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ORIGINAL ARTICLE

Anticancer effects of rosmarinic acid in human oral cancer cells is mediated *via* endoplasmic reticulum stress, apoptosis, G2/M cell cycle arrest and inhibition of cell migration

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Summary

Purpose: *This investigation was undertaken to infer the anticancer effects of rosmarinic acid against human oral cancer cells.*

Methods: *Normal hTRET-OME oral cell line and oral cancer cell line SCC-15 were used in the present study. CDK-8 was used to determine the proliferation of cancer cells. Apoptosis of cancer cells was assessed by DAPI staining method. Flow cytometric procedure was employed to study the cancer cell cycle phase distribution. The migratory potential of cancer cells was estimated by transwell assay.*

Results: *Rosmarinic acid inhibited the proliferation of oral cancer cells and the level of inhibition was dose-dependent. The antiproliferative role of rosmarinic acid was exerted through apoptotis induction and arrest of cell cycle at G2/M phase in oral cancer cells. Treatment of rosmarinic acid also resulted in endoplasmic reticulum stress and affected negatively the migratory potential of cancer cells in a concentration-dependent manner.*

Conclusion: *The results of this study revealed the anticancer potential of rosmarinic acid against the oral cancer cell growth and propagation. The study envisages the importance of natural compounds for their usage against human cancers.*

Key words: *rosmarinic acid, anticancer, apoptosis, proliferation, cell cycle arrest, flow cytometry*

Introduction

The living world is much fascinating not only in the sense that it dwells a vast diversity of living entities but also in the sense that a huge number of naturally occurring compounds are synthesized by the living organisms [1]. These naturally occurring compounds fall in broad categories of primary and secondary metabolites. The secondary metabolites, although considered the accessory natural compounds, are fairly important for the better survival of host organism for their potential to provide a helping hand to their host in the fluctuating environmental conditions [2,3]. Further, they aid in the chemical defense against predators' attacks [4]. The plant kingdom is very diverse and plants are seen to possess an enormous variety of secondary metabolites which benefit the host plant in a number of ways. The plant-derived natural products have long been valued for their health benefitting effects in humans [5,6]. The plant secondary metabolites have been shown to confer positive results on human health and have been used for treating a number of human disorders. The role of plant-derived compounds to be used against human cancers is well recognized [7]. Active research is going on to

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explore, evaluate and understand the anticancer effects of plant-based natural compounds. Phenolics comprise a class of ubiquitously occurring plant secondary metabolites synthesized through shikimic acid and phenyl propanoid pathways [8]. These compounds have bioactive properties and possess health-protective effects. Rosmarinic acid, an ester of caffeic acid, belongs to the phenolic group of plant secondary metabolites and found mainly in plants belonging to Lamiaceae family like *Rosemarinus officinalis* (rosemary) [9]. The anticancer effects of caffeic acid and its phenyl esters against oral cancer are well understood [10-13]. Rosmarinic acid has been proved to possess anticancer properties against different human cancer cell lines [14]. Here, we tried to investigate the anticancer potential of rosmarinic acid against human oral cancer. The oral and pharyngeal cancers are ranked 6th in terms of cancer prevalence. The overall 5-year survival rate of oral cancer is much lower compared with breast and prostate cancers. Hence, researchers look urgently for effective measures of preventing and treating oral cancer. This study was undertaken to investigate the anticancer effects of Rosmarinic acid on human cell oral cancer cells and to explore the underlying mechanism.

Methods

Culture and maintenance of cell lines

Normal hTRET-OME oral cell line and oral cancer cell line SCC-15 were procured from American Type Culture Collection (ATCC,USA). Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific, Waltham, Mass, USA) containing 10% fetal bovine serum (FBS) was used for culturing the cell lines at 37°C with 5% CO_2 in a humidified incubator.

