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Original Article

The cytotoxic effect of quercetin-induced apoptosis on lung metastatic cells from giant cell tumor of bone

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Abstract

The pulmonary parenchyma is the primary site of metastasis for giant cell tumor (GCT) of bone, a benign yet aggressive musculoskeletal tumor. Current treatments, including surgery and antibody therapy, are only partially effective and often lead to significant side effects. This study aimed to evaluate the apoptotic activity of quercetin, a naturally occurring flavonoid with anticancer properties, on metastatic GCT lung cells (TIB-223). The immunophenotype of the TIB-223 cell line was characterized using flow cytometry, revealing positivity for CD166 and CD47 markers and negativity for CD34, CD73, CD117, CD45, and fibroblast markers. The IC50 of quercetin was determined at 91.1 µM through MTT assays, demonstrating its cytotoxic effect in a dose-dependent manner. Apoptosis was confirmed via flow cytometry and Western blotting, showing increased caspase-3 expression after 24 hours of treatment. These findings indicate that quercetin induces apoptosis in metastatic GCT cells and could serve as a basis for developing phytopharmaceutical therapies targeting this pathology.

Keywords: Nutraceuticals, Anti-cancer therapy, Quercetin, Apoptosis, Giant cell tumor.

1. Introduction

Giant cell tumor (GCT) of bone is a frequent type of musculoskeletal tumor generally found in large bones, particularly in the epiphysis of the femur or tibia and metaphysis, with rare, but yet possible, potential to metastasize with augmented osteoclastic activity [1-3]. GCT is reported to be one of the most common benign bone tumors, presents high incidence rates in young adults aged 20 to 40 years old, and has significant potential to become aggressive [4]. It accounts for nearly 20% of benign bone tumors and presents a slight predisposition to occur in females [3], and approximately 6% of cases manifest metastases with a certain tendency to appear in the pulmonary parenchyma [5]. Metastases are slow-growing nodules that can arise singly or multiple times in the lungs [6]. It has been proposed that metastases originate from tumor emboli located in areas of bleeding that lead to the formation of thrombi inside the primary tumor. In contrast, migration to the lungs originates from the cells within the thrombi [7]. However, a clear relationship between thrombus formation in the vascularized areas inside the primary tumor and the circulating tumoral cells' preference for the lung tissue

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has not been established [6, 8, 9].

The symptomatology for GCT is nonspecific and embraces bone and cortical degradation with surrounding tissue softening, pain, and reduced range of motion at the affected joint, weakening the bone and resulting in fracture [2, 5]. The gold standard for the advanced stage of primary GCT is surgery; however, the effectiveness depends on the resection performed. As many as 27% to 65% of patients treated with surgical methods still present tumor remnants [5]. Notably, most pulmonary metastases are detected approximately a year after surgery for resection of the primary tumor [10-12]. For this reason, the approach for metastasis treatment focuses mainly on surgery and treatment with bisphosphonates. [13, 14].

According to previous reports, we can affirm that there are no primary or metastatic GCT treatments that do not result in significant side effects for patients. In particular, surgery is mainly associated with varying degrees of motor disability [15]; tumor recurrence has been reported at a rate of 40-60% [15], while neurological loss has been observed in 12.5% of cases [16]. There have also been reports of tissue necrosis surrounding the affected area [17, 18]. For pharmacological treatments, studies have reported tumor recurrence in 40% of cases after the suspension of treatment [19] and damage to the digestive system [20].

On the other hand, quercetin is a flavonoid found plentifully in nature and is present in daily human diets. It is present in fruits and vegetables and provides a wide range of biological benefits and effects, including anticancer properties, induction of apoptosis, cell-cycle arrest, and inhibition of cell proliferation [21, 22]. In addition, it has been reported that administrating quercetin at different concentrations causes angiogenesis inhibition and tumor growth suppression and limits the carcinogenesis process [21]. To date, there is only one published report in the literature on the use in vitro of quercetin as an antitumor agent for primary GCT of bone, which provided us with background information and the possibility of employing quercetin in metastatic GCT cells in the lung [23].

