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Article

Morphological and biochemical effects of carnosic acid on human hepatocellular carcinoma HepG2 cells

Efectos Morfológicos y Bioquímicos del ácido Carnósico en Células de Carcinoma Hepatocelular Humano HepG2

Aşkin Keskin Kaplan
Department of Family Medicine, Faculty of Medicine, Maltepe University, Istanbul, Turkey.

Hatice Maraş
Cancer and Stem Cell Research Center, Faculty of Medicine, Maltepe University, Istanbul, Turkey

Merve Dilara Kolsan
Cancer and Stem Cell Research Center, Faculty of Medicine, Maltepe University, Istanbul, Turkey

Mustafa Erinç Sítar
Department of Medical Biochemistry, Faculty of Medicine, Maltepe University, Istanbul, Turkey

Ranan Gülhan Aktaş
Director of Cancer and Stem Cell Research Center, Faculty of Medicine, Maltepe University, Istanbul, Turkey

Correspondence to: *Aşkın Keskin Kaplan*
Department of Family Medicine, Faculty of Medicine, Maltepe University, Istanbul, Turkey.
askin.kaplan@maltepe.edu.tr

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ABSTRACT

Background/aim: Autophagic cell death and apoptosis of tumor cells has become one of the main objectives in cancer treatment, whereas tumor cell lines are mainly used in studies for providing important data for the evaluation of potential anti cancer substances. In this study, our objective was to evaluate morphological and biochemical changes including rate of apoptosis and Alpha Fetoprotein (AFP) levels at different concentrations of Carnosic Acid (CA) on Human Hepatocellular Carcinoma HepG2 Cells.

Materials and methods: Human Hepatocellular Carcinoma (7th passage HepG2 cells) Cell lines were cultured on 11 μM D263M schott glass coverslips placed in 12-well plates and

were treated with DMSO, 1, 2.5, 5 and 10 µM concentrations of CA for 24, 48 and 72 hours. Morphological and biochemical data were recorded daily including apoptosis rates demonstrated by Caspase 3, Annexin V expressions under inverted light and Immunofluorescence microscopy, then data were analyzed for statistical significance. AFP, albumin and total protein levels were analyzed spectrophotometricaly for biochemical evaluation.

Results: Our results showed that CA significantly inhibited HepG2 cell proliferation in a dose and time dependant manner and significantly caused the formation of autophagic vacuoles starting from 5µM and reaching significance at 10 µM concentrations. Significant decrease was observed in AFP when 48 and 72 hours expressions were examined, with the lowest level reached at 72 hours in the 10 µM CA group. Additionally, increase in albumin levels reached significance only in the 48 h group whereas non-significant increases were also observed in 24 h and 72 h groups.

Conclusion: Our current study demonstrates significant increase in apoptosis rates by Carnosic Acid mainly at 10µM concentrations, supporting its anticancer effect on HepG2 cells. These findings are also supported by changes in biochemical analyses of Albumin and AFP levels at 10 µM concentrations.

Keywords: Apoptosis; Carnosic Acid; Hepatocellular Carcinoma; HepG2; Rosemarinus Officinalis; Caspase III; Annexin V

1. Introduction

Primary malignant form of liver cancer - Hepatocellular Carcinoma (HCC) is the most common 5th cancer type and the 3rd in terms of mortality worldwide. The annual incidence of HCC in Europe varies from 10 to 15 cases per 100.000, however the incidence is much higher in Asia and Africa as of 50 to 150 cases per 100,000 (Bosch *et al.*, 2004) . For the time being, surgical resection of the tumor is the main treatment in many diagnosed HCC patients and researchers work on cell cultures in order to find possible alternative treatment options (Llovet *et al.*, 2004). In this matter, studies on cancer cell line cultures have become the main area of research conducted to improve diagnosis and treatment for different types of cancers (Wilding and Bodmer, 2014).