Growth and proliferation assay

Cell counting kit-8 (CCK-8, MedChemExpress, China) was used for estimation of proliferative rates of oral cancer cells treated with rosmarinic acid (96%, Sigma-Aldrich) and the proliferation rates were compared with normal treated cells. In brief, the cells were placed in 96-well plates at 1×10^6 cells/well density and cultured for 24h with 0, 2.5, 5, 10, 20, 40, 80 and 180μM rosmarinic acid, after which cell counting kit-8 (CCK-8) 10 μl solution to each well was employed to estimate the proliferation rates at the indicated time intervals. Following 2h incubation at 37°C, absorbance at 450nm was read for each sample with the help of microplate reader.

Analysis of cellular apoptosis

DAPI staining was performed to examine the effect of rosmarinic acid on the viability of oral cancer cells and induction of cell apoptosis. The cells were plated in 12-well plates at a density of 0.6×10^6 cells/well. 0, 10, 20 and 40μM rosmarinic acid was added to each well and cells were incubated at 37°C for 24h. Afterwards, the cells were harvested and washed twice with phosphate buffered saline (PBS), followed by fixing with 4% paraformaldehyde. The DAPI solution was used to stain the cells. Afterwards, the cells were examined for fluorescence measurements using fluorescent microscope.

Cell cycle phase distribution study

The SCC-15 cells were treated with 0, 10, 20 and 40μM rosmarinic acid for 24h at 37°C in 6-well plates with 1×10^6 cells/well. Cell pellets were obtained by centrifugation and subsequently washed with PBS. The cells were then fixed using absolute methanol. This was followed by propidium iodide (PI) treatment and flow cytometry to analyze the cell cycle phase distribution of treated oral cancer cells in comparison to the untreated ones.

Transwell migration assay

After 24h treatment with 10, 20 and 40μM rosmarinic acid, the treated oral cancer cells and untreated cells were plated at a density of 1×10^5 cells in the upper chamber of transwell plate without matrigel coating. The cells were allowed to grow for 24h at 37°C with 5% CO_2. Afterwards, the cancer cells invading the lower chamber were fixed with 70% ethanol and then stained using 0.1% crystal violet solution. Finally the migratory cells were visualized under 100x magnification light microscope and pictures were taken.

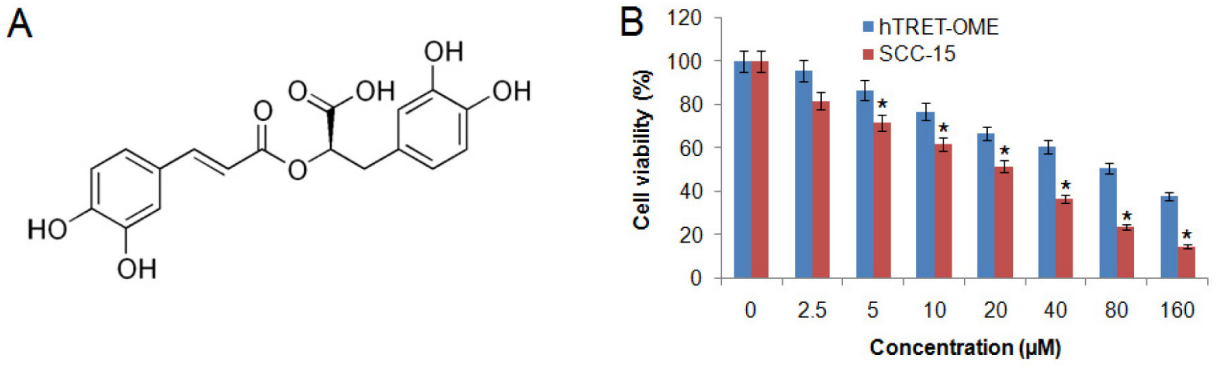

Figure 1. A: Chemical structure of Rosmarinic acid. **B:** Cell viability of normal hTRET-OME and oral cancer SCC-15 cells. The experiments were repeated three times and shown as mean ± SD (*p<0.05).