The effect of quercetin in inducing apoptosis has been observed in various tumor lines, such as large intestine cancer (SW48 cell line), [24] colon cancer (HCT 116, COLO 320, and HT-29 cell lines), [25, 26] Cholangiocarcinoma (ICC cell line), [27] among others. Still, it has not been described yet whether its cytotoxic effect is maintained in lung metastatic cells from GCT of bone.

Considering the available evidence and resources, we hypothesized that quercetin presents cytotoxic effects and can potentially induce apoptosis in the TIB-223 cell line isolated from GCT metastases. Therefore, this study aimed to evaluate the biological activity of quercetin on the GCT cell line (TIB-223, ATCC®), which was isolated from the lung of a 29-year-old male patient with fibrous histiocytoma to generate new knowledge that can be used in the design of phytopharmaceutical therapies. Secondly, we proposed to use flow cytometry to establish the immunophenotype of cell line TIB-223, which has not been previously characterized.

2. Materials and methods

The TIB-223 cell line was acquired from the American Type Culture Collection (ATCC®, MD, USA), and all experiments were carried out after approval by our institute's research and ethics committee.

2.1. Immunophenotype of the cell line

The immunophenotype of the cell line was established using monoclonal antibodies for detecting human antigens specific to tumoral cells. For these purposes, the following monoclonal antibodies were employed: FITC-conjugated CD73 (rat IgG1,κ, Cat# 130-095-183) from Miltenyi Biotec (Bergisch Gladbach, Germany); PE-conjugated CD166 (mouse IgG1,κ, Cat# 559263), FITC-conjugated CD45 (mouse IgG1,κ, Cat# 555482), APC-conjugated CD117 (mouse IgG1,κ, Cat# 341096), FITC-conjugated CD47 (mouse IgG1,κ, Cat# 556045), and PE-conjugated CD34 (mouse IgG1,κ, Cat# 555822), all from BD Pharmigen (CA, USA); and human fibroblast marker (ER-TR7) and FITC-conjugated antibody (rat IgG2a, Cat# SC-73355) from San Cruz Biotechnology (CA, USA). Cells were marked in 1 mL aliquots containing $6x10^5$ cells each. Furthermore, 2 µL of antibody suspension was added to each tube and incubated for at least 30 minutes at 4°C. Results were obtained using a BD FACSCalibur flow cytometer, whereas the number of events considered was 10,000. The resulting data was analyzed using the CellQuest™ PRO software (Becton-Dickinson, CA, USA).

2.2. Cytotoxic assay via MTT

To conduct the cytotoxic assay, we first expanded a vial of the TIB-223 cell line. Once cells reached optimal confluency, they were seeded on a 96-well (FALCON, AZ, USA) plate at a $0.6x10^3$ cell density per well in McCoy's 5A medium (GIBCO, MA, USA) supplied with FBS at 10% (GIBCO, MA, USA) and antibiotic antimycotic at 1% (Santa Cruz, CA, USA). Cells were incubated overnight at 37°C in an atmosphere of 5% CO_2.

The day after, quercetin (Sigma-Aldrich, Darmstadt, Germany) was diluted in DMSO (Sigma-Aldrich, Darmstadt, Germany) and a range of solutions with concentrations of 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, and 220 µM were prepared in McCoy's supplied medium. The medium in the wells was replaced with the corresponding solution, leaving one control. Afterward, the plate was left to incubate during the night.

The day after, the medium was replaced with fresh McCoy's medium with 5 µL of the MTT reagent (R&D systems, NE, USA, Cat# 4890-25-01) per well; then, the plate was incubated at 37°C in an atmosphere of 5% CO_2 for about 3 hours. The wells were aspirated once the incubation was complete, and the formazan precipitate was diluted in DMSO. To measure absorbance, an ELx800 microplate reader with a 490 nm filter was used (BioTek Instruments, CA, USA). The viability percentage of cells was assessed by comparing the absorbance of the control against that of the treated wells to determine IC50.

