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Anti-Cancer Effects of Nanoliposomes Containing *Rosemary* and *Zataria multiflora Boiss* Essential Oils on HepG2 Cell Line under in Vitro Conditions

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ABSTRACT

Background and Objective: In recent years, with increasing interest in the use of natural substances such as essential oils, extensive studies have been conducted to use them more efficiently in the form of nanoliposomes. The present study was conducted to investigate the anti-cancer effects of nanoliposomes containing *Rosemary* and *Zataria multiflora Boiss* essential oils on HepG2 cell line under in vitro conditions.

Methods: In this basic-applied research, nanoliposomes containing *Rosemary* and *Zataria multiflora Boiss* essential oils were used. The mortality rate of HepG2 cell line was calculated using MTT method and IC50 was obtained. The study groups calculated according to IC50 were: control group, the group receiving 79.72 µg/ml *Zataria multiflora Boiss* nanoliposome for 48 hours and the group receiving 53.50 µg/ml *Rosemary* nanoliposome for 72 hours. The rate of apoptosis and necrosis of cancer cells and the type of cancer cell cycle were determined by flow cytometry.

Findings: The results showed that nanoliposomes containing *Rosemary* essential oil after 72 hours (53.50 µg/ml) and *Zataria multiflora Boiss* after 48 hours (79.72 µg/ml) showed the highest capability to inhibit the growth of HepG2 cell line ($p<0.05$). The type of cell mortality was significantly apoptotic compared to the control group, as nanoliposomes containing *Zataria multiflora Boiss* caused apoptosis in 37.3% and *Rosemary* nanoliposomes in 32.1% of cells ($p<0.05$). Inhibition of cancer cells was in the G2 phase, which indicates that nanoliposomes inhibit cancer cells in the cell division phase ($p<0.05$).

Conclusion: The results showed that nanoliposomes containing essential oils have anti-cancer properties, cause cell apoptosis and stop cell growth in the G2 phase of the cell cycle.

Keywords: *Liver Cancer, Nanoliposomes, Essential Oils, Rosemary, Zataria multiflora Boiss, HepG2.*

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Introduction

The use of medicinal plants for the treatment of diseases has long been common due to fewer side effects, easier access and lower costs (1). *Zataria multiflora Boiss* is a genus of mint and a species that belongs to the genus *Zataria* and is native to Iran, Afghanistan and Pakistan (2). Important compounds in *Zataria multiflora Boiss* are Thymol and Carvacrol (3, 4). Extensive studies have been performed regarding the anti-cancer properties of *Zataria multiflora Boiss* (5, 6). In a study in 2020, it was reported that *Zataria multiflora Boiss* essential oil inhibits cell proliferation as well as apoptosis in colon cancer cell line in a time- and dose-dependent manner by increasing intracellular ROS (7). *Rosemary* (*Rosmarinus officinalis L*) is another member of the mint family. Studies show that *Rosemary* has antioxidant properties that are related to the two phenolic diterpene constituents, carnosol and carnosic acid, which are present in the composition of this plant (8). In a study in 2016, it was shown that *Rosemary* extract in lung cancer cells inhibited the proliferation, suppressed viability and increased programmed cell death by suppressing the Akt/mTOR/p70S6K pathway (9). In 2017, a study reported that *Rosemary* extract could inhibit proliferation and reduce viability by causing oxidative stress in liver cancer cells (10).

Essential oils are unstable, volatile, and hydrophobic compounds with low solubility in water, which reduce bioavailability and increase their clearance in the body, resulting in higher necessary dosage, repeated use, and ultimately limitations in therapy. New drug delivery systems offer a new approach to overcoming these limitations (11). Recently, new methods have been introduced to increase the stability and bioavailability of drugs, including encapsulation in liposomes. Liposomes are spherical particles with one or more bilayer membranes that are formed by the accumulation of fat or phospholipid molecules and by consuming energy in an aqueous environment (12). Loading and encapsulation of various drugs inside liposomes has a significant effect on improving the drug effect and their distribution in tissue (13). Since the anti-cancer effects of *Rosemary* and *Zataria multiflora Boiss* essential oils have been proven and the encapsulation of essential oils has been confirmed in previous studies (14, 15). Thus, in the present study, the anti-cancer effects of nanoliposomes containing *Rosemary* and *Zataria multiflora Boiss* essential oils on HepG2 cell line was evaluated under in vitro conditions.

