Medical Research Archives

European Society of Medicine

OPEN ACCESS

Published: December 31, 2023

Citation: Viana Valle AC, de Carvalho AC, at al., 2023. Evaluation of cell viability and nitric oxide release after treatment of Hepatocellular Carcinoma cells (HepG2) with ultradiluted *Ruta graveolens,* Medical Research Archives, [online] 11(12). https://doi.org/10.18103/mra.v11i12.4736

DOI
https://doi.org/10.18103/mra.v11i12.4736

ISSN: 2375-1924

RESEARCH ARTICLE

Evaluation of cell viability and nitric oxide release after treatment of Hepatocellular Carcinoma cells (HepG2) with ultradiluted *Ruta graveolens*

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ABSTRACT

Hepatocellular carcinoma (HCC) is a type of neoplasm of high-frequency occurrence and late diagnosis. Therefore, medicine must act in a multidisciplinary and preventive manner to give patients a better prognosis. Homeopathy appears as a possible therapeutic tool to treat these patients, emphasizing significant results regarding quality of life and longevity. This article aimed to evaluate the effect of homeopathic dilutions of *Ruta graveolens* in the D35 potency on the *in vitro* cell viability and nitric oxide quantification of hepatocellular carcinoma cells (HepG2). The results showed a critical cytotoxic activity and high production of nitric oxide of the cells when the medicine to the culture. These results indicate that Ruta D35 has action potential in inhibiting HepG2 tumor cell growth, being an important and promising therapeutic tool for treating patients diagnosed with hepatocellular carcinoma if these results be confirmed *in vivo*.

Introduction

Of various types of cancer that affect humans and animals, hepatocellular carcinoma (HCC) is among the six most common malignancies. In most cases, the patient is diagnosed at an advanced stage of the disease, which causes the mortality rate to be high, reaching about 830,000 deaths worldwide per year [1]. Infection by hepatitis B (HBV) and C (HCV) viruses, hepatic steatosis, and smoking are among the predisposing factors for HCC. In addition, molecular pathogenesis, endogenous alteration in genetics, epigenetics, and dysregulation of the main signaling pathways strongly contribute to developing HCC [2]. The treatment of this disease includes surgical removal of the affected lobe and also less invasive possibilities, such as immunotherapics. Thus, it requires a multidisciplinary analysis of the best alternative for the patient according to the degree of tumor development [3,4]. In addition, conventional therapies very often cause severe adverse events leading patients to stop their treatment [5,6].

In this context, it is essential to consider the development of complementary therapies that improves the tolerance to treatments that are already on the market [7]. This supportive care is part of an integrative approach to the patient with a view to ensure the quality of life and benevolence. However, it is worth emphasizing the importance of scientific evidence that proves its action [6].

Among the various natural medicines, *Ruta graveolens*, a plant native to Europe, has bioactive substances, such as flavonoids, furocoumarins, and alkaloids. Rutin, a phytochemical compound, is also found in its composition and presents antioxidant, neuroprotective, cardioprotective and even anticarcinogenic effects [7]. However, at high concentrations, this plant is associated with toxicity on the functionality of some organs, such as heart and liver. The cytotoxic activity of the *Ruta graveolens* extract has already been demonstrated in various cancer cell lines as well as its *in vivo* anti-inflammatory and antioxidant effects [8].

To mitigate the cytotoxic effects and use as supportive care treatments, homeopathy has been frequently considered. Its use in oncology as supportive care is relatively recent [9], with *in vitro* and *in vivo* publications being published and beginning to demonstrate the potential of homeopathic dilutions affecting cancer cells [7]. Therefore, there are still concerns about the way of action of homeopathic medicines in cancer cells and this study aimed to evaluate the effect of the homeopathic medicine Ruta D35 in human Hepatocellular Carcinoma cells (HepG2) through *in vitro* cytotoxicity tests.

Method

OBTAINING THE MEDICINE RUTA D35

Ruta D35 was obtained from the Injectcenter Laboratory (São Paulo, Brazil). According to the laboratory, the Hahnemannian Decimal Method was used, as described in the Brazilian Homeopathic Pharmacopoeia. One part of the Mother Tincture of *Ruta graveolens* was mixed with 9 parts of sterile isotonic solution, and succussed 100 times, yielding Ruta D1 ($1\times10-1$). Then, 1 part of Ruta D1 was used with 9 parts of sterile isotonic solution and succussed 100 times, yielding Ruta D2 ($1\times10-2$). The successive dilution continued till Ruta D35 was obtained. This product was then bottled in 1.1 mL ampoules.

