



# Ethanol extract of gabirobeira (*Campomanesia adamantium*) leaves reduces proliferation of osteosarcoma cells *in vitro*

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**ABSTRACT.** Osteosarcoma is the most commonly diagnosed malignant bone tumor in humans, with a higher incidence in children and young people. It is highly aggressive and has a high metastatic potential. Its treatment is based on both chemotherapy and surgical intervention. However, currently used chemotherapeutic agents, such as doxorubicin, have several adverse effects on the patient. Therefore, there is a growing demand for new chemotherapeutic agents that stimulate new researches, such as those involving compounds extracted from plants, such as the gabirobeira. In this study, we aimed to evaluate the cytotoxic effects of ethanolic extract, both crude and ethyl acetate, of gabirobeira leaves on osteosarcoma cells *in vitro*. Cytotoxicity was evaluated using the Trypan blue exclusion method and the IC<sub>50</sub> values were calculated using the tetrazolium reduction method. The ethanolic extract of gabirobeira leaves showed a cytotoxic effect on osteosarcoma cells *in vitro*. The group treated with the crude extract at 1.0  $\mu$ L mL<sup>-1</sup> concentration for 48 hours showed higher cytotoxicity and the lowest IC<sub>50</sub> value for this extract was found in the 24 to 48 hours interval. The ethanolic extract of gabirobeira leaves is cytotoxic for osteosarcoma cells.

**Keyword:** plant extracts; tumor; *Campomanesia adamantium*.

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## Introduction

Osteosarcoma (OS) is a mesenchymal origin highly aggressive malignant bone tumor, with high morbidity and mortality. Besides, it has a high metastatic potential, especially in the lungs (Brown, Schiavone, Gouin, Heymann, & Heymann, 2018; Xie et al., 2017; Evola et al., 2017; Dorfman et al., 2002).

Diagnosis of OS is based on medical history, laboratory, and complementary physical examinations, such as radiography and histopathology (Ritter & Bielack, 2010). Treatment is usually multimodal, including systemic chemotherapy and surgery (Mercatelli, Bortolotti, Bazzocchi, Bolognesi, & Polito 2018). The most commonly used chemotherapeutic agents are cisplatin (CDP), doxorubicin (DOX), methotrexate (MTX), and ifosfamide (IFO) that can be used as neoadjuvant (preoperative) and adjuvant (postoperative) therapy (Mercatelli et al., 2018; Harrison & Schwartz, 2017). Although important for the treatment of patients, these drugs may cause short- and long-term adverse effects, such as nausea, vomiting, alopecia, mucositis, nephrotoxicity, among others (Harrison & Schwartz, 2017). Moreover, its prognosis is reserved for patients with metastases. Survival rates at five years after OS diagnosis for patients with metastases are only 10% to 30%, and for patients without metastases 60% to 70% (Mercatelli et al., 2018).

The incidence of adverse reactions justifies the search for new antineoplastic agents. Recently, the number of researches towards the discovery of new chemotherapeutic agents has grown, often using plants and other natural sources (Zhang et al., 2013). In this regard, the Brazilian Cerrado is considered a source of biological resources (Silva, Boas, Barros, Rodrigues, & Siqueira, 2009). Its flora is rich in fruit species that have great therapeutic potentials, such as *Campomanesia adamantium* (Cambess. O. Berg), popularly known as the guabiroba, guavira, guariroba, guabiroba-do-campo, guabiroba-do-cerrado and guabiroba-lisa (Campos et al., 2017; Pascoal et al., 2014).

*Campomanesia adamantium* is a shrub, with thin and multiple branches, measuring up to 2m in height (Vieira et al., 2011; Amaral, Reis, Ressel, & Pinto, 2016). Its leaves are simple, opposite, oval, or elliptical. It blooms from September to November and has flowers that are white and solitary (Amaral et al., 2016). The fruits ripen from November to December, are round, dark to light green and yellow colors (Vieira et al., 2011; Amaral et al. 2016; Vallilo et al. 2006). They are edible by both animals and the local population and are used in food manufacturing of products such as juices, jams, liqueurs, and jellies (Pascoal et al. 2014; Vallilo, Lamardo, Gaberlotti, Oliveira, & Moreno 2006).