2.3. The apoptotic effect of quercetin was assessed via flow cytometry

Apoptosis was evaluated using the Annexin V-FITC apoptosis Detection Kit (eBioscience, CA, USA, Cat# BMS500FI-300). In a 24-well plate, $1.5x10^4$ TIB223 cells were seeded per well. Once confluency reached 80%, the medium was replaced with 125 and 220 µM solutions of quercetin diluted in DMSO. The plate was incubated during the night at 37°C in an atmosphere of 5% CO_2. A binding buffer was prepared by diluting the 4X stock buffer in distilled water using a 1:4 factor. The cells were collected in a 15 ml tube, washed with PBS, and centrifuged. Afte-

rward, the cells were resuspended in 195 µL of binding buffer 1X, and 5 µL of Annexin V-FITC was added to the cell suspension. Finally, the mix was incubated for 10 minutes at room temperature. After incubation, the cells were washed in 200 µL of binding buffer 1X and resuspended in 190 µL of binding buffer 1X and 10 µL of propidium iodide. Fluorescence-activated Cell Sorting (FACS) analysis was performed using a FACSCalibur flow cytometer (Becton Dickinson, CA, USA).

2.4. Western blotting analysis

For protein analysis, 148,750 cells were seeded per well on a 6-well plate. The following day, the cells were exposed to different doses of quercetin for 24 hours. After the exposition time, the cells were washed with PBS 1X, and then 300 µl of RIPA lysis Buffer System (Santa Cruz Biotechnology, Texas, USA) was added to each well. The cells were scraped, collected, and fragmented via sonication; cells were incubated for 30 min at 4° C. The samples were centrifuged at 15,000 rpm for 20 min at 4°C. Proteins were quantified, and 60 µg were separated using electrophoresis on a 12% SDS-PAGE gel, followed by transfer to a nitrocellulose membrane. The membranes were blocked, and primary antibodies for caspase-3 (Santa Cruz Biotechnology, Texas, USA) and anti-ACTB-Peroxidase (Sigma-Aldrich, MO, USA) were incubated overnight. The day after, the membranes were washed, followed by incubation of the secondary antibody for one hour at room temperature. After another round of washing, images were captured using a C-DiGit® Blot Scanner (LI-COR Biosciences, NE, USA). The relative density of specific bands was then quantified using Image Studio Digits V4.0 (LI-COR Biosciences, NE, USA).

2.5. Statistical analysis

Statistical analysis was performed using the GraphPad Prism version 9.0.1 for Mac OS X, GraphPad Software, San Diego, California, USA. A T-test for independent samples was used to compare percentages of fluorescence obtained from the control and treatment groups. Statistically significant differences were established at $p < 0.05$. Data are presented as mean ± standard deviation.

3. Results

3.1. Establishment of the immunophenotype of metastatic giant cell tumor cell line (TIB-223)

After labeling the cells with the antibodies, we analyzed them using flow cytometry and obtained the following results. (mean ± standard deviation): CD73: 0.83% ± 0; CD166: 87.89% ± 0.01; CD45: 0.54% ± 0.02: CD117: 0.01 % ± 0; CD47: 91% ± 0.02; CD34: 0.46% ± 0.01; and ER-TR7: 0.7% ± 0.08. Histograms with positive results correspond to CD47, found on the surface of the cell membranes of almost all normal cells. CD47 has been identified in gastric cancer tumor cells [28, 29] and hematological cancers like Hodgkin and non-Hodgkin lymphoma [30]. In addition, the marker CD166 for activated T cells has been reported in other types of cancer, such as colon [31], prostate cancer [32], and peritoneal metastases from gastrointestinal cancers [33, 34]. The immunophenotyping results confirm that the TIB-223 cell line is related to other malignant and metastatic tumor cells (Fig.1).

