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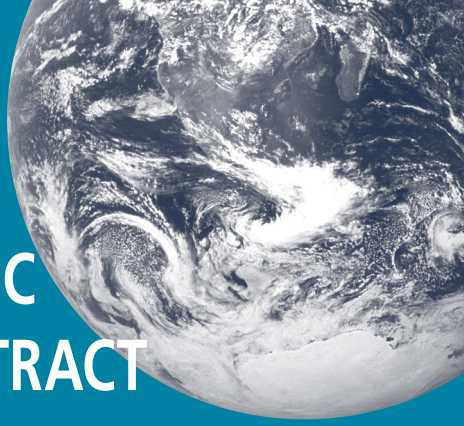

ANTI-PROLIFERATIVE AND APOPTOTIC EFFECTS OF ROSA CANINA FRUIT EXTRACT ON THYROID CANCER CELLS (B-CPAP AND THR.C1-PI 33)

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Abstract – *Objective:* *Thyroid cancer is one of the most common endocrine malignancies. Although most thyroid cancers respond to surgery and treatment, drug-resistant is a problem that necessitates new therapeutic strategies. Rosa canina from the Rosaceae family is suggested as an herbal remedy for numerous human ailments. Due to the need to identify new therapies for thyroid cancer and due to the therapeutic properties of Rosa canina, this study aimed to investigate the effect of Rosa canina fruit extract on two thyroid cancer cell lines (B-CPAP and Thr.C1-PI 33).*

Materials and Methods: *After preparation of hydro-alcoholic extract of Rosa canina fruits and treatment of thyroid cancer cells, the cell survival, and proliferation were evaluated. Apoptosis cell death and nitric oxide production were estimated. Real-time PCR was used to investigate the expression of apoptosis-related genes. Statistical evaluation was performed using one-way analysis of variance (ANOVA) and differences were considered not significant when $p > 0.05$.*

Results: *The extract reduced the viability of the cells in a concentration- and time-dependent manner. Nitric oxide production was decreased in a concentration- and time-dependent manner. The extract stimulated apoptosis cell death by decreasing the expression of Bcl-2 and increasing the expression of Bax, p53, and Caspase 3.*

Conclusions: *This extract can be promising in the treatment of patients with thyroid cancer.*

KEYWORDS: *Rosa canina, Thyroid cancer, Apoptosis, Herbal drug, Nitric oxide.*

INTRODUCTION

Cancer incidence and mortality are rapidly growing worldwide. In many countries, it ranks as the second most common cause of death following cardiovascular diseases[1]. Thyroid cancer is the most common cancer of the endocrine system and mostly affects young adults. Estimates show that its prevalence is 1%–5% in women and 2% in men. It is the 7th and 14th most common cancer in women and men, respectively. There is a significant increase in thyroid cancer incidence over the recent decades and it will replace colorectal cancer as the fourth leading cancer by 2030, followed by breast, prostate, and lung cancers. Thus, such an increase will cause extensive clinical and economic burdens that must be considered[2,3]. Four types of standard treatment are used in thyroid cancer that includes surgery, radiation ther-

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apy, chemotherapy, and hormone therapy. Nevertheless, patients with anaplastic thyroid cancer only have a median survival of 5 months, and less than 20% survive for 1 year after diagnosis[4].

Apoptosis is a particular type of programmed cell death that commonly occurs without the production of inflammation and is characterized by changes in cellular morphology and nuclear distortions including cell membrane blebbing, cell shrinkage, condensation, and fragmentation of nuclei, formation of cytoplasmic blebs, and chromosomal DNA fragmentation. It occurs normally as a homeostatic mechanism to maintain cell populations in tissues. The dysregulation of apoptotic signaling is an important causative factor in carcinogenesis and also contributing to drug-induced apoptosis-resistant. So, induction of apoptosis can be a good strategy to kill cancer cells[5].

Studies have been done on the anti-cancer properties of plant antioxidants such as polydatin[6,7]. More than half of currently used anti-cancer drugs have natural sources, and most of them are of plant origin. Examples of natural drugs that are applied in cancer therapy are vinca alkaloids (vincristine, vinblastine, vindesine, vinorelbine), taxanes (paclitaxel, docetaxel), podophyllotoxin and its derivative (etoposide, teniposide), camptothecin and its derivatives (topothecan, irinothecan), anthracyclines (doxorubicin, daunorubicin, epirubicin, idarubicin)[8].

