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ORIGINAL ARTICLE

Carnosic acid regulates cell proliferation and invasion in chronic myeloid leukemia cancer cells via suppressing microRNA-708

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Summary

Purpose: *Carnosic acid (CA) is an important polyphenol mainly isolated from the famous spice and the medicinal plant Rosmarinus officinalis. CA has been shown to exhibit tremendous pharmacological properties which include, but are not limited to, anticancer, antioxidant and anti-inflammatory activities. The current study was designed to evaluate the anticancer effects of CA against chronic myeloid leukemia (CML) which is one of the rare but deadly malignancies both in men and women.*

Methods: *CML KBM-7 cell line was used in this study. Cell viability was assessed by MTT assay. Apoptosis was detected by DAPI and annexin V/PI staining, cell cycle analysis by flow cytometry and cell invasion by Boyden chamber assay. The microRNA-780 expression was determined by quantitative RT-PCR.*

Results: *Our results indicated that CA exhibits significant anticancer activity on CML KBM-7 cells with an IC_{50} of 25 μM. The anticancer activity was due to induction of apoptosis and cell cycle arrest. Moreover, it was observed that CA inhibits the proliferation and invasion of CML KBM-7 cells which could mainly be due to downregulation of microRNA-780 expression as indicated by the quantitative RT-PCR analysis.*

Conclusion: *Taken together, we propose that carnosic acid could prove a potential lead compound in the treatment of CML and deserves further in vitro as well as in vivo study.*

Key words: *cell invasion, chronic myeloid leukemia, cornosic acid, microRNA-708*

Introduction

Plant extracts and plant-derived natural products have been used in the treatment of a diversity of diseases since immemorable times. In Egypt, India and China a number of terrestrial plant species are being used as a source of medicines and many drugs have been developed from these plants. As per the estimates of WHO around 80% of the world's population is dependent on traditional medicine [1]. Cancer is a deadly disease and is considered as the main public health concern in both developing and developed countries [2]. According to a report there were around 11 million new cancer cases detected, more than 6.5 million deaths and around 25 million cancer patients around the globe [3]. Furthermore, around one in every four deaths across the globe is due to cancer [4]. Therefore, there is an urgent need to develop novel drugs or to explore viable therapeutic targets for the treatment of cancer. Plant polyphenols form a large and diverse group of plant-derived secondary metabolites that have been shown to facilitate antioxidants. From the results of sev-

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eral epidemiological studies it has become quite obvious that intake of polyphenol-rich diets is associated with reduced risk of cancers. This may be attributed to the structure of the polyphenols that enables to interact with several of the cellular components including some of the important enzymes [5]. *Rosmarinus officinalis L.* (rosemary), belonging to Lamiaceae family is an important medicinal plant and forms and important spice. It is a rich source of polyphenols which includes, but not limited, to CA, carnosol (COH) and rosmarinic acid (RA) [6]. Among these polyphenols, CA (Figure 1) has been shown exhibit tremendous pharmacological activities which include its anticancer effects on human leukemia cells, anti-inflamatory activities and inhibition of cancer cell migration [7]. With this background, the present study was designed to evaluate the anticancer effects of CA against CML cells. CML is one of the rare malignancies. The incidence of CML is around one or two in every 0.1 million people annually. CML has been reported to be common in older people. This disease is mainly diagnosed around the age of 65 years. Moreover, CML affects more men than women, but women exhibit a survival advantage over men. There are no significant geographic differences on the incidence of CML. However, the differences in the treatment strategies and the availability of the drugs that are very expensive could account for the differences in mortality rates across several countries [8]. In the current study CA was evaluated in CML cells and it was observed that CA exhibits significant growth inhibitory effects on CML cells via induction of apoptosis and cell cycle arrest. Moreover, CA inhibited cell invasion in via suppression of microRNA-780. Taken together, we conclude that CA may prove beneficial in the treatment of CML and deserves further research endeavours.