Methods

Preparation of essential oils and nanoliposomes containing *Rosemary* and *Zataria multiflora Boiss* essential oils: In this basic-applied study approved with the ethics code IR.KAUMS.MEDNT.REC.1398.017 in Kashan University of Medical Sciences, *Rosemary* and *Zataria multiflora Boiss* essential oils are prepared from Barij Essential Oil Co. The nanoliposomes used in previous studies at Kashan University of Medical Sciences have also been optimized (14, 15).

Evaluation of HepG2 cell viability using MTT method: After culturing HepG2 cell line in 96-well cell culture plates and after reaching 80% density, cell supernatant was removed and the cells were treated with different doses of nanoliposomes containing *Rosemary* and *Zataria multiflora Boiss*. The plates were then incubated for 48 and 72 hours. The study groups included: control group, the group receiving 79.72 µg/ml *Zataria multiflora Boiss* nanoliposome for 48 hours, and the group receiving 53.50 µg/ml *Rosemary* nanoliposome for 48 hours. After the desired time, the cells were first washed with PBS and then MTT solution (0.5 mg/ml in PBS) was added to each well and incubated for 3 hours. During this time, the MTT solution is revived by the succinate dehydrogenase enzyme to produce blue formazan dye, and the number of living cells in each well is directly related to the amount of dye produced. The supernatant was then

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removed and 100 µl of DMSO was added to each well. Finally, the amount of dye was read at 570 nm and the value of IC50 was calculated (16).

Evaluation of apoptosis and necrosis in HepG2 cell line using flow cytometry technique: To determine the number of apoptotic and necrotic cells, cells were stained with Annexin-FITC and propidium iodide (PI) according to the kit instructions. Data analysis was performed using device software and after dividing the two-dimensional curve of FITC-Annexin against PI into four regions of Q1 to Q4: Q1 region: necrotic cells (annexin V-/PI+), Q2 region: cells in the final stages of apoptosis (+annexin V-/PI+), Q3 region: apoptotic cells (annexin V+/PI-) and Q4 region: healthy and specific cells (-annexin V/PI-). To prepare cells after treatment, after 48 hours for *Zataria multiflora Boiss* and 72 hours for *Rosemary* according to IC50 for each nanoliposome, the supernatant of the wells was removed and 400 µl of trypsin was added to each well and after 5 minutes of incubation, 1 ml of culture medium containing DMEM was added to stop the effect of protease and to create cell suspension. The cells were transferred to special flow cytometry tubes and then centrifuged at 400 g for 5 minutes. The supernatant was removed and the cells were washed twice with PBS. Then, 2 µl of Annexin-FITC and 2 µl of PI were added to 50 µl of binding buffer and reached 500 µl by adding deionized distilled water. 100 µl of the above solution containing the desired dyes was added to each tube. After 15 minutes of incubation at room temperature and away from light, centrifugation was performed. The dye-containing solution was removed from the cells and 500 µl of PBS was added to each tube. To evaluate the number of cells reacting with Annexin-FITC and PI, the emitted beams were read at 530 nm and 600 nm using Becton Dickinson BD FACS Calibur Flow Cytometer under radiation effect of 488 nm (17).

Investigation of cell cycle in HepG2 cell line using flow cytometry: DNA-binding dyes are stoichiometric and their binding in the cell depends on the amount of DNA. Cells in the S phase have more DNA than G1 cells and absorb more dye, and the intensity of the fluorescent dye increases as the DNA content doubles. Similarly, the color intensity of DNA cells in G2 phase is twice that of G1 phase. To measure the DNA content in cells of a cell population, DAPI marker was used to compare the distribution of cells in different phases of the cell cycle in drug-treated and negative control samples. After treating the cells, 5×10^5 cells were incubated at 4 °C for 30 minutes in the dark with DAPI solution in 10 µg/ml PBS buffer containing 6% TritonX-100. Then, the fluorescence emission of the marker after excitation was recorded at 359 nm. Emission photons were recorded at 461 nm and the data were analyzed on a two-dimensional curve of cell count using FlowJo software (18).

Flow cytometry data analysis was performed using FlowJo software and statistical analysis of data was performed by one-way ANOVA and Tukey test using SPSS software version 19 and $p\leq0.05$ was considered significant.

