding sites, essential to its conformation and affinity. In this way, these binding sites seem to be a possible target for pharmacological strategies. Currently, it has been demonstrated that flavonoids, such as genistein and quercetin, inhibit the binding of ATP with tyrosine kinases, which is also capable of inhibiting GLUT-1 through this mechanism (21). In fact, the action of flavonoids as GLUT-1 competitive inhibitors is already known and has been studied (25).

Quercetin is a bioactive flavonoid type flavonol which presents antioxidant, anti-inflammatory, and vasodilatory effects, having been proposed as a potentially anticancer agent (26). Studies performed in cell cultures showed that quercetin and its glucosides exhibit anticancer activity in some types of tumor cells, which may be due to their anti/pro-oxidant and/or anti-inflammatory properties, and even to other less explored mechanisms of action (26–30). Recent studies have also shown highly positive results in the use of quercetin in combination with drugs commonly used in clinical practice, which open the door to the possibility of quercetin utility in combination with traditional drugs used in chemotherapy (31–33). Therefore, quercetin can be considered as a potential therapeutic agent to treat PLTs, since it is possible to check its anticarcinogenic effect as a GLUT-1 competitive inhibitor, in addition to its other characteristics. Taking all these characteristics into account, the main goal of this study is the evaluation of anticancer activity of quercetin in three human HCC cell lines that differ in P53 expression and in a human CC cell line.

Materials and methods

Cell cultures

The human HCC cell lines used were HepG2 (wP53), HuH7 (mP53), and Hep3B2.1–7 (P53 null) (34,35). HepG2 and Hep3B2.1–7 cell lines were obtained from American Tissue Cell Collection (ATCC), USA, and HuH7 cell line was obtained from Japanese Collection of Research Bioresources (JCRB), Japan. The human CC cell line TFK-1 was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). This cell line does not express P53 (36). Cells were propagated on adherent cultures at 37°C in 5% CO_2 humidified atmosphere using Dulbecco's Modified Eagle's Medium (DMEM) (Sigma D5648) as culture medium for HCC cell lines and RPMI 1640 (Sigma R4130) for CC line, both supplemented with 100 μM sodium pyruvate (Gibco 11360), 10% fetal bovine serum (Sigma F7524), and 1% antibiotic (100 U/mL of penicillin and 10 μg/mL of streptomycin) (Gibco 15240). These culture media contained 25 mM glucose. For fluorine-18 fluorodeoxyglucose (^{18}F-FDG) uptake studies, cells were also cultured in low-glucose (LG) medium (5 mM) DMEM (Sigma D5523) for HCC cell lines and RPMI 1640 (Sigma R1383) for CC cell line, in order to mimic the normal human blood plasma glucose concentration.

Metabolic activity evaluation

To evaluate the effect of quercetin or sorafenib on metabolic activity, cells were plated in 24-well multiwell plates with 25,000 cells in each well. After 24 h, cells were incubated with different concentrations of quercetin (0.1–25 mM) or sorafenib (0.025–10 mM). Metabolic activity was evaluated 24, 48, 72, and 96 h after incubation with compounds using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) (Sigma M2128) assay as described by Mamede *et al.* (37).

Assessment of the combination effect of quercetin with sorafenib

Chou and Talalay method (38) was used in order to verify the response produced through the combination of quercetin with sorafenib. For this evaluation, 25,000 cells were plated in 24-well multiwell plates and incubated with one-fourth of the half-maximal inhibitory concentration (IC_{50}) value previously obtained for quercetin (9.42 μM for HepG2 cells, 22.65 μM for HuH7cells, 12.59 μM for Hep3B2.1–7 cells, and 12.16 μM for TFK-1 cells). Cells were also treated with different concentrations of sorafenib (0.25–10 mM). Sorafenib was added simultaneously with quercetin and its effect on metabolic activity was evaluated with the MTT assay after 48 h.

Cell survival evaluation

For this study, 500 cells of each cell line were seeded per well in six-well plates. After 24 h, the cells were

incubated with different concentrations of quercetin, taking into account the previously calculated IC_{50} value. Thus, for each cell line, the concentration corresponding to the IC_{50} value, a lower concentration and a higher concentration were used. Therefore, the quercetin concentrations used for HepG2 cell line were 5 μM, 90.60 μM, and 200 μM; for HuH7 cell line5 μM, 37.67 μM, and 100 μM; for Hep3B2.1–7 cell line 5 μM, 50.35 μM, and 100 μM; and finally for TFK-1 cell line 5 μM, 48.65 μM, and 100 μM. Control wells, which were not submitted to treatment, were also reserved. 48 h after exposure to quercetin, cells were washed with phosphate buffered saline (PBS) and a new medium was added. For all these conditions, the effect of quercetin on cell survival was evaluated with the clonogenic assay according to Mamede *et al.* (39).

Flow cytometry

In order to assess the effects of quercetin on cell viability and types of cell death, cell cycle, Bcl-2 associated X protein (BAX), B-cell lymphoma 2 (BCL-2), and GLUT-1 protein expression it was used flow cytometry. The analysis was performed using a 6-parameter, 4-color FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) equipped with a 15-nW argon laser. For each assay, at least 10^4 events were collected using CellQuest software (Becton Dickinson, San Jose, CA) and analyzed using Paint-A-Gate software (Becton Dickinson, San Jose, CA). For each flow cytometry analysis, 10^6 cells were seeded per well, being, 24 h after, incubated with the same concentrations of quercetin used in cell survival for 48 h.

Cell viability and types of cell death

In order to study cell viability, after the incubation of all cell lines with quercetin for 48 h, flow cytometry using annexin V/propidium iodide was performed according to a method already described by Brito *et al.* (17). The quercetin concentrations used for HepG2 cell line were 5 μM, 90.60 μM, and 200 μM; for HuH7 cell line 5 μM, 37.67 μM, and 100 μM; for Hep3B2.1–7 cell line 5 μM, 50.35 μM, and 100 μM; and finally for TFK-1 cell line 5 μM, 48.65 μM, and 100 μM.

Determination of BAX and BCL-2 expression

After incubation of cell lines with quercetin, BAX and BCL-2 protein expression was determined according to Brito *et al.* (17). PE-anti-BAX (sc-20067PE, Santa Cruz) and FITC-anti-BCL-2 (sc-509 FITC, Santa Cruz) antibodies were used for this purpose.

Cell cycle

In order to analyze possible alterations in cell cycle, the cells, after incubation with quercetin and trypsinization, were centrifuged at 1,300 G for 5 min and the supernatant was discarded. Then, 200 μL of ethanol 70% was added to the pellets while vortexing and the tubes were incubated for 30 min at 4°C in the dark. Then, the cells were washed with 2 mL of PBS and centrifuged at 1,300 G for 5 min. The supernatant was discarded and 500 μL of PI/RNase solution (PI/RNase Solution, Immunostep) was added to the pellet and incubated for 15 min in the dark at room temperature. Subsequently, cell cycle detection was performed in the flow cytometer using excitation wavelength of 488 nm.

P53 expression

Western blotting was used to evaluate the expression of P53 protein. This analysis was performed in control cells and in cells incubated with the same concentrations of quercetin used in cell survival evaluation for 48 h. Thus, the quercetin concentrations used for HepG2 cell line were 5 μM, 90.60 μM, and 200 μM; for HuH7 cell line 5 μM, 37.67 μM, and 100 μM; for Hep3B2.1–7 cell line 5 μM, 50.35 μM, and 100 μM; and finally for TFK-1 cell line 5 μM, 48.65 μM, and 100 μM. The protocol used was described by Santos *et al.* (40). The primary antibodies used were anti-P53 (Sc-47698, Santa Cruz) at a dilution of 1:500 and antiactin (A5441, Sigma) at a dilution of 1:10000.

