

ORIGINAL ARTICLE

Detailed studies on the anticancer action of rosmarinic acid in human Hep-G2 liver carcinoma cells: evaluating its effects on cellular apoptosis, caspase activation and suppression of cell migration and invasion

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Summary

Purpose: Liver cancer is one of the most common and highly malignant cancers of the digestive system. The main aim of the present research work was to investigate the anticancer action of rosmarinic acid - a naturally occurring plant secondary metabolite. We also investigated its effects on cell apoptosis, caspase activation, cell migration and cell invasion.

Methods: Cell viability of Hep-G2 liver cancer cells was evaluated by CCK-8 assay while apoptotic studies were carried out by fluorescence microscopy using Hoechst, acridine orange (AO)/ethidium bromide (EB) and Comet assays as well as using annexin-v/propidium iodide (PI) assay for apoptosis quantification. Western blot assay was used to study the effects of rosmarinic acid on apoptosis-related protein expressions including Bax, Bcl-2 and various caspases. In vitro wound healing assay was used to evaluate the effects on cell migration while transwell chambers assay with Matrigel was used to assess the effects of rosmarinic acid on cell invasion.

Results: Rosmarinic acid caused significant reduction in the viability of the human Hep-G2 liver carcinoma cells in a

dose-dependent manner, exhibiting an IC_{50} of 14 μ M in cancer cells. The AO/EB staining assay showed that rosmarinic acid suppressed the viability of cancer cells via induction of apoptotic cell death which was associated with rise in Bax and decrease in Bcl-2 levels. DAPI staining results also confirmed that rosmarinic acid induced apoptosis. The apoptotic cells increased from 5.8% in control to 24.68% at 28 μ M concentration of rosmarinic acid. Rosmarinic acid also caused activation of caspase-3 and 9 along with suppressing liver cancer cell migration and invasion.

Conclusions: The current study shows that rosmarinic acid has a potential to inhibit in vitro cancer cell growth in Hep-G2 cells by triggering apoptosis, caspase activation and suppressing cell migration and invasion and as such this molecule could be developed as a possible anticancer agent provided further studies are carried out.

Key words: rosmarinic acid, liver cancer, apoptosis, cell migration, caspase activation

Introduction

Liver cancer or hepatocellular carcinoma (HCC) is a highly malignant cancers of the digestive system and is also one of the most frequent cancers. HCC is among the top 10 human cancers globally and is included among the top 5 cancers in terms of cancer-related deaths. The majority of the liver cancer cases are presented as HCC, although sec-

ondary liver cancers arise from metastases originating from distant tumors, mostly from the gastrointestinal tract [1-3]. Two principal histological kinds of liver cancer exist, one is hepatocellular carcinoma (HCC) and the other is intrahepatic cholangiocarcinoma (ICC). The major risk factors of liver cancer include chronic infection of hepati-

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tis C virus and hepatitis B virus, while the minor risk factors are aflatoxins in the diet, liver toxins in the water, diabetes mellitus, non-alcoholic fatty liver disease, tobacco use, and betel nut chewing [4,5]. In 2012 alone, it was estimated that about 782,451 new cases of liver cancer were reported and about 746,000 liver cancer-related deaths occurred worldwide. In China, the incidence of liver cancer is much higher as compared to the rest of the world. About 20% of the liver cancer cases in the world are attributed to China and account for about 50% of all newly diagnosed HCC cases and mortality [6,7]. HCC treatment includes surgical resection and liver transplantation which have significantly increased the survival rate of HCC patients. However, the condition also needs long-term adjuvant chemotherapy which becomes a limiting factor for its success because chemotherapy is associated with various adverse side effects and is prone to the development of multidrug resistance. Other therapies for HCC like transcatheter arterial chemoembolization (TACE), radiotherapy, cryo-ablation are also used but are less effective and also result in severe side effects like hair loss, depression of bone marrow, kidney failure, liver failure etc [8-10]. Therefore, there is an immediate need for new, novel, more effective and much cheaper drugs for liver cancer which are also less prone to multidrug resistance. Medicinal plants have always rescued humans by providing blockbuster drugs like taxanes, vincristine, vinblastine among others, to tackle health challenges like cancer. More than 60% of the currently used and clinically approved anticancer drugs come from natural sources most notable medicinal plants [11-13]. In the present research work, we report the anticancer effects of rosmarinic acid in Hep-G2 liver cancer cells along with assessing its effects on cell apoptosis, caspase activation and inhibition of cell migration and invasion