CELL CULTURE AND VIABILITY TEST ASSAY

Human hepatocarcinoma cells (HepG2) were obtained from a commercial bank (BCRJ) and grown in 75 cm^2 culture flasks with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum. The culture flasks were incubated in an incubator chamber 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 Ruta D35 at 70 and 90 µL/mL. The control group was not submitted to any treatment. The plates were incubated for another 48h.

After this period, the culture medium was removed and added to another plate to perform the Griess reaction and indirect evaluation of nitric oxide production by quantifying the samples' nitrite. After adding Griess (50 µL per well), the plate was placed in a spectrophotometer, and the absorbance was read. The result (in µM nitrite) was tabulated, and statistical analysis was performed.

Subsequently, the MTT reagent was added to the plate with the cells, which was placed again in the incubator 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 GraphPrisma Version 9.5.0. Data were analyzed for normality by the Shapiro-Wilk test. Afterward, ANOVA and Dunnett's multiple comparisons test were performed.

Results

The hepatocarcinoma cells (HepG2) were cultured and presented morphology and growth as expected, with an epithelioid appearance and adherence to the plastic.

To evaluate if the homeopathic medicine Ruta D35 had any effect on liver cancer cells metabolism, the viability analysis was performed. Results showed that cell viability decreased ($p<0.0001$) in a dose dependent manner after treating HepG2 cells with Ruta D35 to 28% at 70 and 18% at 90 µL/mL concentrations (Figure 2).

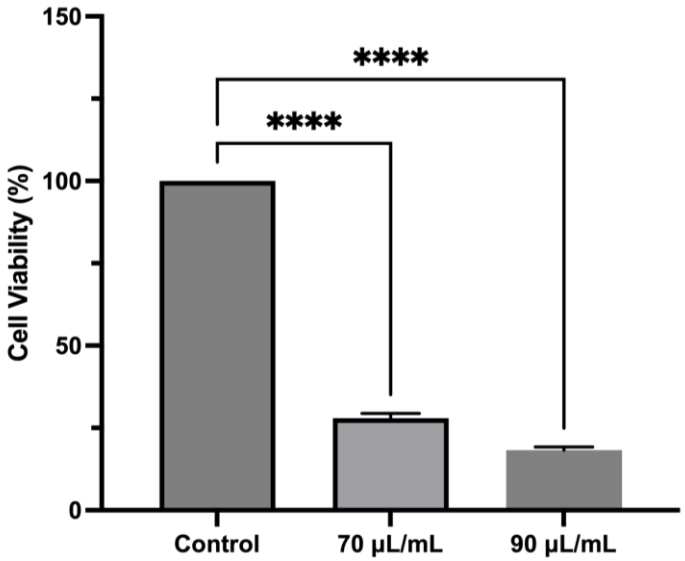

Figure 1. HepG2 cell viability after treatment with Ruta D35 at 70 and 90 µL/mL for 48h in 96-well culture plates. Control had no medicine addition. ****$p<0.0001$.

To reinforce the toxicity of Ruta D35 in the studied liver cancer cells, the production of nitric oxide was analyzed as it is related to the apoptotic levels of cells. The results showed that nitric oxide production increased ($p<0.0001$) from 49µM (control) to 58µM in cells treated with 70 µL/mL and to 57µM in cells treated with 90 µL/mL (Figure 2).