Comet assay

Damage in cell DNA was analyzed with alkaline single-cell gel electrophoresis, comet assay. Briefly, 5×10^5 cells were treated with the two highest concentrations of quercetin used in cell survival evaluation, i.e., 90.60 μM and 200 μM for HepG2 cell line, 37.67 μM and 100 μM for HuH7 cell line, 50.35 μM and 100 μM for Hep3B2.1–7 cell line, and finally 48.65 μM and 100 μM for TFK-1 cell line. The protocol used was described by Santos *et al.* (40).

GLUT-1 expression

For GLUT-1 membrane expression determination, after trypsinization, cells were centrifuged at 1,300 G and further incubated for 15 min at room temperature with 10 μL of PE-anti-GLUT-1 monoclonal antibody (MAB1349, R&D Systems). Then, the cells were washed by centrifugation at 1,300 G for 5 min and detected in a

flow cytometer using the excitation wavelength of 585 nm.

The same protocols described above for BAX and BCL-2 determination using the PE-anti-GLUT1 (MAB1349, R&D Systems) antibody were used for GLUT-1 intracellular expression detection.

^{18}F-FDG uptake studies

Uptake studies using ^{18}F-FDG were performed in HepG2, HuH7, Hep3B2.1–7, and TFK-1 cell lines and in the same lines previously incubated with 37.67 μM, 90.60 μM, 50.35 μM, and 48.65 μM quercetin, respectively, for 1 h. The uptake studies were performed according to an already described protocol (41,42). Cell suspensions with 2×10^6 cells/mL were prepared. Then, 925 Bq/mL of ^{18}F-FDG was added to each cell suspension. 1, 5, 15, 30, 45, 60, 90, and 120 min after radiopharmaceutical addition, duplicate samples of 200 μL of cell suspension were collected in microtubes with iced PBS to determine the tracer uptake. The samples were centrifuged (5,600 G for 60 s) to separate the pellet from the supernatant. Radioactivity of cell pellets and supernatants were measured separately with well counter (Capintec CRC-15 W) in counts per minute (CPM) in order to determine the ^{18}F-FDG uptake percentage by the cells, according to the following equation.

$$\%\ \text{Uptake} = \frac{\text{CPM}_{\text{pellet}}}{\text{CPM}_{\text{pellet}} + \text{CPM}_{\text{supernatant}}} \times 100$$

Equation 1: Calculation of uptake percentage.

Statistical analysis

Statistical analysis was performed using IBM® SPSS® v. 20.0 software (IBM Corporation, Armonk, New York, USA). The normality of quantitative variables distribution evaluation was performed according to Shapiro-Wilk test. Parametric tests were used in case there was a normal distribution and nonparametric tests were used otherwise.

In the analysis of metabolic activity according to MTT assay, the experimental data obtained were fitted to a sigmoidal dose-response model using OriginLab v. 8.0 software in order to calculate the IC_{50} value.

The comparison of values obtained between different therapeutic conditions within the same cell line and between cell therapy for the same condition lines was performed according to analysis of variance (ANOVA) factor with multiple comparisons using Bonferroni correction.

In the type of cell death evaluation, cell cycle analysis, and BAX/BCL-2 ratio, the comparison between two different treatment conditions for each cell line was performed through an ANOVA factor if any there was normality and homogeneity of variance or Kruskal-Wallis test otherwise. Multiple comparisons were performed using Bonferroni correction.

In comparing between therapeutic conditions of clonogenic capacity and GLUT-1 expression, the comparison with the control was performed according to Student's *t*-test for an average; while in the comparisons between the other conditions, the ANOVA test of a factor or the Kruskal-Wallis test (test selection according to the above criteria) was used. Multiple comparisons were obtained according to Bonferroni correction. In the comparison of GLUT-1 membrane and cytoplasmic expression for each cell line, Student's *t*-test for paired samples or the corresponding nonparametric Wilcoxon test was used.

In the analysis of ^{18}F-FDG uptake, the experimental values obtained were fitted to an exponential model using OriginLab v. 8.0 software. The parameters were compared using the ANOVA test of a factor with multiple comparisons according to Bonferroni correction. A significance level of 0.05 was considered for all comparisons.

Results

Effect of quercetin on metabolic activity

Through the analysis shown in Table 1 it can be seen that quercetin has an inhibitory effect on metabolic activity in all the studied cell lines, depending on the incubation time and the concentration of compound.

Observing the IC_{50} values, it was noted that for shorter incubation times HepG2 was the most sensitive to cell line incubated with quercetin. However, by increasing the incubation time, the lowest IC_{50} values were obtained with the Hep3B2.1–7 cell line. For the HepG2 cell line, it was found that there were statistically significant differences between the IC_{50} values obtained after 24 and 48 h ($p < 0.05$), 24 and 72 h ($p < 0.01$), 48 and 96 h ($p < 0.01$), and finally between 24 and 96 h ($p < 0.001$) of incubation. Regarding the IC_{50} values obtained for the HuH7 cell line, there is a statistically significant difference among all values obtained ($p < 0.05$), except between 48 and 72 h of incubation. It was also found that among the IC_{50} values obtained for the Hep3B2.1–7 cell line, statistically significant differences occur systematically ($p < 0.01$) with the exception of the values acquired after 72 and 96 h of incubation. Finally, concerning IC_{50} values calculated for TFK-1 cell line, statistically significant differences have been observed ($p < 0.001$) between the value obtained after 24 h and other incubation times. In turn, comparing the IC_{50} values obtained after 48 h of incubation with quercetin in the various cell lines under study, differences

Table 1. IC_{50} values and confidence intervals obtained for HepG2, HuH7, Hep3B2.1–7, and TFK-1 cell lines when treated with quercetin for different incubation times. The obtained r^2 values were always equal or greater than 0.9.

	Quercetin							
	HepG2		HuH7		Hep3B2.1–7		TFK-1	
Cell line	IC_{50} (μM)	Confidence interval	IC_{50} (μM)	Confidence interval	IC_{50} (μM)	Confidence interval	IC_{50} (μM)	Confidence interval
24 h	45.53	[36.62; 56.61]	174.26	[156.85; 193.61]	87.07	[65.67; 115.44]	100.77	[82.39; 123.24]
48 h	37.67	[31.24; 39.34]	90.60	[79.35; 93.81]	50.35	[46.23; 64.06]	48.65	[33.56; 53.22]
72 h	33.06	[26.32; 35.91]	72.21	[52.08; 81.29]	27.88	[20.06; 31.42]	41.11	[26.97; 47.89]
96 h	29.02	[24.38; 30.91]	61.61	[53.36; 65.20]	26.63	[23.21; 27.99]	39.26	[32.09; 42.24]

with statistical significance ($p < 0.001$) were found among the values obtained for the cell lines HepG2 and HuH7, Hep3B2.1–7 and HepG2, HuH7 and Hep3B2.1–7, and HuH7 and TFK-1.

Cell survival

The treatment with different concentrations of quercetin induced significant reductions on colony forming ability of all the cell lines studied (Fig. 1).

For HCC cell lines, with only 5 μM quercetin, a reduction of about 20–25% on the capacity of colony formation was observed in relation to the control, always with statistically significant differences ($p < 0.05$). For the other two concentrations of quercetin used, an absence of colony formation was found.

