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**NORDIHYDROGUAIARETIC ACID AS NEW THERAPEUTIC  
APPROACH IN HEPATOCELULAR CARCINOMA**

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## **NORDIHYDROGUAIARETIC ACID AS A NEW THERAPEUTIC APPROACH IN HEPATOCELULAR CARCINOMA**

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## II. Abstract

Cancer is the second leading cause of death worldwide, making this disease responsible for 12.5% of deaths. Moreover, liver cancer is one of the most common malignant tumor worldwide and HCC represents 70% to 85% of the total liver cancer burden worldwide. Consequently, the pharmaceutical industry has focused on developing anticancer drugs more potent and effective that moves annually, billions of euros. In fact, the history of anti-cancer therapy is closely related to natural products, which represent over 60% of medicines used in this pathology. Since the twentieth century, clinical trials were developed in order to identify, develop and approve new active compounds derived from plants, which show anti-cancer activity.

Nordihydroguaiaretic acid (NDGA) is a phenolic compound produced by *Larrea tridentate*, a creosote bush of Mexico and the American southwest, known for its antioxidant and anti-inflammatory properties. Recently, it has also shown that this compound inhibits cell growth and induces apoptosis in different types of cancer, in both cell culture and animal models. However, the mechanisms involved in its anti-tumorigenic and anti-proliferative effects, namely in hepatocellular carcinoma (HCC), are not clear.

The aim of this study was to test the cytotoxic and antiproliferative effects of NGDA in different HCC cell lines, as well as to study the mechanisms involved in the process of cell death.

For this purpose, three HCC cell lines with different etiologies and p53 levels, the HUH-7, Hepg2 and Hep3 cells, were incubated in the absence and presence of the natural compound NGDA. The antiproliferative effect was assessed by the *Alamar Blue* assay and cell death was analyzed by flow cytometry using Annexin V and Propidium iodide. . We also evaluated oxidative stress levels in these cells upon incubation with NGDA by analyzing the intracellular ROS levels by flow cytometry using specific fluorescent probes, DHE and DCF-DHA, respectively for superoxide anion and peroxides.

Our results showed that NGDA have antiproliferative and cytotoxic effects in a dose, cell and time dependent manner inducing cell death in all cell lines, preferentially

by apoptosis. This effect seems not to be mediated by the increase in intracellular ROS levels.

In sum, our results suggest that NGDA may constitute a new potential therapeutic approach in HCC treatment owing to its pro-oxidant properties when used in high concentrations.

**KEYWORDS:** hepatocellular carcinoma; nordihydroguaiaretic acid (NDGA). oxidative stress; reactive oxygen species; apoptosis.

### III. Resumo

O cancro é a segunda principal causa de morte no mundo, sendo responsável por 12,5 % do total das mortes. Os cancros do fígado são dos tumores malignos mais comuns a nível mundial, incluindo o carcinoma hepatocelular que representa 70 a 85% destes. Consequentemente, a indústria farmacêutica tem-se centrado no desenvolvimento de terapêutica anti-neoplásica mais potente e eficaz num mercado que movimenta, anualmente, milhões de euros. Na verdade, a história da terapêutica oncológica está intimamente relacionada com os produtos naturais, que representam mais de 60 % dos fármacos utilizados nesta patologia. Desde o século XX, vários ensaios clínicos foram desenvolvidos a fim de identificar, desenvolver e aprovar novos compostos ativos derivados de plantas, que apresentam atividade antitumoral.

O Ácido Nordihidroguaiarético (NDGA) é um composto fenólico produzido por *Larrea tridentata*, um arbusto de creosoto do México e do Sudoeste Americano, conhecido pelas suas propriedades antioxidantes e anti-inflamatórias. Recentemente, foi ainda demonstrado que pode inibir o crescimento de células tumorais e induzir apoptose em diferentes tipos de cancro, quer em estudo *in vitro* como em modelos animais. No entanto, os mecanismos envolvidos nos seus efeitos antitumorais e anti-proliferativos, nomeadamente no carcinoma hepatocelular, ainda não foram esclarecidos.

Assim, o objetivo deste estudo foi avaliar os efeitos citotóxicos e antiproliferativos do composto natural NDGA, em diferentes linhas celulares de carcinoma hepatocelular.

Para tal, três linhas celulares de carcinoma hepatocelular com diferentes etiologias e níveis de *p53*, HUH-7, HepG2 e Hep3B, foram incubadas na ausência e na presença do composto NDGA. O efeito antiproliferativo foi avaliado pelo teste de *Alamar Blue*, enquanto a morte celular foi analisada por citometria de fluxo, utilizando Anexina V e Iodeto de propídeo. Também foram avaliados os níveis de espécies reativas de oxigénio intracelulares por citometria de fluxo, recorrendo a sondas fluorescentes específicas, DHE e DCF-DHA, para os aniões superóxido e peróxido respectivamente.

Os resultados desta investigação mostraram que o NGDA tem efeitos antiproliferativos e citotóxicos de forma dependente da dose, da linha celular e do tempo, ocorrendo a morte em todas as linhas de células, preferencialmente por apoptose. A morte celular parece não ser mediada por um aumento dos níveis intracelulares de espécies reativas de oxigênio. Concluindo, o NGDA pode constituir uma potencial nova abordagem terapêutica no tratamento do carcinoma hepatocelular, devido às suas propriedades pró-oxidantes, quando utilizados em elevadas concentrações.

**PALAVRAS-CHAVE:** Carcinoma hepatocelular; Ácido Nordihidroguaiarético (NDGA). Stress oxidativo; espécies reativas de oxigênio; apoptose.