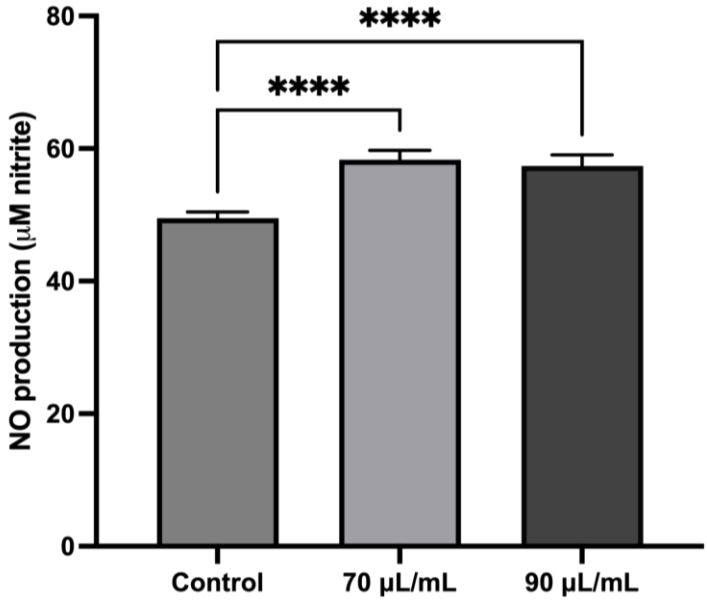

Figure 1. Nitric oxide (NO) production in HepG2 cells after 48h of treatment with Ruta D35 in 96-well plates. Control had no medicine addition. ****$p<0.0001$.

Discussion

Hepatocellular carcinoma is a prevalent type of cancer with a high mortality rate since it is usually only diagnosed in advanced stages of the disease [10]. As patients may present severe adverse events while submitted to conventional treatments, alternatives must be evaluated, like homeopathy, a type of medicine that brings benefits specially related to human welfare without side effects [7]. Homeopathy is undoubtedly used for years in supportive care treatments, but its action in cancer cells has been recently evaluated [11]. In this study, the action of the homeopathic medicine Ruta D35 was evaluated in hepatocellular carcinoma cells (HepG2) through *in vitro* tests of cell viability and quantification of nitric oxide. The decrease in cell viability when cells were in contact with Ruta D35 indicates this medicine acts right in cell metabolism. The MTT assay used in this study identifies cells mitochondrial action [12] so the decrease of this marker indicates that cells are under intense stress as result of the contact with the medicine. Fuselier et al. (2022) described the effect of a homeopathic medicine *R. graveolens* based in melanoma through *in vivo* and *in vitro* tests and confirmed its anticancer activity, corroborating the findings reported in the present study. According to the authors, the homeopathic medicine decreases the migration of cancer cells acting on the plasma membranes, blocking intracellular calcium influx and inducing a significant destruction of actin filaments[7].

Among the different bioactive compounds present in this plant, phytosterols are known to present anticancer and dose-dependent anti-inflammatory activity. Baker et al. proved the action of these substances present in *R. graveolens* extracts against colorectal cancer, breast cancer, lung cancer, and hepatocellular carcinoma tumor cells (HepG2) [9]. In this way, the effects of the extract of this plant have already been showed, now our study evaluated the cell-based effects of the homeopathic medicine and presented the cytotoxic mechanism against liver cancer cells.

The cellular mechanism of Ruta D35 was not yet developed, but the nitric oxide (NO) test was also performed to evaluate if homeopathic R. graveolens had effects on NO production by HepG2 cells. Nitric oxide (NO) is a molecule which plays a crucial role in regulating several vital processes for the body, such as the metabolism of various substances, neurotransmission, immunity, cardiovascular function, among others. NO dilates vessels and stimulates angiogenesis, while at the same time has cytotoxic and immune-modulatory properties [13]. Changes in its signaling are a central feature of many significant disorders, including cardiovascular disease, diabetes, and cancer [14]. Studies suggest that when tumor infiltrating immune cells generate NO, they contribute to tumor killing [15,16], therefore, the higher the nitric oxide release, the higher the apoptosis index (antitumor effect)[17], result that could be observed in our study, when the presence of the medicine in cells promoted higher release of NO and higher cytotoxicity. At high steady state levels, NO can be mutagenic and carcinogenic, but also cytotoxic to established tumor cells [14]. On the other hand, at low-to-medium levels, NO can foster tumor persistence and progression by activating pro-growth/migratory signaling or by inhibiting tumor suppression signaling [15]. This has been observed for many different malignancies, including breast, prostate, bladder, cervical, and gliomas [16].

Conclusion

Our results presented the ability of Ruta D35 to act against HepG2 (hepatocellular carcinoma) tumor cells in *in vitro* tests. The medicine tested decreased cell viability and increased the release of nitric oxide. The trials demonstrated a relationship between tumor cells death and the increase in the level of nitric oxide released, as demonstrated in the literature. Therefore, Ruta D35 may be an ally in treating hepatocellular carcinoma.

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