Methods

CCK-8 cell viability assay

The Hep-G2 liver cancer cells were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were maintained in RPMI-1640 medium (Gibco, USA) at 37°C with 95% humidity and 5% CO₂. The proliferation of Hep-G2 cells was examined by CCK-8 (Cell Counting Kit-8) assay obtained from Dojindo Laboratories, Kumamoto, Japan. The Hep-G2 cells were initially treated with 0, 2.5, 5, 10, 20, 40, 80, 160 and 320 μM of rosmarinic acid after which 20 μl CCK-8 was slowly added to the cell culture plates. The cell plates were then incubated for 12 h at 37°C. The absorbance was measured at 450 nm wavelength using a microplate reader (BioTek Instruments, USA). Cell cytotoxicity was measured from the observed absorbance.

DAPI and Comet assay for apoptosis

We employed fluorescence microscopy using DAPI as staining agent to study the apoptotic effects exerted by rosmarinic acid in Hep-G2 liver carcinoma cells. The Hep-G2 cells at a cell density of 2×10⁵ cells per ml were cultured in 6-well plates and then subjected to the treatment with increasing doses of rosmarinic acid followed by incubation for 24 h at 37°C. After that, 20 μl of cell culture were placed onto glass slides and stained with DAPI. The slides were then cover-slipped and scanned under fluorescence microscope (Nikon Corporation, Tokyo, Japan) to evaluate apoptotic changes. Comet assay which determines the DNA damage was performed by alkaline single cell gel electrophoresis according to the guidelines of the method formerly published in the literature [14].

Acridine orange/ethidium bromide (AO/EB) staining assay for apoptosis

The Hep-G2 human liver cancer cells at a cell density of 2×10⁵ cells/ml were placed into 6-well plates and cultured for 24 h. The cells were exposed to treatment with different concentrations (0, 7, 14, 28 μM) of rosmarinic acid and incubated for 24 h. Subsequently, 20 μl of cell culture were placed onto glass slides and

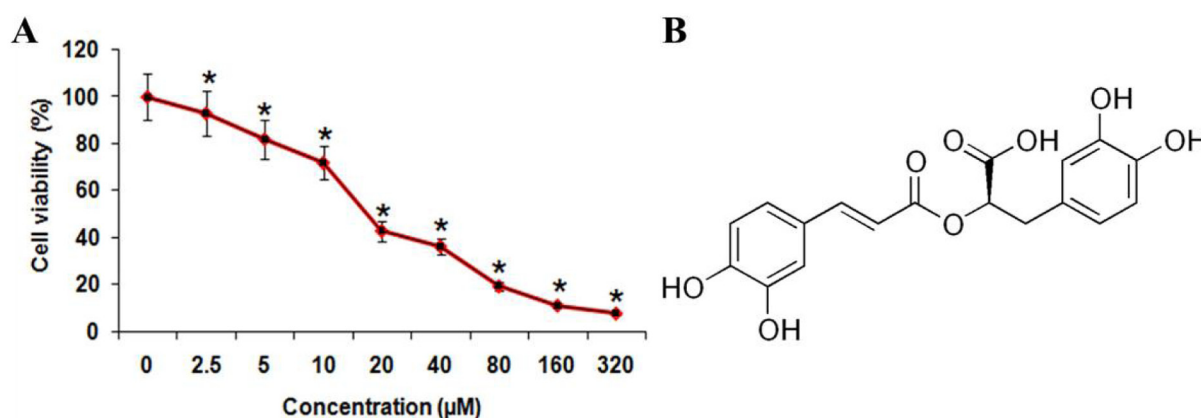


Figure 1. A: CCK-8 assay showing the effects of Rosmarinic acid on the viability of the Hep-G2 cancer cells. The experiments were performed in triplicate and shown as mean±SD (*p<0.05). **B:** Chemical structure of Rosmarinic acid.

stained with AO/EB separately (20 μ l). The slides were then covered with cover slips and inspected under fluorescence microscope (Nikon Instruments Inc., NY, USA) for evaluating apoptotic changes.