Rosa canina is a perennial plant that belongs to the Rosacea family. It has been traditionally accepted as a medicinal plant. Many studies showed the biological potencies of this plant including anti-inflammatory, anti-cancer, immunomodulatory, anti-microbial, antioxidant, pain modulation, anti-diabetic, anti-hyperlipidemic, neuroprotective, genoprotective, anti-obesity, skin-whitening, and anti-biotic resistance reversal activity as well as exerting a positive impact on the osteoarthritis, anxiety, depression, recognition memory, urinary and reproductive systems disorders, and neutrophil respiratory burst. Although, the precise mechanism of action for these properties is not completely understood. Due to the lack of toxicity and side effects, this plant has been recognized as a valuable complementary remedy for various diseases[9]. This research aimed to study the anti-cancer features of Rosa canina hydro-alcoholic extract on thyroid cancer cells.

MATERIALS AND METHODS

Cell Line and Reagents

For this *in vitro* study, B-CPAP and Thr.C1-PI 33 cancer cell lines were purchased from the Pasteur Institute of Iran (Tehran, Iran). Trypan blue, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), Annexin V-FITC, propidium iodide (PI), and trypsin were purchased from Sigma-Aldrich Chemical Co (St. Louis, MO, USA). Roswell Park Memorial Institute (RPMI) 1640 medium and fetal bovine serum (FBS) were purchased from Gibco-Invitrogen Co. (Carlsbad, CA, USA). The study was approved by the Ethical Committee of Kermanshah University of Medical Sciences, Kermanshah, Iran (Code: IR.KUMS.REC.1399.574).

Herb extract preparation

Rosa canina fruits were collected in the winter of 2021 from Kermanshah city in the western part of Iran and identified in terms of species by a botanist (Kermanshah University of Medical Sciences, Kermanshah, Iran). The fruits were dried and powdered, and 15 g of the powder was dissolved in 150 mL of 70% ethanol for 48 h in the dark. Then it was filtered through filter paper (Whatman, grade 42) and dried. The resulted powder was dissolved in a serum-free cell culture medium and passed through a 0.22 µm filter before use[10].

Cell culture

The cells were seeded in 25 cm^2 cell culture flasks containing RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, and 1% penicillin/streptomycin under standard cell culture conditions (37ºC and 5% CO_2). They were detached with trypsin/EDTA solution and harvested by centrifugation.

The half maximal inhibitory concentration measurement

The cell death as measured by trypan blue and MTT assays. The cells were cultured in 96-well plates (1.5×10^4 cells/well). After attachment, the media containing different concentrations of the extract (6.25, 12.5, 25, 50, 100, 200, 400, and 800 µg/ml) were added to the wells and incubated for 24, 48, and 72 h. For trypan blue assay, the cells were harvested and resuspended in 1 ml of phosphate-buffered saline (PBS). A total of 10 µl 0.4% trypan blue was mixed with 10 µl of cell suspension. The mixture was applied to a hemocytometer and the number of stained and non-stained cells were counted under a light microscope. The percentage of viable cells was calculated[11].

In the MTT assay, after treatment, the media were removed and frozen for lactate dehydroge-

nase (LDH) and nitric oxide (NO) assays. Thereafter, 20 µL of MTT (5 mg/mL) solution was added to each well and then incubated for 4 h. The colored formazan crystals which were produced from MTT were dissolved in DMSO for 10 min. Then the optical density (OD) values were measured at 570 nm with a microplate reader, which is directly proportional to the number of living cells in culture. The percentage of cell viability was estimated according to the following formula[12]:

Cell viability (%)=[OD570, 630 (sample)/ OD570, 630 (control)]×100

The half-maximal inhibitory concentration (IC_{50}) values were calculated using nonlinear regression using GraphPad Prism version 8.0 software[12].

Cytotoxicity assay

LDH is a stable cytosolic enzyme that can be detected in a cell culture medium when the plasma membrane is broken. To evaluate the cytotoxicity of the extract on cancer cells, LDH activity in the medium was detected by a colorimetric LDH test kit supplied by Abcam (Cambridge, MA, USA). Shortly, the culture media from the MTT assay was gathered and centrifuged at 250 g for 10 min, and LDH activity was estimated per the manufacturer's instructions.