#### **IV. Abbreviations List**

5FC: 5-fluorocytosine

AV: Annexin V

DCF: Dichlorofluorescein

DCFH2 –DA: Diacetate 2 , 7- diclorodihydrofluorescein

FBS: Fetal bovine serum

FC: Flow cytometry

HBV: Hepatitis B Virus

HCC: hepatocellular carcinoma

HCV: Hepatitis C Virus

IC50: Half-maximal inhibitory concentration

LOX: Lipoxygenase

MIF: Mean intensity of fluorescence

NGDA: Nordihydroguaiaretic Acid

PBS: Phosphate buffer solution

PI: Propidium iodide



## V. Introduction

In men, liver cancer is the fifth most common neoplasm in the world and the second most common cause of cancer-related death. In women, it is the seventh most common cancer and the sixth leading cause of cancer death. An estimated 748,300 new liver cancer cases and 695,900 deaths occurred worldwide in 2008 (Jemal A, 2011). Most cases of HCC (>80%) occur in sub-Saharan Africa and in Eastern Asia, with typical incidence rates of more than 20 per 100,000 individuals. Southern European countries, (such as Spain, Italy and Greece) tend have mild-incidence levels (10.0 to 20.0 per 100,000 individuals), whereas North America, South America, Northern Europe, and Oceania have a lower incidence of HCC (<5.0 per 100,000 individuals) (El-Serag H, 2012).

The etiology of HCC can explain these geographical differences in incidence. The elevated prevalence of chronic hepatitis B (HBV) or C (HCV) virus and aflatoxin B1 intake from contaminated food, in some parts of Asia and Africa, are the reason of the high HCC incidence rates in these countries (Jemal A, 2011; Severi T, 2010). In western countries with low-risk of HCV infection, alcoholic cirrhosis, haemochromatosis and nonalcoholic fatty liver disease, associated with obesity, account for the majority of liver cancer (Jemal A, 2011; Llovet JM, 2003).

Phenotypically and genetically, HCC is very heterogeneous, possibly in part due to the heterogeneity of etiologic factors implicated in its development, the complex functions of the liver cell, and the advanced stage at diagnosis (Spangenberg HC, 2008). As for most types of cancer, hepatocarcinogenesis is a multifactorial and multistep process involving different genetic and epigenetic alterations, chromosomal aberrations, mutations, and altered molecular pathways, including oxidative stress, that result in the deregulation of key oncogenes and tumor-suppressor genes involved in several signaling pathways (Llovet JM, 2008). However, the molecular contribution of the different factors, their interaction and exact sequence in hepatocarcinogenesis, including the development of preneoplastic lesions and progression to HCC, are still poorly understood (Makoto M, 2011).

Current therapeutic strategies for HCC can be divided into potentially curative therapies as surgical interventions (tumor resection and liver transplantation), percutaneous interventions (ethanol injection, radiofrequency thermal ablation)

(Spangenberg HC, 2008), and palliative therapies as transarterial interventions (embolization, chemoperfusion, or chemoembolization), systemic chemotherapy with experimental tamoxifen and doxorubicin and other experimental strategies, including gene and immune therapy (Alves RC, 2011). In advanced HCC, Sorafenib, a multikinase inhibitor, that block the VEGF and PDGF-dependent angiogenesis, showed an increase on overall survival. However, this drug can't be use in all tumors and have no negligible side effects (Severi T, 2010). So, new therapeutic approaches are needed for this kind of cancer.



**Figure 1** – *Larrea tridentate*  
(Adapted from wikipedia.org)

Since the twentieth century, clinical trials were developed in order to identify, develop and approve new active compounds derived from plants, which show anti-cancer activity, like nordihydroguaiaretic acid (NDGA) (Lambert J, 2005).

NDGA is a phenolic compound produced by *Larrea tridentate* (fig.1), a creosote bush of Mexico and the Southwest United States, used for many years as natural medicine in various diseases, as infections, rheumatism and cancer (Arteaga S, 2005). This compound is known for its anti-oxidant properties. In fact, it eliminates reactive Oxygen Species (ROS) *in vitro*. Other propriety assigned to this compound is its anti-inflammatory capacity through the inhibition of 5-lipoxygenase and, consequently, the redution in leukotriens synthesis (Eads D, 2009).

Some studies also demonstrated that this compound inhibits cell growth and induces apoptosis in different types of cancer, in both, cell culture and animal models (Seufferlein T, 2002). However, the mechanisms involved in its anti-tumorigenic and anti-proliferative effects are not clear. *In vitro* studies have shown that NDGA suppresses tumor growth by inhibiting metabolic enzymes and tyrosine kinase phosphorylation, which is overexpressed in certain types of cancer cells (Lu JM, 2010; Floriano-Sánchez E, 2006). These data suggest NGDA could have a possible role in cancer therapy.

## **VI. Objectives**

The aim of this study was to test the therapeutic efficacy of NGDA in different HCC tumor cells lines with different etiologies, as well as to study the mechanisms involved in the process of cell death.

For these purposes, we evaluated three topics:

- The cytotoxic and antiproliferative effect of NGDA in three HCC cell lines with different etiologies, the HUH-7, Hepg2 and Hep3b cells, using the Alamar Blue assay.
- The type of cell death induced by NGDA, using Annexin V and Propidium iodide staining analyzed by Flow Cytometry.
- The involvement of oxidative stress in the observed cytotoxicity, through the evaluation of the intracellular ROS accumulation assessed by the fluorescent probes DCFH2-DA and DHE, respectively for superoxide anion and peroxides.

## **VII. Materials & Methods**

### **VII.1. Cell line culture conditions**

For this study, three hepatocarcinoma cell lines with different etiologies were used: HUH-7, HepG2 and Hep3B.

- The human HUH-7 cell line, offered by Professora Doutora Maria Conceição Pedroso Lima from Center for Neuroscience and Cell biology, University of Coimbra, was established by Nakabayashi and Sato (1982) from hepatoma tissue of a 57-years-old Japanese male with well differentiated hepatocellular carcinoma expressing high levels of mutated p53 (Carlioni V, 2005).