Annexin V-FITC/PI assay for apoptosis

The Hep-G2 cells at a density of 2×10^5 cells/well were initially treated with different doses of rosmarinic acid for 24 h. The cells were then resuspended and washed twice with phosphate buffered saline (PBS) and then assayed with a cell apoptosis detection kit obtained from KGA-107; Keygene, China. The cells were incubated with 200 μ l of binding buffer followed by addition of 10 μ l of annexin V-fluorescein isothiocyanate solution and 10 μ l solution of PI. The cells were then incubated for 10 min in the dark and finally examined by FACSCalibur flow cytometry (FACSCalibur; BD Biosciences, China).

Cell migration assay

The Hep-G2 cells at a cell density of 1×10^5 cells/ml were plated in 6-well plates for 12 h, they were cultured with increasing doses of rosmarinic acid for 24 h. Then, a sterile 20 μ l pipette was used to make a straight cell-free wound in the wells and the suspended cells were thrown out. The liver cancer cells were cultured continuously and maintained in RPMI-1640 medium and finally the migration of Hep-G2 cells was observed by using optical microscope after every 12 h.

Cell invasion assay

The impact of rosmarinic acid on the invasion ability of Hep-G2 liver cancer cells was evaluated by transwell chambers with Matrigel having coating of polyvinylpyrrolidone-free polycarbonate filter (6-mm pore size). The cells at a density of 1×10^5 cells/ml were shifted to upper chamber of a Matrigel-coated chamber (purchased from BD Biosciences, USA) having RPMI-1640 medium free from FBS. The cells were then treated with increasing doses of rosmarinic acid for 24 h. The lower compartment contained only medium having FBS (20%). Using paraformaldehyde (4%), the migrated cells were fixed and then stained with 1.55% crystal violet dye for 40 min. Inverted microscope was used to count the number of invaded cells at 200x magnification.

Western blot analysis

The Hep-G2 cells were firstly washed with ice-cold PBS and then lysed in RIPA lysis buffer encompassing the protease inhibitor. Around 40 μ g of proteins from each cell culture were then loaded on the SDS-PAGE. The protein contents were calculated using a bicinchoninic acid protein quantitative kit (Boster, China). The gels were then shifted to nitrocellulose membranes and exposed to treatment with primary antibody at 4°C for 24 h. Then the membranes were incubated with HRP-conjugated secondary antibody for 60 min at 37°C. Enhanced chemiluminescence reagent (ImmunoCruz™ Western Blotting Luminol Reagent, Santa Cruz Biotechnology, USA) was used to visualise the protein bands. Finally the signal was detected by Odyssey Infrared Imaging System (LI-COR, USA). Actin was used as control for normalisation.

Statistics

Data are shown as mean \pm SD. Statistical analysis was done using Student's *t*-test with GraphPad prism 7 software. $P < 0.05$ suggested significant difference.

Results

Rosmarinic acid exerted potent cytotoxic effects in Hep-G2 human liver cancer cells

CCK-8 assay for cell viability was used to examine the cytotoxic effects of rosmarinic acid in Hep-G2 human liver cancer cells. The graphical representation of the cell cytotoxicity induced by rosmarinic acid is shown in Figure 1A and indicates that the cell viability of these cancer cells was drastically reduced as the dosage of rosmarinic acid increased from 0 to 320 μ M. The IC_{50} value of rosmarinic acid was calculated and was found to be IC_{50} of 14 μ M. Figure 1B shows the structure of rosmarinic acid and clarifies that it is a plant polyphenol.