Western blotting

Using RIPA lysis and extraction buffer (Thermo Fisher Scientific, Waltham, Mass, USA), total proteins were isolated from untreated oral cancer cells and cancer cells treated with 10, 20 and 40μM rosmarinic acid for 24h. Bradford method was used to quantify the protein concentrations. About 45μg of total proteins from each sample were separated electrophoretically on 10% SDS-PAGE. The gel was blotted to nitrocellulose membrane and the membrane was then exposed to specific primary antibodies followed by exposure to secondary antibodies. ECL reagent was used for the detection of bands corresponding to proteins of interest. The protein expression procedures were normalized with human GADPH protein.

Statistics

The experiments were performed in triplicate and expressed as mean±SD. Graphpad Prism 7 software was used to perform *t*-test. A p value less or equal to 0.05 was taken as an indicator of statistically significant difference.

Results

Inhibition of oral cancer growth by rosmarinic acid through cell apoptosis

To assess the growth inhibitory effects of rosmarinic acid against the oral cancer cells, normal (hTRET-OME) and cancer (SCC-15) oral cells were treated with 0, 2.5, 5, 10, 20, 40, 80 and 160μM concentrations of rosmarinic acid for 24h (Figure 1A). Using CCK-8 kit, the proliferation rates of cells were determined. It was found that the viability of SCC-15 cancer cells decreased proportionally with the increasing doses of rosmarinic acid with an IC_{50} value ranging between 20 and 40μM (Figure 1B). The inhibitory effects were less pronounced against the normal oral cells indicating that rosmarinic acid selectively inhibits the growth of oral cancer cells. To confirm whether the anti-growth effects are due to the induction of apoptosis, the SCC-15 cells were treated with 0, 10, 20 and 40μM rosmarinic acid for 24h and processed for nuclear morphology assessment through DAPI staining. The cancer cells were seen with clear nuclear deformation, indicative of apoptotic cell death (Figure 2). Moreover, the effects were more prominent at higher doses of rosmarinic acid. Further support was obtained from western blotting results where it was found that the positively regulating apoptotic proteins Bax and active Caspase-3 were upregulated under rosmarinic acid (Figure 3). However, the protein concentration of pro-caspase-3 remained unchanged, meaning that the treatment of rosmarinic acid promotes the cleavage of pro-caspase-3 to generate caspase-3 and thus prompts the oral cancer cells to go for apoptotic cell death. Bcl-2 protein was seen to be decreasing with increasing treatment concentration increasing the Bax/Bcl-2 ratio. The results infer that the rosmarinic acid induces apoptosis in oral cancer cells and thus reduces their proliferation rates.

Rosmarinic acid promotes endoplasmic reticulum oxidative stress

The anticancer effects of rosmarinic acid were also investigated for its potential to induce the oxi-

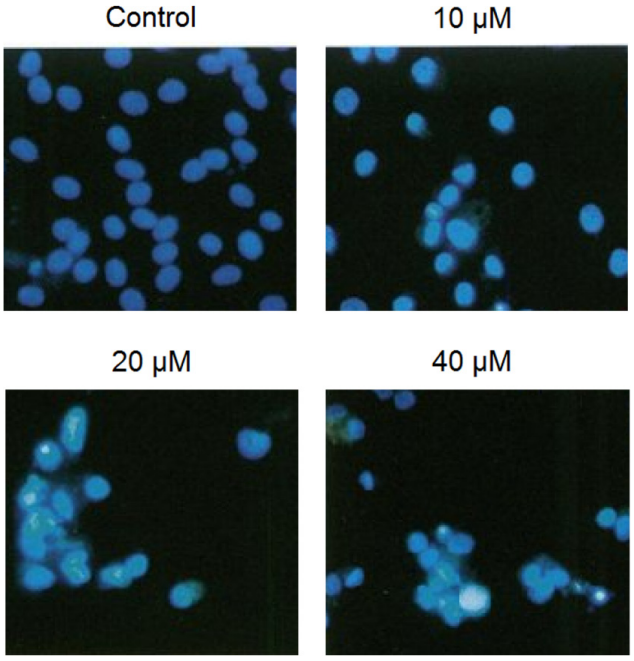

Figure 2. DAPI staining of oral cancer SCC-15 cells showing Rosmarinic induction of apoptosis in a dose-dependent manner. The experiments were repeated three times.