Regarding Hepatocellular carcinoma, Hep G2 cells, one of the HCC cell lines, which are frequently used in cancer treatment studies, was for the first time obtained in 1979 from the biopsy material of a patient who had been diagnosed with liver cancer. Moreover HepG2 cells show similarities to normal cells and are frequently used by researchers to understand liver cancer phenotypes (Aden *et al.*, 1979).

Recently in many studies, plant and herb extracts have shown potential beneficial anti-cancer and anti-oxidant effects due to their capacity to prevent, reverse or inhibit certain processes of carcinogenesis (Yesil-Celiktas *et al.*, 2010). Rosemary (Rosemarinus officinalis L.) a perennial herb from the Lamiaceae family, typical of the Mediterranean region, is known to have hepatoprotective, antiproliferative, antiviral, anti angiogenic, antimicrobial, anti fibrotic, antioxidant, antinociceptive, antidiabetic and anticancer effects (González-Vallinas *et al.*, 2015, Petiwala *et al.*, 2013, Petiwala and Johnson, 2015, Slameňová *et al.*, 2002, Alexandrov *et al.*, 2006, Altin and Sloan, 1997, Cheung & Tai, 2007, Shabtay et al., 2008, Moore *et al.*, 2016, Borrás-Linares *et al.*, 2015, Huang & Zheng, 2006, Li *et al.*, 2010, Kim *et al.*, 2005, Hozayen *et al.*, 2014).

Accordingly, the antioxidant properties of rosemary are related to the presence of antioxidant phenolic substances, mainly Carnosic Acid (CA) and Carnosol (Slameňová *et al.*, 2002, Alexandrov *et al.*, 2006, Cheung & Tai, 2007, Shabtay *et al.*, 2008, Cuvelier *et al.*, 1994). CA has shown to enhance the inhibitory effects of 5 FU on cell proliferation and sensitize 5 FU resistant cells (González-Vallinas

et al., 2015). Also CA has the potential to inhibit growth in HepG2 cells without increasing ROS production (Akın *et al.*, 2019). However, some in vitro studies have shown that the antioxidant activity of CA is three times more potent than of carnosol (Cuvelier *et al.*, 1996). In recent studies the inhibitory effects of CA was shown in lung, colon, breast and prostate cancer cells, but its effect on liver cancer is not precisely known (González-Vallinas *et al.*, 2015, Petiwala *et al.*, 2013, Petiwala & Johnson, 2015, Shao *et al.*, 2019).

Gao *et al.* have shown that CA causes autophagic cell death in Human Hepatocellular Carcinoma HepG2 Cells (Gao *et al.*, 2015). However, in other tumor cell lines, they could not obtain any results related to apoptosis which is the main mechanism used by this plant molecule(Yuan & Kroemer, 2010). Current data clearly demonstrates the anticancer activity of CA on liver cancer tumor cell lines of human or rat hepatic tumors. Although CA has shown to induce apoptosis in HCC cells in some studies, a conclusive clear result has not yet been reached due to the limited number of studies.

For this reason in the present study, we aimed to evaluate the morphological characteristics of HepG2 cells for the rate of cell apoptosis after cell cultures were exposed to CA at variable concentrations in a time – dose dependant manner by Caspase-3 and Annexin-V methods under inverted light and Immunofluorescence microscopy. Additionally we also simultaneously analyzed changes in AFP, albumin and total protein levels biochemically to support our findings.

2. Materials and methods

Cell Culture

7th passage HepG2 cells (American Type Culture Collection, USA) in 89% Dulbecco's Modified Eagles Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% antibiotic (Streptomycin + Penicillin) were cultured in an incubator (Maltepe University, Panasonic, Istanbul) containing 5% CO2 at 37°C. Cells were cultured on 11 µM D263M schott glass coverslips placed in 12-well plates for morphological analysis. Live images of HepG2 cells were evaluated with inverted light microscope daily (Maltepe University, Zeiss) under 40×, 200× and 400× magnification and growth cultures were collected at the end of the determined processes to measure the amount of secretion for biochemical analyses.