- The human Hep-G2 cell line, offered by Professora Doutora Filomena Botelho, from Biophysics/Biomathematics of the Faculty of Medicine, University of Coimbra, was established by Aden *et al.*, (1979) from a liver tumor biopsy obtained from a 15 years old Caucasian male, express small amounts of wild type p53 (Müller Martina, *et al.*, 1997).

- The human Hep3b cell line, offered by Professora Doutora Maria Conceição Pedroso Lima from Center for Neuroscience and Cell biology, University of Coimbra, was established by Knowles *et al.*, (1983) from hepatoma tissue of a 8-years-old black male infected by Hepatite B virus is deficient of p53 (Müller Martina, *et al.*, 1997).

The cell lines were grown in DMEM contained with L-glutamine 2mM (Gibco – Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (FBS)(Gibco – Life Technologies), penicillin 100U/mL and streptomycin 100µg/mL(Gibco – Life Technologies), and maintained at 37°C in a humidified incubator containing 5% CO<sub>2</sub>. For the assays, cells were seeded 24h before incubation with the natural compound at a density of 50000 cells per cm<sup>2</sup>.

### **VI.2. Evaluation of cell viability**

To evaluate the antiproliferative and cytotoxic effect of NGDA in HCC cells lines, cells were cultured in monotherapy during 72 hours in absence and presence of

NGDA (Sigma Aldrich, St. Louis, MO, USA) in concentrations ranging from 10 to 250 mM.

Cell viability was evaluated at 24, 48 and 72 hours after addition of compound, by Alamar Blue assay. The Alamar Blue (resazurin) assay is a colorimetric growth indicator based on the detection of redox metabolic activity. Metabolic activity of cells leads to a reduction of the resazurin compound from the oxidized form (non-fluorescent, blue) to the reduced form (fluorescent pink). For this purpose, the medium was replaced by 250 µl of culture medium containing 10% Alamar Blue (Resazurin 0.1 mg / ml in PBS) and cells were incubated about 3 hours at 37 °C (O'Brien J, 2000). After incubation, we collected 200µL of supernatant from each well and transferred to 96 well-plates and read the absorbance in a spectrophotometer Mediators PhL luminometer (Mediators Diagnostika, Vienna, Austria) at 570 nm (oxidized form) and 600 nm (reduced form) wavelengths.

Finally cell viability was determined as a percentage of viable cells relative to control, using the following formula:

$$\frac{[(A570 - A600)_{\text{treated cells}}] - [(A570 - A600)_{\text{blank}}]}{[(A570 - A600)_{\text{control}}] - [(A570 - A600)_{\text{blank}}]} \times 100$$

### VI.3. Analysis of cell death

In order to analyze the type of cell death induced by NDGA, flow cytometry using Annexin V/propidium iodide labelling was performed. In apoptotic cells, the membrane phospholipid phosphatidylserine from the inner leaflet undergoes translocation to the outer leaflet of the plasma membrane, being exposed to the extracellular medium. The Annexin V, a phospholipid-binding protein of about 36 kD, has a high affinity for phosphatidylserine and binds to cells when this phospholipid is exposed. Thus, Annexin V may be conjugated to fluorochroms such as APC, while keeping its affinity for phosphatidylserine and thus serving as a probe to cell analysis by flow cytometry. Since externalization of phosphatidylserine occurs in the early stages of apoptosis staining with Annexin V- APC can identify early apoptotic cells. Propidium iodide (PI) is used to score necrotic cells through the emission of red fluorescence

binding to DNA and RNA. This dye can only traverse the plasma membrane of necrotic cells, but not of apoptotic cells. The viable cell membrane is impermeable to this dye.

After an incubation period of 48h with different conditions, cells were trypsinized, centrifuged at 300g for 5 min and resuspended in phosphate buffer (PBS) to obtain a density of  $1 \times 10^6$  cells / ml. Untreated and treated cells were washed with PBS (centrifuged at 300g for 5 min), resuspended in 500  $\mu$ l of Annexin V binding buffer and incubated for 10 min at room temperature with 5  $\mu$ l of Annexin V-APC (Santa Cruz Biotechnology) and 2  $\mu$ l of Propidium iodide (Aubry JP, 1999). The cells were analysed in a FACS Calibur (Becton Dickinson) flow cytometer equipped with an argon ion laser emitting at 488nm. Green fluorescence of Annexin V-FITC was collected with a 525nm band pass filter and red fluorescence of PI with a 610nm band pass filter. The results were expressed in % of viable (V), initial apoptotic (IA), late apoptotic/necrotic (LA/N) and necrotic (N) cells.

#### **VI.4. Evaluation of reactive oxygen species levels**

To determine the levels of reactive oxygen species, we used two probes of stable nonfluorescent lipid permeable compounds: dihydroethidium (DHE, Molecular Probes, Eugene, OR) and 2',7'-dichlorodihydrofluorescein diacetate (DCFH<sub>2</sub>-DA) (Invitrogen), to detect superoxide anion ( $O_2^{\cdot-}$ ) and peroxides (hydrogen peroxide,  $H_2O_2$ ) levels, respectively. The DHE is able to move freely across cellular membranes and after reaction with superoxide anion, form ethidium, an oxidized product which fluoresces in red (Owusu-Ansah E, 2008).. The acetylated form of DCFH<sub>2</sub>, DCFH<sub>2</sub>-DA, can cross the cell membrane easily and after deacetylation by cellular esterases, this compound may be oxidized by peroxides to form the fluorescent probe 2',7'- dichlorofluorescein (DCF), which after excitation at 480 nm fluoresces in green at 520 nm (Brömme H, 2008).

After an incubation period of 48h with the different conditions, cells were incubated with 2  $\mu$ l of DHE (10  $\mu$ M ) or 1  $\mu$ l of DCFH<sub>2</sub> -DA (10  $\mu$ M) for 30 min at 37 °C and then washed with PBS by centrifugation at 300xg for 5 min.

