Study of carcinogenic activity of carnosic acid and rosmarinic acid in cancer cell line of Hep- G۲

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Abstract

Background: As anti-apoptotic properties of polyphenols have been proved in past years. In this study, carcinogenic activity of carnosic acid and rosmarinic acid in human hepatoma carcinoma cells (Hep- G۲) were studied.

Method: In this experimental study, Hep -G۲ cells were cultured in DMEM supplemented containing bovine fetal serum and antibiotics. Cells with double dilution were then cultured from ۰ to ۷۰ µM for ۲٤ h and viability of cells was determined by MTT method. In order to evaluate activity of caspase۳ and ۹ enzymes after ۲٤ hours of incubation of the cells with treated materials, cells were centrifuged and cell lysis solution was added. This mixture was centrifuged and then an appropriate substrate was added into each enzyme and after incubation, absorbance of the resulted solution was read at ٤۰٥ nm using a spectrophotometer device. To measure the level of ceramide, a recombinant acid ceramidase enzyme and naphthalene-۲, ۳-dialdehyde, which is fluorescent and is connected to sphingosine resulted from acid ceramidase, were added to the cell extract and was ultimately determined by HPLC.

Results: Carnosic acid increased cell viability and caused no induction of apoptosis to a dose-dependent species in Hep-G۲ cells by reducing ceramide levels and decreasing activity of caspase ۳ and caspase ۹ enzymes. Rosmarinic acid in concentrations of up to ٥۰ µM decreased cell viability by increasing ceramide levels and activity of caspse-۳ and caspase ۹ enzymes.

Conclusion: Although in most cases, polyphenols have caused no induction of apoptosis and have decreased cell viability percentage. However in some cases, they have affected inversely and have caused cell growth.

Keywords: Carnosic acid, Rosmarinic acid, Ceramide, Cell line Hep-G۲, Caspse-۳ and ۹.

Introduction

Hepatocellular carcinoma (Hcc) is a type of liver cancer, which often occurs after infection with hepatitis B and C viruses and cirrhosis that is due to alcohol consumption in most cases. Hepatocellular carcinoma like other cancers occurs when a mutation occurs in the cellular machinery and leads to uncontrolled cell proliferation or it occurs as a result of cell avoiding apoptosis phenomenon (Kumar et al, ۲۰۰۳).

As lot of one-year fruits, vegetables and herbs play an important role in the initiation and also progression of cancer, countless patients around the world use medicine herbs to maintain health. Therefore, scientists deeply consider biological properties, health care power of these products (Abdullaev et al, ۲۰۰۰; Abdullaev, ۲۰۰۱). Two substances of rosmarinic acid and carnosic acid, which are in rosemary plant (Rosmarinus *officinalis. L*), have special properties such as anti-cancer, anti-inflammatory and antioxidant properties (Jin min et al, ۲۰۱٤; Shahadat Hoosa et al, ۲۰۱٤).

In recent research, it has been found that there is a relation between increasing levels of total ceramide in cancerous and malignant tumor tissue (Levy & Futerman, ۲۰۱۰). Accordingly, this study is aimed to study whether rosmarinic acid and carnosic acid have an effect on Hep-G۲ cell viability and if they are effective, dose the change in the metabolism of ceramides by these two substances explain effect of these substances on metabolism of these cells.

Materials & Methods

In this study, two substances of rosmarinic acid and carnosic acid were purchased from Sigma-Aldrich and concentrations of ۰, ۱۰, ۲۰, ۳۰, ۴۰, ۵۰, ۶۰ and ۷۰ µM of these two substances were prepared and were cultured in ۹۶-well culture plates with ۵۰۰۰ cells for ۲۴ h and the effects of extracts on cell viability was determined by MTT test.

Cell Culture: Liver cancer cells (Hep-G۲) were prepared from Razi vaccine and serum research institute (Hesarak, Iran). Then, they were cultured in DMEM medium with ۵٪ fetal bovine serum (FBS), penicillin and streptomycin, the cells were incubated at ۳۷ °C, humidity ۹۰٪ and ۵٪ of $CO_۲$. After ۲۴ hours, the cells were exposed to different concentrations of two compounds, at the same time, triple control samples without treatment was considered.

