Medical Research Archives

ESMED
European Society of Medicine

OPEN ACCESS

Published: July 31, 2023

Citation: Valle ACV, de Carvalho AC, et al., 2023. Evaluation of cell viability and nitric oxide release after treatment of human hepatocellular carcinoma cells with a homeopathic compound of Graviola (*Anonna muricata*) and Purple Ipe (*Handroanthus impetiginosus*), Medical Research Archives, [online] 11(7). https://doi.org/10.18103/mra.v11i7.4091

DOI
https://doi.org/10.18103/mra.v11i7.4091

ISSN: 2375-1924

RESEARCH ARTICLE

Evaluation of cell viability and nitric oxide release after treatment of human hepatocellular carcinoma cells with a homeopathic compound of Graviola (*Anonna muricata*) and Purple Ipe (*Handroanthus impetiginosus*)

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ABSTRACT

Homeopathy has become increasingly known and used in various treatments, showing excellent clinical results. One of its most common indications is to help treat chronic diseases, directly improving patients' quality of life, especially those with cancer. Hepatocellular carcinoma is a disease that depends on different factors for its development and the success of its treatment. In this context, searching for new therapeutic tools is essential, and homeopathy seems promising. Therefore, *Anonna muricata* (soursop/graviola) and *Handroanthus impetiginosus* (purple ipe) have been studied due to their action potential against tumor cells. This study aimed to evaluate the effect of these medicines combined in 1.1mL ampoules, in homeopathic dilutions, on HepG2 (hepatocellular carcinoma) cells through *in vitro* tests. Cells were cultivated in 75 cm^2 culture bottles in an oven with controlled temperature and CO_2 quantity (37 °C, 5% CO_2). Then, cells were plated in 96-well plates where treatment was performed using the products at different concentrations. After 48 hours of treatment, cell viability analyses were performed by MTT and quantification of the nitric oxide released using the Griess reaction. Treatment with the *Anonna muricata* D5 ($1x10^{-5}$) + *Handroanthus impetiginosus* D5 ($1x10^{-5}$) formulation resulted in decreased cell viability and increased nitric oxide production by these cells. These findings indicate that this medicine showed an antitumor effect and can be an ally in treating this type of pathology.

Introduction

Homeopathy was first invented in Germany in 1796, introducing the concept of minimum doses and that the more one dilutes and succusses a given substance, the more active it becomes. Since then, there has been a higher search for this therapy as a complementary medicine to traditional methods of orthodox medicine to alleviate the side effects of conventional drugs in the various treatments[1].

Within this context, homeopathy has improved patients' quality of life, especially cancer patients who are submitted or not to conventional treatments[2]. Among the different types of cancer, hepatocellular carcinoma (HCC) is a type that affects many people and has a high mortality rate. It occurs primarily in patients who develop cirrhosis[3]. Also, hepatitis B and C virus infection, liver steatosis, and smoking can be considered predisposing factors that increase this disease incidence, in addition to the epigenetics and dysregulation of some signaling pathways[4].

The treatment of cancer patients is becoming more challenging due to lifestyle, environmental contamination, and acquired resistance to the drugs of choice for their treatment. Therefore, some natural medicines have been evaluated for their action potential against different types of cancer, especially Hepatocellular Carcinoma.

For example, *Anonna muricata* is a plant found in Brazil and has been studied for years because of its antitumor potential. Its *in vitro* and *in vivo* activity was evaluated for certain types of cancer, such as pancreatic adenocarcinoma and colon, lung, prostate, and breast cancer[5]. Besides *Anonna muricata* , *Handroanthus impetiginosus* has bioactive compounds with proven cytotoxic activity against cancer cells. β-lapachone, a naphthoquinone[6], and lapacol, which act in the glycolysis process by inhibiting tumor cell growth[7], can be found in plant extracts and demonstrate interesting activity in the cell cycle of cancer cells. Based on this scenario, the present study evaluated the *in vitro* activity of *Anonna muricata* + *Handroanthus impetiginosus* in Hepatocellular Carcinoma (HepG2) cells using the homeopathic dilution D5 ($1x10^{-5}$). Cell viability and the amount of nitric oxide released after treating the cells with the medicine were evaluated.

