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RESEARCH ARTICLE

Cell Viability Assessment by MTT After Treatment of Hepatocellular Carcinoma Cells (HepG2) with *Chelidonium majus*

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ABSTRACT

Hepatocellular carcinoma is a type of cancer with a high mortality rate since it is primarily diagnosed in an advanced stage. Therefore, searching for therapies that help in this treatment becomes increasingly important. Therapies comprising Complementary Medicine have become more popular and have shown beneficial effects in treating various types of cancer. Homeopathy, a therapy established for more than 200 years to treat various diseases, has become the target of several studies, especially regarding chronic diseases, which are difficult to control by conventional medicine. This study evaluated the effect of the homeopathic dilution of *Chelidonium majus* D35 ($1x10^{-35}$) in hepatocellular carcinoma cells (HepG2). Cells were cultivated in an oven at 37 °C, 5% CO2, and then trypsinized and plated in 96-well plates for product addition. *Chelidonium majus* D35 was added in triplicate to each well at three different concentrations. The concentrations tested were 20, 40, and 60 µL/mL. After 48 hours of incubation with the product, cell viability was measured by MTT, and it was possible to observe a decrease in the groups that received the treatment compared to the control.

A decrease in cell viability was recorded compared to the control of cells without treatment, indicating a cytotoxic effect of *Chelidonium majus* D35 on tumor cells. Different studies confirm this result due to the evaluation of biologically active compounds present in extracts of *Chelidonium majus* D35 that control cell proliferation and induce apoptosis. The results of the *in vitro* tests performed were satisfactory.

Introduction

Hepatocellular carcinoma (HCC) is a relatively common type of tumor. It has a high mortality rate due to late diagnosis associated with the disease advance and the lack of therapeutic options that contemplate the cure or even the clinical improvement of patients[1].

Existem tanto fatores externos como infecções ou doenças que favorecem para o surgimento do carcinoma quanto fatores genéticos e de desregulação de vias de sinalização 2.

The hepatocellular carcinoma treatment is performed according to the cancer stage and the patient's health conditions. In cases of disease at an initial stage, it is preferred to do resection, transplantation, and local ablation. Patients in intermediate stages are the first candidates for TACE, and those with advanced disease will first receive systemic therapies[3].

Therefore, as relevant therapeutic tools, complementary therapies have been emphasized daily in doctors' and veterinarians' offices, prioritizing the relief of clinical signs of the disease and side effects of conventional drugs. Homeopathy stands out among these therapies for promoting the well-being of patients through the administration of dynamized medicines, which promote the rebalancing of the organism[4]. Due to all the excellent and proven results, especially in patients with chronic diseases, Homeopathy has increasingly become an important ally in integrative therapies. It is based on the "Law of Similars" principle, in which a substance that causes specific effects can also be used in low doses to treat them. Therefore, dilution steps (potencies) are performed to obtain the homeopathic medicine.[5] This alternative medicine may improve the quality of life of patients undergoing treatment for various types of cancer. [6]

Among the origin of homeopathic medicines are those of plant origin, which have established use for treating various diseases and their undeniable scientific evidence as herbal medicines. Based on this knowledge and the Homeopathic Medical Matter, *Chelidonium majus* (Papaveraceae family), originating in the European continent, is indicated in cases of liver diseases, usually accompanied by severe local pain and jaundice, among other clinical signs[7]. Furthermore, according to NAWAZ et al. (2022) & SHEN et al. (2022), *Chelidonium* herbal extracts have anticancer action by decreasing cell proliferation and inducing apoptosis. Chelerythrine (CHE) is the main compound present in this plant, and studies have shown its antibacterial, antiparasitic, anti-inflammatory, antidiabetic, and antiplatelet activities. It also relieves ulcers caused by alcohol intake 8. In addition, studies have shown that *Chelidonium majus* has anticancer action by preventing cell proliferation and inducing apoptosis 9,10.

This study evaluated the effect of the D35 ($1x10^{-35}$) homeopathic dilution of *Chelidonium majus* in hepatocellular carcinoma cells (HepG2) after cell culturing and treatment with this medicine, using the MTT *in vitro* cell viability test.