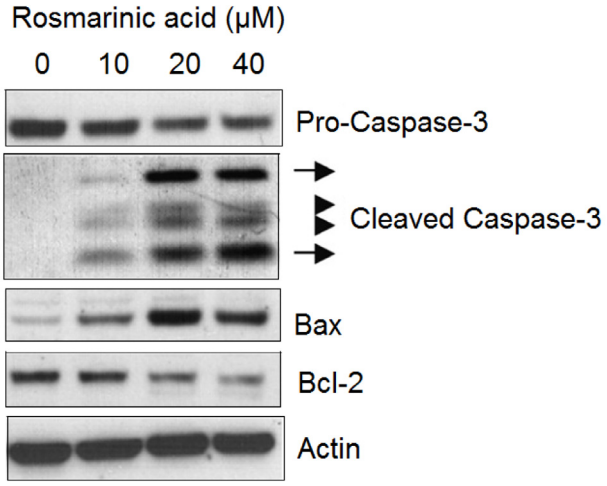

Figure 3. Western blots showing that Rosmarinic acid increases the expression of cleaved caspase-3 and Bax and decreases Bcl-2 expression dose-dependently in oral cancer SCC-15 cells. The experiments were repeated three times.

dative stress in endoplasmic reticulum (ER). For elucidating the relationship, the protein expressions of ER-stress marker proteins were studied under 0, 10, 20 and 40μM concentrations of rosmarinic acid. The cancer cells exhibited higher protein concentrations of all the marker proteins studied like PERK, p-PERK, IRE 1, ATF4 and CHOP (Figure 4). Thus, it is clear that rosmarinic acid inhibits the oral cancer cell growth by inducing the ER-stress.

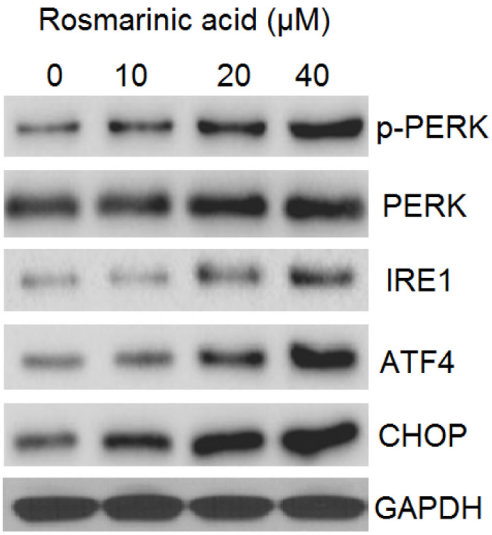

Figure 4. Western blots showing that Rosmarinic acid increases the expression of p-ERK, IRE1, ATF4 and CHOP in a dose-dependent manner in oral cancer SCC-15 cells. The experiments were repeated three times.