In traditional medicine, this plant is used as antidiarrheal, antimicrobial, antioxidant, antirheumatic, antitumor, as well as in urinary tract infections, reducing cholesterol levels and treating diabetes. Studies have demonstrated its antimicrobial, antiproliferative, anti-inflammatory and antinociceptive activity (Campos et al., 2017; Pascoal et al., 2014; Viscardi et al., 2017; Ferreira et al. 2013; Fernandes et al. 2015), besides the cytotoxic potential of its leaf extract. Chalcone cardamonin, isolated from the ethanolic extract of leaves, has been reported to have antiproliferative activity in prostate PC-3 cancer cells (Campos et al., 2017; Pascoal et al., 2014).

Therefore, in this study, we aimed to assess the *in vitro* cytotoxic effects of gabirobeira leaf extract on OS cells culture to identify compounds with antineoplastic potential and, possibly, demonstrate a viable alternative to the currently chemotherapy treatments.

## Material and methods

**Plant material and extraction** - The crude ethanolic extract of gabirobeira leaves (EEG) was obtained from dry and fragmented leaves of *C. adamantium* using the maceration technique. The botanical material, in this case gabirobeira leaves, was obtained in the *Universidade Federal de Goiás* (UFG), Campus Jataí, Jataí, GO, Brazil (16° 17' 26.6" S, 048° 57' 21.1" W) and identified by the Prof. Dr. Luiza Francisca de Souza.

The leaves were washed and oven-dried at 35°C for seven days to eliminate moisture and stabilize the enzymatic content. Subsequently, they were ground in an electric knife mill. Extraction occurred by macerating the plant material in absolute alcohol at a rate of 2,200 mL of alcohol 480 g<sup>-1</sup> of powder for 72 hours at room temperature. The extract was then filtered and concentrated in a rotary evaporator (Model Ika-Werke) at a constant temperature of 45°C.

To obtain the ethyl acetate extract (EAE), the crude extract was submitted to the solid-liquid extraction process. The extract was pelleted after being placed on the silica gel. Subsequently, it was placed on a chromatographic column with acetate added to the saturation point to obtain the ethyl acetate fraction.

**Ethics** - This study was approved by the Research Ethics Committee of the *Universidade Federal de Goiás* (CEP/UFG).

**Cells and culture** - OS cells (MG-63, BCRJ 0173, Lot 000888, Passage 104), from the ATCC (American Type Culture Collection - Manassas, VA, USA), were obtained at the Rio de Janeiro Cell Bank (UFRJ - Rio de Janeiro, Brazil). They were placed in a humidified CO<sub>2</sub> incubator at 37°C and 5% CO<sub>2</sub> atmosphere. Cultivation in bottles was performed in Dulbecco's Modified Eagle Medium (DMEM) plus 20% fetal bovine serum, amphotericin B and L-glutamine solution (all from Cultilab, Campinas, Brazil) according to the method of Yu et al. (2014).

**Experimental design** - After the cell culture, the cells were quantified in the Neubauer chamber and seeded in 96-well plates containing 100µL DMEM medium at concentrations of 5x10<sup>4</sup> e 1x10<sup>4</sup>, respectively, for the evaluation of cell viability (CV) using the Trypan blue exclusion method and for the CV assay using the tetrazolium reduction method [MTT (3- (4,5-dimethyl-2-thiazolyl) -2,5-diphenyl-2H-tetrazolium)]. The plate was placed in a humidified CO<sub>2</sub> incubator at 37°C and 5% CO<sub>2</sub> atmosphere for 24 hours.

The medium was then discarded and the wells treated and not treated with the gabirobeira leaves extract (EEG and EAE) for 24 (G<sub>24</sub>), 48 (G<sub>48</sub>) and 72 (G<sub>72</sub>) hours, at concentrations of 0.1 µL mL<sup>-1</sup>, 1 µL mL<sup>-1</sup>, 10 µL mL<sup>-1</sup> and 100 µL mL<sup>-1</sup>. Moreover, the negative control group (CG) was treated only with dimethyl sulfoxide (DMSO, Cultilab, Campinas, Brazil), aiming to verify whether cell death in the groups treated with the extracts is solely due to their effect since the extracts are diluted in DMSO.

After treatment with the extract, the medium was discarded and cells submitted to the following analyses: assessment of CV using the Trypan blue exclusion method and CV assay using the tetrazolium reduction method. Both assays were performed in three independent experiments and triplicates.