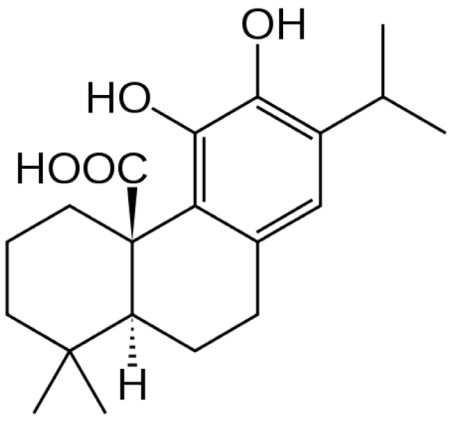

Figure 1. Chemical structure of carnosic acid.

Methods

Cell line and culture conditions

DAPI, RNase A triton X-100 and dimethylsulfoxide (DMSO) were obtained from Sigma-Aldrich Co, USA. Primary and secondary antibodies were purchased from Santa Cruz Biotechnology Inc (Dallas, TX, USA). Fetal bovine serum (FBS), RPMI-1640 medium, L-glutamine, antibiotics were obtained from Sigma-Aldrich, USA. Human chronic myeloid leukemia KBM-7 cell line was purchased from Type Culture Collection of Chinese Academy of Sciences, Shanghai, China. The cells were cultured in RPMI-1640 medium containing 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin and maintained in a humidified atmosphere containing 5% CO_2.

Antiproliferative assay

To assess the cytotoxicity of CA on KBM-7 cells, the cells were seeded in 96-well plates at a density of 1×10^5 and incubated with varying concentrations of CA. Then, after 48 hrs, 25 µl of 5 mg/ml MTT stock solution was added. This was followed by incubation of the plates for 4 hrs. Thereafter, the medium was removed and the formazan crystals were dissolved by adding 50 µl of DMSO. Finally, the cytotoxicity was assessed by measuring the absorbance at 540 nm with the help of microplate spectrophotometer.

Apoptosis assay

CML KBM-7 cancer cells were seeded at α density of 2×10^5 cells/well in 6-well plates, treated with different concentrations (0, 12.5, 5 and 25 µM) of CA and incubated for 24 hrs. DAPI staining was carried out by incubating the cells in 6-well plates for 24 hrs. The cells were then washed with PBS, fixed in 10% formaldehyde and washed again with PBS. The DAPI-stained cells were then examined by fluorescence microscope. For annexin V/PI a similar procedure as that of DAPI was followed except for the cells stained with annexin V/PI and investigated by flow cytometry.

Cell cycle analysis

For estimating the distribution of KBM-7 cells in different phases of the cell cycle, the CA-treated cells were harvested and washed twice with PBS. Thereafter, the cells were fixed with 70% ethanol for about an hr and then washed again with PBS. The cells were finally resuspended in propidium iodide solution (50 µl/ml) and RNase1 (250 µg/ml). This was followed by incubation for 30 min at room temperature and fluorescence-activated cell sorting (FACS1) cater-plus cytometer using 10,000 cells/group.

Matrigel invasion assay

Invasion was determined using Matrigel®-coated invasion chambers (BD Biosciences, Singapore). Untreated or treated KMB-7cells that reached the lower surface of the membrane were stained with crystal violet (CV). Images of CV-stained cells were captured by a phase contrast microscope.The resultant crystal vio-

let complex was dissolved in 10% acetic acid and the absorbance was measured at 600 nm to determine the extent of invasion.

Quantitative real time PCR analysis

RNeasy kit was used for the extraction of RNA and the whole protocol was carried out as per the instructions of the manufacturer. Thereafter, cDNA was synthesized with the help of RevertAid cDNA synthesis kit (Fermentas, USA) as per the manufacturer's protocol. For expression analysis, qRT-PCR was carried out in three biological replicates in ABI StepOne Real time using SYBR Green Master Mix (Fermentas, USA). The $^{\Delta\Delta}$-CT method was employed for the relative quantification of the expression of the genes. Actin was used as a reference gene.