Study of cells viability: Cells viability was tested by MTT [۳-(۴, ۵-dimethylthiazol-۲-yl) - ۲, ۵-diphenyl-۲H-tetrazolium bromide] (Xiang et al, ۲۰۱۰). Briefly, cells were plated at ۵۰۰۰ cells per ۹۶-

well culture plates and were treated with different concentrations of two compounds of rosmarinic acid and carnosic acid. The supernatant medium was discarded. The cells were placed in a solution of MTT (۵ ml in PBS) for ۴ h and after preparing a solution of formazan by ۱۰۰ µl of DMSO, absorbance was read at ۵۷۰ nm using a spectrophotometer.

Evaluation of caspse-۳ enzyme activity: To measure the activity of caspase-۳ enzyme, after ۲۴ hours of incubation of the cells with the treated material and centrifugation, cell lysis buffer was added to samples. Then the lysate mixture was centrifuged. A solution containing AC-DEVD-PNA substrate was added to the supernatant solution and incubated. Absorbance of supernatant solution resulting from separation of p-nitroanilide was measured at ۴۰۵ nm.

Evaluation of caspse-۹ enzyme activity: To measure the activity of caspase-۹ enzyme, after ۲۴ hours of incubation of the cells with the treated material and centrifugation, cell lysis buffer was added to samples. Then the lysate mixture was centrifuged. A solution containing AC-DEVD-PNA substrate was added to the supernatant solution and incubated. Absorbance of resulted supernatant solution was measured at ۴۰۵ nm.

Evaluation of cellular ceramide level: To measure the level of ceramide, the recombinant ceramidase acid enzyme was first added to cell extract that as a result, ceramide in the sample was fully hydrolyzed and converted to sphingosine. A special substance called naphthalene-۲, ۳-dialdehyde (NDA), which is fluorescent, was connected to sphingosine. Sphingosine-NDA was then separated from cell extracts by reverse phase HPLC and was determined in fluorescence detector. Ceramide level is exactly as same as sphingosine obtained by HPLC in the same sample.

Statistical analysis: Each test was repeated three times and comparison of average data with the control and treatments with each other was obtained using SPSS software (ANOVA test).

Results

Hep-G۲ cells under two treatments of rosmarinic acid and carnosic acid with different concentrations showed dose dependence differences with each other.

Under treatment with carnosic acid, cell viability was enhanced with increasing concentration of this treatment. In rosmarinic acid treatment, a reduction in percentage of cell viability was observed by increasing the concentration from ۵۰ to ۷۰ µM.

Results of MTT color measurement by measuring optical density (OD) based on the concentration of used extracts compared with the molecular amplification rate were obtained as a plot (Figure ۱).