Results

Evaluation of the effect of nanoliposomes containing *Rosemary* and *Zataria multiflora Boiss* essential oil on cell viability: The viability of HepG2 cells exposed to different concentrations of nanoliposomes containing *Rosemary* and *Zataria multiflora Boiss* essential oils was measured in a dose-dependent manner compared to the control group. Based on the diagram, the effective drug concentration for 50% reduction in cell growth and proliferation was 79.72 µg/ml for *Zataria multiflora Boiss* nanoliposomes for 48 hours and 53.50 µg/ml for *Rosemary* nanoliposomes for 72 hours (Diagram 1). These doses were used in all subsequent experiments in this study.

Evaluation of the effect of nanoliposomes containing *Rosemary* and *Zataria multiflora Boiss* essential oils on cell cycle: The results of cell cycle test showed that the treated cells were more concentrated in G2

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phase, which indicates the inhibitory effects of cancer cell growth by nanoliposomes containing *Rosemary* and *Zataria multiflora Boiss* (Diagram 2).

Comparing the results of the cell cycle, it was found that the accumulation of cells in the G1 phase in the control group was significantly higher than the groups of *Rosemary* and *Zataria multiflora Boiss* nanoliposomes. However, in G2 phase, accumulation of cells in nanoliposome groups containing *Rosemary* and *Zataria multiflora Boiss* was significantly higher than the control group (Diagram 3). Furthermore, no significant difference was observed in phase S.

Evaluation of cell viability in the presence of nanoliposomes containing *Rosemary* and *Zataria multiflora Boiss* essential oils using flow cytometry: The results of flow cytometry on control samples showed that 94.7% of cells were alive and only 4.40% of cells were affected by apoptosis (Figure 1). Furthermore, 48 hours after the cells were exposed to nanoliposomes containing *Zataria multiflora Boiss*, 46.63% of cells were alive and 37.3% of the cells were affected by apoptosis (Figure 2). The results showed that in the cells located next to the *Rosemary* nanoliposome for 72 hours, on average, 63.03% of cells were alive and 32.1% of the cells were affected by apoptosis (Figure 3). The rate of apoptosis in the groups receiving the drugs was significantly higher than the control group (p=0.001). Moreover, no significant difference was observed in the final phases of apoptosis and necrosis between the study groups (Diagram 4).