The fluorescent intensity of DHE and DCF was measured by flow cytometry (FL-2, 620nm band pass filter and FL-1, between 500 and 530nm band pass filter,

respectively). The results are expressed as Mean Intensity Fluorescence (MIF) which is proportional to the respective ROS expression levels and represent the mean  $\pm$  SD of two independent experiments.

### **VI.5. Statistical analysis**

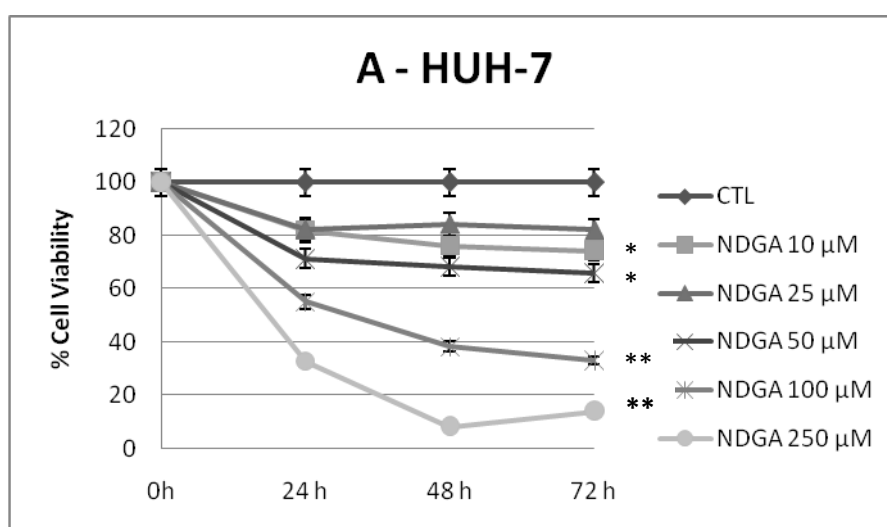
Statistical analyses were performed using GraphPad Prism software, version 5.0 (GraphPad Prism software, Inc., San Diego, CA). Statistically significant differences ( $p < 0,05$ ) between the experimental groups were determined by Student's t test.

## VIII. Results

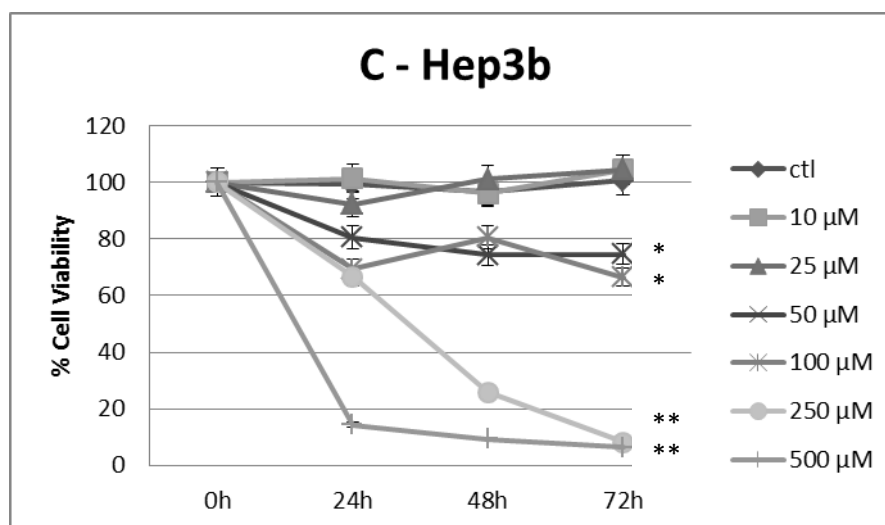
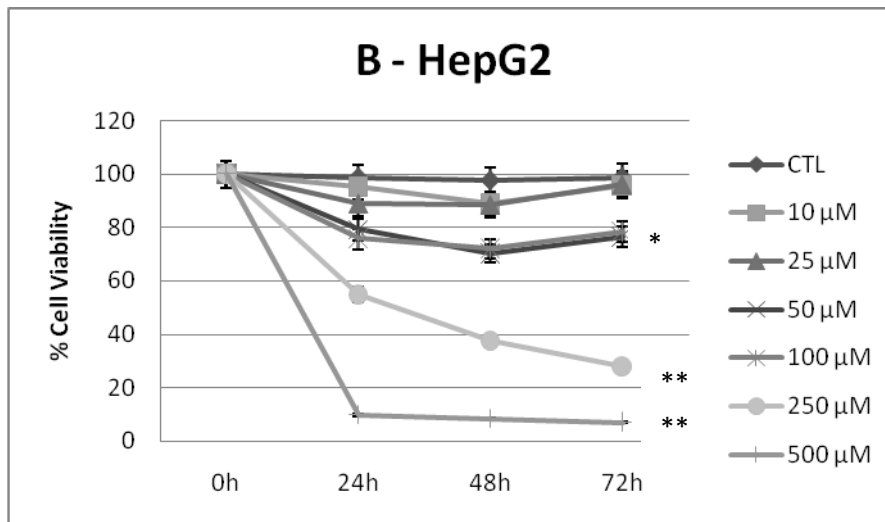
### VII.1. Evaluation of the antiproliferative effect of NDGA

In order to evaluate the effect of NDGA in HUH-7, HepG2 and Hep3b cell lines viability/proliferation, cells were cultured in absence and in presence of the natural compound for up 24, 48 and 72 hours with increasing concentrations of compound. The antiproliferative effect was evaluated by the Alamar Blue assay as described in material and methods section.