Rosmarinic acid activates programmed cell death (apoptosis) pathway in Hep-G2 cells

Fluorescence microscopy along with flow cytometry using DAPI, AO/EB, annexin V-FITC/PI staining agents were used to study the mode of

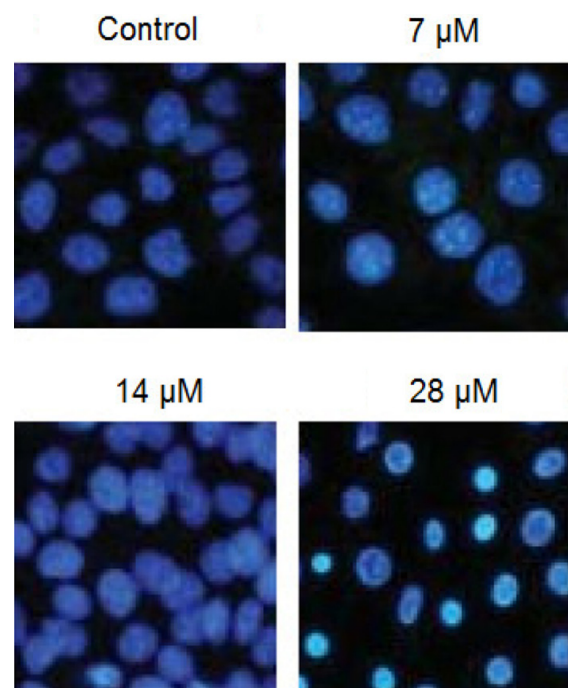


Figure 2. Fluorescence microscopy studies using (4',6-diamidino-2-phenylindole) DAPI staining, showing rosmarinic acid-induced programmed cell death as indicated by enhanced chromatin condensation and nuclear fragmentation with increasing rosmarinic acid dose. The experiments were performed in triplicate.

action of cell death induced by rosmarinic acid. It was shown that the percentage of the DAPI positive cells showed a significant upsurge reminiscent of the apoptosis in the Hep-G2 cells featured by chromatin condensation and nuclear fragmentation. Increasing doses of rosmarinic acid at 0, 7, 14, 28 μM led to a significant chromatin condensation shown by DAPI fluorescence (Figure 2). Figure 3 shows the Comet assay results revealing that

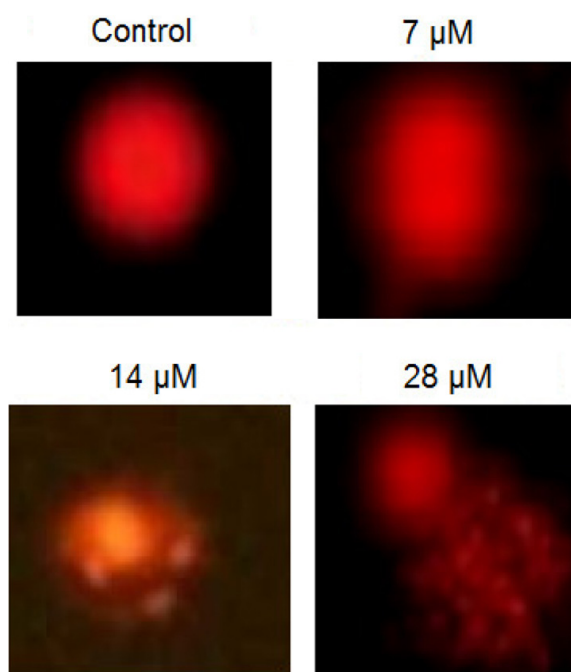


Figure 3. Fluorescence microscopy studies using Comet assay to investigate the DNA-damaging effects of rosmarinic acid on Hep-G2 cells. The results showed increasing DNA fragmentation dose-dependently.