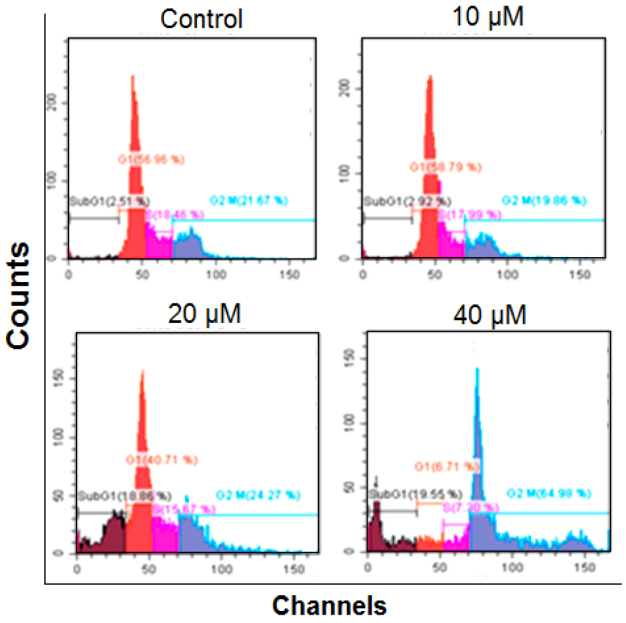

Figure 5. Flow cytometry showing that Rosmarinic acid blocks the SCG-15 cells in the G2/M phase of the cell cycle in a concentration-dependent manner. The experiments were repeated three times.

Cell cycle arrest in rosmarinic acid treated oral cancer cells

To elucidate whether rosmarinic acid has any effect on the cell cycle of oral cancer cells, the SCC-15 cancer cells were treated with 0, 10, 20 and 40μM rosmarinic acid and cell cycle phase distribution was determined through flow cytometry. The results indicated that the percentage of G2/M phase cells increased with increasing rosmarinic concentration which was 21.67, 19.86, 24.27 and 64.95 under 0, 10, 20 and 40μM treatment concentration, respectively (Figure 5). This signified that rosmarinic acid promoted cell cycle arrest in oral cancer cells which was further confirmed by western blotting results. Additionally, the expression of cyclin B1 was decreased in SCC-15 cells dose-dependently upon exposure to rosmarinic acid (Figure 6).

Rosmarinic acid inhibits the migration of oral cancer cells

The transwell assay-based assessment of oral cancer cell migration revealed that migration of

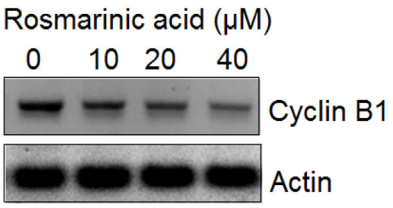

Figure 6. Western blots showing that Rosmarinic acid inhibitis the expression of cyclin B1 in oral cancer SCC-15 cells concentration-dependently. The experiments were repeated three times.

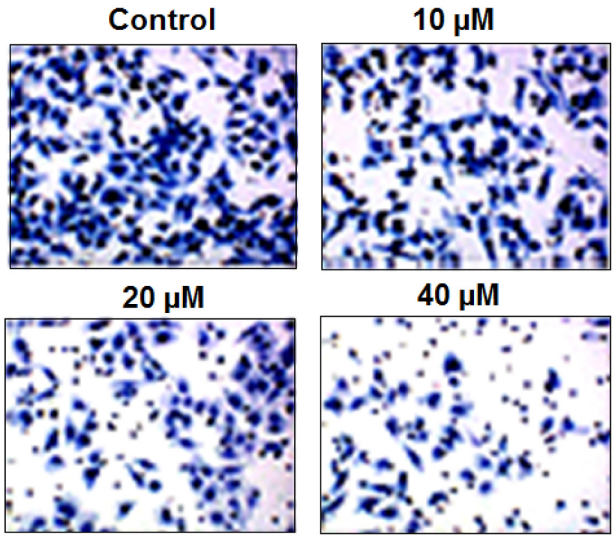

Figure 7. Transwell assay showing the effects of different concentrations of Rosmarinic acid on the migration of the oral cancer SCC-15 cells. The experiments were repeated three times.