3.2. Evaluation of half-maximal inhibitory concentration (IC50)

MTT assays were performed to evaluate the antiproliferative effects of quercetin on giant cell tumor metastatic cells (TIB-223). After performing the experiments, the IC50 was determined at 91.1 µM. Figure 2 illustrates a significant decrease in cell viability in a dose-dependent manner.

During the MTT assay, yellow tetrazolium was reduced to purple formazan, corresponding to active, viable living cells. Higher cell viability was observed in the control group containing fresh medium, resulting in increased formazan precipitation. However, comparing the results in Fig.2 with Fig.3, it can be observed that as we increase the quercetin doses, formazan precipitation decreases, leading to a reduction in absorbance and, thus, in the percentage of cell viability. This suggests that quercetin has a cytotoxic effect on the TIB-223 cell line.

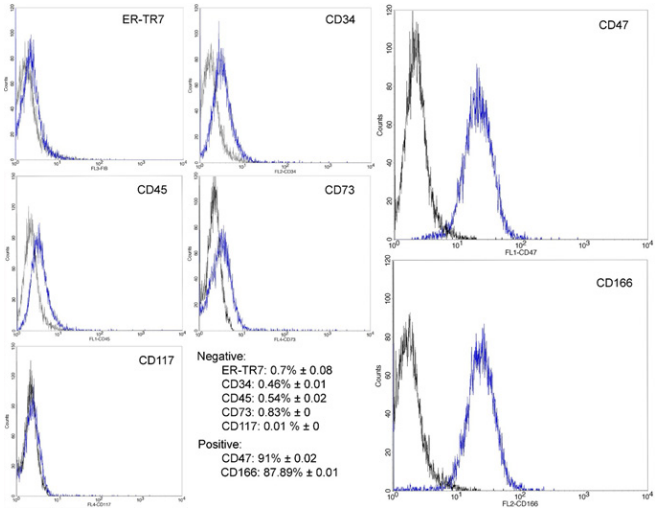

Fig. 1. Immunophenotype of a giant cell tumor retrieved from the fibrous histiocytoma of the 29-year-old male patient (TIB-233). The results are presented according to the positivity percentage of fluorescence for each conjugated fluorochrome with each antibody compared with events counted. The black peak corresponds to the autofluorescence of the cells, whereas the blue represents the fluorescence of the marked cells with each antibody.

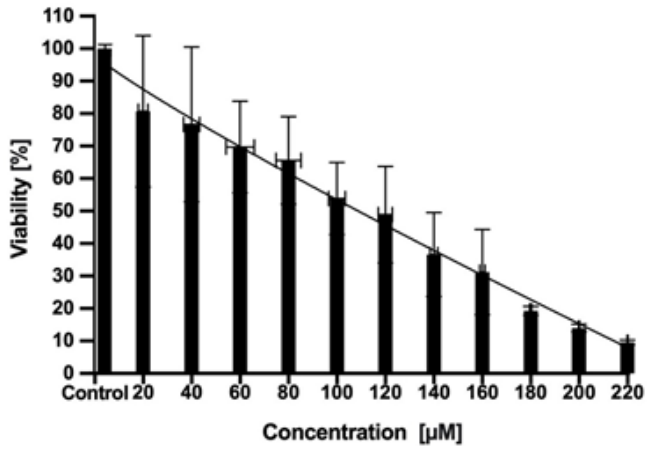

Fig. 2. The percentage of cellular viability at different quercetin concentrations was assessed through the MTT assay read at 490 nm. IC50 was determined at 91.1 µM. The results are presented in average ± standard deviation.

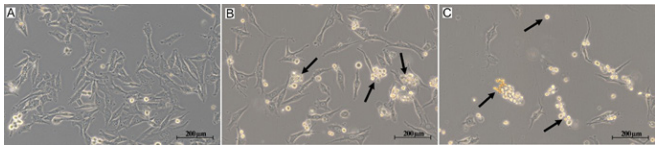

Fig. 3. Microphotographs of TIB-223 cells after being treated with quercetin at different doses: (A) cells without treatment (control group); (B) cells after 24 hours of being treated with a dose of 91.1 µM (IC50); and (C) cells after 24 hours of receiving a 220 µM dose. Cell number decreases in cells treated with quercetin (B and C), with a contrasting increase in cellular debris (black arrows) and loss of homogeneous cell morphology.