In relation to TFK-1 cell line, the treatment with the lowest concentration of quercetin used also induced a significant decrease (about 20%) on survival factor ($p < 0.05$). For the other concentrations, a significant loss of

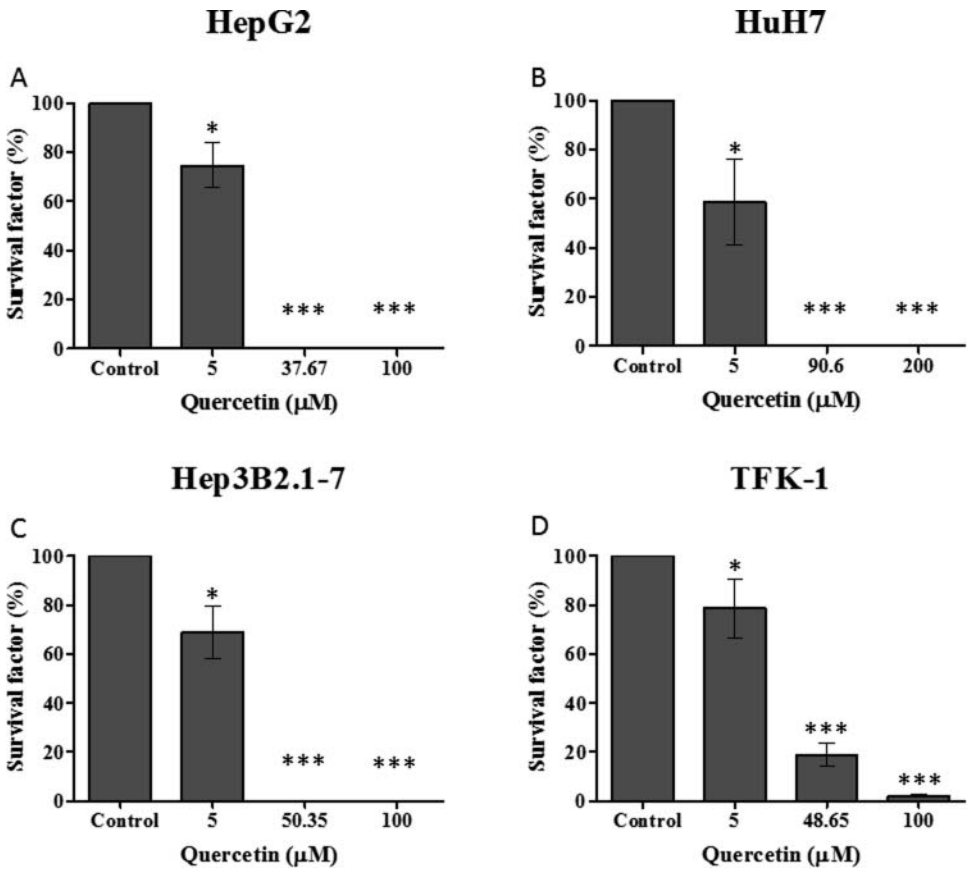

Figure. 1. Analysis of cell survival factor in response to the treatment with quercetin by HepG2 (A), HuH7 (B), Hep3B2.1–7 (C), and TFK-1 (D) cell lines using clonogenic assay. The percentage of colonies relative to control as well as the respective mean and standard deviation of six independent experiments are expressed. Significant differences relative to control are identified with *. The use of * represents $p < 0.05$ and *** represents $p < 0.001$.

ability to form colonies greater than 80% ($p < 0.001$) was noted.

Cell viability and cell death

As a response to incubation with quercetin, the viability of the HepG2 cells decreases, which occurs simultaneously with a tendency to activate cell death through apoptosis as it is shown in Fig. 2A. Statistically significant differences ($p < 0.001$) were only shown between the control condition and the highest concentration of quercetin used in relation to the percentage of viable cells. For the HuH7 cell line (Fig. 2B), when cells were incubated with the two lower concentrations of quercetin, the cell viability remained similar to those of control cells. However, when the quercetin concentration was increased to 200 μM, the cell viability decreased in a statistically significant manner ($p < 0.05$) to about 40%. Apoptosis was the predominant type of cell death when the quercetin concentration corresponded to its IC_{50} value, and a balance between apoptosis and necrosis occurred for the other two concentrations tested. Statistically significant differences were also observed between cell populations undergoing initial apoptosis and the control at the concentration of 200 μM ($p < 0.05$). In turn, it was verified in the Hep3B2.1–7 cell line (Fig. 2C) that when cells were incubated with the concentration of quercetin that corresponds to the IC_{50} value, only about 45% of the cells remained viable, with statistically significant differences in relation to the control cells ($p < 0.001$). About 20% of the remaining cells died by necrosis and about 35% by apoptosis, the latter being significantly different from the control value ($p < 0.05$). There were also no statistically significant differences between the populations of viable cells in control condition and at those incubated with 100 μM quercetin ($p < 0.05$) and between those incubated with 5 μM and 100 μM quercetin, and among populations at an initial apoptosis ($p < 0.05$) and necrosis ($p < 0.01$) between control and cells incubated with 100 μM quercetin. Concerning TFK-1 cell line (Fig. 2D), it can be observed that quercetin did not induce a very evident increase in cell death, because even when cells were treated with 100 μM this compound, cell viability was around 65%. Nevertheless, there were statistically significant differences ($p < 0.05$) among populations of viable cells in the control condition and those incubated with 100 μM quercetin. There were also statistically significant differences in initial apoptotic cells between control and cells incubated at 100 μM quercetin ($p < 0.05$) and cells in late apoptosis/necrosis as well as between the control and the cells incubated with 100 μM quercetin ($p < 0.05$).

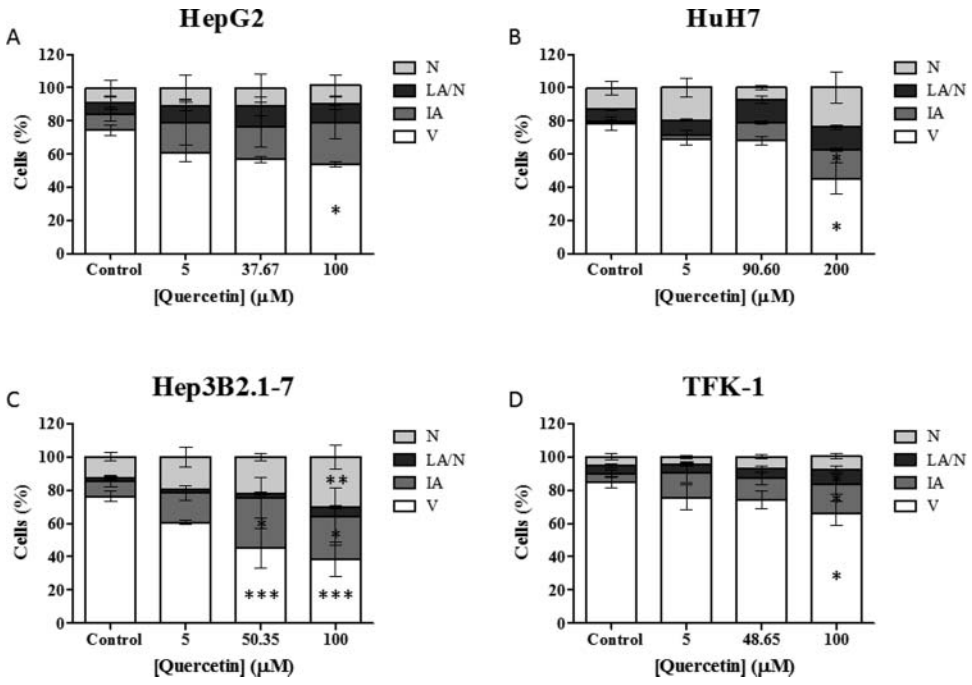

Figure 2. Analysis of cell viability and different types of cell death by flow cytometry using double staining with annexin V - fluorescein isothiocyanate (AV-FITC) and propidium iodide (PI) in HepG2 (A), HuH7 (B), Hep3B2.1–7 (C), and TFK-1 (D) cell lines when treated with three different concentrations of quercetin during 48 h. The results represent the percentage of viable cells (V), cells in initial apoptosis (AI), cells in late apoptosis/necrosis (AT/N), and cells in necrosis (N). Results express the mean and standard deviation of four independent experiments. Statistically significant differences in relation to control are represented by *. The use of * represents $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