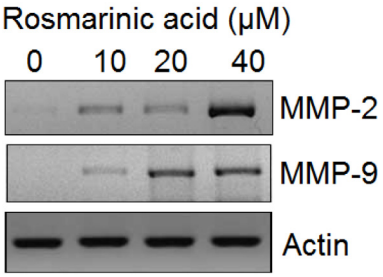

Figure 8. Western blots showing the effects of different concentrations of Rosmarinic acid on the MMP-2 and MMP-9 expression in oral cancer SCC-15 cells. The experiments were repeated three times.

cancer cells was remarkably inhibited by rosmarinic acid treatment. The extent of restriction of cell migration by rosmarinic acid increased with increasing doses of the molecule (Figure 7). The western blotting of matrix metalloproteinases (MMP-2 and MMP-9) showed that the rosmarinic acid exerted its antimigratory effects on oral cancer cells by decreasing the protein concentrations of both MMP-2 and MMP-9 dose-dependently (Figure 8).

Discussion

Plant phenolics constitute a highly diverse class of secondary metabolites [15]. The phenolic compounds are seen to possess antioxidant properties and are thus considered as health protective molecules in humans [16]. Many of these compounds are shown to have anticancer potential [17,18]. Hence, this group of plant secondary metabolites might act as a potential source of plant-based anticancer molecules and subsequent drug discovery. Researchers in recent times have undertaken exploratory studies to look for the phenolic compounds which can serve as efficient anticancer agents against particular human cancers. In such sort of exploratory study, the present work was designed to assess the anticancer potential of rosmarinic acid against the human oral cancer. Oral cancer is ranked among the most prevalent human cancers [19]. The rosmarinic acid is an ester of caffeic acid and has previously been proven to possess anticancer action [20]. In our study, we confirmed that rosmarinic acid is active in preventing the growth of oral cancer cells, indicative of its already reported anticancer potential [21]. Rosmarinic acid induced apoptotic protein mediated apoptosis in oral cancer cells. The ratio of Bax/Bcl-2 apoptosis-related proteins was shown to increase under rosmarinic acid treatment, which acts as a positive signal for induction of apoptotic cell death and increases the concentration of caspase-3 protein [22]. Cell division is regulated by a group of cyclin proteins, cyclin B1 being central to this mechanism [23]. The rosmarinic acid declined the cyclin B1 concentration in oral cancer cells thus causing the mitotic cell cycle arrest. ER is a vital organelle because of the functions it serves inside the human cells. The oxidative stress in ER makes it to malfunction and causes cell death [24]. The markers of ER-stress are a set of proteins like PKR-like endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF6), inositol-requiring 1 (IRE 1) and ClEBP homologus protein (CHOP). The increase of these proteins by rosmarinic acid thus further highlights its anticancer potential against oral cancer. The matrix metalloproteinases (MMPs) are important for cancer cell metastasis and angiogenesis [25]. The decline in MMPs protein concentrations by rosmarinic acid supports the role of rosmarinic acid as anticancer compound against the human oral cancer.

Conclusion

Summing up, the results of the current study shed light on the potential of rosmarinic acid against the human oral cancer. The efficacy of its anticancer effect may be enhanced through semi-synthetic chemistry approaches and it may act as a crucial lead molecule for discovery of more efficient drugs against oral cancer.

Conflict of interests

The authors declare no conflict of interests.

References

1. Oparin AI. The origin of life. Courier Corporation; 2003 Feb 20.
2. Pichersky E, Gang DR. Genetics and biochemistry of secondary metabolites in plants: an evolutionary perspective. Trends Plant Sci 2000;5:439-45.
3. Cipollini ML, Levey DJ. Secondary metabolites of fleshy vertebrate-dispersed fruits: adaptive hypotheses and implications for seed dispersal. Am Naturalist 1997;150:346-72.
4. Wink M. Plant breeding: importance of plant second-

ary metabolites for protection against pathogens and herbivores. Theoret Applied Genetics 1988;75:225-33.