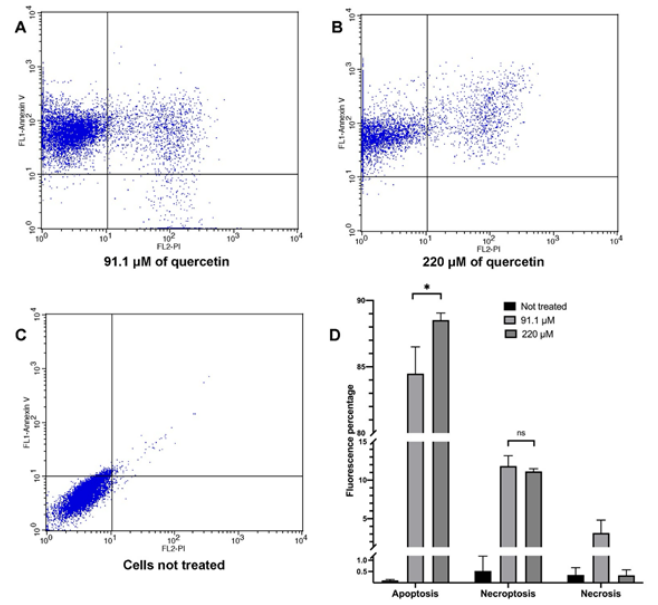

Fig. 4. Apoptotic and necrotic effects on TIB-223 cells were determined using the Annexin V/PI staining kit. (A) Cell culture after 91.1 µM of quercetin treatment during 24 hours in the control cell culture, where a few positive cells for necrosis and necroptosis are observed and where the population of apoptotic cells (blue) predominates in the upper left quadrant of the dot plot. (B) Cell culture after 220 µM of quercetin treatment for 24 hours. For this analysis, the positive cells (blue) are concentrated in the population of apoptotic cells (upper left quadrant) and to a lesser proportion in the cell population of cells in necroptosis (upper right quadrant). (C) Cells were not treated after 24 hours of cellular culture. Here, we found that most of the cells were concentrated in the lower left quadrant, being negative for annexin V and propidium iodide, with an average percentage of 99.02% ± 1.03, which indicates a high viability of these cells. (D) Comparative analysis of the different treatments to which TIB-223 cells were exposed highlights a statistically significant increase between the concentrations of 91.1 µM vs 220 µM when analyzing apoptosis (p= 0.001), as well as a non-significant decrease in necroptosis between concentrations 91.1 µM vs 220 (p= 0.58). Finally, there is also a significant decrease in necrosis when comparing the concentration of 91.1 µM vs 220 (p= 0.001). * $p < 0.05$ are significantly different; ns, are not significantly different.

3.3. Quercetin's effect on cell apoptosis and necrosis was evaluated via flow cytometry.

The apoptotic and necrotic effects of quercetin on TIB-223 were measured using annexin V to evaluate apoptosis and propidium iodide to evaluate necrosis and double labeling (annexin V/propidium iodide (PI) to assess necroptosis) via flow cytometry.

The exposure of TIB-223 cells to 91.1µM of quercetin for 24 hours resulted in a mean value of 84.48% ± 2.2 for apoptosis, 3.14% ± 1.67 for necrosis, and 11.84% ± 1.67 for necroptosis. In cells exposed to 220 µM of quercetin, a significant decrease in apoptosis was observed 0.33% ± 0.24. For necroptosis, the values remained similar, with an average of 11.14% ± 0.35. Finally, for untreated cells, significantly lower values were obtained for all markers, with an average value for apoptosis of 0.11% ± 0.04, for necrosis 0.33% ± 0.24, and for necroptosis with a value of 0.53% ± 0.66 (Fig.4).