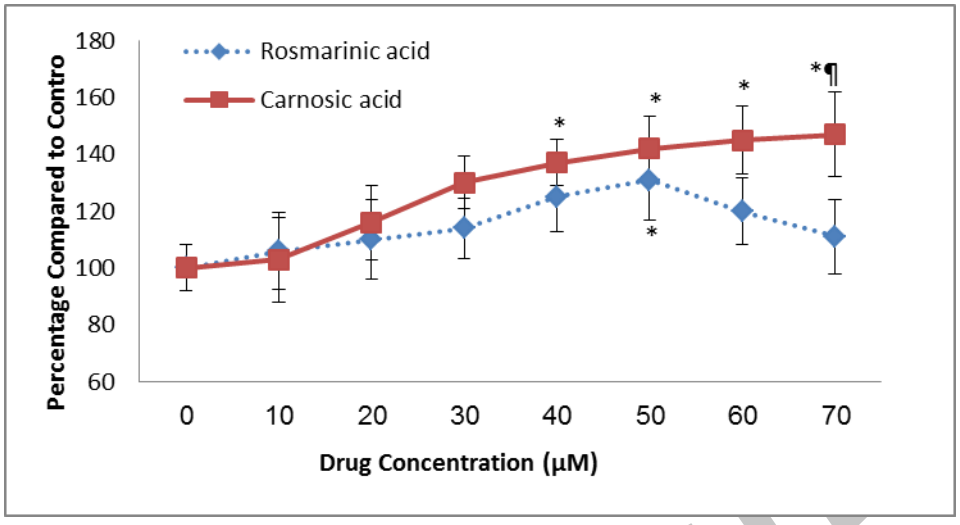

Figure ۱. The effect of various concentrations of two studied substance on Hep-G۲ cells viability after ۲٤ h of incubation.
*** Significant difference compared to control (P <۰,۰٥); significant difference of a treatment compared to other treatment (P <۰,۰٥).**

The effect of two treatments of rosmarinic acid and carnosic acid on caspse-۳ enzyme activity: rosmarinic acid acid treatment of Hep-G۲ cell line reduced caspase-۳ enzyme activity in a dose-dependent trend. In rosmarinic acid treatment, it was observed that the enzyme activity of caspase-۳ following a downward trend was increased with increasing concentration of treatment from ۴۰ to ۷۰ µM. The results of evaluation of caspase-۳ enzyme activity under two treatments of rosmarinic acid and carnosic acid are seen in Figure ۲.

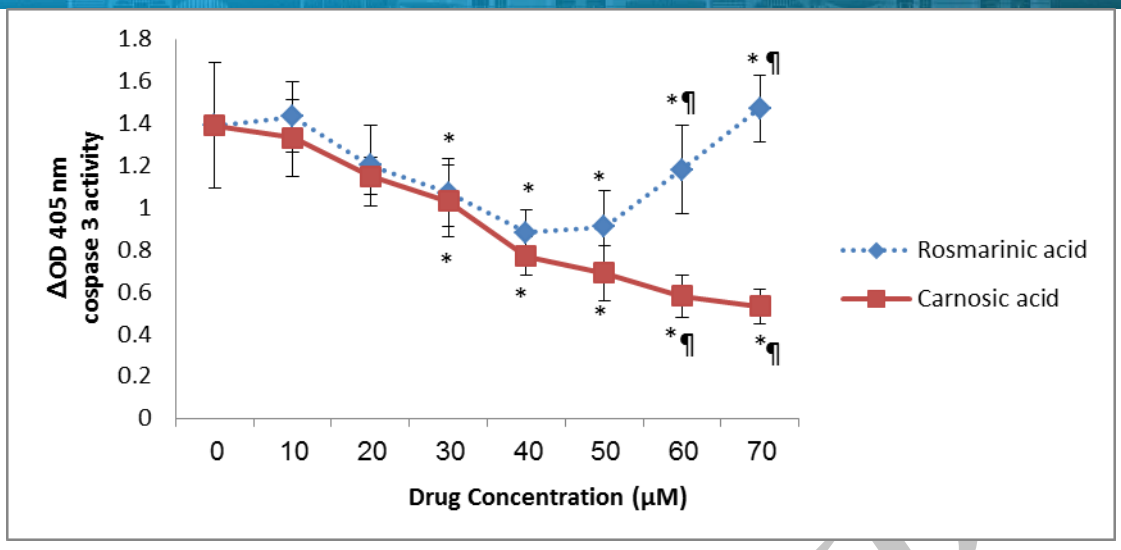

Figure ۲. Caspse-۳ enzyme activity in Hep-G۲ cells line treated with various concentrations of two substances of rosmarinic acid and carnosic acid.
*** Significant difference compared to control (P <۰,۰۵); significant difference of a treatment compared to other treatment (P <۰,۰۵)**

Evaluation of caspse-۹ enzyme activity: Carnosic acid treatment of Hep-G۲ cells reduced caspase۹ enzyme activity in a dose-dependent trend.

In rosmarinic acid treatment, it was observed that the enzyme activity of caspase-۹ following a downward trend was increased with increasing concentration from ۵۰ to ۷۰ µM. Figure ۳ demonstrates the results of evaluation of caspase-۹ enzyme activity.