Method

Obtaining *Anonna muricata* D5 + *Handroanthus impetiginosus* D5

The medicine was purchased from the Injectcenter® laboratory and was manufactured according to the Hering decimal scale by the Hahnemanian method, as described in the German Homeopathic Pharmacopoeia. The Mother Tincture was used as the starting point to prepare the tested substance (*Anonna muricata* D5 + *Handroanthus impetiginosus* D5). 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 *Anonna muricata* D1 ($1x10^{-1}$). Then, 1 part of *Anonna muricata* D1 was used with 9 parts of the inert ingredient and succussed 100 times, yielding *Anonna muricata* D2 (1×10^{-2}). The successive dilution continued till *Anonna muricata* D5 ($1x10^{-5}$) was obtained. The same process was performed with the *Handroanthus impetiginosus* mother tincture until dilution D5. After this process, the medicines were mixed in a 1:1 ratio, resulting in the *Anonna muricata* D5 + *Handroanthus impetiginosus* D5 formulation. This product was then bottled in 1.1 mL ampoules.

Cell culture, viability assay, and nitric oxide determination

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 10,000 cells per well. After 24 hours of incubation under the conditions described above, these cells were treated with *Anonna muricata* D5 + *Handroanthus impetiginosus* D5 at 70 and 90 µL/mL. The control group was not submitted to treatment. The plates were incubated again for another 48h in an oven. 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. 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 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 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

Cell viability decreased after treating HepG2 cells with *Anonna muricata* D5 + *Handroanthus impetiginosus* D5 at concentrations of 70 and 90 µL/mL (Table 1).

Table 1. Cell viability (%) after contact with the homeopathic medicine *Anonna muricata* D5 + *Handroanthus impetiginosus* D5 at different concentrations.

Cell Viability (%)	
70 µL/mL	90 µL/mL
25	20
31	15
23	17

Furthermore, *Anonna muricata* D5 + *Handroanthus impetiginosus* D5 caused an increase in nitric oxide production by tumor cells (Table 2).

Table 2. Production of nitric oxide by tumor cells in contact with the medicine *Anonna muricata* D5 + *Handroanthus impetiginosus* D5 at different concentrations.

Nitric Oxide Production (µM nitrite)		
Control	70 µL/mL	90 µL/mL
47	58	58
47	51	52
47	54	54
45	56	56

Data were considered normal (parametric), and the statistics showed that the medicine was highly cytotoxic at the concentrations tested (Figures 1 and 2). Also, there was high nitric oxide production when the medicine was added to the tumor cell culture.

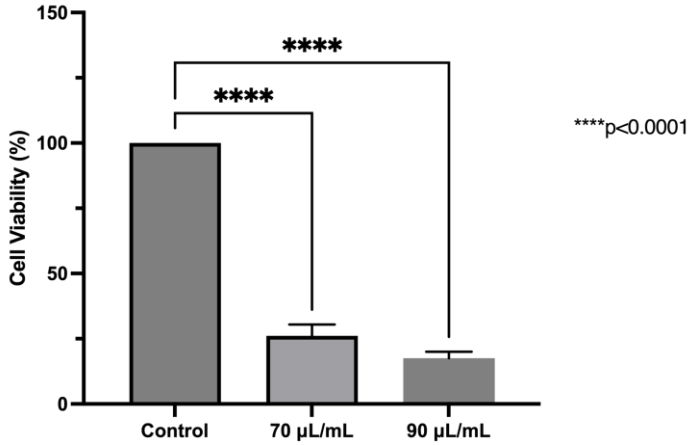

Figure 1. HepG2 cell viability after treatment with *Anonna muricata* D5 + *Handroanthus impetiginosus* D5 at 70 and 90 µL/mL for 48h in 96-well culture plates. The control group did not receive any treatment.

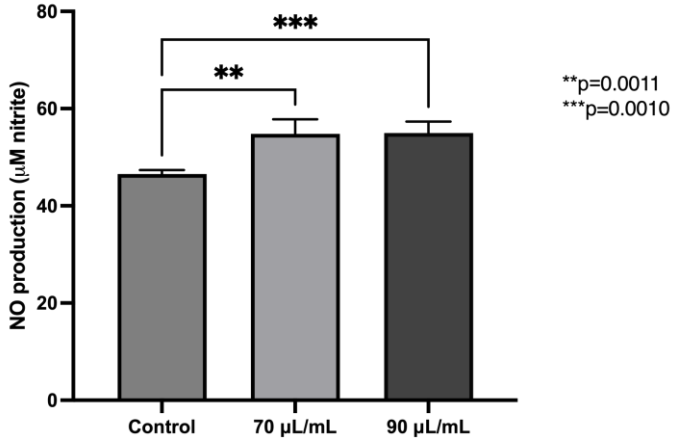

Figure 2. Nitric oxide (NO) production in HepG2 cells after 48h of treatment with *Anonna muricata* D5 + *Handroanthus impetiginosus* D5 in 96-well plates. Control was performed with no addition of the medicine.