**Cell viability using the Trypan blue exclusion method** - For the assessment of CV using the Trypan blue exclusion method, the technique used was adapted from Mosman (1983) and Peres et al. (2015). After

treatment, the wells were washed twice with 100  $\mu\text{L}$  phosphate-buffered saline (PBS, Cultilab, Campinas, Brazil). Then, the cells were resuspended using 100  $\mu\text{L}$  of trypsin (Cultilab, Campinas, Brazil) in each well. The plates were then kept in the humidified  $\text{CO}_2$  incubator for five minutes, homogenized and thus, remained for a further five minutes in the incubator. Soon after, trypsin was inactivated with 100  $\mu\text{L}$  DMEM.

Subsequently, the material was centrifuged at 1400 rpm for 10 minutes and after that, the medium was discarded and cells resuspended in 1 mL DMEM. From this new solution, 40  $\mu\text{L}$  were transferred to microtubes microcentrifuge containing 40  $\mu\text{L}$  Trypan blue (Trypan blue - Sigma-Aldrich, St. Louis, USA). Then, to assess the CV, 10  $\mu\text{L}$  of this new solution was instilled in a slide to be read in the Luna Automated Cell Counter. Cytotoxicity (CT) was estimated by the equation:

$$\%CT = 100 - [(\text{abs of treatment}/\text{abs of negative control}) \times 100]$$

Where CT is cytotoxicity and abs is absorbance.

We analyzed the values of each group using the Scott-Knott test. From the results, we calculated the CV, CT and cell size (CS) means for each group of exposure time ( $G_{24}$ ,  $G_{48}$ , and  $G_{72}$ ) and both extracts (EEG and EAE).

Cell viability assay using the tetrazolium reduction method (TRM) – The cell viability assay using TRM [3-(4,5-dimethyl-2-thiazolyl) -2,5-diphenyl-2H-tetrazolium] was performed by adapting the technique described by Yu et al. (2014). After treatment, 10  $\mu\text{L}$  TRM was added in each well, previously diluted in phosphate-buffered saline (PBS, Cultilab, Campinas, Brazil) at the rate of 500mg TRM to 100mL PBS, and plates kept in a humidified  $\text{CO}_2$  incubator for 3 hours. Afterwards, 50  $\mu\text{L}$  of 10% sodium dodecyl sulfate (SDS, Vivantis Biochemical) was added to stop the reaction and kept the plates at room temperature for 24 hours. Following, a 10% SDS solution was prepared using 1g SDS, 9mL 0.01N hydrochloric acid solution, 0.15 mL hydrochloric acid, and 499.85 mL distilled water.

Thereafter, the optical density of the wells was quantified through a spectrophotometer (Awareness Technology Inc/Stat Fax 2100, 425-540 nm, Palm City, FL, USA) and the concentration value that inhibits 50% of cell viability (Inhibitory concentration -  $\text{IC}_{50}$  in  $\mu\text{L mL}^{-1}$ ) was determined using the GraphPad Prism statistical program (GraphPad Software, San Diego, CA, USA).

## Results and discussion

The Trypan blue exclusion method is used to evaluate the cytotoxicity by measuring cell viability. This analysis allowed the identification of non-viable cells. Viable cells did not allow the Trypan blue dye to penetrate the intracellular medium due to their membrane integrity. However, the non-viable cells, due to their damaged membranes, allowed the passage of dye and were stained blue.

From this assay, we calculated the CV, CT and CS (cell size) mean values at the concentrations used (0.1, 1, 10 and 100  $\mu\text{L mL}^{-1}$ ) and at the three exposure times ( $G_{24}$ ,  $G_{48}$  e  $G_{72}$ ) of both extracts (EEG and EAE). Regarding the EAG, we found no statistical difference for the three parameters between the concentrations used for groups  $G_{24}$  and  $G_{72}$ , as well as the CS in  $G_{48}$ . However, CV and CS for  $G_{48}$  showed a statistical difference between the 0  $\mu\text{L mL}^{-1}$  and 0.1  $\mu\text{L mL}^{-1}$  concentrations in relation to the 1, 10 and 100  $\mu\text{L mL}^{-1}$  concentrations, as shown in Table 1.