3.4. Apoptotic effector caspase-3 up-regulation analysis with Western Blotting

We examined the apoptotic effector caspase-3 using Western blotting to elucidate whether quercetin could activate it. The results showed that after 24 hours of treatment with various concentrations of quercetin, the concentration of 220 µM was the most effective *(** $p=0.000017$)*, whereas the 91.1 µM quercetin concentration achieved statistical significance *(* $p=0.005$)*. The caspase-3 protein increased in a dose-dependent manner, and both are effective in upregulating this protein expression compared to the control (Fig.5).

4. Discussion

This study demonstrated the biological effects of quercetin on metastatic GCT cells and its inhibitory effects. In addition, the immunophenotype of the cell line positive for CD47 and CD166 was established. In healthy tissues, these markers have been described as corresponding to human peripheral blood lymphocytes and human fibroblasts,

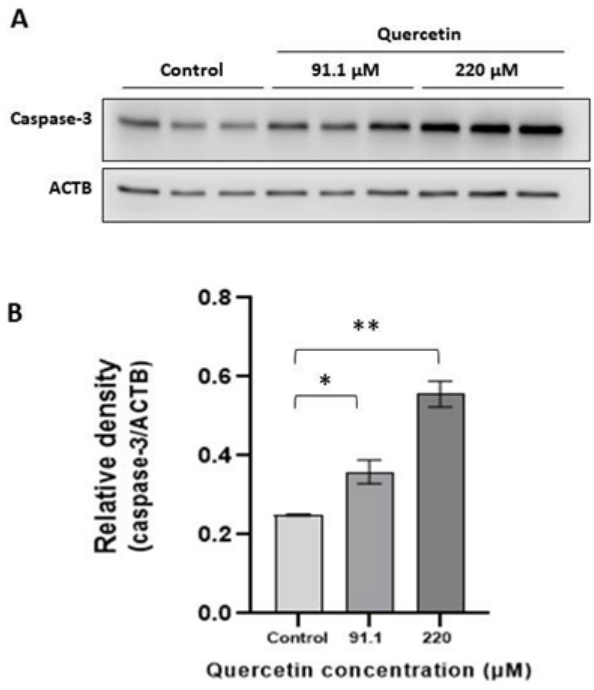

Fig. 5. Effect of quercetin on caspase-3 in a giant cell tumor. A) Western blot analysis of caspase-3 in giant cell tumor (TIB223) treated for 24 h with quercetin. Bands were normalized to β-actin intensity. B) Densitometric analysis represented a relationship between caspase-3 intensity and actin intensity. After 24h of treatment with quercetin, the caspase-3 protein increased significantly compared with the control in a dose-dependent manner.

respectively [6, 8]. Ultrastructural studies have confirmed three cell types in the primary GCT, where the mononuclear spindle-shaped tumor cells show fibroblastic morphology at the ultrastructural level. This corresponds to the immunophenotype of the cell line, which in this study refers to metastatic cells from the primary tumor. Additionally, these two markers have been reported in other types of metastatic cancer, such as prostate and gastric [32-34].

The MTT assays evidenced a high level of cytotoxicity, which can be correlated to the results of the Annexin V/PI assay, as cytotoxicity induced by apoptosis predominantly using the IC50 (91.1 µM) or even higher doses, such as 220 µM, where this trend of cytotoxicity induced by apoptosis remains constant (see Figure 4D).

In a previous work described by Estrada-Villaseñor et al. [23], cells were isolated directly from a primary GCT of bone and were exposed to quercetin. The IC50 was established to be 120µM, and it was observed that most of the cells were found in necroptosis and secondarily in necrosis. On the other hand, for GCT cells (TIB-223) isolated from lung metastasis, we found the IC50 at 91.1 µM and with a majority population in the process of death by apoptosis (84.48%) and secondarily in necroptosis (11.84%) and to a lesser proportion in necrosis (3.14%); both studies were made at 24 hours after treatment. From these results, we hypothesize that metastatic cells are significantly more sensitive to quercetin (28.9 µM less). As cells migrate from the primary bone tumor to colonize the lung tissue, they undergo metabolic changes that facilitate cell migration. These changes affect the cell membrane's structure and may favor quercetin's absorption into the cells. At the same time, most TIB-223 cells went into apoptosis, as illustrated in Figure 4A.