NO assay

The Rosa canina extract effect on NO production levels in cells was estimated from the accumulation of the stable NO metabolite, nitrite (NO2-) by Griess assay. In this assay, the culture supernatant (100 µl) from various treatment groups and Griess reagent (100 µl) were mixed, and the absorbance was measured at 540 nm. The amount of nitrite was calculated from a $NaNO_2$ standard curve (0-200 µM)[13].

Apoptosis assays

Quantitative estimation of cell death was done by flow cytometry technic using an Annexin V-FITC staining. Briefly, after being treated with plant extract at IC_{50} concentration, the cells were harvested and washed twice with PBS and suspended in 400 µL 1× Annexin V binding solution. 5 µL Annexin V-FITC solution was added to the cell suspension, and the cells were mixed and incubated on ice for 15 min in the dark. Then, 10 µL of PI staining solution was added, mixed, and incubated on ice for 5 min in the dark. Cells were analyzed for fluorescence with a flow cytometer.

The percentage of DNA fragmentation of treated cells with IC_{50} values after 24 h was calculated by diphenylamine assay as described by Cohen and Duke[14]. The OD of samples was measured at 600 nm by a spectrophotometer. The percentage of DNA fragmentation was calculated utilizing the following formula:

Percentage of DNA fragmentation = [OD660 (A) /OD660 (B) − OD660 (A)] × 100

Real-time PCR

The effect of the extract on apoptosis-related gene expression levels was evaluated by real-time PCR. Total RNA from control and treated cells was extracted by TRIzol reagent supplied by Thermo Fisher Scientific (Waltham, MA, USA). The quantity and quality of the RNAs were verified by nanodrop spectrophotometer and electrophoresis using 1% agarose gel, respectively. Complementary DNA (cDNA) synthesis was carried out by taking 1 µg RNA using a cDNA synthesis kit supplied by Vivantis Technologies (Selangor DE, Malaysia) according to the manufacturer's protocol. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was served as an internal control. Thermal cycler conditions were as follow: 15 min at 50°C for cDNA synthesis, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C to denature the DNA, and 45 s at 60°C to anneal and extend the template. Real-time PCR was performed using SYBR Premix Ex Taq Technology supplied by TaKaRa Bio Inc. (Otsu, Shiga, Japan) on the Applied Biosystems StepOne Real-time PCR System. All the primers were designed using GeneRunner software, checked in NCBI Primer Blast, and purchased from CinnaGen Co. (Tehran, Iran). The primer sequences were as follow:

Bax Forward: 5′-CCTGTGCACCAAGGTGCCG-GAACT-3′
Reverse: 5′-CCACCCTGGTCTTGGATCCAGC-CC-3′
Bcl-2 Forward: 5′-TTGTGGCCTTCTTTGAGTTC-GGTG-3′
Reverse: 5′-GGTGCCGGTTCAGGTACTCAGT-CA-3′
p53 Forward: 5′-TAACAGTTCCTGCATGGGCG-GC-3′
Reverse: 5′-AGGACAGGCACAAACACG-CACC-3′
Caspase 3 Forward: 5′-CAAACTTTTTCAGAGG-GGATCG-3′
Reverse: 5′-GCATACTGTTTCAGCATGGCAC-3′
GAPDH Forward: 5′-TCCCTGAGCTGAACGG-GAAG-3′
Reverse: 5′-GGAGGAGTGGGTGTCGCTGT-3′