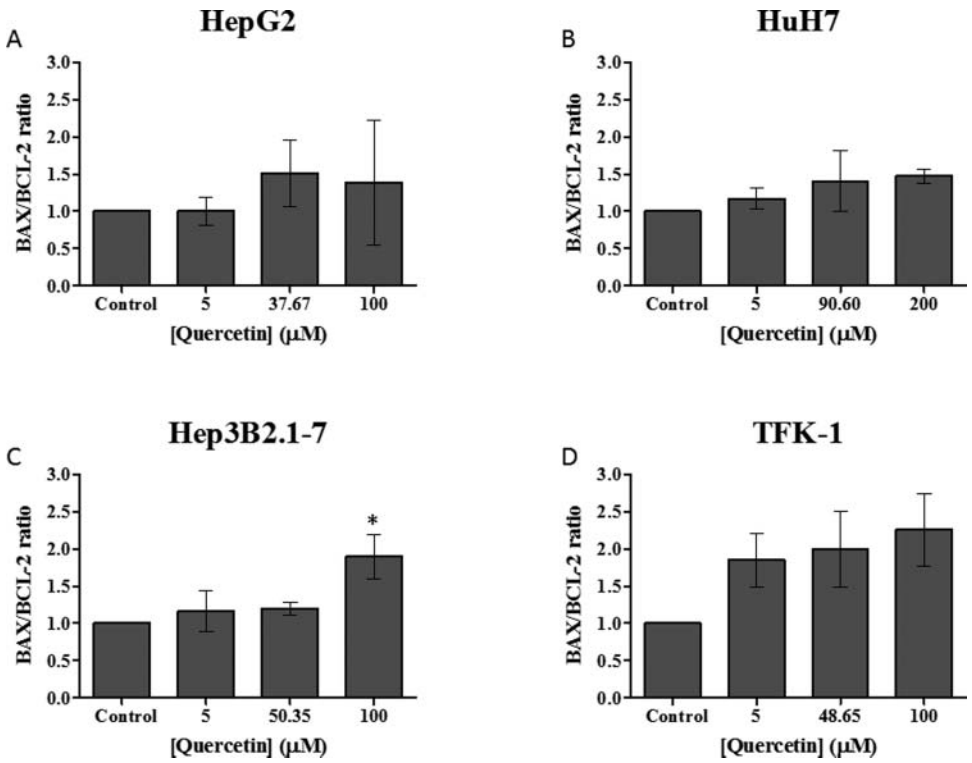

Figure 3. Analysis of BAX/BCL-2 expression in response to quercetin in HepG2 (A), HuH7 (B), Hep3B2.1–7 (C), and TFK-1 (D) cell lines. The results express the mean and standard deviation of four independent experiments and are normalized relative to the control condition. Significant differences from control are represented by * that represents $p < 0.05$.

BAX/BCL-2 ratio

Observing Fig. 3, it can be seen that the treatment with quercetin has a tendency to induce an increase in the BAX/BCL-2 ratio in all the cell lines studied. For HepG2, HuH7, and Hep3B2.1–7 cell lines, when the cells were incubated with quercetin, an increase in BAX/BCL-2 ratio was observed although without significant differences. Regarding Hep3B2.1–7 cell line, a gradual increase of BAX/BCL-2 ratio in response to the treatment with quercetin, with statistical significance between control condition and cells incubated with 100 μM quercetin was observed ($p < 0.05$).

Cell cycle analysis

Figure 4A shows the results obtained by cell cycle analysis of HepG2 cell line after incubation with different concentrations of quercetin. The results revealed the presence of a pre-G0 peak or apoptotic peak for the highest concentrations of quercetin tested, showing statistically significant differences between control cells and cells incubated with 100 μM quercetin ($p < 0.05$) and between cells incubated with 5 μM quercetin and 100 μM compound ($p < 0.05$). Also, a statistically significant increase in cell population in the G0/G1 phase between the control condition and the cells incubated with 37.67 μM quercetin ($p < 0.05$) and between this condition and the highest concentration of quercetin ($p < 0.05$) was observed, suggesting an arrest of the cell cycle in this phase. With increasing concentration of quercetin, a decrease in the S phase population was also observed, with significant differences between control condition and concentration of 100 μM ($p < 0.05$), and between concentrations of 5 μm and 100 μM ($p < 0.05$). There was also a slight increase in G2/M phase population, only with significant differences between the cells incubated with 37.67 μM and 100 μM quercetin ($p < 0.01$). For HuH7 cell line (Fig. 4B), the presence of the apoptotic peak for the highest concentrations of quercetin was also observed, with statistically significant differences between the populations in the pre-G0 phase in the control condition, and the cells incubated with 200 μM quercetin ($p < 0.01$) and between cells incubated with 5 μM and 200 μM compound ($p < 0.05$). A slight increase of the cell population in the G0/G1 phase was also observed when the cells were incubated with 90.60 μM and 200 μM quercetin, with statistically significant differences between the results for concentrations of 5 μM and 90.60 μM ($p < 0.05$). At the same time, a cell population increase in the G2/M phase took place, with statistically significant differences between the

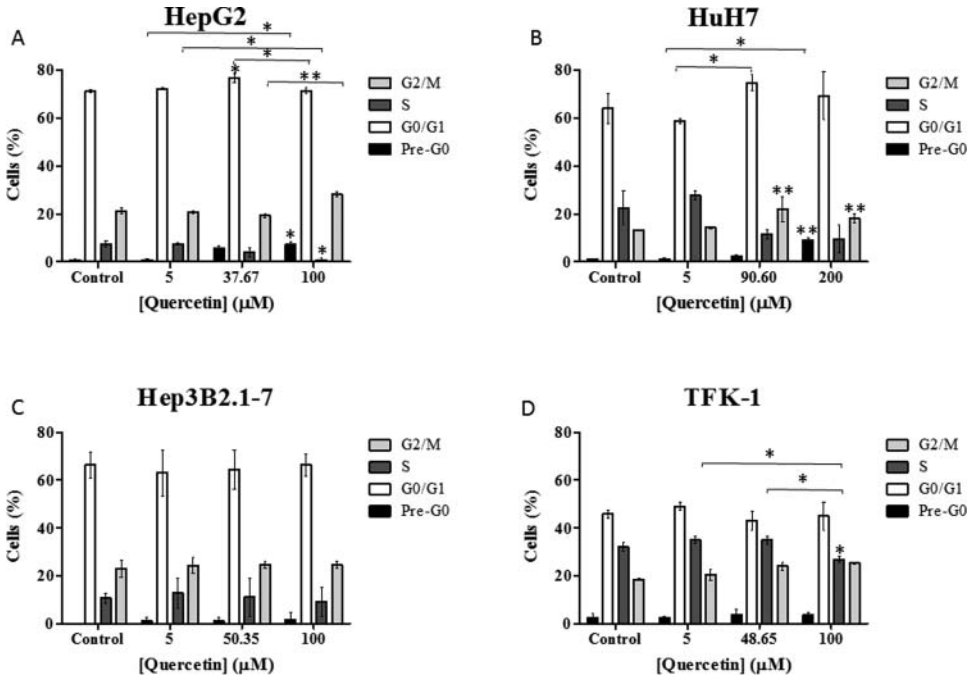

Figure 4. Cell cycle analysis for HepG2 (A), HuH7 (B), Hep3B2.1–7 (C), and TFK-1 (D) cell lines for the different conditions tested. The figure shows the populations in the pre-G0, G0/G1, S, and G2/M phases. For each condition, the results were expressed as percentages of cells. The results express the mean and standard deviation of four independent experiments. Significant differences from control are represented by *. The use of * represents $p < 0.05$ and ** represents $p < 0.01$.

control and the concentration corresponding to IC_{50} value ($p < 0.01$) and between the control and the highest concentration of quercetin used ($p < 0.05$). With respect to the Hep3B2.1–7 cell line (Fig. 4C), in response to the treatment with quercetin, only a decrease in the percentage of cells in S phase and the presence of the pre-G0 peak were observed. Regarding TFK-1 cell line (Fig. 4D), with the increase in the concentration of compound, there was a decrease in S phase cell population with statistically significant differences between the cells incubated with 5 μM and 100 μM quercetin ($p < 0.05$) and between 48.65 μM and 100 μM quercetin ($p < 0.05$). A gradual increase of the population in G2/M phase with the increase of quercetin concentration was also observed, with statistically significant differences between the control and the highest concentration of quercetin used ($p < 005$).