Discussion

Homeopathic medicines have gained attention in oncology treatments mainly because they provide a better quality of life for these patients and reduce the side effects caused by conventional treatments that often do not contemplate a cure, not even relieve the symptoms[8].

The scientific community has widely studied plant-derived medicines as alternatives to conventional drugs to treat various diseases, such as cancer. Within this context, *A. muricata*, popularly known as soursop and graviola, has biologically active compounds, and its use is established for treating cancer patients by popular medicine. Metabolites such as alkaloids, anthraquinones, saponins, terpenoids, flavonoid phytosterols, phenols, coumarins, lactones, and tannins are found in this plant[9]. They are also called annonaceous acetogenins (ACG) and have cytotoxic activity for the cell cycle by inhibiting the mitochondrial complex I[10,11].

Hadisaputri et al. evaluated the action of fractions of ethanolic extract, ethyl acetate, n-hexane, and water from the leaves of *A. muricata* against breast cancer cells. According to the authors, it was possible to confirm that *A. muricata* induced apoptosis by decreasing the Bcl-2 mRNA expression and increasing the caspase-9 and caspase-3 mRNA expression. The cells showed rupture and loss of membranes and nuclei[12]. The same effect was observed by Naik et al., who used the methanolic extract of *A.muricata* in MCF-7 cells[13].

In addition to the antitumor benefit of *A. muricata*, studies report its antibacterial activity. Ngemenya et al. evaluated the action of this plant extract against infections caused by *Salmonella sp.* and observed high bacteriostatic activity without causing toxicity to the control cells of the study[14].

Treating cancer is challenging in medicine due to multidrug-resistant cell lines. However, a study with different extracts of medicinal plants, including *A. muricata*, showed that this plant has high efficiency even in treating multidrug-resistant carcinogenic lines, making it very promising in treating complementary medicine[15].

H. impetiginosus, in its turn, popularly known as purple ipe, has an important antitumor potential due to the presence of β-lapachone, an ortho-naphthoquinone. This bioactive compound is well-known to inhibit topoisomerase I and induce NAD(P)H:quinone oxidoreductase. According to Gomes et al., *H. impetiginosus* extracts demonstrated excellent cytotoxic capacity in phase II clinical trials for treating pancreatic cancer. These authors also highlight the extraordinary activity of this extract against several types of malignant tumors, such as lung, pancreas, and melanoma cancer[16].

The effects of β-lapachone were also evaluated in different colon cancer cells (CT26 and MC38). In this study, the authors observed that the compound induced cellular apoptosis and inhibited colorectal lung metastasis[17].

Therefore, the *Anonna muricata* and *Handroanthus impetiginosus* plants' antitumor effect was evaluated in this study in HepG2 cells (hepatocellular carcinoma) using the homeopathic dilution D5 ($1x10^{-5}$) and showed excellent results. It was possible to observe that the progression of tumor cells is associated with changes in the expression of cell cycle proteins causing these cells to begin their proliferation disorderly. This finding is corroborated by Salem et al. (2022), who demonstrated the activity of *Anonna muricata* extracts in cell cycle arrest, controlling cell proliferation[18]. Additionally, Dias et al. described the potential of *Handroanthus impetiginosus* extracts to stop the cell cycle in the G2/M stage in cancer cells, followed by the activation of apoptotic proteins and, consequently, cell death[6].

In the present study, these effects were observed in evaluating cell viability after treatment of HepG2 cells with *Anonna muricata* D5 + *Handroanthus impetiginosus* D5, which decreased cell viability compared to the control (cells without treatment).

In addition to the decrease in cell viability, a certain increase in the nitric oxide release was observed after treatment of the cells with *Anonna muricata* D5 + *Handroanthus impetiginosus* D5. The nitric oxide role is important because low levels of this gas can stimulate the proliferation of cancer cells, and high levels indicate the occurrence of apoptosis[19]. Therefore, the nitric oxide level increase in the in vitro test is consistent with the expected decrease in cell viability.

Conclusion

After performing the *in vitro* tests, it can be proven that the homeopathic formula *Anonna muricata* D5 + *Handroanthus impetiginosus* D5 decreased cell viability and increased the release of nitric oxide by the Hepatocellular Carcinoma cells. As a consequence, the medicine caused cell death in the HepG2 line and can be an ally in treating this type of tumor.

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