**Table 1.** Mean values of cell size (CS), cell viability (CV), and cytotoxicity (CT) parameters for each concentration group of the crude ethanol extract of gabirobeira leaves (EEG) in relation to the exposure time. Data were calculated using the Scott-Knott test from the values obtained from the Luna Automated Cell Counter in CV assessment using the Trypan blue exclusion method.

Exposure time	Parameter	EEG concentration				
		0 $\mu\text{L mL}^{-1}$	0.1 $\mu\text{L mL}^{-1}$	1 $\mu\text{L mL}^{-1}$	10 $\mu\text{L mL}^{-1}$	100 $\mu\text{L mL}^{-1}$
24 hours	%CS	100a	99.35a	93.7a	99.47a	100.19a
	%CV	100a	62.6a	71.19a	96a	62.6a
	%CT	100a	123.62a	128.42a	111.15a	131.62a
48 hours	%CS	100a	112.67a	101.97a	107.52a	106.22a
	%CV	100a	85.7a	57.26b	25.79b	40.29b
	%CT	100b	137.56b	185.6a	219.04a	200.17a
72 hours	%CS	100a	100a	96.97a	94.43a	88.65a
	%CV	100a	118.61a	99.89a	82.82a	76.34a
	%CT	100a	102.48a	114.51a	136.05a	132.56a

For EEG, we found no statistical difference between the concentrations used (0.1, 1, 10 and 100  $\mu\text{L mL}^{-1}$ ) at the three exposure times ( $G_{24}$ ,  $G_{48}$  and  $G_{72}$ ) in any of the three analyzed parameters (CS, CV and CT), according to Table 2.

**Table 2.** Mean values of cell size (CS), cell viability (CV) and cytotoxicity (CT) parameters for each concentration group of the acetate extract of gabirobeira leaves (EAE) in relation to the exposure time. Data were calculated by the Scott-Knott test from the values obtained from the Luna Automated Cell Counter in the CV assessment using the Trypan blue exclusion method. For the assay, we performed three independent experiments and in triplicates.

Exposure time	Parameter	EAE concentration				
		0 $\mu\text{L mL}^{-1}$	0.1 $\mu\text{L mL}^{-1}$	1 $\mu\text{L mL}^{-1}$	10 $\mu\text{L mL}^{-1}$	100 $\mu\text{L mL}^{-1}$
24 hours	%CS	100a	76.63a	88.79a	87.88a	88.32a
	%CV	100a	119.58a	151.19a	163.95a	96.86a
	%CT	100a	117.56a	94.96a	107.97a	113.67a
	%TC	100a	81.67a	84.39a	96.08a	82.16a
48 hours	%CS	100b	56.14a	116.29a	95.47a	83.14a
	%CV	100a	132.5a	90.92a	104.43a	116.3a
	%CS	100a	96.6a	94.54a	90.99a	88.22a
72 hours	%CV	100a	89.62a	92.26a	61.59a	80.34a
	%CT	100a	129.97a	101.86a	142.63a	117.56a

Regarding CV and CT, groups  $G_{24}$  and  $G_{48}$  submitted to treatment with EEG showed higher CV and lower CT compared to EEG. However, the CV of  $G_{72}$  was lower and CT higher when submitted to the EAE. Moreover, regarding CV,  $G_{24}$  and  $G_{48}$  groups showed a statistical difference between the extracts, while  $G_{72}$  showed no difference. Regarding CT, only  $G_{48}$  showed a statistical difference between the treatments used. Regarding CS, groups  $G_{24}$ ,  $G_{48}$  and  $G_{72}$  treated with EEG had higher CS compared to groups treated with EAE. However, groups  $G_{24}$  and  $G_{72}$  showed no statistical difference between the extracts used, unlike  $G_{48}$ , as shown in Figure 1.

Given these results, it appears that EEG has higher antiproliferative potential than EAE, because it showed a lower CV and higher CT. Moreover, when analyzing the cell groups treated with EEG, we verified that  $G_{48}$  showed higher potential than  $G_{24}$  and  $G_{72}$ . Finally, among the concentrations used in  $G_{48}$ , the concentrations 1, 10 and 100  $\mu\text{L mL}^{-1}$  showed significant results. However, we highlight the result at 1  $\mu\text{L mL}^{-1}$  since it is the smallest concentration and therefore, in theory, has fewer adverse effects.