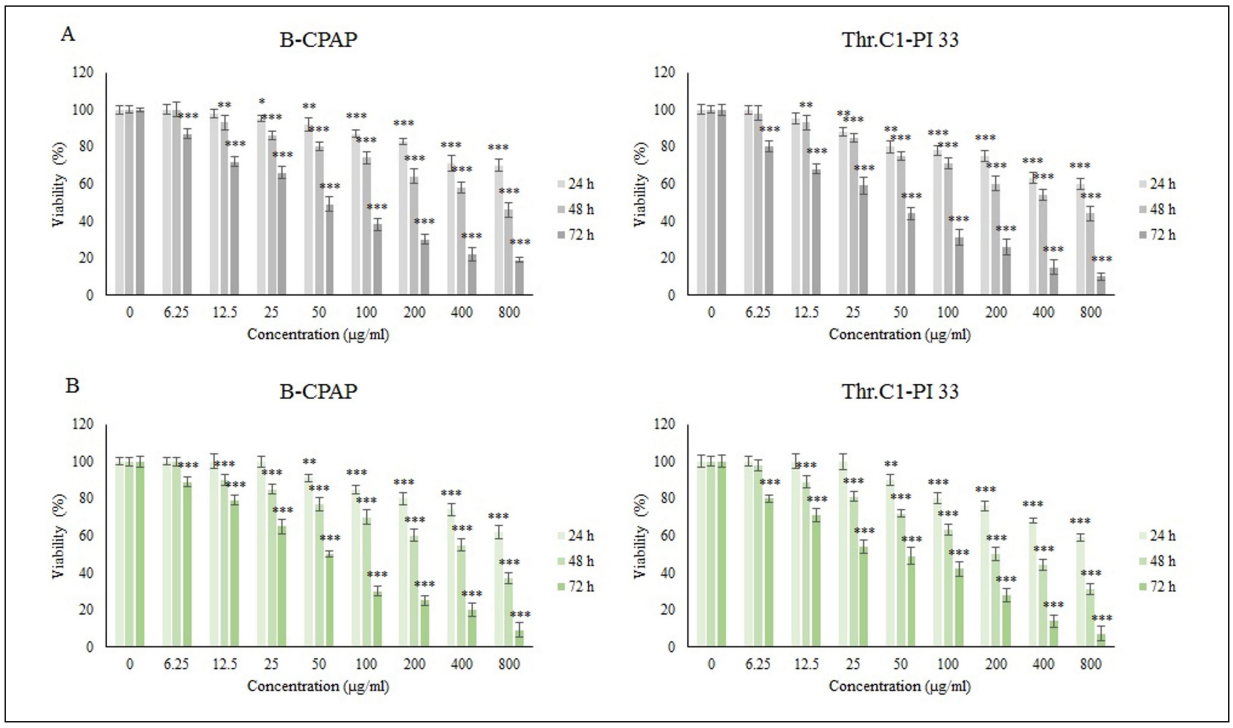

Fig. 1. The effects of Rosa canina extract on thyroid cancer cells. Cell proliferation was determined using A. The MTT assay, and B. Trypan blue staining. The data are expressed as the percentage of control cells as the means ± SD. *; $p < 0.05$, **; $p < 0.01$, and ***; $p < 0.01$ compared with control.

Statistical Analysis

All experiments were performed in triplicate and repeated independently at least three times. The data are presented as mean ± standard deviation (SD). Statistical evaluation was performed using one-way analysis of variance (ANOVA) with SPSS version 16.0 (SPSS Inc., Chicago, IL, USA) software, and differences were considered not significant when $p > 0.05$.

RESULTS

Anti-proliferative and Cytotoxic Effects of Rosa canina

The effect of the extract on cell viability was evaluated using the MTT and trypan blue assays after 24, 48, and 72 h (Figure 1 A and B). The IC_{50} values for both cell lines were listed in Table 1. The results showed that the cytotoxic effects of the extract on both cell lines were in a concentration- and time-dependent manner and the Thr.C1-PI 33 cells were more sensitive to the extract as revealed by IC_{50} values. Also, for thyroid cancer cells, the LDH activity in the cell culture medium increased with the increasing concentration of the extract (Figure 2). Therefore, Rosa canina's cytotoxic effect is achieved with plasma membrane injury.

Rosa Canina Effect on NO Production Level

The effects of different concentrations of Rosa canina extract on thyroid cancer cells after 24, 48, and 72 h of treatment showed concentration- and time-dependent manner decrease in NO production (Figure 3).

Apoptosis Induction Effect of Rosa Canina

Apoptosis measurement showed the significant differences between control and extract-treated cells. In B-CPAP cells 94.1% of control were viable cells, 0.63% were early apoptotic cells, and 3.55% were late apoptotic cells. The treatment group showed a significant increase both in the early (5.10%) and in the late (10.50%) apoptotic cells (total percentage of apoptotic cells, 15.60%). In Thr.C1-PI33 control cells, 93.70%, 0.78%, and 4.38% of cells were viable, early apoptotic, and late apoptotic cells, respectively. The extract also

TABLE 1. IC_{50} values for Rosa canina extract in the thyroid cancer cell lines.