Western blotting

Total protein from the treated and untreated leykemia cells was isolated in RIPA lysis buffer. Equal protein extracts from each group were run on SDS PAGE and then transferred to a polyvinylidene fluoride membrane. This was followed by blocking with 5% non-fat milk and incubation at room temperature for 1 hr. Thereafter, the membranes were incubated with a specific primary antibody at 4°C overnight. This was followed by washing in washing buffer and incubation for 1 hr with the suitable secondary antibody. The protein bands of interest were visualized by an ECL Advanced Western Blot Detection Kit (Fermentas, USA).

Statistics

All experiments were carried out in triplicate and expressed as mean ± SD. GraphPad 7 software was used for statistical analysis. The values were considered significant at *p<0.01, **p<0.001 and ***p<0.0001.

Results

Antiproliferative effects of CA on KBM-7 cells

To investigate the effect of CA on the cell viability of KBM-7 cells, the cells were treated with different concentrations of CA and subjected to MTT assay (Figure 2). The results indicated that CA exhibited an IC_{50} of 25 µM. Moreover, the antiproliferative effects of CA were found to be concentration-dependent.

CA induced apoptosis in KBM-7 cells

To investigate if the anticancer effect of CA is due to apoptosis, we carried out DAPI staining (Figure 3A-B). The results showed that the CA triggered apoptosis in KBM-7 cells. To find the percentage of apoptotic cell population we carried out annexin V/PI staining and the results clearly showed that the apoptotic cell populations increased from 2.96% in the control to 38.52% at 50 µM concentration (p<0.0001; Figure 4).

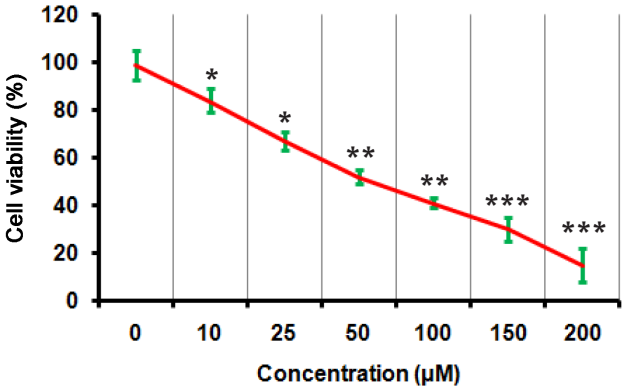

Figure 2. Carnosic acid exerted antiproliferative effects on KBM-7 cells as determined by MTT assay. Experiments were carried out in triplicate and the values were considered significant at *p<0.01, **p<0.001 and ***p<0.0001.