Moreover, the increase in CT, consequently the reduction in CV, progressively occurred for EAE. However, for EEG, a progressive increase of CT occurred only between  $G_{24}$  and  $G_{48}$ , while between  $G_{48}$  and  $G_{72}$ , the CT decreased. This fact, together with the absence of statistical difference between extracts in  $G_{72}$ , may suggest that the cytotoxic effect of crude extract decreases with certain exposure time.

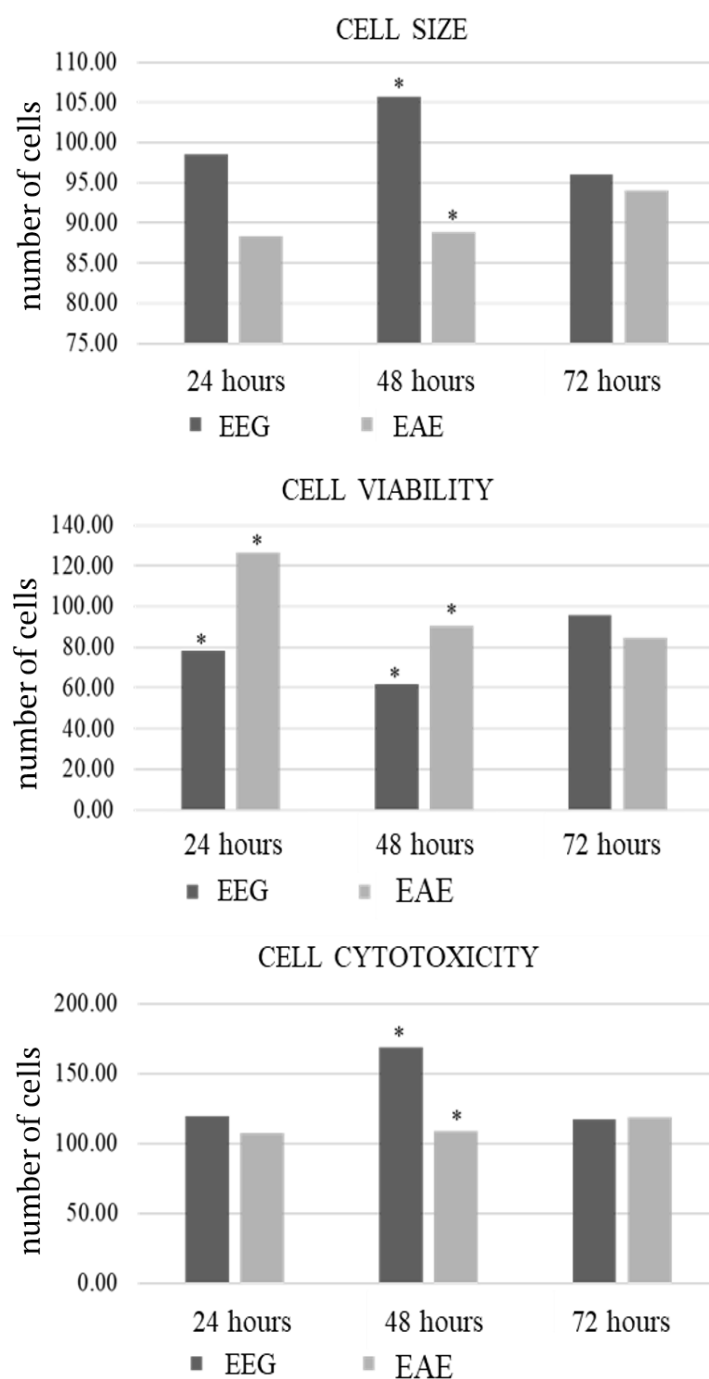
The concentration required to inhibit 50% of cells in vitro was determined by calculating the  $\text{IC}_{50}$  values from TRM cell viability assay data using the GraphPad Prism statistical program. The TRM assay was one of the first methods to be accepted for cell viability and cytotoxicity. It is a colorimetric test, where the absorbance measurements are carried out at the end of the test. TRM uses tetrazolium, a yellow compound, that is cleaved by mitochondrial dehydrogenases and reducing agents present in formazan crystals of viable cells, a blue compound, insoluble in aqueous solution. Crystals accumulate in hydrophobic cytoplasmic domains, such as lipid droplets. Thus, tetrazolium acts as an indicator of intracellular reducing potential (Pascua-Maestro et al., 2018).  $\text{IC}_{50}$  values are shown in Table 3. The  $\text{IC}_{50}$  for EEG progressively increased with time exposure to extract. However, its  $G_{48}$  value was within the proposed range of best cytotoxicity.