As we can observed in Figure 2, NDGA induced a decrease in cell viability in a dose, time and cell type dependent manner. In fact HUH-7 cells seem to be more sensitive to NDGA, as the half-maximal inhibitory concentration (IC<sub>50</sub>) value was achieved after 24 hours of incubation with the lowest dose range of the compound, 100 to 250  $\mu$ M compared with the other cell lines, Hepg2 and Hep3b (250-500  $\mu$ M for both cells). After 48 and 72 hours incubation, the IC<sub>50</sub> value in HUH-7 cells is between 50 to 100  $\mu$ M. In Hepg2 and in Hep3b cells, the IC<sub>50</sub> at 48 and 72 hours was achieved at a higher concentration range, being for both cells between 100 to 250  $\mu$ M. However, after 72 hours of incubation the highest reduction in cell viability (10 to 15%) is achieved with the some dose in HUH-7 and Hep3b cells, (250  $\mu$ M), while in Hepg2 is necessary a higher dose (500  $\mu$ M) to obtain the some result.





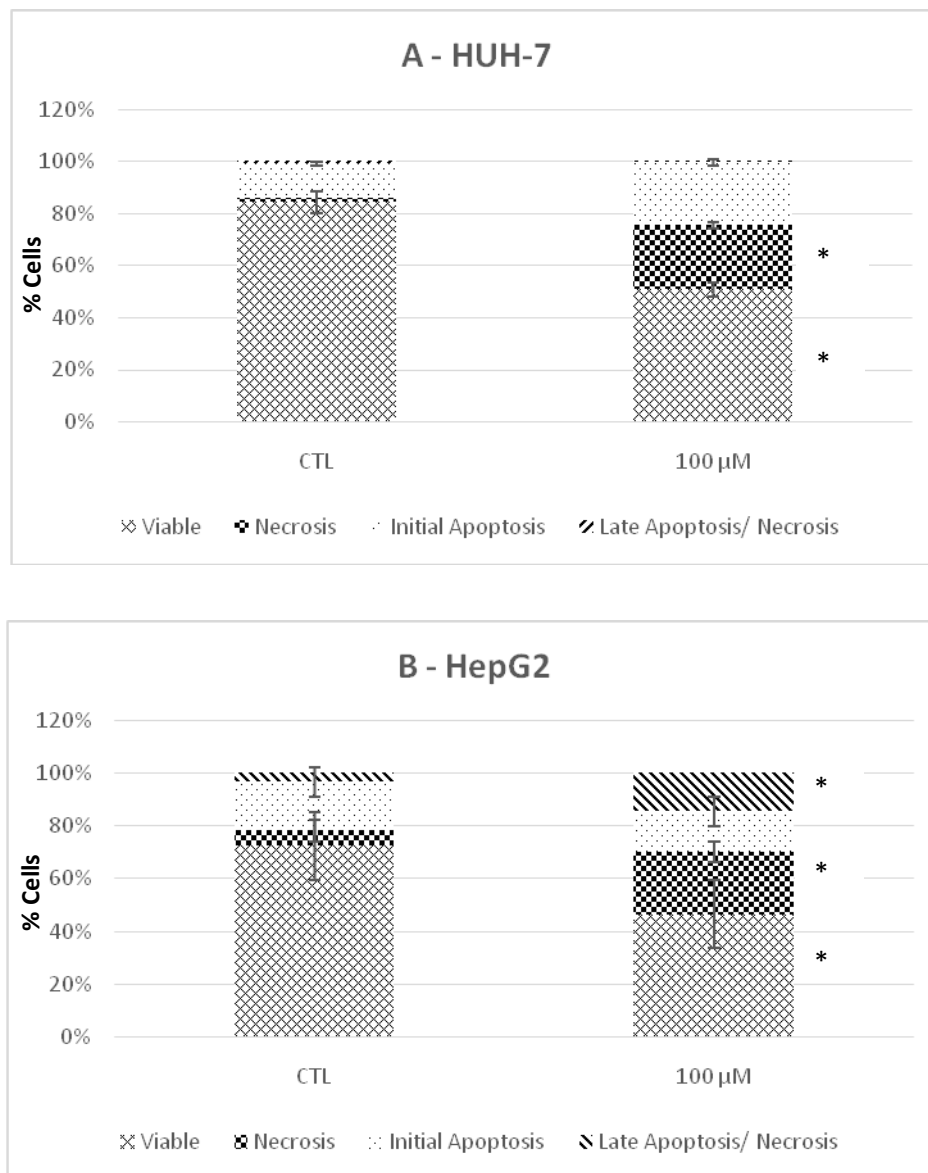


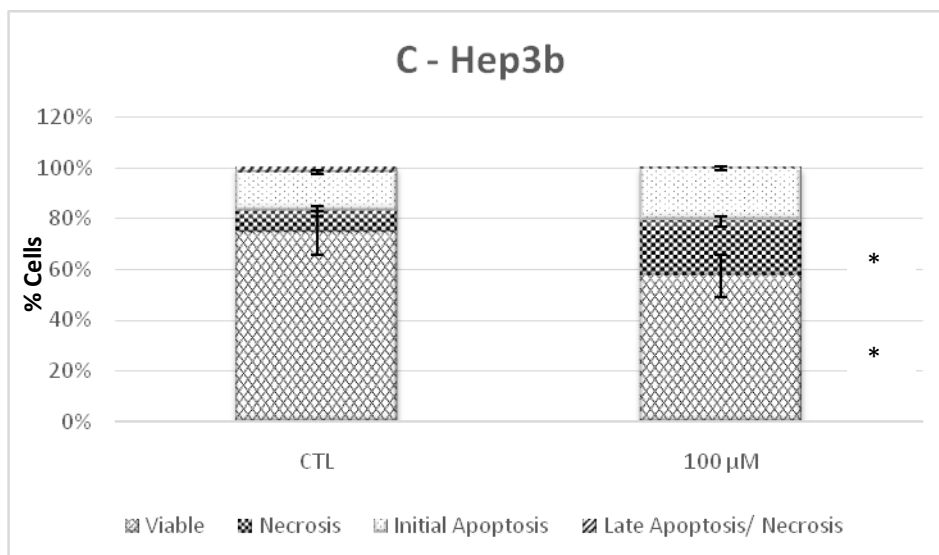
**Figure 2 – Dose- time response curves of NDGA in HUH-7, Hepg-2 and Hep3B cells.** Cell lines were incubated in absence (control) and presence of several concentrations of NDGA. Cells viability was evaluated during 72h of incubation with increasing concentrations of NGDA as represented in figure. Results are expressed in percentage (%) of the control and represent the mean  $\pm$ SD of 3 independent experiments. \* $p < 0,05$ , \*\* $p < 0,01$