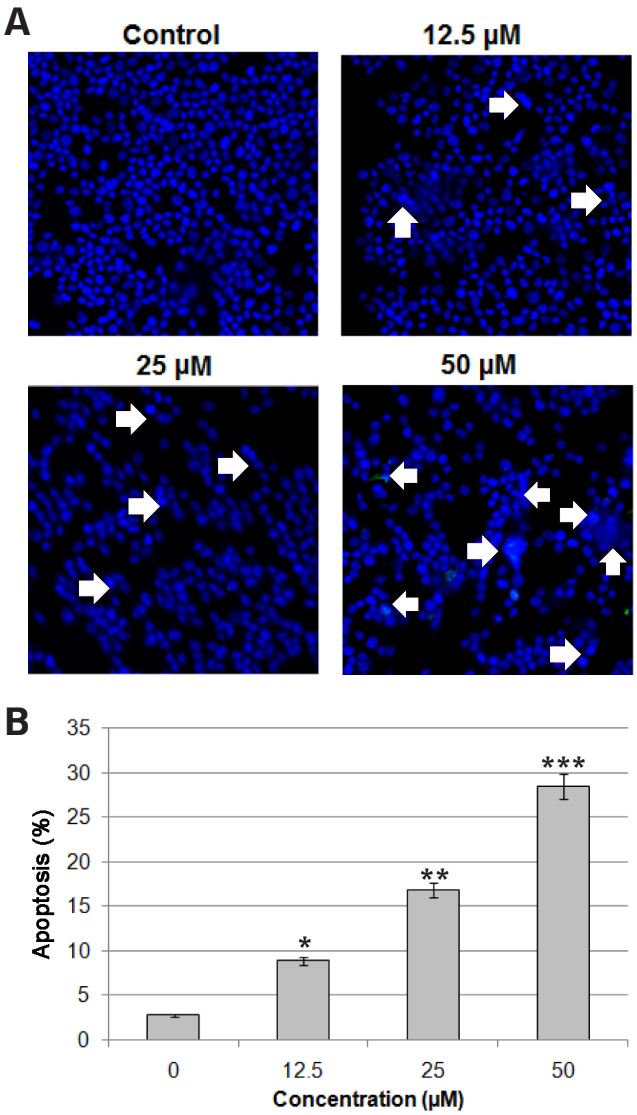

Figure 3. Carnosic acid induced apoptosis in KBM-7 cells in a concentration-dependent manner. **A:** DAPI staining. Arrows show the apoptotic cells; **B:** Estimation of apoptotic cells. Experiments were carried out in triplicate and the values were considered significant at *p<0.01, **p<0.001 and ***p<0.0001.

CA triggered G2/M cell cycle arrest in KBM-7 cells

To investigate if the inhibitory effects on G2/M cell growth of CA were due to cell cycle arrest, KBM-7 cells were treated with different concentrations of CA and the percentage of cells in each cell cycle phase were estimated by flow cytometry (Figure 5). The results of our study indicated that treatment of KBM-7 with CA increased the percentage of cells in the G2/M phase from 13.4% in untreated cells to 48.3% in CA-treated cells at 50 µM concentration ($p<0.0001$). Moreover, CA induced cell cycle arrest in a concentration-dependent manner.

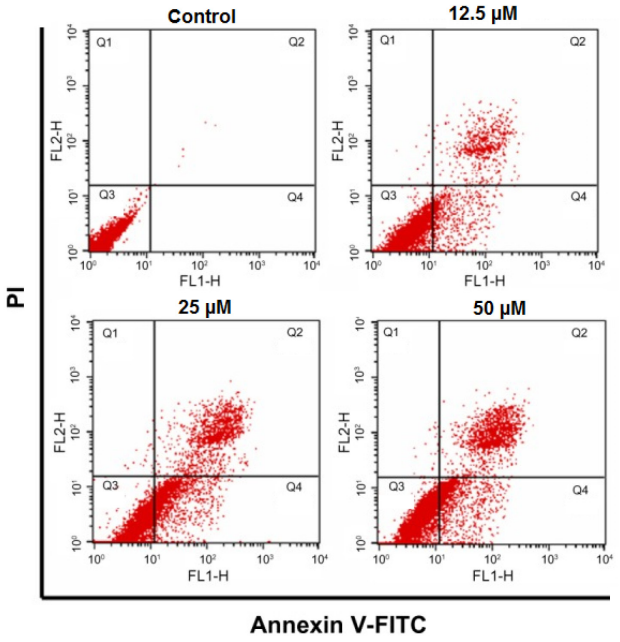

Figure 4. Apoptotic cell populations were determined by annexin V/PI staining. The experiments were carried out in triplicate. The Figure shows that the apoptotic cells increased with the increase in carnosic acid concentration.