Carnosic Acid preparation:

Powdered Carnosic Acid (CA TCI C2488) was dissolved in DMSO at a concentration of 1 mg/ml (20 mg CA + 20 ml DMSO). The prepared stock solution was made ready for use after filtering through a sterile filter. Cells were exposed to CA dissolved in Dimethyl Sulfoxide (DMSO) at doses of 1 µM, 2.5 µM, 5 µM and 10 µM for 72 hours. Two separate control groups were made by cells grown directly in the growth culture and by adding DMSO at the same percentages of the experimental groups to the growth culture. Then, each group was compared at 24, 48 and 72 hours for morphological, functional and biochemical properties.

Biochemical measurements:

Biochemical measurements were done by examining culture media fluid samples from HepG2 cells after centrifugation at the end of 24, 48 and 72 hours of the experiment. Albumin, alpha fetoprotein (AFP) and total protein parameters were measured by auto analyzers according to instructions of the manufacturers (Siemens Dimension RxL Max Germany and Roche Hitachi Cobas Switzerland).

Total lipid analysis was applied directly with an ELISA kit after first extractions were performed. For the AFP analyses, 10 µL samples were enabled to react with biotinylated monoclonal AFP specific antibody and ruthenium complex labeled monoclonal AFP-specific antibody (Tris (2,2'-bipyridyl) ruthenium (II) complexes) using sandwich ELISA principle. The complex was then converted to solid phase by adding streptavidin-coated micro particles. Magnetic fields, ProCell/ ProCellM and voltage were applied to the reaction mixture respectively and results were observed and calculated on a two-point calibration curve. For albumin analysis, after 17 µL of sample, 76 µL particle reagent volume and 76 µL antibody reagent volume was obtained, biochromatic turbidimetric reading was performed by spectrophotometric reading at 340 and 700 nm at 37 ° C in the auto-analyzer. For total protein analysis, after 350 µL reagent volume and 50 µL ready diluent volume of 10 µL sample, spectrophotometric readings were performed on the auto-analyzer with biochromatic endpoint reading at 600 and 700 nm at 37°C.

Cell Fixation

At the end of every 24 hour, HepG2 cells were fixed with Cellfix, and then incubated in the refrigerator at +4 °C until staining.

Monitoring Morphological Changes in Cells

To observe the integrity of the nucleus, cytoplasm and proliferation rates of cells, invert light microscopy was used for examining and monitoring morphological changes by photographing at different magnifications. Live Visualization was done using Phase Contrast Microscopy and cells were photographed live at ×40, ×100, ×200 and ×400 magnifications at 24, 48 and 72 hours.

Immunofluorescence (IF) microscopy:

Fixed HepG2 cells were blocked with bovine serum albumin (Capricorn, Germany) and incubated with anti caspase-3 antibody (1:100 dilution, abcam) and APC Annexin V (1:100 dilution, Biolegend) overnight at 4 °C. The cells were rinsed next day with PBS three times and incubated with goat anti-rabbit IgG DyLight 488 (1:200, Thermo Scientific, USA) and goat anti-mouse IgG DyLight 488 (1:200, Thermo Scientific, USA) for 90 min at 37 °C. After rinsing three times with PBS, the cells were counter stained with Hoechst 33258 (Thermo Scientific) for labeling the nuclei. Images were taken with a laser scanning confocal microscope (LSM 700, Zeiss).

Statistic Analysis

All data are expressed as mean ± standard error of the mean. Statistical evaluation was performed by one-way analysis of variance (ANOVA) test using GraphPad Prism V.8.01 and post-hoc Tukey's analyses were carried out to find groups whose mean differences were significant. Data were pooled from at least three independent experiments.