P53 expression

From Fig. 5A it can be found that Hep3B2.1–7 and TFK-1 cell lines do not express P53. Moreover, compared to HepG2 cell line, the HuH7 cell line markedly expresses P53, i.e., 4 times more.

Figure 5 also shows the analysis of P53 expression in response to the treatment with quercetin. This analysis was only performed for HepG2 and HuH7 cell lines since the other two cell lines did not express P53 (Fig. 5A). According to Fig. 5, incubation with various concentrations of quercetin tested induced a decrease in P53 expression for HepG2 cell line (Fig. 5B) and for HuH7 cell line (Fig. 5C). Statistically significant differences were observed ($p < 0.05$) between the control condition and the cells incubated with 5 μM quercetin in the case of HepG2 cell line, and between control and all the concentrations of quercetin tested for HuH7 cell line ($p < 0.01$).

DNA damage

Through a qualitative analysis it can be seen that the cell lines studied are susceptible to DNA damage when subjected to treatment with quercetin. Since an increase in the length of the tail of the comet with the highest concentration of quercetin was observed, it is inferred that the increase in the compound concentration promoted a greater extent of damage in DNA (Fig. 6).

Graphs in Fig. 7 show the differences in the tail moment (parameter that correlates % of DNA in the tail and tail length) of comets obtained for each tested conditions, for all the cell lines studied. It can be seen that the increase in quercetin concentration causes an increase in the tail moment of the HepG2, Hep3B2.1–7, and TFK-1 cell lines; in other words, incubation with this compound induces DNA damages in these cell lines. Differences with statistical significance ($p < 0.001$) were observed

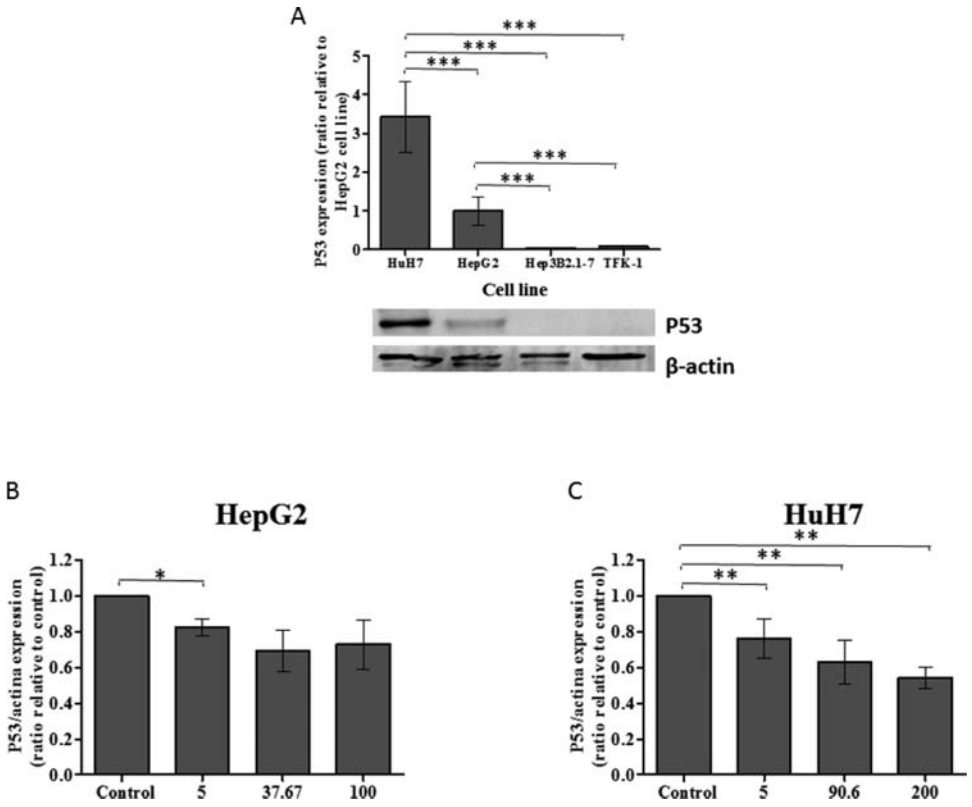

Figure 5. (A) P53 expression in relation to β-actin in HuH7, HepG2, Hep3B2.1–7, and TFK-1 cell lines. The results express the mean and standard deviation of four independent experiments and are normalized relative to the HepG2 cell line. (B and C) P53 expression in relation to β-actin in HepG2 (B) and HuH7 (C) cell lines in response to the treatment with quercetin. The results express the mean and standard deviation of four independent experiments and are normalized relative to control. Significant differences from control are represented by * representing $p < 0.05$, ** representing $p < 0.01$, and *** representing $p <$ 0,001.

between the negative control and the two concentrations of quercetin tested in the case of HepG2 cells, and between the negative control and the highest concentration of quercetin tested in the case of Hep3B2.1–7 and TFK-1 cell lines.

GLUT-1 expression

Regarding GLUT-1 expression, for the HepG2 cell line it was found that the increase in quercetin concentration is associated with an increase in GLUT-1 membrane expression and a decrease in its cytoplasmic expression, as shown in Fig. 8A. The decrease in cytoplasmic expression of this transporter presents statistically significant differences between the control and the treatment with 100 μM quercetin ($p < 0.05$). As the concentration of quercetin increased, there was also an increase in membrane expression of GLUT-1 for HuH7 cell line (Fig. 8B) associated with a decrease in cytoplasmic expression of this transporter. There are statistically significant differences in GLUT-1 cytoplasmic expression between the control and the treatment with 5 μM quercetin ($p < 0.001$) and between control and the treatment with 90.60 μM quercetin ($p < 0.05$). With regard to Hep3B2.1–7 (Fig. 8C) and TFK-1 (Fig. 8D) cell lines, with the increase in concentration of quercetin an increase in GLUT-1 membrane expression can be observed. In the case of TFK-1 cells, statistically significant differences occur in the membrane expression of this transporter between the control and the cells incubated with 5 μM quercetin ($p < 0.05$) and between the cells incubated with 5 μM and 100 μM quercetin ($p < 0.05$). In relation to GLUT-1 cytoplasmic expression, an increase with the increase of the compound concentration was observed.

^{18}F-FDG uptake

Results shown in Fig. 9 indicate that for all the cell lines studied, when cells were preincubated with quercetin, there was a decrease in the percentage of ^{18}F-FDG uptake. The decrease in the radiotracer uptake under study occurs, for both glucose concentrations, in the culture medium.

Graphs in Fig. 9 were used to determine A_{max} (% of maximum uptake) and T_{50} (time to reach half of the maximum uptake) parameters shown, for each condition, in Table 2. With respect to the A_{max} parameter, differences were