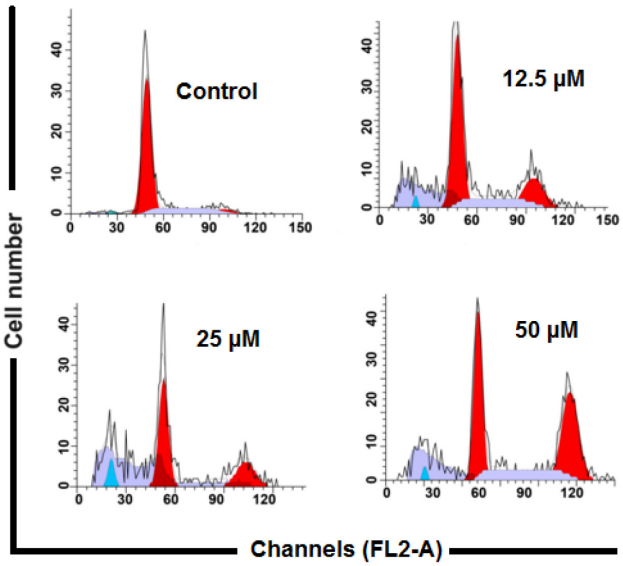

Figure 5. Carnosic acid triggered G2/M cell cycle arrest of KBM-7 cells as determined by flow cytometry. Experiments were carried out in triplicate. The Figure shows that the cells accumulated in the G2 phase of the cell cycle in a concentration-dependent manner.

CA inhibited the invasion of KBM-7 cells

The invasive properties of KBM-7 cells were found to be significantly inhibited in the presence of CA, as determined by Boyden chamber Matrigel assay. The invasive properties of KBM-7 cells were reduced by around 50% in presence of 25 µM CA ($p<0.0001$; Figure 6A-B).

CA treatment suppressed the expression of microRNA-708 in KBM-7 cells

To find out if CA inhibits cell invasion by effecting the expression of microRNA-708, we determined the expression of microRNA-708 in treated and untreated KBM-7 cells. The results of quantitative RT-PCR analysis showed that the expression of microRNA-708 was significantly suppressed upon treatment with 25 µM concentration of CA (Figure 7).

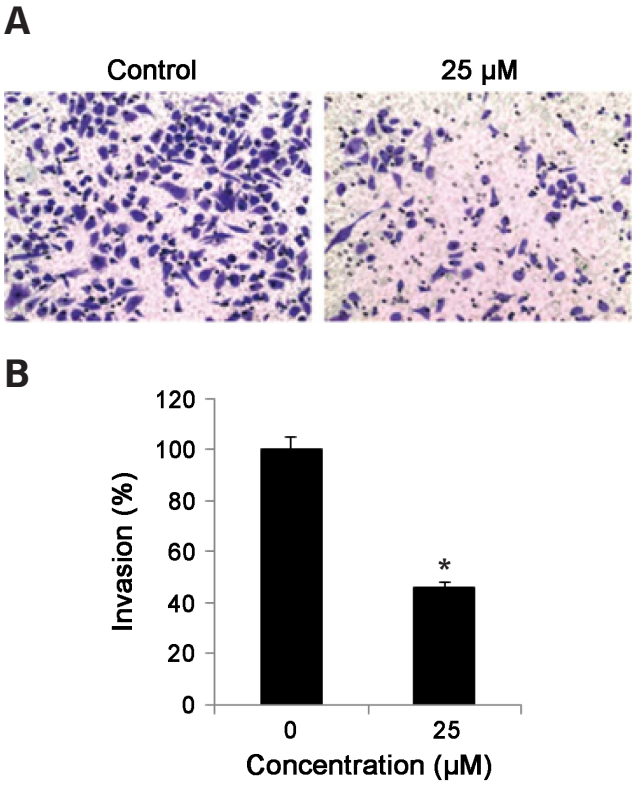

Figure 6. Carnosic acid inhibited the invasion of KBM-7 cells. **A:** Determination of cell invasion by Boyden chamber assay; **B:** Estimation of percent invasion of KBM-7 cancer cells. Experiments were carried out in triplicate and the values were considered significant at *$p<0.01$.