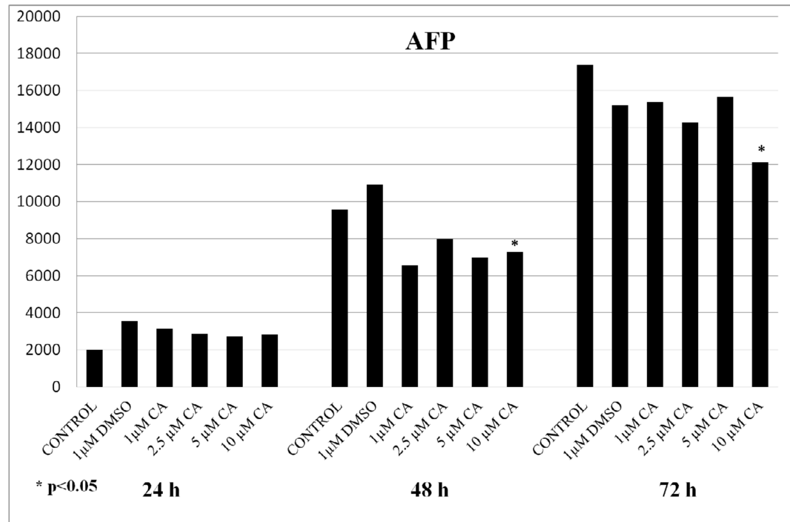
3. Results

Biochemical analysis results:

AFP expressions increased time-dependently when all experimental groups were evaluated within themselves. When 48 and 72 hours expressions were examined, statistically significant decrease was observed in AFP with the lowest content in 72 hours in the in the 10 µM CA group ($p<0.05$). Besides,

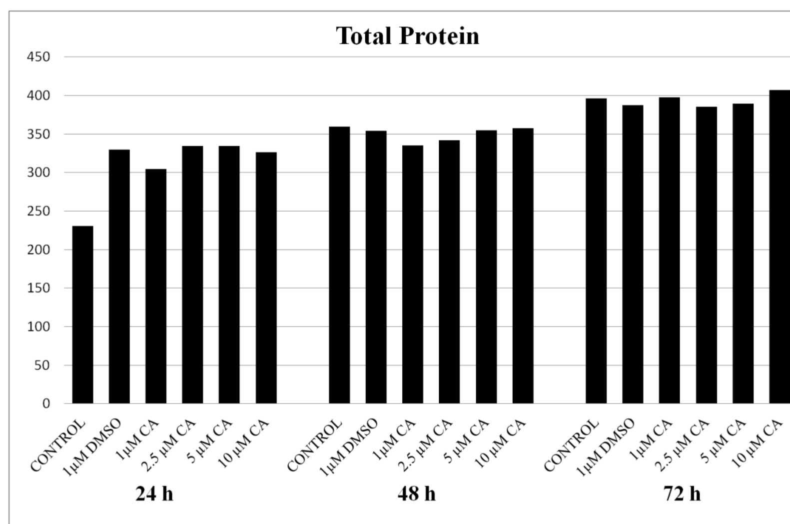
the AFP level in 72-hour DMSO experimental group was over the measurable limit as shown in Figure 1.

Figure 1:
Alpha Fetoprotein (AFP) levels at 24, 48 and 72hours, dose depended on CA.



When each experimental group was evaluated within itself, total protein expressions also had a time dependant increase, similar to that of AFP. Increase was observed in 24 h values for 2.5 µM, 5 µM and 10 µM CA groups. However in the 48 and 72 hour CA experimental groups, similar values with the control groups were observed, and highest values were obtained in the 72 hour 10 µM CA group with no statistical significance as shown in Figure 2.

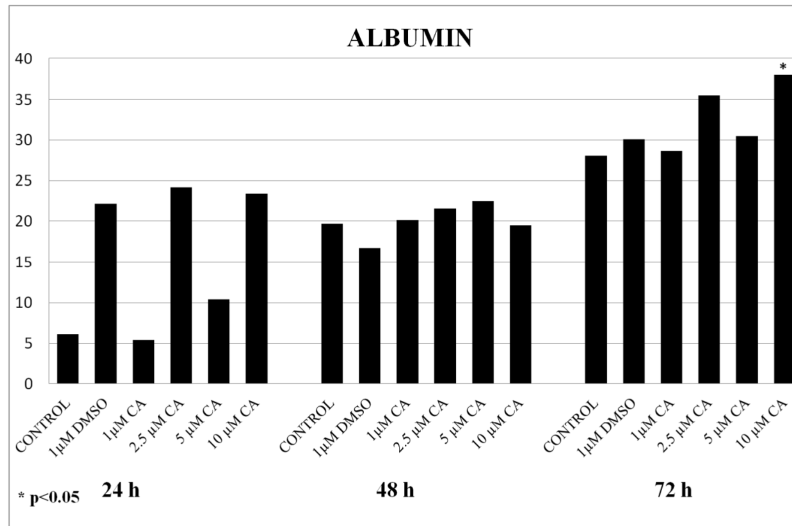
Figure 2:
Total protein (UCFP) levels at 24, 48 and 72hours, dose depended on CA.