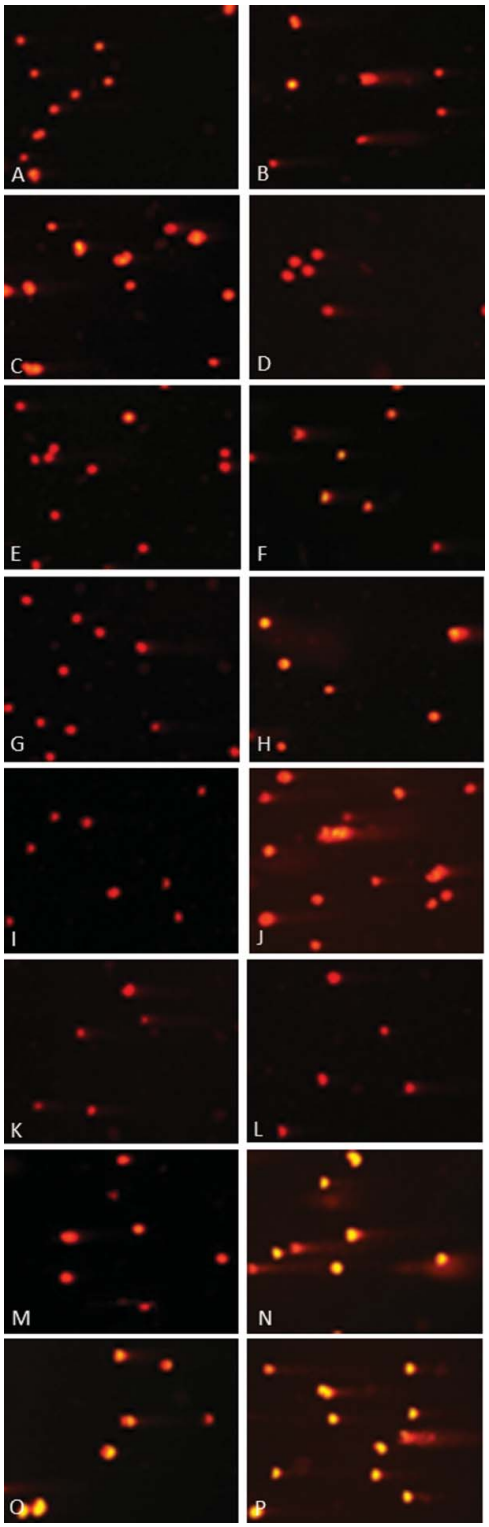

Figure 6. Representative images of comets obtained with cell lines HepG2 (A to D), HuH7 (E to H), Hep3B2.1–7 (I to L), and TFK-1 (M to P), for each condition to which the cells were subjected: (A, E, I, and M) negative control, cells not subject to treatment, (B, F, J, and N) positive control, cells subjected to 20 nM hydrogen peroxide, and the other conditions were incubated for 48 h with quercetin (C) 38 μM, (G) 91 μM, (K) 50 μM, (O) 49 μM, (D, L and P) 100 μM, and (H) 200 μM.

statistically significant ($p < 0.01$) for HepG2 cell line between control cells and cells preincubated with quercetin, when cells were grown in LG medium. Regarding HuH7 cell line, throughout the preincubation with quercetin, decrease in the value of the A_{max} parameter compared to control when cells were grown in high-glucose (HG) medium ($p < 0.05$) was observed; the same occurs when cells were cultured in LG medium ($p < 0.01$). In the case of Hep3B2.1–7 cell line no statistically significant differences in A_{max} parameter were observed between the treated cells and the control ones for both formulations of cell culture medium. Finally, for TFK-1 cells, there are statistically significant differences between the parameter A_{max} obtained for the control cells and cells preincubated with quercetin ($p < 0.001$) when cells were grown in LG medium. With respect to T_{50} parameter, there were no statistically significant differences between control values and those obtained in cells preincubated with quercetin for all the cell lines studied.

Effect of sorafenib on metabolic activity

Based on the studies with sorafenib, it was found (Table 3) that it is able to inhibit the metabolic activity of all the studied cell lines in a time- and concentration-dependent way. In general, the TFK-1 cell line is the most sensitive to sorafenib and the most resistant to the HuH7 cell line. For the IC_{50} values obtained for the HepG2 cell line, there are statistically significant differences between the values determined for all the incubation times tested, with $p < 0.01$ between the IC_{50} values obtained after 72 h and 96 h of incubation and $p < 0.001$ for other combinations. Regarding HuH7 cell line, there are statistically significant differences ($p < 0.001$) between all the IC_{50} values determined except between the values obtained after 72 and 96 h of incubation. With respect to Hep3B2.1–7 and TFK-1 cell lines, the IC_{50} values obtained differ with statistical significance between the IC_{50} values obtained after 72 and 96 h of incubation ($p < 0.05$) and between the values determined for the other incubation times ($p < 0.001$). Comparing the IC_{50} values obtained after 48 h of incubation with sorafenib for the various cell lines, it was found that there are statistically significant differences between all combinations, with $p < 0.01$ between the values obtained for the HuH7 and Hep3B2.1–7 cell lines and $p < 0.001$ for all other combinations.

Evaluation of the combined effect of quercetin with sorafenib

Table 4 shows the IC_{50} values determined by the combined treatments of quercetin with sorafenib. In Tables 1, 3, and 4, it can be seen that that for all the cell lines

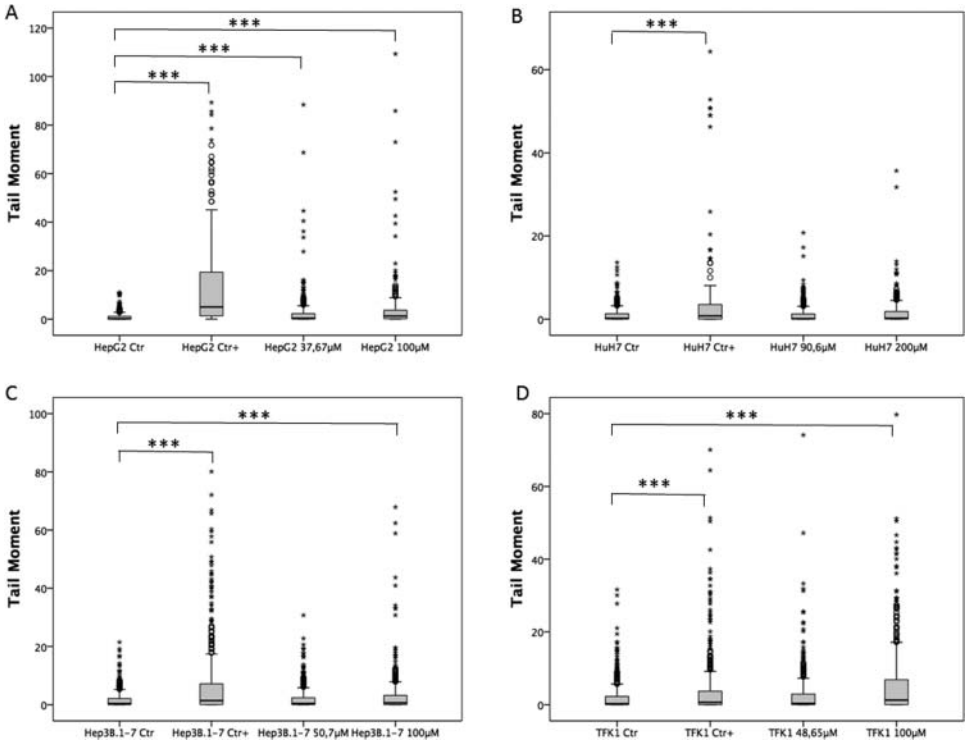

Figure 7. Tail moment, obtained for HepG2 (A), HuH7 (B), Hep3B2.1–7 (C), and TFK-1 (D) cell lines for each condition tested. The outliers are marked with ○ and *. Results represent the median and standard deviation of at least 60 comets obtained from three independent experiments. Statistically significant differences are indicated by *** that represents $p < 0.001$.

studied, the addition of quercetin induces a decrease in the IC_{50} value of sorafenib. Using the determined IC_{50} values, using Chou and Talalay method (38), it was possible to determine the combination indices (CI) shown in Table 5. According to this method, when CI < 1 synergy occurs, when CI > 1 the effect is antagonistic and when CI = 1the effect is additive. According to Table 5 it was found that the combined treatment induces synergy