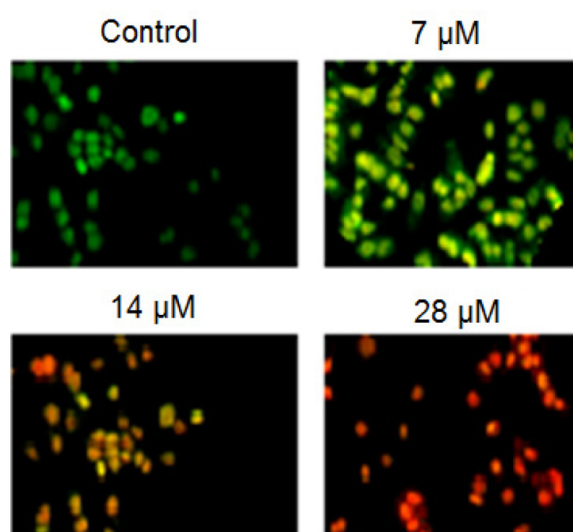


Figure 4. Acridine orange/ethidium bromide (AO/EB) staining showing rosmarinic acid induces apoptosis in Hep-G2 cancer cells dose-dependently. The experiments were performed in triplicate.

as compared to the untreated control cells which indicated intact DNA with no signs of DNA fragmentation, rosmarinic-treated cells exerted potent DNA damaging effects in these liver cancer cells in which these cells with DNA damage appear as comets. The DNA fragmentation in these cells was shown to be concentration-dependent as can be easily seen from longer comet tails at higher rosmarinic acid doses. The results of AO/EB staining are depicted in Figure 4 and reveal that the number of cells with orange/yellow fluorescence (apoptotic cells) increased significantly with increasing rosmarinic acid dosage. Untreated cells only showed green fluorescence indicating no signs of apoptosis. Finally, flow cytometry measurements quantified the percentage of cells which had undergone apoptosis at each tested dose of the molecule. In-

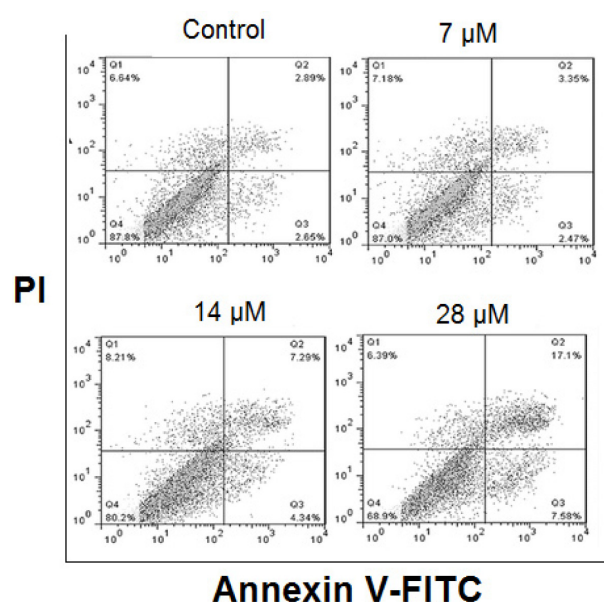


Figure 5. Flow cytometry and annexin V/PI staining showing the apoptotic effects induced by rosmarinic acid in Hep-G2 cells at varying doses of the molecule.

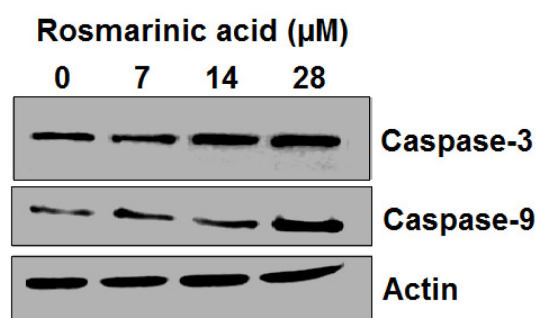


Figure 6. Evaluation of the effects of rosmarinic acid on the expression of caspase-3 and caspase-9 using western blot method. The expression levels of both caspase-3 and caspase-9 were found to increase as the molecule dose increased. The experiments were performed in triplicate.

ing doses of rosmarinic acid at 0, 7, 14, 28 μM doses increased the percentage of apoptotic cells from 5.54% in the control group to 5.83, 11.63 and 24.68% respectively (Figure 5).