## VII.2. Cell Death Analysis

Since tumor cell death mechanisms can interfere with the therapeutic strategy, we also analysed the cytotoxic effect induced by NDGA by studying cell death process by flow cytometry using the AV/PI incorporation as represented in Figure 3. In this figure the extent of apoptosis and necrosis, were evaluated after 48 hours of incubation with 100  $\mu$ M of NDGA.

As can be seen in Figure 3, NDGA induced a decrease in HCC cells viability inducing cell dead mainly by initial or late apoptosis and/or necrosis in a cell type dependent manner. Furthermore, and according with the previous studies, NDGA induced in HUH-7 cells the highest cytotoxic effect when compared with the other cell lines. In fact, in HUH-7 cells (Graph A) we observed a decrease in cell viability about 33,7% and an increase in cell dead mainly by initial apoptosis and necrosis about 10,9% and 23,4%, respectively. In HepG-2 cells (Graph B), we observed a decrease in cell viability about 25,9% and an increase in cell dead mainly by late apoptosis and necrosis about 11, 2% and 17,2%, respectively. Finally, in Hep3b cells (Graph C), it is possible to observed a decrease in cell viability about 17,0% and an increase in cell dead mainly by late apoptosis and necrosis about 18, 6% and 13,2%, respectively.





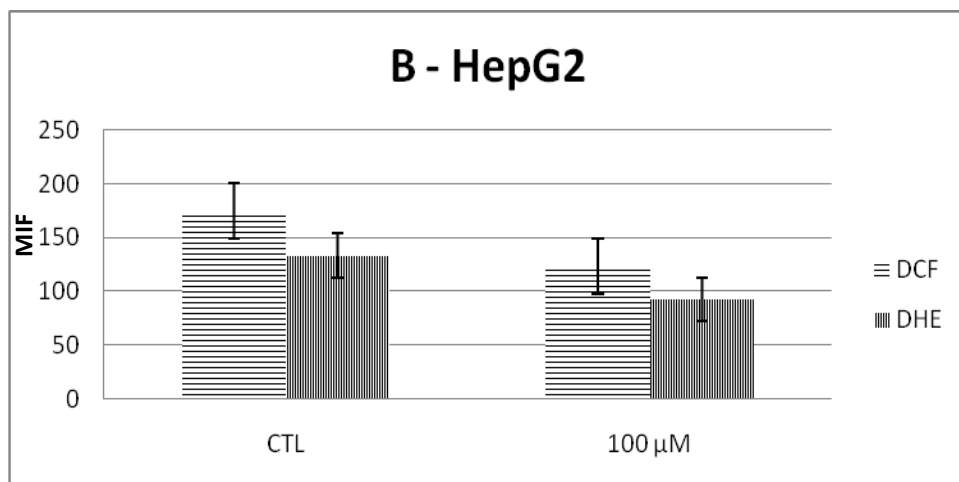
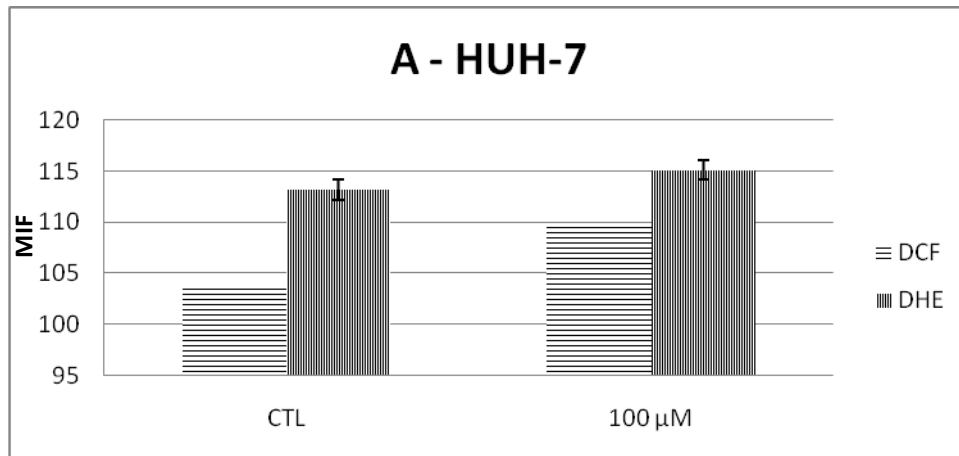
**Figure 3 – Cell death analysis by flow cytometry in HCC cell lines treated with NDGA.** HUH-7, HepG2 and Hep3B HCC cells were incubated in absence (CTL) and in presence of 100μM NDGA. Cell viability and death were assessed by FC using Annexin V and propidium iodide staining as described in material and methods. Alive cells are AV/PI negative; early stages of apoptosis are AV positive and PI negative and cells in late stages of apoptosis are AV/PI positive. Necrotic cells are AV negative and PI positive. Results represented were obtained after 48h of incubation and represent the mean ± SD of 2 independent experiments. \* p<0,05