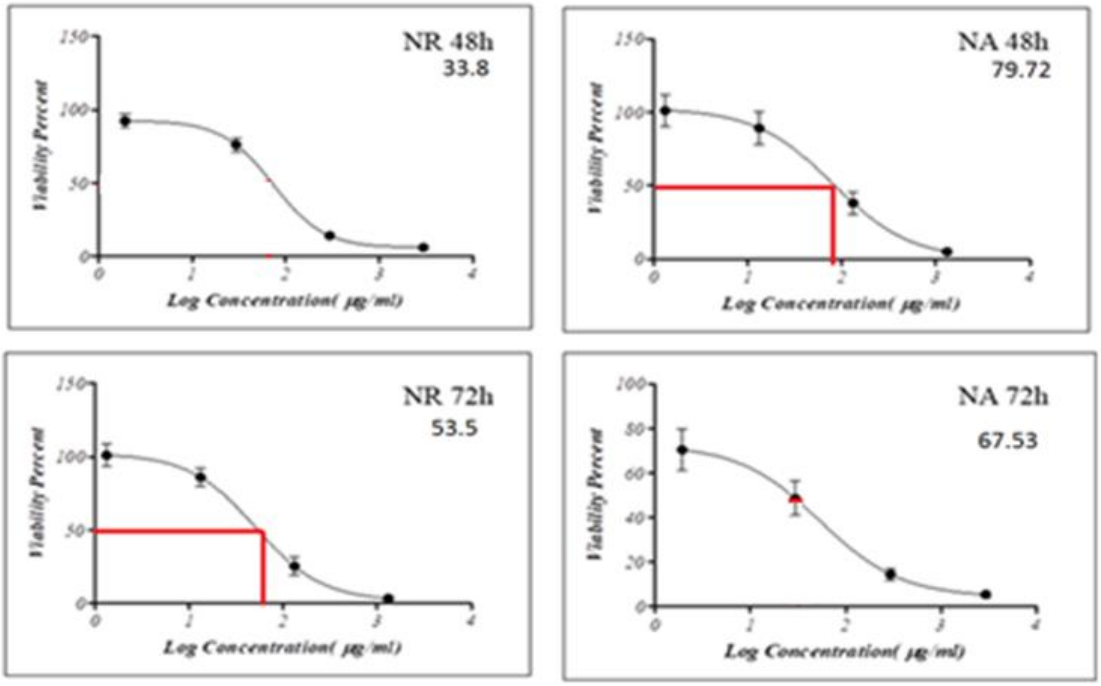

Diagram 1. IC50 of nanoliposomes containing *Rosemary* and *Zataria multiflora Boiss* essential oils on HepG2 cell line after 48 and 72 hours. Inhibitory effect of nanoliposomes containing *Rosemary* and *Zataria multiflora Boiss* essential oils on cell viability as a function of concentration and time (repeated 3 times). NA= nanoliposomes containing *Zataria multiflora Boiss* essential oils, NR= nanoliposomes containing *Rosemary* essential oils.

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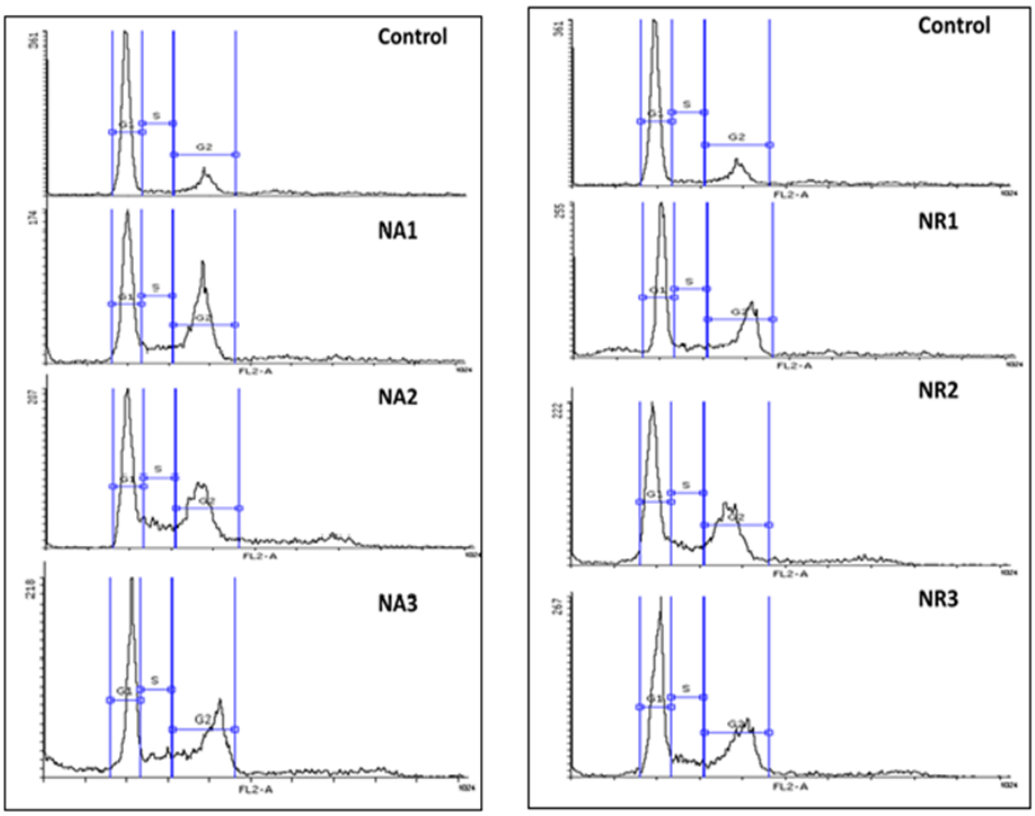

Diagram 2. HepG2 cell cycle due to proximity to nanoliposomes containing *Zataria multiflora Boiss* after 48 hours and *Rosemary* nanoliposomes after 72 hours (repeated 3 times). **NA= nanoliposomes containing *Zataria multiflora Boiss* essential oil, NR= nanoliposomes containing *Rosemary* essential oil.**