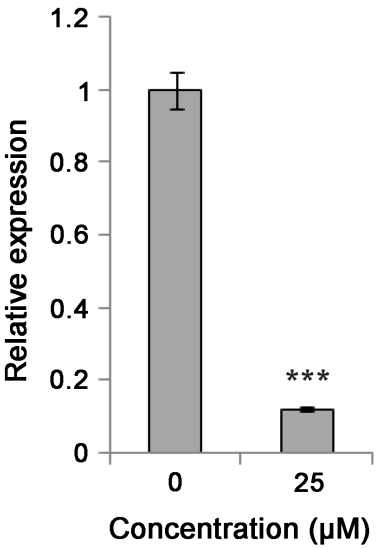

Figure 7. Relative expression of microRNA-708 in KMB-7 cells treated at 0 and 25μM concentrations of CA, as determined by quantitative RT-PCR. Experiments were carried out in triplicate and the values were considered significant at ***p<0.0001.

Discussion

CML is one of the rare but deadly malignancies. The incidence of CML is around one or two in every 0.1 million people every year. It has been reported that CML affects men more frequently than women, and women exhibit a survival advantage over men [8]. The treatment options for CML are limited and the existing drugs used for its treatment are expensive. Besides, the availability of these drugs forms a bottleneck in the treatment of CML [8]. Therefore the current study was designed to evaluate the anticancer activity of CA against CML KBM-7 cells. The results of MTT assay showed that CA exhibited a dose-dependent activity against KBM-7 cells with an IC_{50} of 25 μM. In order to find the underlying reason for the anticancer activity of CA we carried out DAPI staining and observed that CA caused apoptosis in KBM-7 cells in a dose-dependent manner and the cells displayed apoptotic characteristics such as white color nuclei indicative of apoptosis. Further, annexin V/PI staining showed that the apoptotic cell populations increased significantly from 2.96% in the control to 38.52% at 50 μM concentration.

Apoptosis is an important mechanism by which harmful or cancer cells are removed from the body [9]. Our results are well supported by previous investigations wherein several plant-derived anticancer agents, such as taxol and podophyllotoxin, exert their anticancer activities by inducing apoptosis in cancer cells [10-12]. Another mechanism by which a number of anticancer agents exert their anticancer effects is the cycle arrest [13]. Therefore, we also investigated the effect of CA on the cell cycle distribution of KBM-7 cells. Our results showed that CA triggered G2/M cell cycle arrest in KBM-7 cells in a dose-dependent manner. Furthermore, one of the important properties of cancer cells is their ability to invade other tissues. This is referred to as local invasion and metastasis and the molecules that can inhibit this procedure are considered as potential anticancer agents [14]. Therefore, we also investigated the effect of CA on the invasion capability of KBM-7 cells. Our results showed that CA inhibited the invasion of KBM-7 cells as evidenced from the results of Boyden chamber assay.

MicroRNAs include a large family of small non-coding RNAs that are involved in the inhibition of protein synthesis by binding to different sites on 3'-untranslated region (3'-UTR) of target genes. Therefore, microRNAs exhibit remarkable biological functions in a diversity of cellular processes [10]. Among several human microRNAs associated with cancers microRNA-708 has been reported to be principally upregulated [10] thus we investigated the expression of microRNA-708 in the control and CA-treated KBM-7 cells. Our results showed that CA treatment suppressed the expression of microRNA-708. Some authors [15] have shown that suppression of microRNA-708 in lung cancer cells inhibits their proliferation, migration and invasion. Hence, we believe that the antiproliferative effects of CA on KBM-7 cells might be via suppression of microRNA-708.

Conclusion

From the results of this study we conclude that CA is a potent anticancer agent that induces antiproliferative and cell migration inhibitory effects on KBM-7 cells via suppression of microRNA-708 expression. The present study therefore paves way for further evaluation of CA *in vivo*.

Conflict of interests

The authors declare no conflict of interests.

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