Method

Obtaining *Chelidonium majus* D35

The Mother Tincture was used as the starting point to prepare the tested substance (*Chelidonium* D35). The Hahnemannian Decimal Method was used, as described in the Brazilian Homeopathic Pharmacopoeia. One part of the active ingredient was mixed with 9 parts of the inert ingredient, using a sterile isotonic solution, and succussed 100 times, yielding *Chelidonium* D1 (1×10^{-1}). Then, 1 part of *Chelidonium* D1 was used with 9 parts of the inert ingredient and succussed 100 times, yielding *Chelidonium* D2 (1×10^{-2}). The successive dilution continued till *Chelidonium* D35 was obtained. This product was then bottled in 1.1 mL ampoules.

Type of study

The tests performed in this report used a randomized *in vitro* controlled study.

Cell culture and MTT test assay

Human hepatocarcinoma cells (HepG2) were obtained from a commercial bank and grown in 75 cm^2 culture flasks with Dulbecco's Modified Eagle Medium (DMEM) supplemented with an antibiotic. The culture flasks were incubated in an oven at 37 °C, 5% CO_2, and the culture medium was changed every 48 hours until the cells reached a confluence between 60-80%.

Subsequently, these cells were trypsinized and plated in 96-well plates at 1 x 10^4 cells per well. After 24 hours of incubation under the conditions described above, these cells were treated with *Chelidonium majus* D35 at 20, 40, and 60 µL/mL. The control group was not submitted to treatment. The plates were incubated for another 48h in an oven.

After incubation, the treatment medium was removed from the wells, and the MTT reagent was added. Then, the plate was placed back in the oven at 37 °C, 5% CO_2, for 4 hours. After this period, DMSO was added to the wells, and absorbance was read in a spectrophotometer. The results were tabulated, and cell viability (%) was quantified relative to the control treatment.

Statistical analysis

Statistical analysis was performed by GraphPrism Version 9.5.0. Data were analyzed for normality by the Shapiro-Wilk test. Afterward, ANOVA and Dunnett's multiple comparisons test were performed.

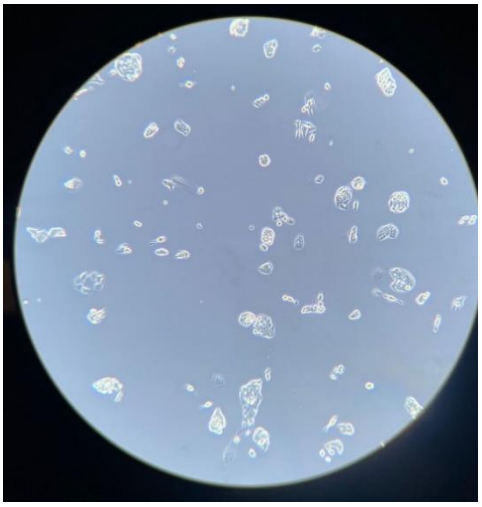

Figure 1 HepG2 cells grown in 75 cm² culture flasks in an oven at 37 °C, 5% CO_2. Photo taken from an inverted microscope with 100x magnification

Results

Obtaining *Chelidonium majus* D35

The homeopathic *Chelidonium majus* D35 was obtained as expected, and the solution was bottled in 1.1 mL sterile ampoules.

Cell culture

Cells presented morphology and growth as expected during cell culture in the 75 cm² flasks. Figure 1 shows HpeG2 cell culture with approximately 10-20% confluence.

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Cell viability

A dose-response effect was observed after treating the HepG2 cells with homeopathic *Chelidonium majus* D35 at 20, 40, and 60 µL/mL. Cell viability decreased as concentration increased, reaching values below 50% at the highest concentration evaluated.

Data were considered normal (parametric), and the statistics showed that the medicine was highly cytotoxic at the concentrations tested.