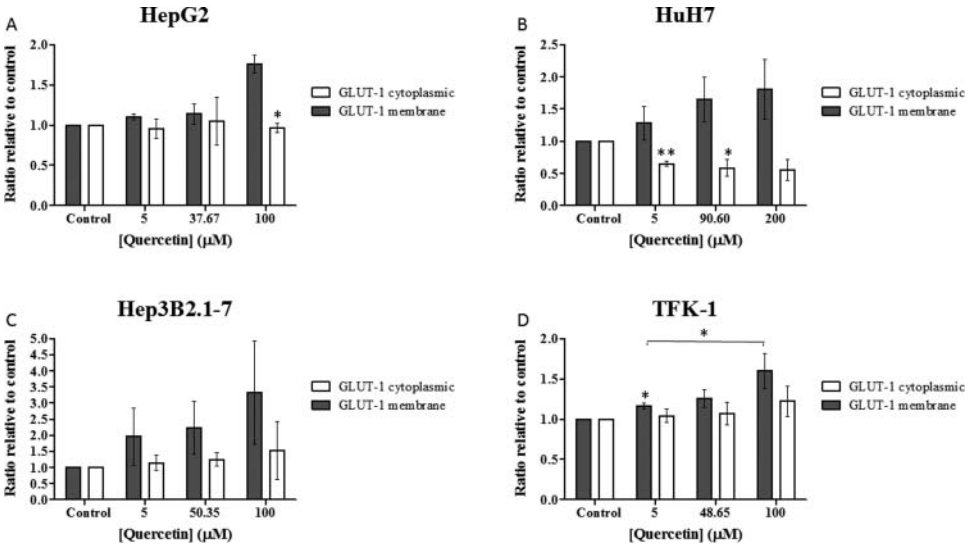

Figure 8. Analysis of GLUT-1 membrane and cytoplasmic expression, in response to the treatment with quercetin, in HepG2 (A), HuH7 (B), Hep3B2.1–7 (C), and TFK-1 (D) cell lines. For each condition, the results are expressed as ratio relative to control and they express the mean and standard deviation of at least three independent experiments. Significant differences from control are represented by *. The use of * represents $p < 0.05$ and ** represents $p < 0.01$.

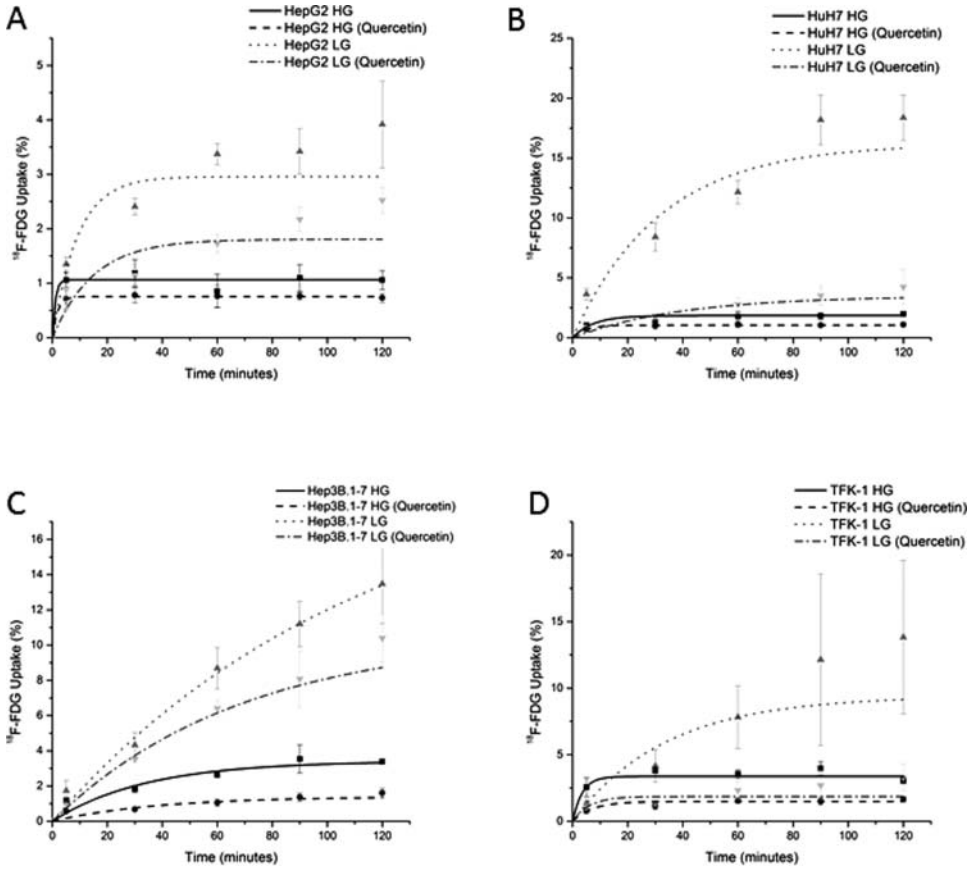

Figure 9. ^{18}F-FDG uptake profiles by HepG2 (A), HuH7 (B), Hep3B2.1–7 (C), and TFK-1 (D) cell lines in control cells and in the same cell lines preincubated with quercetin. The cell lines were cultivated in HG medium and in LG medium. Results are expressed as the mean and standard deviation of four independent experiments performed in duplicate.

for all the HCC cell lines studied, but not for TFK-1 cell line.

Discussion

Flavonoids, including quercetin, have been suggested as potent anticancer agents (21,31,43). One mechanism of action currently assigned to flavonoids is the competitive GLUT-1 inhibition; it is known that in HCC and CC, GLUT-1 has a direct role not only in glucose transport but also in tumorigenesis (21,22,24). In our study it was found that quercetin has an antiproliferative effect both in HCC and CC cell lines studied in a way that is dependent on compound concentration and time of incubation. This result is in conformity with other studies found in the literature on several types of cancer, including HCC and CC (28,44–47). For longer incubation times, clonogenic assay showed that the treatment with different concentrations of quercetin induced a highly significant decrease in colony forming ability in all the cell lines studied, which emphasizes the results obtained by MTT assay. In fact, in other in vitro studies on other types of cancer, it was also found that quercetin has a noticeable antiproliferative effect for longer incubation times (48,49). According to cell viability and cell death analysis, when cell death occurs it is mainly by apoptosis which is in agreement with the literature, as several studies in various types of cancer indicate that quercetin induces cell death by apoptosis (50,51). The treatment with quercetin is related with the increase of the BAX/BCL-2 ratio in the majority of the cell lines studied. These results corroborate those obtained in the analysis of cell viability and cell death, where apoptosis induced by quercetin is present in all the cell lines studied. Furthermore, our results are in agreement with the literature regarding the fact that quercetin leads to an increase in BAX expression with a concomitant decrease in expression of antiapoptotic proteins such as BCL-2, suggesting that this compound induces apoptosis through the mitochondrial pathway (52,53). In fact, this result suggests a possible activation of the mitochondrial pathway of apoptosis in response to treatment with quercetin; however, this theory should be subjected to a more intensive study. In relation to the cell cycle analysis, it was found, in all the cell lines studied, that the presence of apoptotic peak increases as the quercetin concentration increases. These

Table 2. A_{max} and T_{50} parameters and standard error values obtained for HepG2, HuH7, Hep3B2.1–7, and TFK-1 cell lines for each condition. The r^2 values obtained were always equal or greater than 0.9.