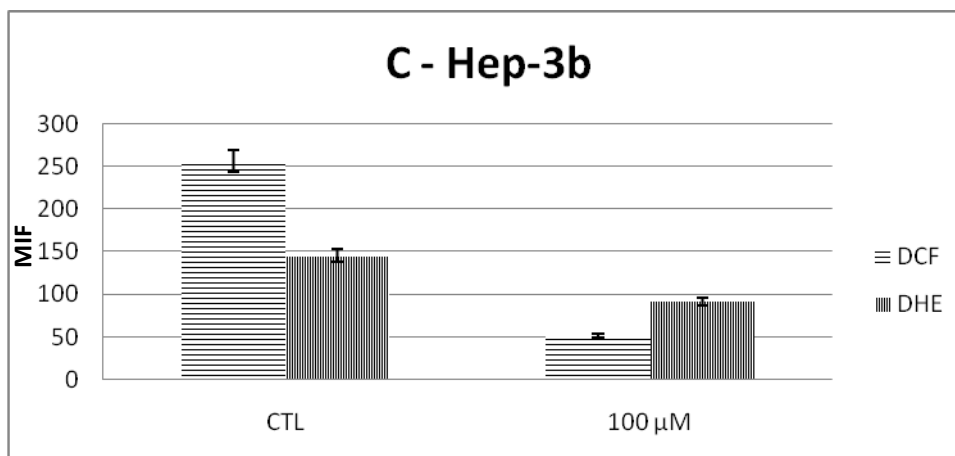
### VII.3. Evaluation of ROS levels

In order to assess the influence of oxidative stress in the cytotoxicity induced by the natural compound NDGA, the levels of reactive oxygen species (ROS), namely the intracellular expression levels of peroxides (hydrogen peroxide, H<sub>2</sub>O<sub>2</sub>) and superoxide anion (O<sub>2</sub><sup>-</sup>), were evaluated in HCC cell lines treated with 100 μM of NDGA for 48 hours by flow cytometry using the fluorescent probes DHE and DCF, respectively (Figure 4).

From the analysis of Figure 4, we observe firstly that HUH-7 cells are those with lower basal peroxide and superoxide anion levels (about 103 MIF) while Hep3b had the highest (about 250 MIF). However, in HUH-7 cells treated with NDGA 100μM (Graph A) we observed a slightly increase in DCF and DHE fluorescence comparing with non-treated cells (about 109 and 115 MIF, respectively), indicating an increase in intracellular expression levels of ROS (peroxides and superoxide anion, respectively).

Contrariwise, in the other cell lines, HepG2 and Hep3B, a decrease in DCF and DHE fluorescence was observed when compared to respective controls (Figure 4, Graph B and C, respectively), more evident in Hep3B (Graph C).





**Figure 4 – Evaluation of intracellular peroxides and superoxide anion levels in HCC cell lines by flow cytometry upon incubation with 100 uM NGDA. HUH-7, HepG-2 and Hep3B cells** were incubated during 48h in absence (CTL) and in presence of 100μM NGDA. Intracellular expression of peroxides (hydrogen peroxide, H<sub>2</sub>O<sub>2</sub>) and superoxide anion (O<sub>2</sub><sup>•-</sup>) was evaluated by flow cytometry by determining the mean fluorescence intensity (MIF) of DCF and DHE, respectively, as described in material and methods. Results are expressed as mean ± SD and represent the MIF variation comparing to the control of 2 independent experiments. (No statistical significance)

## **IX. Discussion and Conclusion**

Hepatocarcinogenesis is a multistep process involving genetic and epigenetic mechanisms which contribute to alteration of numerous signaling pathways leading to deregulation of cell proliferation and resistance to cell death (Thorgeirsson S G *et al.*, 2002; Zhu, J, 2006).

The p53 gene may be the most important gene in human hepatocarcinogenesis. Then loss or inactivation of p53 may contribute to the genetic instability and allows genetically damaged and senescent cells to continue to replicate their DNA increasing the damage allowing them to escape apoptosis. Furthermore, many chemotherapeutic agents cause DNA damage and activate the p53 pathway to induce growth arrest and apoptosis. On account of that, the status of p53 is therefore crucial to the response of HCC to some therapies (chemotherapy or radiation therapy) (Yong-Song G *et al.*, 2006).

The liver is the major detoxification reservoir, receiving toxic substances with carcinogenic potential, from virtually the entire body, that has a common denominator: formation of Reactive Oxygen Species (ROS). ROS are highly reactive and can induce direct damage to many important cellular constituents, such as DNA, lipids and proteins. The formation of oxidative DNA adducts, conditioning genomic instability, aneuploidy, DNA amplification and activation of cell-cycle check points (Kuo M, 2006) contributing to hepatocarcinogenesis. On the other hand, several chemotherapeutic agents induced apoptosis in ROS dependent manner.

Despite all existent therapies for HCC, only a few are potentially curative, as tumor resection, liver transplantation and percutaneous interventions that can result in complete responses and improved survival in a large proportion of patients. However, these invasive therapies are limited by tumor size, the presence of multiple lesions, and impaired function in the case of cirrhotic livers, a scarcity of organ donations and by occurrence of transplant relapse (Spangenberg HC, 2008). In advanced disease, not eligible to the use of potentially curative therapies, systemic treatment has been suggested as beneficial to some patients, especially with the use of tamoxifen and doxorubicin (Alves RC, 2011).

Until recently, there wasn't any available therapy that prolonged overall survival in patients with advanced HCC, indicating the need for new therapies. Recently, HCC natural history was altered by the advent of new targeted drugs, as Sorafenib, a TKI. In 2010 less than 40% of patients in the western world fulfilled criteria for curative treatment (resection, transplantation, local ablation) and only 20% were eligible for chemoembolization. So, there is an urgent need for new therapeutic strategies for HCC (Avila MA, 2006).

Therefore, in this study, we evaluated the therapeutic potential of natural bioactive compounds, such as nordihydroguaiaretic acid (NDGA), in three HCC cell lines with different p53 status, HUH-7 (mutated p53), HepG2 (lower level of wild-type p53) and Hep3B (deleted p53).