When albumin data were analyzed, 24 and 72 hours of the 1 µM CA experimental groups were similar to that of the control group. There was a significant increase in 24 and 72 hours and a non significant increase in 48 hours in the 2.5 µM CA experimental group, compared to the control group

($p<0.05$). In the 5 µM CA experimental group, similar increases were obtained with the control group. However, the 10 µM CA group had significantly greater albumin levels compared to that of the control group at 24 hours. The values were also higher but not significant at 48 hours and 10 µM CA group represented the highest value among all other 72-hour groups as shown in Figure 3.

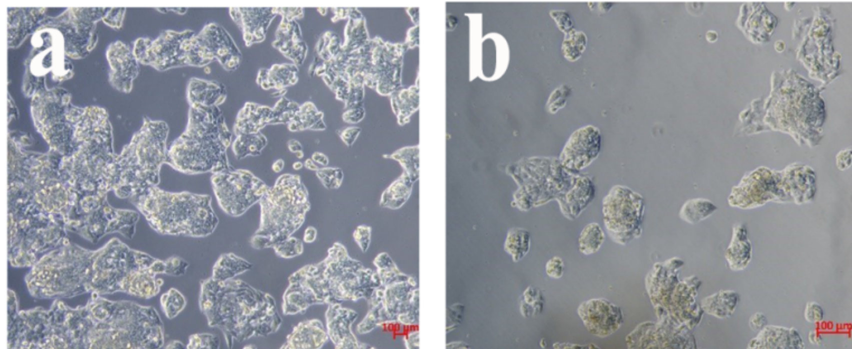
Figure 3:
Albumin levels at 24, 48 and 72hours, dose depended on CA.



Cell Viability Results:

Significant dose-related tendency to decrease in viability rates from the 24th to the 72nd hour was observed and only the 10 µM CA group showed a significant decrease at 24 hours compared to the control group as shown in Figure 4.

Figure 4:
Images of the HepG2 cells under the inverted microscope - (a) Control group, (b) 10 µM CA group.



Morphological Comparison of Annexin-V expressions:

Increase in Annexin-V expression levels were observed in 2,5 µM and 10 µM experimental groups when compared to the control group at 24 hours ($p<0.05$), however when compared at 48 and 72

hours, the 10 µM CA group had statistically significant increases compared to all the other four groups as shown in Figure 5a,b.

Figure 5a:
immunofluorescence images of Hep G2 cells, showing the expression of Annexin V. Cell nuclei were stained with Hoechst.