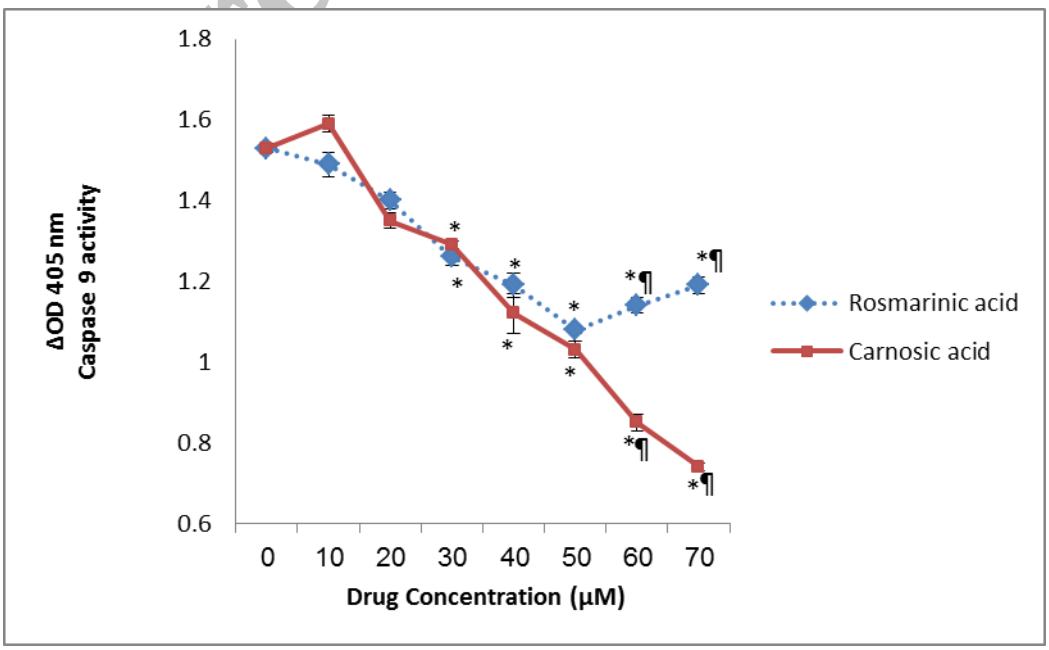

Figure ۳. Caspse-۹ enzyme activity under treatment with two substance of rosmarinic acid and carnosic acid in Hep-G۲ cells line.
*** Significant difference compared to control (P <۰,۰۵); significant difference of a**

Evaluation of ceramide level in Hep-G۲ cells: Carnosic acid treatment of Hep-G۲ cells reduced ceramide level in a dose-dependent trend. In addition treatment of these cells with rosmarinic acid following a downward trend showed an increasing trend from concentration of ۵۰ µM. The results of evaluation of ceramide level in Hep-G۲ cells are observed in Figure ۴.