	24 h	*48 h*	*72 h*
B-CPAP	1737.69	372.09	63.49
Thr.C1-PI 33	1176.65	284.67	38.62

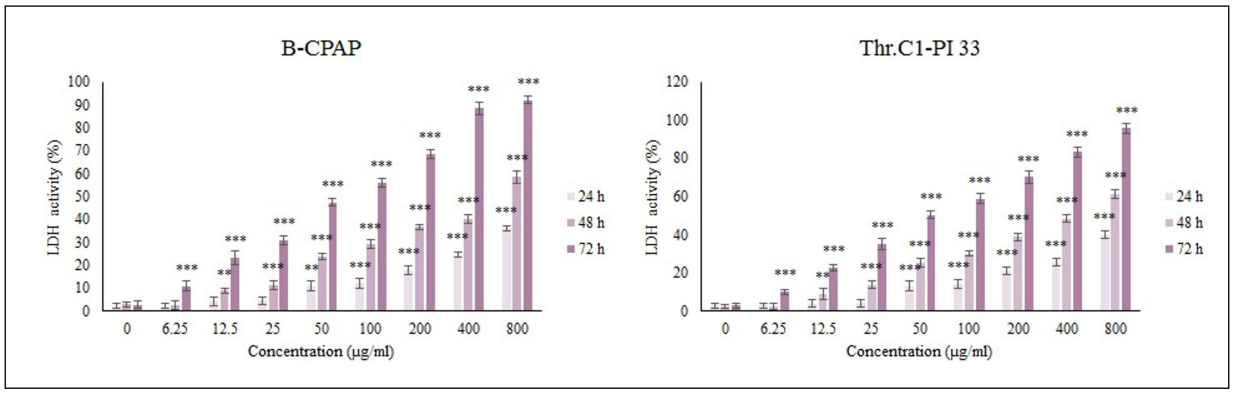

Fig. 2. The effects of Rosa canina extract on thyroid cancer cells. Cytotoxicity was determined using LDH activity assay. The data are expressed as the percentage of control cells as the means ± SD. *; $p < 0.05$, **; $p < 0.01$, and ***; $p < 0.01$ compared with control.

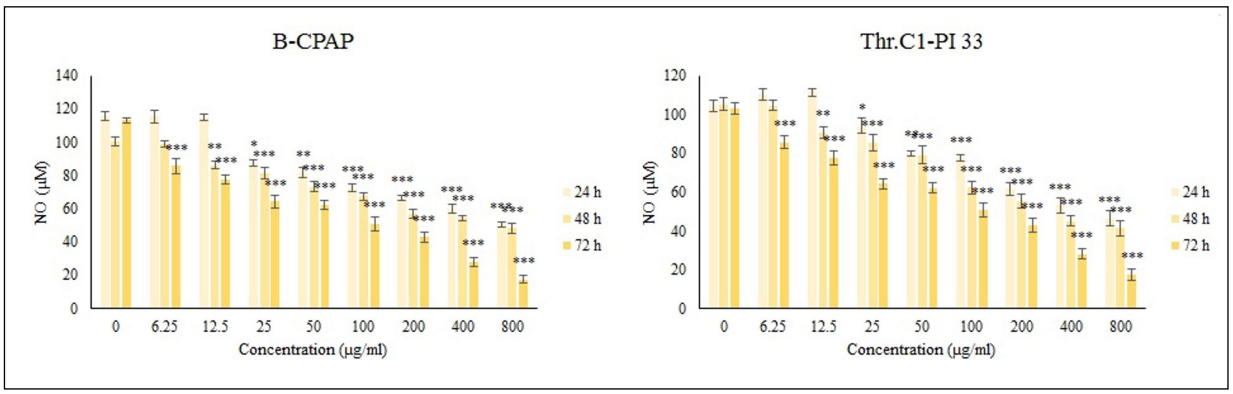

Fig. 3. The effects of Rosa canina extract on Nitric oxide production by thyroid cancer cells. The data are expressed as the percentage of control cells as the means ± SD. *; $p < 0.05$, **; $p < 0.01$, and ***; $p < 0.01$ compared with control.

caused a significant apoptosis induction (81.4% of viable cells, 4.76% early apoptotic, and 7.51 % late apoptotic) (Figure 4).

The spectroscopic apoptosis assay after 24 h treatment showed that the apoptosis cell death was increased by the extract and this increment was statistically significant in 25, 50, 100, 200, 400, and 800 µg/ml for B-CPAP cell line and 12.5, 25, 50, 100, 200, 400, and 800 µg/ml for Thr.C1-PI 33 cell line. Here again Thr.C1-PI 33 cell line was more sensitive to the extract (Figure 5).