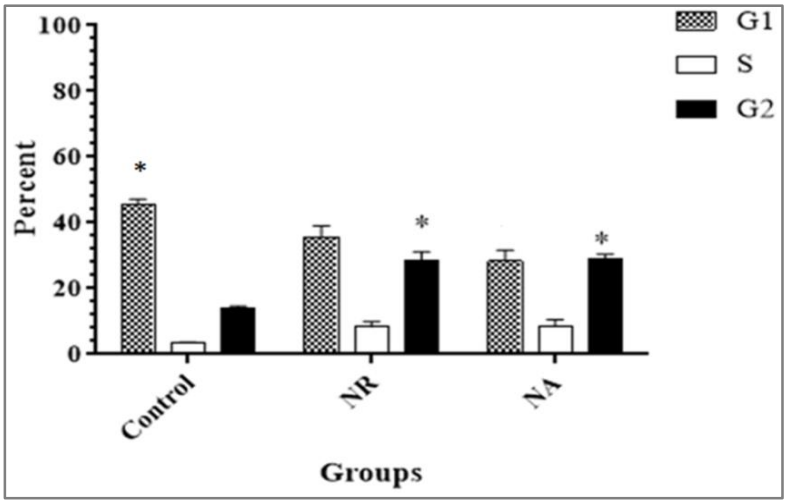

Diagram 3. HepG2 cell cycle affected by nanoliposomes containing *Rosemary* and *Zataria multiflora Boiss* essential oils in comparison with the control group. **NA= nanoliposomes containing *Zataria multiflora Boiss* essential oil, NR= nanoliposomes containing *Rosemary* essential oil. *p<0.05 There was a significant difference between the control group and the groups treated with nanoliposomes containing *Zataria multiflora Boiss* and *Rosemary* essential oils.**

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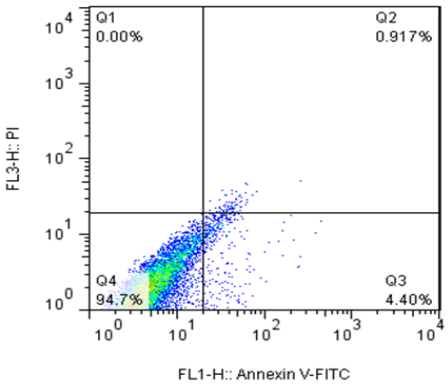

Figure 1. Evaluation of cell viability in control group using flow cytometry (repeated 3 times). **NA= nanoliposomes containing *Zataria multiflora Boiss* essential oil, NR= nanoliposomes containing *Rosemary* essential oil.**

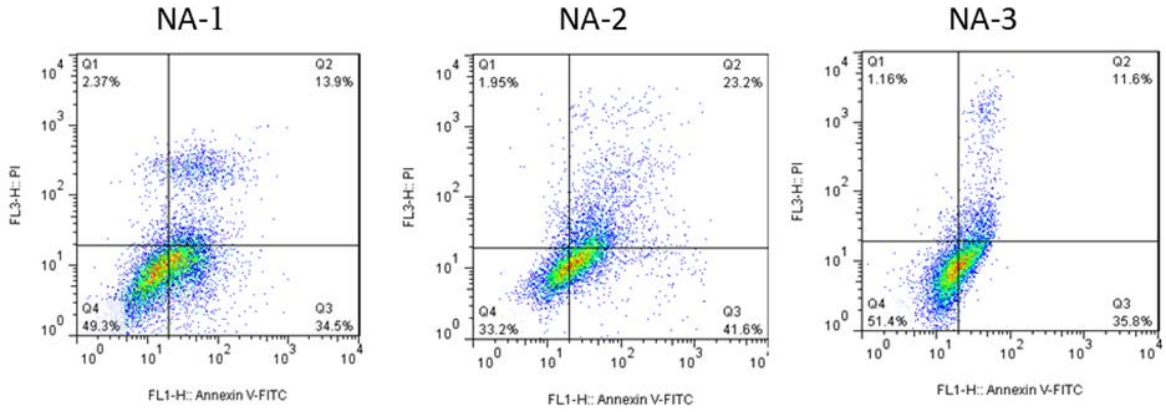

Figure 2. Evaluation of cell viability in the presence of nanoliposomes containing *Zataria multiflora Boiss* essential oil after 48 hours using flow cytometry (repeated 3 times). **NA= nanoliposomes containing *Zataria multiflora Boiss* essential oil, NR= nanoliposomes containing *Rosemary* essential oil.**