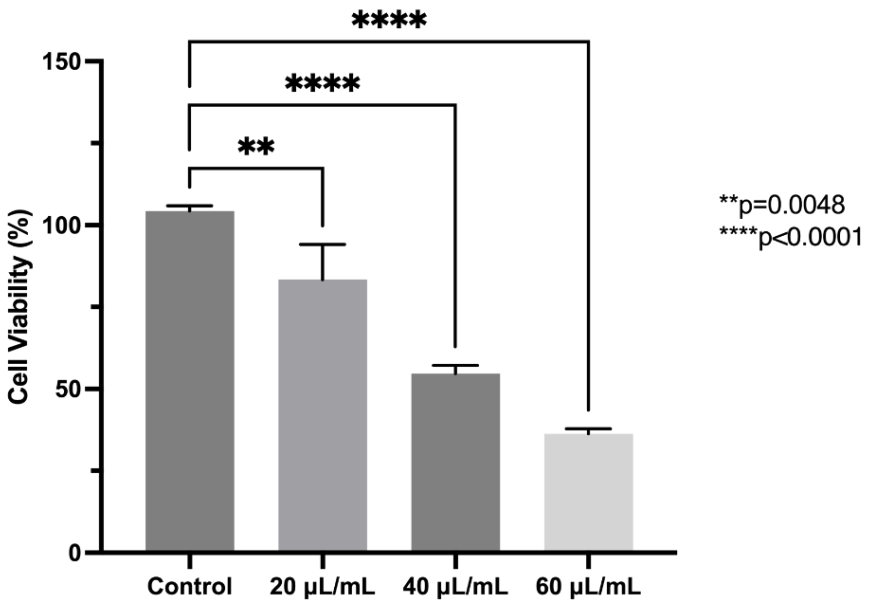

Figure 1.2 *Cellular viability of HepG2 after a 48h treatment with Chelidonium majus D35 at 20, 40, and 60 µL/mL. Control was comprised of untreated cells.*

Discussion

This study reported the cell viability decrease and death of hepatocellular carcinoma cells (HepG2) after treatment with *Chelidonium majus* in the D35 ($1x10^{-35}$) homeopathic dilution. Cell death increased with the increase of the medicine concentration. Therefore, the lower concentration caused less damage to HepG2 cells than the intermediate and higher concentrations.

Chelidonium majus has already been studied due to its antitumor activity. Among the bioactive metabolites present in this plant, chelerythrine (CHE), a substance considered toxic to the host, can be found when in large quantities. CHE

is an inhibitor of the protein kinase C, a critical cell proliferation and survival regulator.

Lin et al. (2022) demonstrated that CHE could induce oxidative stress, mitochondrial dysfunction, cell cycle arrest, endoplasmic reticulum stress, DNA damage, and cell apoptosis in some *in vitro* HepG2 cells. The present study recorded that the cytotoxicity induced by the treatment with *Chelidonium majus* D35 ($1x10^{-35}$) in HepG2 cells is dose-dependent, as also described by Lin et al. (2022). It also proved the effectiveness of this homeopathic medicine by inducing responses similar to those demonstrated by extracts containing ponderal doses of the plant and/or bioactive metabolites in isolation.

Protoberberine is another substance in *Chelidonium majus* that can induce apoptosis. Warowicka et al. (2019) tested protoberberine in HeLa and C33A cells. These authors observed the induction of apoptosis in treated cells through changes in the cell membrane, activation of intracellular caspases, disruption of mitochondrial membrane, and generation of reactive oxygen species.[12]

Apoptosis induction caused by *Chelidonium majus* was also evaluated at the gene expression level, and studies demonstrate an increase in the BAK1, BAD, and BNIP3 expression in the presence of this plant extract. The Bak protein synthesized by the BAK1 gene acts on the formation of mitochondrial transmembrane channels and mediates the release of pro-apoptotic factors from the intra-mitochondrial space, including cytochrome c. In its turn, the BAD protein increases the pro-apoptotic activity of Bak. Finally, BNIP3 acts by synthesizing proteins that neutralize those that are anti-apoptotic.[13]

Another study evaluated the action of *Chelidonium majus* on A431 human epidermoid carcinoma cells and identified that its extract induces apoptosis through caspase activation and NF-κB inhibition via a MAPK-independent pathway.[14]

The cytotoxicity of *Chelidonium majus* was also compared with *Mahonia aquifolium* and *Canadian Sanguinaria*, and the authors observed that *Chelidonium majus* exhibited higher cytotoxicity to melanoma cell lines compared to the other extracts. [15]

Based on these findings, it is possible to observe that *Chelidonium* majus has confirmed antitumor action in different cell lines and induces apoptosis. This effect was observed in the present study since there was a decrease in the HepG2 cell viability after treatment with the homeopathic *Chelidonium majus* D35.

All these results corroborate the findings of the present study indicating the cytotoxic activity of the medicine in tumor cells.

Conclusion

The treatment with *Chelidonium majus* D35 effectively induced HepG2 tumor cell death. Although more studies need to be carried out to evaluate the action mode of this medicine on liver tumor cells, this medicine may be a candidate to be included in the treatment of hepatocellular carcinoma.

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