Analysing the results obtained, it was found by the Alamar Blue assay that NDGA induced a decrease in cell viability in a dose, time and cell type dependent manner, being the HUH-7 cells the more sensitive cells compared with the other cell lines, Hepg2 and Hep3b. In fact, the half-maximal inhibitory concentration (IC50) value in HUH-7 cells was achieved after 24 hours of incubation with the lowest dose range of the compound. These results may be related with molecular and genetics differences between HCC cell lines, namely p53 state.

In response to cellular stress, p53 binds as a tetramer to diverse DNA targets activating the expression of genes involved in cell-cycle arrest or apoptosis. Mutations of p53 gene alter p53's tertiary structure, impairing DNA binding (Yong-Song *et al.*, 2006) interfering with p21 expression (among others), a key mediator of G1/S transition (Peng Gao *et al.*, 2011), and BAX, a proapoptotic protein.

It might also be noted that the reduction in cell viability is dependent on the concentration of NDGA and the time of incubation of the cells with the compound as a decrease in cell viability was greater for higher concentrations of NDGA, and for incubation periods longer. The IC50 values of NDGA were higher the shorter incubation time, showing the influence of incubation time on the effect of NDGA.

Using flow cytometry, it was possible to see that the reduction in cell viability is accompanied by a cytotoxic effect of NDGA. According to the results, it was found that

cell death occurred primarily through necrosis, however an increase in early apoptosis was observed in HUH-7 after treatment with 100  $\mu$ M NDGA. This results may be related with the ability of NDGA to induces at this concentration and in this cell line, the formation of reactive oxygen species, peroxides and superoxide anion. In the other two HCC cell types, Hepg2 and Hep3b, a decreases in ROS level was observed. However, the results obtained in these cell lines may be related with the drug concentration used as, at 100 $\mu$ M of NDGA, with didn't observed a great decrease in cell viability.

These results are in concordance with literature since previous studies by other authors found that the NDGA has the ability to inhibit growth and induce apoptosis in several types of cancer cells either in culture or in animal models. In fact, a study developed by Tang and colleagues (1996), using a monocytic cell line, showed NDGA-induced apoptosis is associated with lipid peroxidation and the depletion of glutathione and unrelated to LOX activity. Two years later, Danta *et al*, showed that activation of caspase-3 was evident after treatment with NDGA.

In same year, Moody and colleagues (1998), using cell lines and human lung cancer nude mice, showed that the NDGA inhibited the growth of cells *in vitro*, and delayed the growth of established tumors in mice. When the tumors were removed and examined, it was found that tumors from mice treated with NDGA were more apoptotic cells in the tumors of mice receiving no treatment with this compound (Brömme H, 2008).

Moreover, this compound can inhibit Lipoxigenase (LOX), whose products, as hydroperoxy and hydroxy fatty acids, have been implicates as important elements in the regulation of tumor cell growth by modulating cell proliferation and apoptosis. So, blocking LOX activity could induce apoptosis in some cell lines. (Biswal S, 2000).

A wide variety of cellular processes are affected by nordihydroguaiaretic acid (NDGA). Many of these effects are related with its action as an antioxidant or free radical scavenger, however NDGA can inhibit tumor growth by inhibiting receptor tyrosine kinase phosphorylation. In addition, NDGA induces apoptosis dependently and independently of its activity as a LOX inhibitor, either sensitizing malignant tumor cells to tumor necrosis factor-related apoptosis inducing ligand (TRAIL)-induced apoptosis



through DR5 up-regulation or through the mitochondrial pathway, through BCL-2 proteins family (Plaza C *et al.*, 2008).

Also, Seufferlein and colleagues (2002) have shown that NDGA inhibits anchorage-independent growth of pancreatic cancer cells (SW 580) and cervix cells (C4- I) *in vitro* and inhibits the growth of established tumors in athymic mice at concentrations of 15 and 30 mM. They also found that this compound induced apoptosis in both types of cancer cells *in vitro* and *in vivo*. The authors studied the possible mechanisms related to the effects of NDGA and found that this compound inhibits the expression of cyclin D1 in cancer cells (Seufferlein T, 2002). This protein promotes cell cycle progression, lying constitutively expressed in pancreatic cancer and cervix cells. Thus, the growth inhibitory effect of NDGA by cancer cells may be associated with this molecular mechanism. In this study it was found that NDGA causes disruption of the actin cytoskeleton, inducing apoptosis in these cells (Arteaga S, 2005).

Recent studies showed NDGA also suppressed mTORC1 (suppressing amino acids- and insulin-stimulated mTORC1 and acting like rapamycin to disrupt mTOR-Raptor interaction) downstream signaling such as expression of cyclin D1, hypoxia-inducible factor- $\alpha$  and VEGF, and prevented proliferation in breast cancer cells. (Zhang Y, 2012).

In conclusion, NDGA has been shown to reduce cell viability of human hepatocellular carcinoma cells by inducing apoptosis and/or necrosis, and its effect depends on the cell type, drug concentration and time of incubation. The cytotoxic effect obtained in HUH -7 cells seems to be caused through the ability of NGDA to induce the formation of reactive oxygen species. The differences observed between the HCC cell lines studied could be explain by the molecular and genetics differences: HepG2 and Hep3B showed a higher expression levels of CD34 and Thy-1, associated with higher proliferation activity, and, in the other hand, HUH-7 cells are the only with a TP53 mutation. (Jabari S, 2009) The stabilization of p53 may be partially responsible for apoptosis induction or cell cycle arrest at the G1 phase found in NDGA-treated cells (Peng Gao *et al.*, 2011).

For future, we suggest to study the variation of mitochondrial membrane potential after NGDA treatment for detecting apoptosis caused by mitochondrial

modifications due to oxidative stress, and analyze cell cycle effects and variation of cyclin D1 levels to distinguish the cytotoxic and antiproliferative effect. Moreover, it is necessary to discover the potential effect of NGDA used as co-adjuvant therapeutic agent to potentiate actual chemotherapy in order to decrease its doses and side effects.

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