Rosa Canina Effect on Apoptotic-related Genes

The expression of some apoptosis-related genes was evaluated using real-time PCR. p53 was upregulated in cells treated with the extract. The results of real-time PCR also suggested a downregulation of Bcl-2 and upregulation of Bax mRNA expression after 24 h exposure to the extract (Figure 6).

DISCUSSION

The current study aimed to evaluate the effects of Rosa canina extract on thyroid cancer cells. First, the potentials of eight different concentrations of extract (6.25, 12.5, 25, 50, 100, 200, 400, and 800 µg/ml) to promote cell death were examined. The results revealed that Rosa canina hydroalcoholic extract lowered cell viability in a time- and concentration-dependent manner. Further, this research showed that Rosa canina extract induced apoptosis in thyroid cancer cells.

Apoptosis or programmed cell death is a highly regulated autonomous process that is required during development. Impaired as well as increased apoptotic signaling can be implicated in a variety of diseases (cancer, autoimmune disease, neurodegenerative diseases). One of the main hallmarks of cancer is apoptosis evasion. The prevention of cancer is one of the main functions of apoptosis. Therefore, inducers of apoptosis can be considered as therapeutic agents in cancer[15].

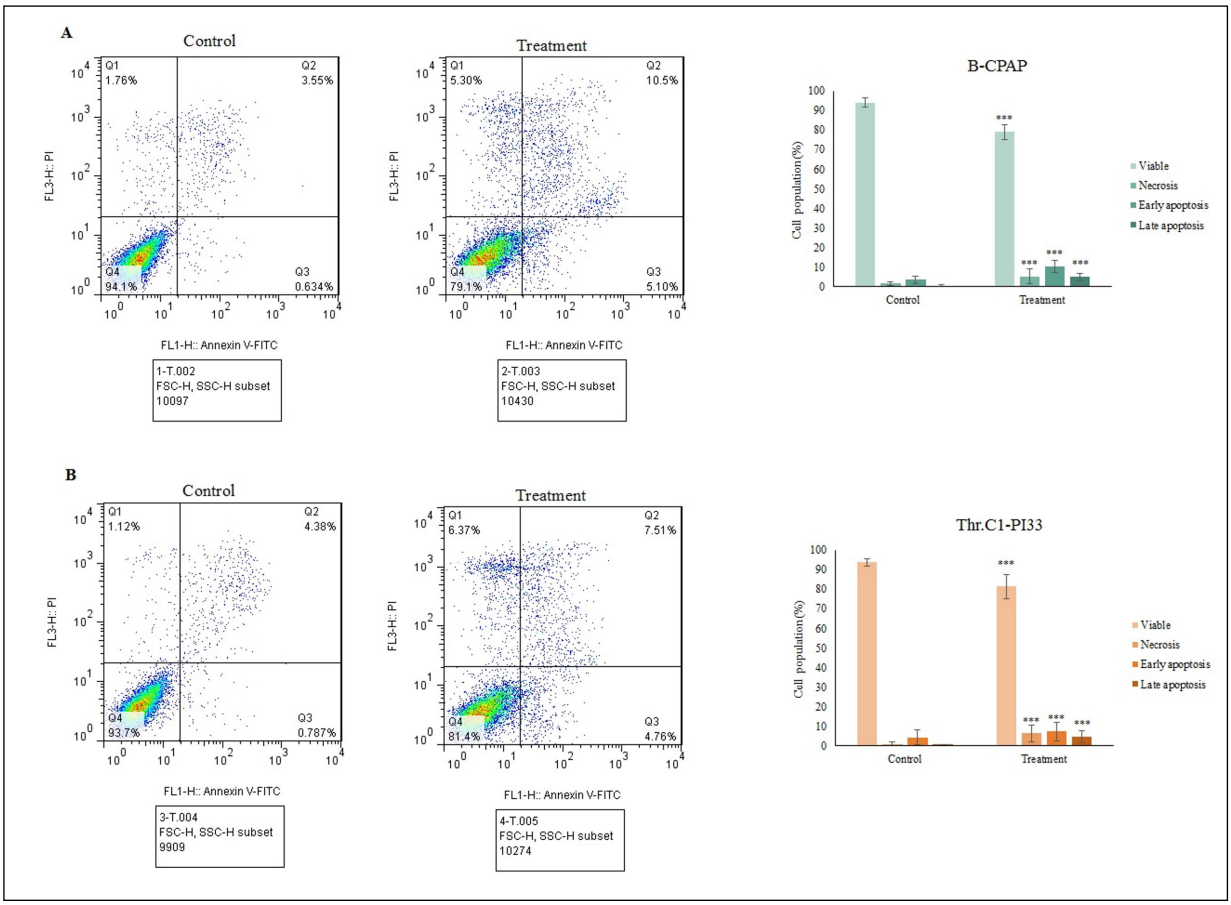

Fig. 4. Apoptosis rates by flow cytometry of (A) B-CPAP and (B) Thr.C1-PI 33 thyroid cancer cells, treated with Rosa canina extract for 24 h. Negative control cells received no treatment. Bar charts represent the percentage of intact, necrotic, early, and late apoptosis cells. The data are expressed as the percentage of control cells as the means ± SD. *; $p < 0.05$, **; $p < 0.01$, and ***; $p < 0.01$ compared with control.

Rosa canina significantly changed transcriptional expression patterns of some apoptosis-related genes. The expression level of Bcl-2 was decreased by extract treatment. These results were in agreement with the findings of the apoptosis assay.

p53 directly induces Bax transcription. Additionally, Bax induction by stress-activated p53 can overcome the anti-apoptotic effects of Bcl-2, and cells deficient for Bax are resistant to certain stimuli known to promote p53-dependent apoptosis. Thus, p53-mediated regulation of the ratio of

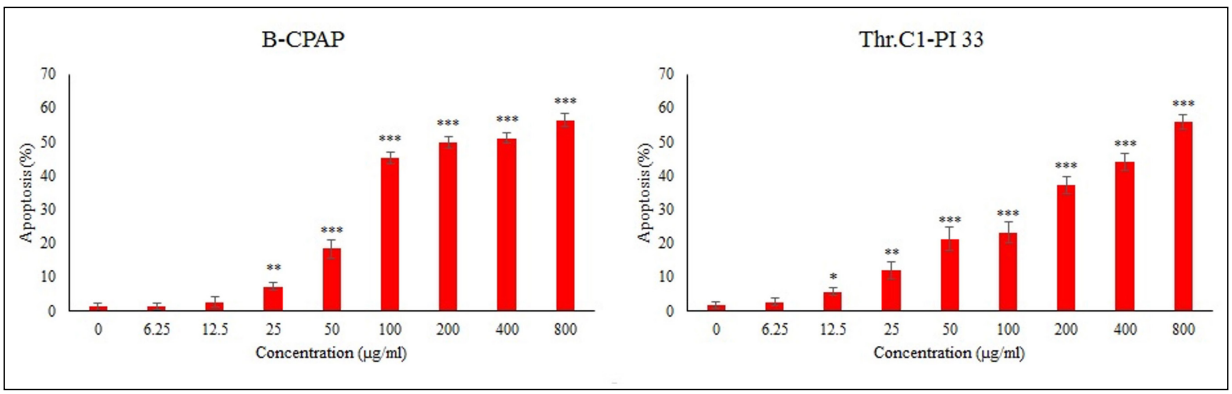

Fig. 5. The effects of Rosa canina extract on apoptosis cell death in thyroid cancer cells. The data are expressed as the percentage of control cells as the means ± SD. *; $p < 0.05$, **; $p < 0.01$, and ***; $p < 0.01$ compared with control.