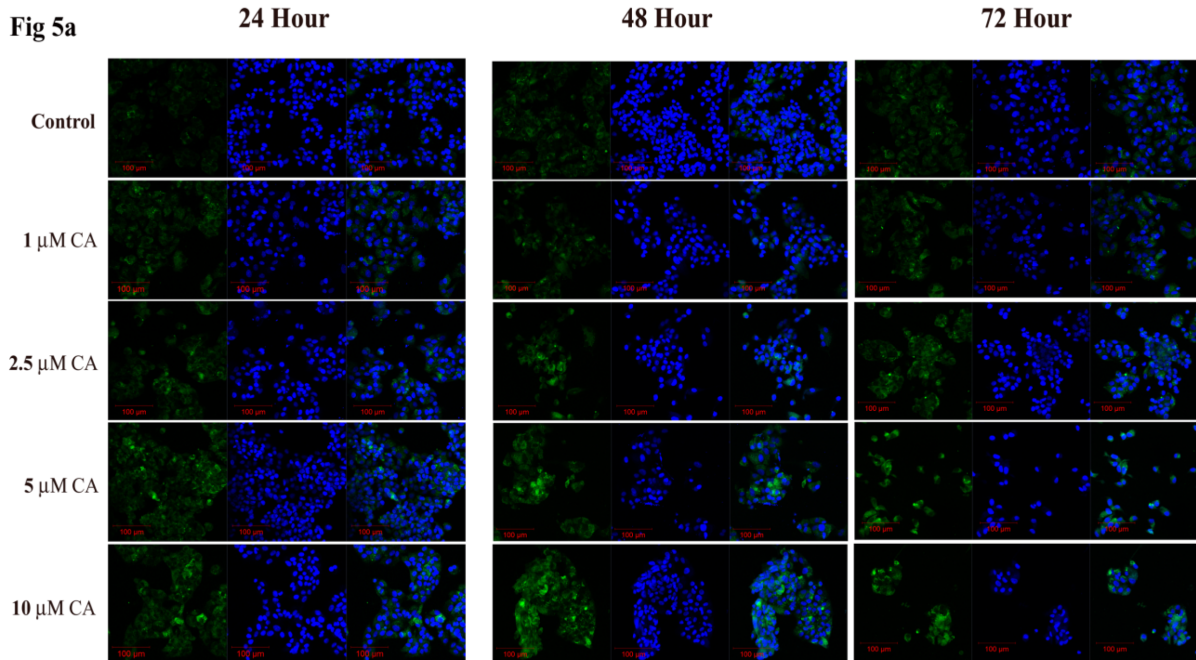
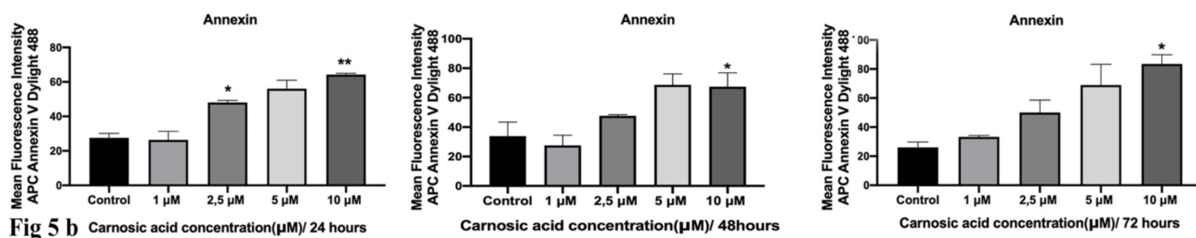


Figure 5b:
Comparison of HepG2 cell groups marked with Annexin V and exposed to different concentrations of CA at 24, 48, and 72 hours.



Morphological Comparison of Caspase-3 expressions:

There was a statically significant increase in Caspase-3 expression levels of the 2.5 µM CA group when compared to the control group at 24 h ($p<0.05$). However in the 10 µM CA group, significant high levels were observed when compared to the other four groups at 48 and 72 hours as shown in Figure 6 a,b. ($p<0.05$)

Figure 6a:
immunofluorescence images of Hep G2 cells, showing the expression of Caspase-3. Cell nuclei were stained with Hoechst.