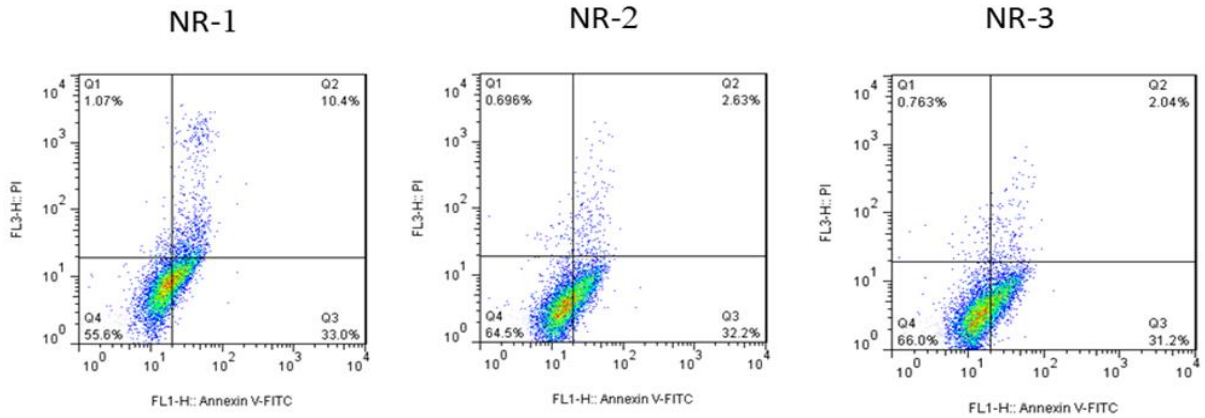

Figure 3. Evaluation of cell viability in the presence of nanoliposomes containing *Rosemary* essential oil after 72 hours using flow cytometry (repeated 3 times). **NA = nanoliposomes containing *Zataria multiflora Boiss* essential oil, NR = nanoliposomes containing *Rosemary* essential oil.**

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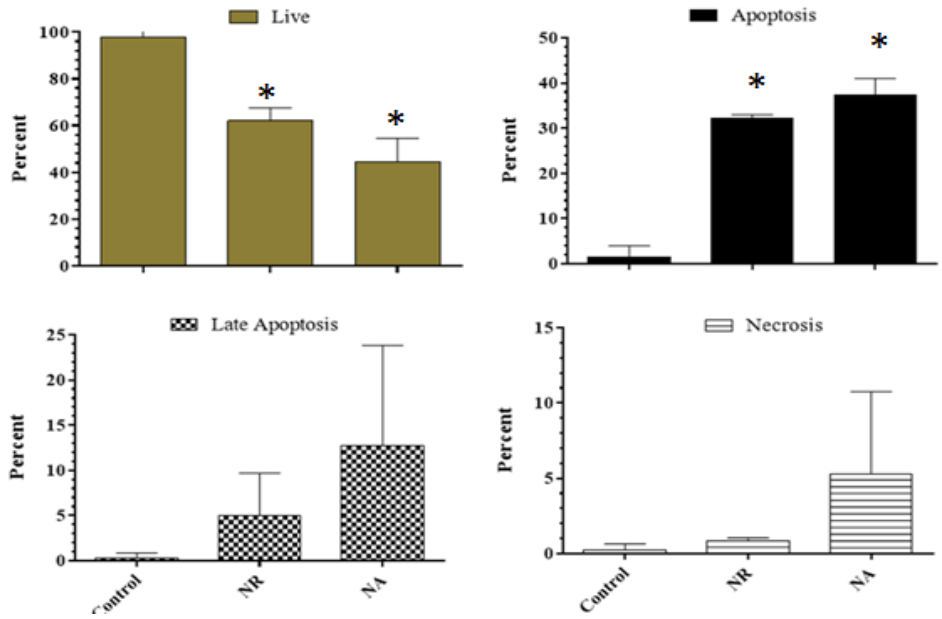

Diagram 4. Comparison of flow cytometry results of HepG2 cells after proximity to nanoliposomes containing *Rosemary* and *Zataria multiflora Boiss* essential oils in comparison with the control group (repeated 3 times). **NA= nanoliposomes containing *Zataria multiflora Boiss* essential oil, NR= nanoliposomes containing *Rosemary* essential oil. *p<0.05 Significant difference compared to the control group.**