Cell line	Cell culture medium	Condition	Parameter	Average	Standard error
HepG2	HG	Control	A_{max}	1,07	0,06
			T_{50}	0,71	1,56
		Quercetin	A_{max}	0,76	0,01
			T_{50}	1,25	0,35
	LG	Control	A_{max}	2,96	0,31
			T_{50}	6,37	2,29
		Quercetin	A_{max}	1.81	0.36
			T_{50}	10.26	5.57
HuH7	HG	Control	A_{max}	1.85	0.14
			T_{50}	5.22	2.65
		Quercetin	A_{max}	1.04	0.03
			T_{50}	3.10	0.60
	LG	Control	A_{max}	16.19	3.43
			T_{50}	21.96	11.32
		Quercetin	A_{max}	3.49	1.51
			T_{50}	27.19	17.95
Hep3B2.1–7	HG	Control	A_{max}	3.42	0.40
			T_{50}	23.46	10.10
		Quercetin	A_{max}	1.40	0.81
			T_{50}	26.45	26.68
	LG	Control	A_{max}	20.56	8.89
			T_{50}	78.94	47.13
		Quercetin	A_{max}	10.59	4.31
			T_{50}	47.83	25.06
TFK-1	HG	Control	A_{max}	3.37	0.20
			T_{50}	2.42	2.02
		Quercetin	A_{max}	1.49	0.11
			T_{50}	4.98	1.60
	LG	Control	A_{max}	9.41	2.82
			T_{50}	23.40	8.65
		Quercetin	A_{max}	1.86	0.36
			T_{50}	4.20	2.51

results corroborate those previously discussed, where it was found that apoptosis was the type of cell death predominantly induced accompanied by an increase in the BAX/BCL-2 ratio. For HepG2 and HuH7 cells, it was also observed that apoptosis occurs in a P53-independent manner. Although apoptotic cell death caused by DNA damage is often mediated by P53, there are other proteins that may be involved in this mechanism, such as P63 and P73 (54,55). Consistent with our results, a study carried out in 2009 by Chien and colleagues on breast cancer cells showed that quercetin induced apoptotic cell death accompanied by a decrease in P53 expression (56). Hep3B2.1–7 and TFK-1 cell lines do not express P53; therefore, the effect of quercetin on P53 expression in these cell lines was not examined. It was also noted that with the exception of HuH7 cells, cell death was also accompanied by damages at the DNA level. Regarding cell cycle, it was also noted that the treatment with quercetin induces arrests in the G0/G1 and G2/M phases which is in accordance with the arrests already observed in other cancer cell lines in response to treatment with this compound (51,57,58). Regarding GLUT-1 expression, as the concentration of quercetin increases, an increase in the membrane expression of this transporter, accompanied by a decrease in the cytoplasmic expression, was observed. In fact, some studies

Table 3. IC_{50} values and confidence intervals obtained for HepG2, HuH7, Hep3B2.1–7, and TFK-1 cell lines when treated with sorafenib for different incubation times. The obtained r^2 values were always equal to or greater than 0.9.

	Sorafenib							
	HepG2		HuH7		Hep3B2.1–7		TFK-1	
Cell line	IC_{50} (μM)	Confidence interval	IC_{50} (μM)	Confidence interval	IC_{50} (μM)	Confidence interval	IC_{50} (μM)	Confidence interval
24 h	17.01	[15.48; 18.69]	22.79	[25.36; 30.45]	22.95	[20.42; 25.80]	8.82	[8.10; 9.60]
48 h	6.62	[5.46; 7.10]	17.98	[13.32; 20.05]	13.08	[10.78; 14.03]	4.63	[4.07; 4.85]
72 h	4.63	[4.13; 5.20]	9.53	[8.23; 11.03]	4.60	[4.02; 5.27]	3.24	[2.71; 3.46]
96 h	3.46	[2.80; 4.28]	11.94	[11.00; 12.96]	3.37	[3.02; 3.76]	2.24	[1.76; 2.44]

Table 4. IC_{50} values and confidence intervals obtained for HepG2, HuH7, Hep3B2.1–7, and TFK-1 cell lines when treated in combination with quercetin and sorafenib. The r^2 values obtained were always equal to or greater than 0.9.

Cell line	IC_{50} (μM)	Confidence interval (sorafenib) (μM)
HepG2	9.42 quercetin + 2.92 sorafenib	[1.95; 3.38]
HuH7	22.65 quercetin + 3.08 sorafenib	[1.28; 4.23]
Hep3B2.1–7	12.59 quercetin + 5.91 sorafenib	[4.97; 6.29]
TFK-1	12.16 quercetin + 3.99 sorafenib	[2.63; 4.64]

have shown that flavonoids and isoflavones are potent inhibitors of glucose influx, and it has been demonstrated that flavonoids (such as quercetin) inhibit the binding of ATP with tyrosine kinase and also competitively inhibit GLUT-1 through this mechanism (31,59). Thus, the competitive inhibition of GLUT-1, at the membrane level, can lead to GLUT recruitment from the cytoplasm to the membrane in order to supply the cell's glycolytic requirement. To corroborate these results, the ^{18}F-FDG uptake studies demonstrated that, in general, there was a higher radiotracer uptake when cells were grown in LG medium; in all the cell lines studied, there was a decrease in ^{18}F-FDG uptake percentage when cells were previously incubated with quercetin compared with control cells, although this is not accompanied by inhibition of GLUT-1 membrane expression. These data are in agreement with the results obtained in clinical practice, demonstrating that blood glucose levels affect the diagnostic value of ^{18}F-FDG PET (60). On the other hand, ^{18}F-FDG is a radiolabeled glucose analogue which is uptaken by tumor cells mainly using GLUTs 1 and 3 (61,62). Therefore, the decrease in ^{18}F-FDG uptake percentage when cells were previously incubated with quercetin indicates that, in fact, as already described in the literature, quercetin is a competitive inhibitor of GLUT-1 that can decrease GLUT-1 function but not expression (21,31,59). Sorafenib also induced a decrease in metabolic activity of all the cell lines studied. In fact, the results obtained with all HCC cell lines in response to sorafenib demonstrate, although with some differences among them, that they are generally sensitive to this drug. This is in agreement with what is observed in clinical practice, where sorafenib is, nowadays, the systemic therapy most widely used in HCC treatment (9,63). Interestingly, TFK-1 was the most sensitive cell line to sorafenib. This result becomes very promising because sorafenib is not used in clinical practice for CC treatment. Some studies have shown that sorafenib has a diversified field of action. In addition to being considered an inhibitor of vascular endothelial growth factor receptor 1 (VEGFR-1), VEGFR-2, and VEGFR-3, it can act on various other aspects, e.g., as an inhibitor of some members of mitogen-activated protein kinase (MAPK) family, more particularly of rapidly accelerated fibrosarcoma (RAF) kinase, as well as tyrosine kinase receptors (19,64). In fact, mutations in the MAPK signaling pathway are one of the most frequent genetic alterations in CC. More specifically, in this signaling pathway, mutations in the rat sarcoma viral oncogene (RAS) and B-RAF are common to CC, which makes sorafenib a drug with promising potential in the treatment of this type of cancer (65). For the combined treatment there was synergy effect in HCC cell lines but not for CC cell line. Quercetin has the potential to inhibit epidermal growth factor (EGFR), which is mainly responsible for the resistance that some HCCs have to sorafenib (66,67). Thus, quercetin may sensitize HCC cells to sorafenib through this mechanism and this kind of combination therapy can provide very useful advances in the HCC treatment.

Table 5. Combination index obtained for HepG2, HuH7, Hep3B2.1–7, and TFK-1 cell lines by the Chou and Talalay method.

	Combination index			
	HepG2	HuH7	Hep3B2.1–7	TFK-1
Quercetin + Sorafenib	0.69	0.42	0.70	1.11

Conclusions

It was found that quercetin and sorafenib have the ability to inhibit the metabolic activity of all cell lines studied and that this effect was dependent on the compound concentration and the time of incubation. The combination of quercetin with sorafenib was effective in HCC cell lines. In addition, quercetin induces a decrease in the ability to form colonies of all cell lines studied. The analysis of cell viability concluded that treatment with quercetin decreases the viability of all cell lines and that cell death occurs mainly by apoptosis in a P53-independent manner, accompanied by an increase in BAX/BCL-2 ratio and, in HCC cells, the appearance of preapoptotic peak. The cell cycle analysis led to the conclusion that for all the studied cell lines, in response to quercetin, there was a cell cycle arrest in the G0/G1 and G2/M phases. It was also found that glucose content in the medium influences the ^{18}F-FDG uptake profile with a decrease in the radiotracer uptake of cells previously incubated with quercetin. In parallel, it was found that quercetin induced an increase in GLUT-1 membrane expression.

In summary, the results seem to show that quercetin inhibits the function but not the expression of this transporter; i.e., this is a competitive inhibition. Together,

these results indicate that GLUT-1 is a therapeutic target for PLTs, with quercetin being an option to be considered as part of the key to solve this problem.

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Conflict of Interest: None.

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