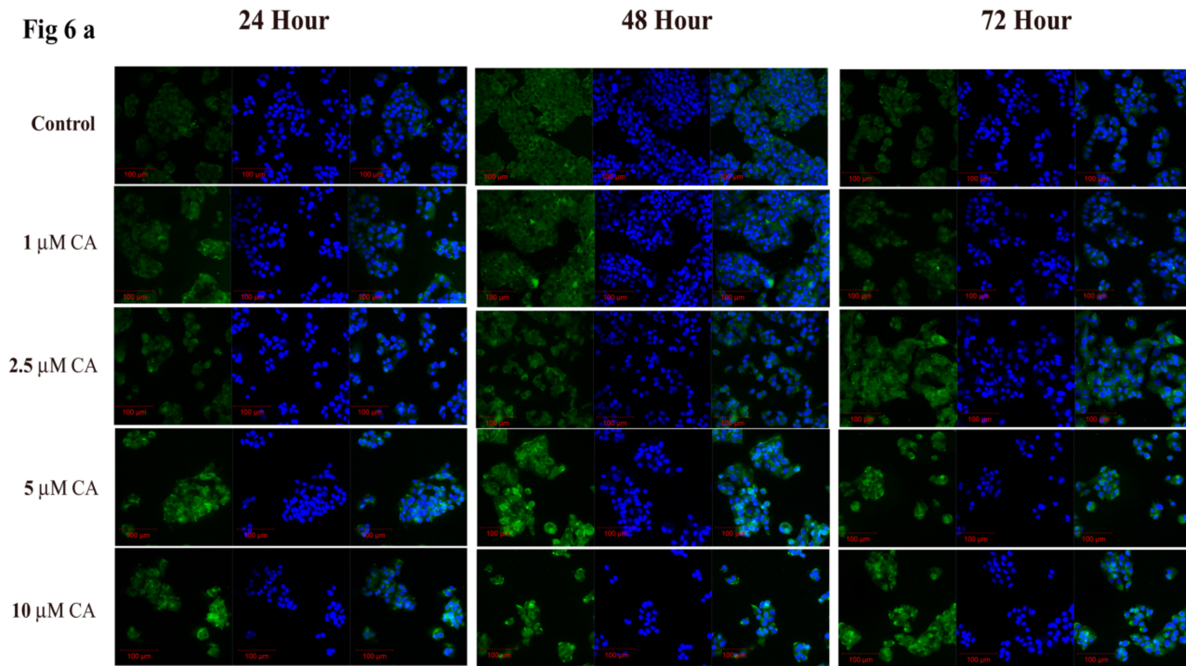
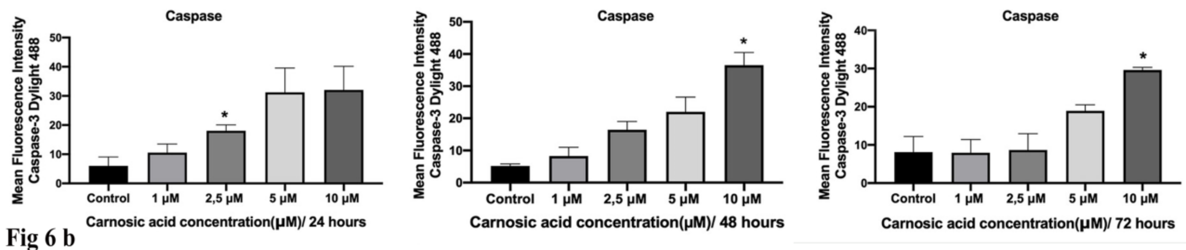


Figure 6b:
Comparison of HepG2 cell groups marked with Caspase-3 and exposed to different concentrations of CA at 24, 48, and 72 hours.



4. Discussion

In this study, the anti-proliferative activity of Carnosic Acid (CA) in HepG2 cells and its underlying molecular mechanism of inducing autophagic cell death were investigated. As a natural product being one of the main polyphenolic diterpene of Rosemary, CA has multiple biological properties and provides a biochemical basis for therapeutic selectivity. Programmed cell death plays an important role in tumor development, with apoptosis currently viewed as the most important death modality (Yuan and Kroemer, 2010). Several previous studies have reported that Carnosic Acid exhibits anti-cancer activities through inducing apoptosis (González-Vallinas *et al.*, 2015, Petiwala *et al.*, 2013, Petiwala & Johnson, 2015). It should be noted that, in our study CA significantly inhibited HepG2 cell proliferation in a dose and time-dependant manner, and our results showed that CA significantly induced HepG2

cell apoptosis. However, CA at given dosages except 10 μM concentration, did not significantly cause any morphological changes on HepG2 cancer cells, while CA at 10 μM concentration led to important functional changes with promising efficacy in HepG2 hepatocellular carcinoma cells.