Rosmarinic acid modulated the expressions of apoptosis-related proteins

The apoptotic effects of rosmarinic acid were finally confirmed by carrying out experiments using western blot assay. The results are shown in Figures 6 and 7 and reveal that rosmarinic acid led to upsurge of expression levels of both caspase-3 and caspase-9 dose-dependently hinting at the apoptotic cell death induced by rosmarinic acid. Further the expression of Bax was shown to increase while as that of Bcl-2 was shown to decrease. Both these counter effects were seen to follow rosmarinic acid dose (Figure 7).

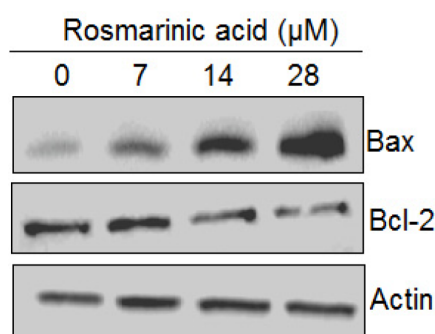


Figure 7. Effects of rosmarinic acid on the expression levels of apoptosis-related proteins like Bax and Bcl-2 using western blot assay. The Figure shows that the expression levels of Bax enhanced with increasing molecule dose, in contrast to Bcl-2 levels indicating apoptotic cell death. The experiments were performed in triplicate.

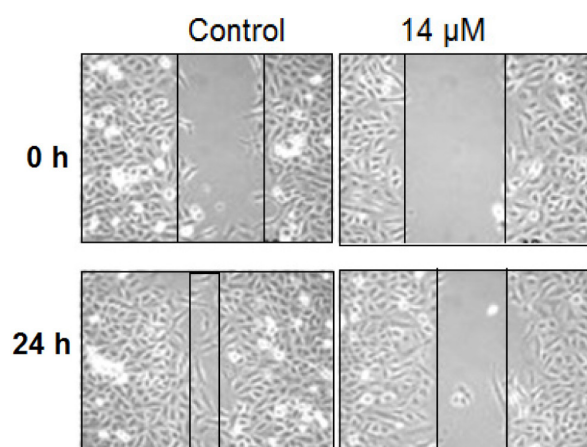


Figure 8. *In vitro* wound healing assay for evaluation of effects of rosmarinic acid on the Hep-G2 cancer cell migration. The experiments were performed in triplicate. Rosmarinic acid was shown to induce significant cell migration inhibitory effects. The experiments were performed in triplicate.

Rosmarinic acid suppressed cell migration and invasion in Hep-G2 cells

In vitro wound healing assay for cell migration and transwell assay for cell invasion were performed to evaluate the anti-metastatic effects of rosmarinic acid in Hep-G2 liver cancer cells. Figure 8 shows the cell migration results and reveal that rosmarinic acid suppressed dose-dependently cell migration ability in these liver cancer cells even at a dose of 14 μM as was clearly visible from the wound width at this dose. Figure 9 shows that rosmarinic acid also led to a dose-dependent inhibition of Hep-G2 cell invasion tendency. Both these results convincingly point out to the fact that rosmarinic acid may find applications as an anti-metastatic drug which can stop the spread of tumor cells to neighbouring tissues.