Also, our results showed that caspase-3 protein was significantly upregulated at 24 h after treatment with quercetin (91.1 µM). In contrast, as previously reported with primary cells GCT of bone (120µM, 24 hours to treatment), the caspase-3 did not present significant differences compared to the control, and the cellular damage was reported mainly by necroptosis, autophagocytosis, and secondary necrosis [23].

Similar to our findings, Saravani et al. (2024) [35] reported that Psidium guajava extract had positive effects on blood parameters and showed low toxicity at high doses. This further supports the potential of natural compounds as a therapeutic avenue, suggesting that the observed effects of quercetin are not unique and may be part of a broader trend of beneficial impacts from natural sources.

Our findings demonstrate that quercetin induces apoptosis in metastatic GCT cells, possibly through its antioxidant activity. Similarly, Hosseini et al. (2024) [36] found that methanol extracts from Zeravschania membranacea, Zeravschania aucheri, and Aegopodium tribracteolatum (Apiaceae family) exhibited significant antioxidant properties, attributed to their high phenolic and flavonoid content, including quercetin. This reinforces the potential of natural antioxidants in influencing cellular processes relevant to cancer.

These results suggest that caspase-3 mediates apoptosis in metastatic GCT cells induced by quercetin. However, the mechanism and role of quercetin in GCT are still unclear and require further study.

This study demonstrates that quercetin, a readily available and producible antioxidant, effectively induces cell death in metastatic GCT of bone, primarily through apoptosis. The observed cytotoxic effects and caspase-3 activation in TIB-223 cells suggest that quercetin may disrupt mitochondrial membrane potential, leading to the release of pro-apoptotic factors. Furthermore, the immunophenotypic characterization of TIB-223 cells, revealing the presence of CD166 and CD47 markers, aligns with other metastatic tumor cells, reinforcing the potential of quercetin as a targeted therapy. These findings support further investigation into quercetin as a phytopharmaceutical agent and warrant preclinical studies in animal models to validate its efficacy and safety in treating metastatic GCT. Further research should explore the synergistic effects of quercetin with existing treatments like surgery and antibody therapy to improve outcomes for patients with this challenging malignancy.

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Conflict of interest
The authors declare no conflicts of interest.

Consent for publication
The author read and proved the final manuscript for publication.

Availability of data and material
The data supporting this study's findings are available from the corresponding author upon reasonable request.

Authors' contribution
Conceptualization, C.L.-S. and A.B.L.-A.; methodology, A.E.M.-R., J.R.C.-G., and M.d.R.A.-G.; validation, L.S.-C., C.L.S.-C., E.P.F.-B., A.B.L.-A., and M.d.R.A.-G.; formal analysis, E.P.F.-B. and G.L.-H.; investigation, A.E.M.-R., C.L.S.-C., L.S.-C., G.L.-H., and J.R.C.-G.; resources, C.L.S.-C., E.P.F.-B., B.C.C.-G., and G.L.-H.; data curation, C.L.-S.; writing original draft preparation, C.L.-S., A.E.M.-R., J.R.C.-G., and A.B.L.-A.; writing review and editing, C.L.-S and A.B.L.-A.; visualization, M.d.R.A.-G., B.C.C.-G., and I.U.B.-O.; Quality control of raw materials, B.C.C.-G. and I.U.B.-O., supervision, C.L.-S., L.S.-C., A.B.L.-A., and I.U.B.-O.; project administration, C.L.-S.; funding acquisition, C.L.-S. All authors have read and agreed to the published version of the manuscript.

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Ethics approval and consent to participate
This work was approved by the research and ethics committee of the National Institute of Rehabilitation "Luis Guillermo Ibarra Ibarra" (INR LGII 52/19).

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