Discussion

The results of this study showed that the anti-cancer effects of nanoliposomes containing *Rosemary* essential oil were quite significant compared to the control group. Flow cytometry results also confirmed the induction of apoptosis in HepG2 cells by *Rosemary* nanoliposomes. In various laboratory studies, the anti-cancer effects of *Rosemary* extract on different cancer cell lines of the colon, pancreas, breast, prostate, lung, bladder, liver and leukemia have been expressed and its anti-cancer effects have been shown to reduce cell proliferation and viability and induce apoptosis in a dose- and time-dependent manner (19). In the study of Shabani et al., the results showed that *Rosemary* extract had the ability to inhibit the growth of mammary gland cancer cells in dogs and was most effective at a dose of 25 mg/ml within 48 hours after treatment. The results also showed high expression of BAX gene as an apoptosis-promoting gene in the same drug dose and time interval (20). In one study, the results showed that oral administration of *Rosemary* extract reduced the size of colon tumors in mice by increasing the expression of GCNT3 gene, along with epigenetic changes and decreasing the expression of gene encoding miR-15b (21). Administration of oil-soluble extract for 4 weeks inhibited the formation of HCT116 xenograft tumor in rats by increasing Nrf2 gene expression (22). The Nrf2/ARE signaling pathway is important in protecting cells against carcinogenesis and slows down the carcinogenesis process by neutralizing reactive oxygen species and carcinogens. *Rosemary* extract expresses proteins of this pathway such as sestrin2 and it also increases oxygenase-1 in colon cancer cells (23). Biochemical analysis of serum in Sprague-Dawley rats treated with *Rosemary* extract showed that the extract had a significant anti-cancer effect by altering gene signaling and proteins β-catenin, K-ras and c-myc (24). *Rosemary* extract inhibited leukemia cancer cell line expression, mRNA expression and AKT 1 protein. This protein in the PI3K/AKt signaling pathway increases the survival of this cell line. Stopping the cell cycle prevents further cell proliferation, and *Rosemary* extract can stop the cell cycle in a

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number of cancer cells and increase the retinoblastoma-dependent gene 2, which regulates entrance to cell cycle (25). *Rosemary* extract increases the production of nitric oxide and tumor necrosis factor in pancreatic and liver cancer cells, which indicates an increase in cell death and nitric oxide-dependent apoptosis (26, 27).

The results of this study also showed that essential oil and nanoliposomes containing *Zataria multiflora Boiss* essential oil have cytotoxic effects on HepG2 cancer cell line. In addition, the results of flow cytometry showed that nanoliposomes containing *Zataria multiflora Boiss* essential oil induced apoptosis in HepG2 cells, which is completely consistent with the results of previous studies. Based on the results of a study that evaluated the reduction in the bioavailability of cancer cells treated with *Zataria multiflora Boiss* essential oil, it was found that this essential oil inhibits the growth of squamous cell carcinoma caused by head and neck tumors in humans under in vitro conditions (28). A study by Kim et al. confirmed the traces of cyclooxygenase and oxidative stress enzymes during cell growth and differentiation, including cancer, and found that hydroalcoholic extracts of *Zataria multiflora Boiss* contain compounds that inhibit these enzymes and reduce oxidative stress (29). Liang et al. also found that the flavonoids in *Zataria multiflora Boiss* activated PPARɣ, thereby inhibiting 2-COX expression (30).

One of the features of this study and the point of difference with most studies is the use of nanoliposomes in this study. According to the results of a recent study, the cytotoxic effects of *Rosemary* and *Zataria multiflora Boiss* in the form of nanoliposomes were significantly higher than the essential oils of these plants. The results of this study demonstrated the cytotoxic effect of nanoliposomes containing *Rosemary* and *Zataria multiflora Boiss* essential oils on HepG2 cancer cells. *Rosemary* and *Zataria multiflora Boiss* nanoliposomes induce apoptosis in the HepG2 cancer cell line. Furthermore, cancer cells stop in the G2 phase when exposed to *Rosemary* and *Zataria multiflora Boiss* nanoliposomes in the G2 phase, indicating the role of the drug in preventing cell proliferation. In line with the results of the present study, in a study in 2021, it was reported that treatment of MCF-7 and Hella cells with mitomycin coated with *Rosemary* nanoemulsion, induced apoptosis in cultured cancer cells even at low doses of mitomycin (31).

According to the results of recent studies and other studies in this field, it is suggested that medicinal plants and especially the nano form of these compounds be used as an appropriate adjunctive therapy to cancer treatment.

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