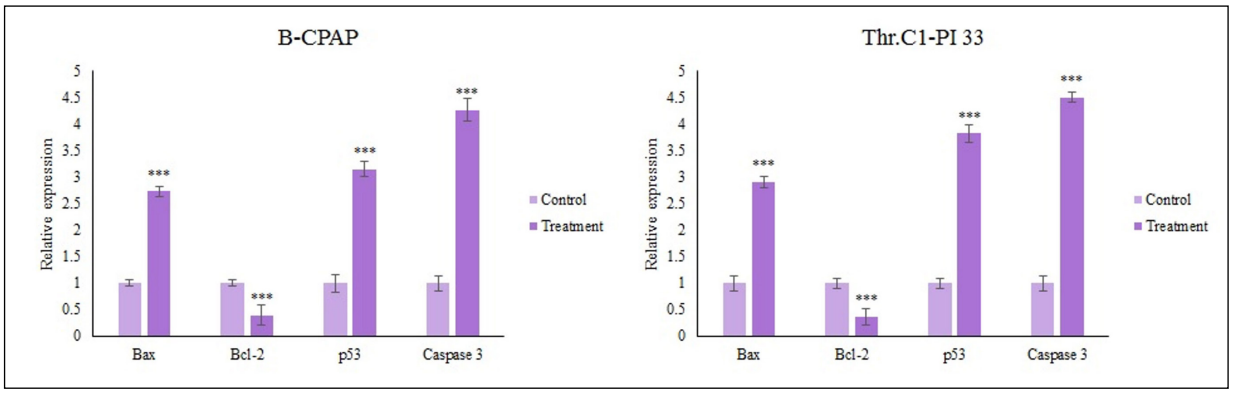

Fig. 6. The effects of Rosa canina extract on apoptotic-related genes expression in thyroid cancer cells. the cells were treated with 200 µg/ml extract for 48 h. The data are expressed as the percentage of control cells as the means ± SD. *; $p < 0.05$, **; $p < 0.01$, and ***; $p < 0.01$ compared with control.

Bax *vs*. Bcl-2 protein level can influence the fate of a cell in response to stress. Increased Bax /Bcl-2 proportion up-regulates Caspase 3 expression and causes apoptosis cell death[16].

NO is a pleiotropic molecule critical to a number of physiological and pathological processes and plays a dual and critical role in cancer pathogenesis. This research indicated that Rosa canina extract significantly decreased NO generation by thyroid cancer cells. NO is a signaling molecule with multiple regulatory effects. So, modulation of NO production in cancer cells can probably be a good procedure to achieve anti-cancer effects. NO concentration can alter the proliferation, vascularization, invasion, chemo-and radiotherapy sensitivity, and immune reactivity of cancer cells[17].

Also, NO is a dual role in apoptosis. In some cell types, NO can promote apoptosis, whereas in other cells NO inhibits apoptosis. Understanding the regulatory mechanisms of NO in apoptosis and carcinogenesis will provide important clues to the diagnosis and treatment of tissue damage and cancer. The pro-apoptotic and anti-apoptotic roles of NO have been described in different studies. NO is an endogenous suppressor of apoptosis. It can induce cytoprotective stress proteins, inhibit cGMP-dependent apoptotic signal transduction, suppress Caspase activity, and inhibit cytochrome c release. NO also suppresses Caspase activation and apoptotic morphology in neurons. However, there has not been any study on the role of NO in thyroid cell apoptosis. Our data indicated that Rosa canina extract decreased NO level and may eliminate the anti-apoptotic effect of NO in thyroid cells[18].

Natural antioxidants from Rosa canina were separated by solid-phase extraction (SPE) into ascorbic acid, flavonoids, and phenolic acids. These fractions were then tested for their anti-proliferative effect on three human cancer cell lines including HeLa (cervix epitheloid carcinoma), MCF7 (breast adenocarcinoma, estrogen receptor-positive), and HT-29 (colon adenocarcinoma). Data showed the lowest IC_{50} values for flavonoids fraction. However, the ascorbic acid fraction did not decrease the growth of tumor cells. So, only polyphenols contribute to Rosa canina anti-proliferative activity. The anti-cancer property of total extract, ascorbic acid, neutral polyphenols, and acidic polyphenols of Rosa canina on the colon cancer cell line (Caco-2) was investigated. All of them, in both low and high concentrations, inhibited cell proliferation and caused disturbances in the cell cycle resulting in concomitant cell death by the apoptotic pathways. Also, in the WiDr colon cancer cell line, Rosa canina extract presented a selective cytotoxic effect compared with normal colon cells. The extract induced cell cycle arrest at the S phase and apoptosis via reduced mitochondrial membrane potential. Also, telomerase expression was reduced by Rosa canina extract[19-21]. Based on these studies and the results of the present study, this natural plant could be an effective component of functional foods for patients with cancer.

CONCLUSIONS

Rosa canina is potentially beneficial for the further development of new chemotherapeutic agents. The present data open a new possible approach in the cure of thyroid cancer. Future studies are needed to investigate if Rosa canina provides results in *in vivo* models.

Conflict of interest:
The authors declare that there is no conflict of interest.

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Author's Contributions:
T C.J. designed the experiments, supervised the research, and co-authored the manuscript. R.N., E.A.J., and M.K. performed the cell culture experiments. I.R. and M.Z. analysed data and wrote the manuscript. F.Kh.H. carried out one gene expression analysis of this work. All authors read and approved the final manuscript.

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