The high  $\text{IC}_{50}$  value in  $G_{72}$  corroborates the results found in the cytotoxicity evaluation, in which there was less cell death compared to  $G_{48}$ . It reinforces the theory that the action of this extract decreases with the exposure time. Thus, it is necessary to investigate the antiproliferative activity of this extract by consecutive treatments throughout the exposure time.

A pertinent consideration for *C. adamantium* extract is the relationship between the plant material used and its cytotoxic potential. In the antiproliferative activity analysis of the leaves and roots aqueous extract in leukemia, we found  $\text{IC}_{50}$  value of 40 and 80  $\mu\text{g mL}^{-1}$ , respectively, in Jurkat cells (neoplastic T lymphocytes) (Silva et al., 2009). Thus, although the cytotoxic potential of gabirobeira leaves has already been demonstrated (Silva et al., 2009; Campos et al., 2017), other types of botanical materials are need to be studied.

Another crucial factor is the fraction of the extract used. EAE showed lower cytotoxic potential than EEG, suggesting that the compounds responsible for this effect are lower in the acetate fraction. The ethanolic

extract of *Mesona Palustris* BL also showed higher cytotoxicity,  $IC_{50}$  value of  $146.0 \mu\text{g mL}^{-1}$ , compared to ethyl acetate,  $IC_{50}$  value of  $182.96 \mu\text{g mL}^{-1}$  (Widyaningsih, 2012).



**Figure 1.** Graphical representation of cell size (CS), cell viability (CV) and cell cytotoxicity (CT) mean values obtained by the Trypan blue exclusion assay. Osteosarcoma cells were treated with ethanolic extract of gabirobeira leaves (EEG) and acetate ethanolic extract of *Campomanesia adamantium* leaves (EAE) at concentrations of 0.1, 1, 10 and  $100 \mu\text{L mL}^{-1}$  for 24, 48 and 72 hours. For the assay, three independent experiments in triplicates were performed for each extract. \*Statistical difference between crude and acetate extracts.

**Table 3.** Concentration values necessary to inhibit 50% ( $IC_{50}$  in  $\mu\text{L mL}^{-1}$ ), calculated through the cell viability using the tetrazolium reduction method (TRM). MG-63 osteosarcoma cells were subjected to treatment with ethanolic extract of gabirobeira leaves (EEG) and acetate ethanolic extract of *C. adamantium* leaves (EAE) at concentrations of 0.1, 1, 10 and  $100 \mu\text{L mL}^{-1}$  for 24, 48 and 72 hours. For the assay, three independent experiments in triplicates were performed for each extract.

Extract	24 hours	48 hours	72 hours
EEG	$2.46 \mu\text{L mL}^{-1}$	$9.49 \mu\text{L mL}^{-1}$	$35.73 \mu\text{L mL}^{-1}$
EAE	$79.44 \mu\text{L mL}^{-1}$	$0.07 \mu\text{L mL}^{-1}$	$0.24 \mu\text{L mL}^{-1}$

The cell line employed in the analyses is another factor to consider. In leukemia, the aqueous extract of *C. adamantium* leaves showed no significant effect on peripheral blood mononuclear cells, while in Jurkat cells, it showed cytotoxic activity. The same study demonstrated that vinblastine promoted cell death in both peripheral mononuclear blood and Jurkat cells, but presented distinct  $IC_{50}$  values, respectively 25.9 and 9.5  $\mu\text{g mL}^{-1}$  (Silva et al., 2009).

On that basis, we emphasize the need to evaluate the cytotoxic potential of gabirobeira leaves through other fractions of the extract, as well as in other OS lines and other neoplasms. Besides, it is important to evaluate other types of botanical materials of this plant such as the roots, since the results suggest that this plant may constitute a new alternative to the currently used chemotherapeutic agents.

## Conclusion

From the results obtained, we conclude that the ethanolic extract of gabirobeira leaves (*Campomanesia adamantium* (Cambess. O. Berg)) has a cytotoxic effect on MG-63 osteosarcoma cells, more pronounced after 48 hours of treatment.

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