Discussion

Apoptosis or programmed cell death is a well organised biochemical process which helps eradicating damaged cells which otherwise can cause diseases. Apoptosis is one of the most well studied biochemical processes mainly because of its role in normal as well as in several pathological conditions including cancer. Apoptosis is characterized by several morphological as well as biochemical changes within the cells. The morphological changes of apoptosis include cell membrane damage, cellular contraction, and membrane blebbing, while

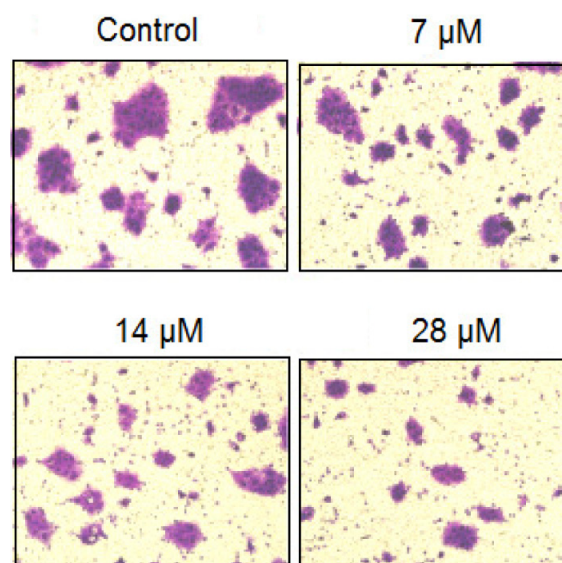


Figure 9. Transwell assay using Matrigel for evaluation of effects of rosmarinic acid on the Hep-G2 cancer cell invasion. The experiments were performed in triplicate. Rosmarinic acid was shown to induce significant cell invasion inhibitory effects. The experiments were performed in triplicate.

biochemical changes include activation of several caspases including caspase 3 and caspase 9, activation of Bax protein, and inhibition of Bcl-2 protein, DNA fragmentation and protein degradation. The most noticeable feature of apoptosis is the activation of caspases which are essentially cysteine proteases that can slice numerous important cell proteins that degrade the nucleus. These caspases can also trigger DNAases which catalyse nuclear DNA damage. Several of the naturally occurring plant molecules have been reported to induce apoptotic cell death in a wide range of cancer cell lines [15-17]. In the current study we investigated the anti-cancer and apoptosis-inducing effects of rosmarinic acid in Hep-G2 cell line along with its effects on cell migration and invasion. Rosmarinic acid has also been shown to exert anticancer effects in human prostate cancer cells by cell cycle arrest and inducing apoptosis by modulating the expression of HDAC2 [18]. This molecule in combination with Adriamycin has been reported to induce apoptosis by activating mitochondrial-mediated signalling pathway in Hep-G2 and Bel-7402 cells [19].

In the current study, we found that the cell viability of these Hep-G2 cancer cells was drastically reduced as the dosage of rosmarinic acid increased from 0 to 320 μM . Increasing doses of rosmarinic acid at 0, 7, 14, 28 μM doses led to a significant chromatin condensation shown by DAPI fluorescence. Comet assay results revealed that as compared to the untreated control cells which indicated intact DNA with no signs of DNA fragmentation, acid rosmarinic acid-treated cells exerted potent DNA damaging effects in these liver cancer cells in which these cells with DNA damage appear as com-

ets. The results of AO/EB staining revealed that the number of apoptotic cells increased significantly with increasing rosmarinic acid dosage. Untreated cells showed no signs of apoptosis. Flow cytometry measurements quantified the percentage of cells which had undergone apoptosis at each tested dose of the molecule. Increasing doses of rosmarinic acid at 0, 7, 14, 28 μM doses increased the percentage of apoptotic cells from 5.54% in the control group to 5.83, 11.63 and 24.68%, respectively. Rosmarinic acid led to increased expression levels of both caspase-3 and caspase-9 dose-dependently hinting at the apoptotic cell death induced by the molecule. Further, the expression of Bax was shown to increase while that of Bcl-2 was shown to decrease. Rosmarinic acid was also shown to inhibit both cell migration and invasion in Hep-G2 liver cancer cells.

Conclusion

In conclusion, the results of this study showed that the cytotoxic effects induced by rosmarinic acid in Hep-G2 cells are mediated via apoptosis induction, caspase activation and inhibition of cell migration and invasion.

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Conflict of interests

The authors declare no conflict of interests.

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