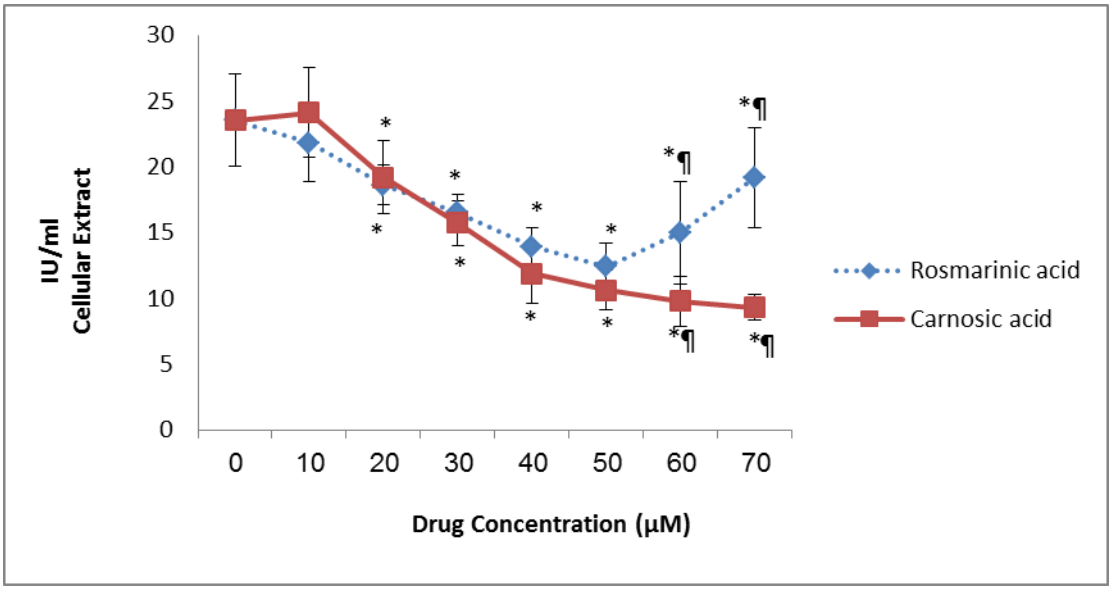

Figure ۴. Ceramide level in Hep-G۲ cells line treated with various concentrations of rosmarinic and carnosic acid.
*** Significant difference compared to control (P <۰,۰۵); significant difference of a treatment compared to other treatment (P <۰,۰۵).**

Discussion

The sphingomyelin/ceramide signaling pathway is an evolutionary system conserved from yeast to humans (Nardini et al, ۲۰۰۱). Function of ceramide has been reported as a secondary messenger in this pathway and inducing different responses depending on the type of cells including proliferation, distinction, growth arrest or mostly apoptosis (Nardini et al, ۲۰۰۱). In order to better understand the effective role of polyphenols in many pathological conditions involved in ceramide pathway (Nardini

et al, ۲۰۰۱), the effects of rosmarinic acid and carnosic acid on Hep-G۲ cell line have been studied in this study. Carnosic acid has increased Hep-G۲ cells growth by increasing the dose. Rosmarinic acid after a decreasing trend has increased Hep-G۲ cells growth from concentration of ۵۰µ M. Hep-G۲ cells growth can be attributed to the reduction of ceramide levels (Nrddini et al, ۲۰۰۱).

Caspases are a family of cysteine proteases that are central regulators of apoptosis. Among caspase family members, caspase-۳ is one of important performer caspses in running apoptosis. Activation of caspse-۳ is considered as a conventional marker of apoptotic cells. Activation of performer caspses such as caspse-۳ and caspse-۷ can cause disintegration of nuclear cytoplasmic substrates and fragmentation of DNA (Xiang et al, ۲۰۱۵). In this study, it has also found that carnosic acid in a dose-dependent trend has reduced activity of caspase۳ enzyme and as a result, cell viability of Hep-G۲ cells has been increased.

Rosmarinic acid has increased enzyme activity after a decreasing trend from concentration of ۵۰ µM that seems is due to increasing cell viability because of an increase in ceramide levels and cell's tendency toward the apoptotic process. In this study, the activity of the caspse ۹ enzyme, which is an initiator or signaling caspase and its main duty is activating performer caspases (Kohler et al ۲۰۰۳), has been decreased with increasing concentration of carnosic acid that reduction of activity of caspase-۹ is also because of reducing the percentage of cell viability and decreasing activity of caspase-۳ enzyme. As a result of treatment by rosmaric acid for active caspase ۹ as caspase ۳ is obtained.

The findings show that levels of ceramide in treated carnosic acid dose dependant was decreased that this decrease may be due to increasing the activity of enzyme ceramidase acid that due to break down the ceramide (Park & Schuchman, ۲۰۰۶) and so attributed reduce the activity of enzyme sphingomylinase Because of ceramide production from sphingomyelin by dividing (Goni & Alonso, ۲۰۰۲; Pavoine & Pecker, ۲۰۰۹). Rosmaric acid treatment after a decline of ۵۰ -µmolar concentrations to be increase ceramide levels that cause it seems to reduce the activity of the enzyme acid ceramidase and increased activity of the sphingomylinase at this concentration.

Conclusion

Although in most cases, polyphenols have resulted in induction of apoptosis and decreased cell viability, but in some cases they have inversely affected and caused cell growth.

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