It has been reported in a previous study that CA decreases human lung fibroblast (HLF) viability with IC50 value of 17.13±1.06 μM, without any cytotoxic effect (Bahri *et al.*, 2017), for this reason this suggests that correct dosing of CA is an important factor for showing significant results. Besides, our concern was not to have a cytotoxic effect on HepG2 cells during our 72 h study and this explains our intention for using a concentration ranging from 1 to 10 micro mol of CA for observing dose time dependant effects.

Regarding biochemical analyses, a decrease in AFP secretion by HepG2 cells was observed, which implies to a decrease in carcinogenic activity and normal cell functioning, thus suggesting anticancer activity of CA. However, CA also caused significant increase in albumin secretion levels which is another marker for normal hepatic cell function. These findings also may suggest Carnosic Acid to have a differentiative effect on HepG2 cells to acquire normal cell function characteristics due to its anti-cancer properties.

5. Conclusion:

In conclusion, our study demonstrates significant increases in apoptosis rates by Carnosic Acid at mainly 10μM concentrations, which is also supported by significant biochemical changes, thus could be confirming its anticancer effect in Human Hepatocellular Carcinoma HepG2 cells.

6. Conflict of Interest:

None.

7. Funding:

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8. Acknowledgements:

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RESUMEN

Antecedentes / objetivos: La muerte celular autofágica y la apoptosis de células tumorales se ha convertido en uno de los principales objetivos en el tratamiento del cáncer, mientras que las líneas celulares tumorales se utilizan principalmente en estudios para proporcionar datos importantes para la evaluación de posibles sustancias anticancerígenas. En este estudio, nuestro objetivo fue

evaluar los cambios morfológicos y bioquímicos, incluida la tasa de apoptosis y los niveles de alfa fetoproteína (AFP) a diferentes concentraciones de ácido carnósico (CA) en células de carcinoma hepatocelular humano HepG2.

Materiales y métodos: Carcinoma hepatocelular humano (HepG2).Las líneas celulares se cultivaron en cubreobjetos de vidrio Schott D263M de 11 µM colocados en placas de 12 pocillos y se trataron con DMSO, concentraciones de CA 1, 2,5, 5 y 10 µM durante 24, 48 y 72 horas. Los datos morfológicos y bioquímicos se registraron diariamente, incluidas las tasas de apoptosis demostradas por Caspasa 3, las expresiones de Anexina V bajo luz invertida y microscopía de inmunofluorescencia, luego se analizaron los datos para determinar la significación estadística. Los niveles de AFP, albúmina y proteínas totales se analizaron espectrofotométricamente para evaluación bioquímica.

Resultados: Nuestros resultados mostraron que CA inhibió significativamente la proliferación de células HepG2 de una manera dependiente de la dosis y el tiempo y causó significativamente la formación de vacuolas autofágicas comenzando desde 5 µM y alcanzando significancia a concentraciones de 10 µM. Se observó una disminución significativa en la AFP cuando se examinaron las expresiones de 48 y 72 horas, alcanzando el nivel más bajo a las 72 horas en el grupo de CA 10 µM. Además, el aumento en los niveles de albúmina alcanzó significación solo en el grupo de 48 h, mientras que también se observaron aumentos no significativos en los grupos de 24 hy 72 h.

Conclusión: Nuestro estudio demuestra un aumento significativo en las tasas de apoptosis por el ácido carnósico principalmente a concentraciones de 10 µM, lo que respalda su efecto anticancerígeno en las células HepG2. Estos hallazgos también están respaldados por cambios en los análisis bioquímicos de los niveles de albúmina y AFP a concentraciones de 10 µM.

Palabras Clave: Apoptosis; Ácido Carnosico; Carcinoma Hepatocelular; HepG2; Rosemarinus Officinalis; Caspasa III; Anexina V