5. Carpenter SR, Mooney HA, Agard J et al. Science for managing ecosystem services: Beyond the Millennium Ecosystem Assessment. Proceed Natl Academy Sci 2009;106:1305-12.
6. Raja A, Gajalakshmi P, Raja MM. Drugs from the natural bio sources for human disease. Int J Pharmacol 2010;6:360-3.
7. Kaur R, Kapoor K, Kaur H. Plants as a source of anticancer agents. J Natl Prod Plant Resour 2011;1:119-24.
8. Laura A, Moreno-Escamilla JO, Rodrigo-García J, Alvarez-Parrilla E. Phenolic compounds. In:Postharvest physiology and biochemistry of fruits and vegetables. 2019, pp 253-271, Woodhead Publishing.
9. Tsimogiannis D, Oreopoulou V. Classification of Phenolic Compounds in Plants. In: Polyphenols in Plants 2019, pp 263-284, Academic Press.
10. Prasad NR, Karthikeyan A, Karthikeyan S, Reddy BV. Inhibitory effect of caffeic acid on cancer cell proliferation by oxidative mechanism in human HT-1080 fibrosarcoma cell line. Molec Cell Biochem 2011;349:11-9.
11. Huang MT, Smart RC, Wong CQ, Conney AH. Inhibitory effect of curcumin, chlorogenic acid, caffeic acid, and ferulic acid on tumor promotion in mouse skin by 12-O-tetradecanoylphorbol-13-acetate. Cancer Res 1988;48:5941-6.
12. Lee YJ, Liao PH, Chen WK, Yang CC. Preferential cytotoxicity of caffeic acid phenethyl ester analogues on oral cancer cells. Cancer Lett 2000;153:51-6.
13. Watabe M, Hishikawa K, Takayanagi A, Shimizu N, Nakaki T. Caffeic acid phenethyl ester induces apoptosis by inhibition of NFκB and activation of Fas in human breast cancer MCF-7 cells. J Biol Chem 2004;279:6017-26.
14. Yesil-Celiktas O, Sevimli C, Bedir E, Vardar-Sukan F. Inhibitory effects of rosemary extracts, carnosic acid and rosmarinic acid on the growth of various human cancer cell lines. Plant Foods Hum Nutrition 2010;65:158-63.
15. Croteau R, Kutchan TM, Lewis NG. Natural products (secondary metabolites). Biochem Mol Biol Plants 2000;24:1250-319.
16. Rice-Evans C, Miller N, Paganga G. Antioxidant properties of phenolic compounds. Trends Plant Sci 1997;2:152-9.
17. Newmark HL. Plant phenolics as potential cancer prevention agents. In: Dietary Phytochemicals in Cancer Prevention and Treatment 1996, pp 25-34. Springer, Boston, MA.
18. Roleira FM, Tavares-da-Silva EJ, Varela CL et al. Plant derived and dietary phenolic antioxidants: Anticancer properties. Food Chem 2015;183:235-58.
19. Khalili J. Oral cancer: risk factors, prevention and diagnostic. Exp Oncol 2008;30:259-64.
20. Hossan MS, Rahman S, Bashar AB, Jahan R, Al-Nahain A, Rahmatullah M. Rosmarinic acid: A review of its anticancer action. World J Pharm Sci 2014;3:57-70.
21. Moon DO, Kim MO, Lee JD, Choi YH, Kim GY. Rosmarinic acid sensitizes cell death through suppression of TNF-α-induced NF-κB activation and ROS generation in human leukemia U937 cells. Cancer Lett 2010;288:183-91.
22. Salakou S, Kardamakis D, Tsamandas AC et al. Increased Bax/Bcl-2 ratio up-regulates caspase-3 and increases apoptosis in the thymus of patients with myasthenia gravis. In vivo 2007;21:123-32.
23. Harashima H, Dissmeyer N, Schnittger A. Cell cycle control across the eukaryotic kingdom. Trends Cell Biol 2013;23:345-56.
24. Fu J, Zhao L, Wang L, Zhu X. Expression of markers of endoplasmic reticulum stress-induced apoptosis in the placenta of women with early and late onset severe pre-eclampsia. Taiwan J Obstet Gynecol 2015;54:19-23.
25. Curran S, Murray GI. Matrix metalloproteinases in tumour invasion and metastasis. J Pathol 1999;189:300-8.