doi.org/10.36721/PJPS.2025.38.4.REG.13514.1

Rosemary reduced estrogen receptor (ESR1) protein mediated cycle arrest and apoptosis: A potential hormonal dependent anticancer mechanism of the plant in the MCF-7 breast cancer cells

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Abstract: Breast cancer is a global health issue, driving the development of various treatment methods, including anticancer drugs. The unfavorable side effects of these medicines, especially in targeting hormones, have increased interest in naturally occurring therapy options due to their potential for many targets and less side effects. Rosemary (*Salvia rosmarinus*) extract may treat breast cancer naturally. The study used MCF7 cells and found that the rosemary extract exhibited significant anticancer activity, with an IC_{50} value of 12.5µg/ml. The anticancer effect of the rosemary extract was primarily mediated by apoptosis, which was induced by a substantial reduction in the expression of the estrogen receptor (ESR_1) protein. The downregulation of ESR_1 led to G_0/G_1 cell cycle arrest and apoptosis through the activation of downstream pathways, resulting in increased expression of TP53 and BAX, while reducing Bcl_2 levels, ultimately leading to apoptosis. Moreover, the study confirmed the extract's ability to induce apoptosis by demonstrating a noticeable increase in DNA damage in the treated cancer cells, as assessed by DNA fragmentation and comet assay. These results demonstrate rosemary extract's potent breast cancer-fighting properties. The study concludes that rosemary extract has potent anticancer effects on breast cancer cells.

Keywords: Rosemary (ROSM); human breast cancer cell line; ESR1, TP53-Bcl2-BAX-gene expression; apoptosis**;** receptor binding

Submitted on 02-10-2024 – Revised on 04-12-2024 – Accepted on 18-12-2024

INTRODUCTION

Rosemary (*Salvia rosmarinus*) is a medicinal herb with various bioactive components that affect practically all of the body's organs. Therefore, the plant was chosen in 2000 as a herb of the year by the International Herb Association. The plant is an aromatic herb with many volatile oils, of which eucalyptol, camphor, and bornyl acetate are the chief volatile ingredients (Mohammed *et al*., 2020). The plant also contains many biologically active polyphenols, including phenolic acids and flavonoids. (de Macedo *et al*., 2020) In addition, rosemary has been reported to contain resinous materials, triterpenes, and tannins. The plant's traditional applications and participation in the modern medicine and food industries are mostly attributed to its polyphenolic and volatile oil contents (Aziz *et al*., 2022). ROSM is used for several medical applications, including its topic and systemic application as a tonic for hair and circulation (Ahmed and Babakir-Mina, 2020). ROSM has beneficial effects on the heart, liver, and respiratory dysfunctions (de Macedo *et al*., 2020). Cancer cells proliferate rapidly and are resistant to apoptosis. Cancer cells must modify critical signaling pathways engaged in proliferation and survival to avoid homeostasis. Plant-derived compounds, including dietary components, are still being studied. Natural product research can help with the development of new anticancer agents as well as the discovery of different mechanisms. Rosemary extract has reduced inflammation, managed diabetes, and controlled cancer progression. Carnosic and rosmarinic acids are plentiful in rosemary extract (Brindisi *et al*., 2020).

As an important material, rosemary's volatile oil is the most studied component of the plant. The literature demonstrated significant variations in the plant essential oils related to environmental conditions, including biotic and abiotic factors (Jene *et al*., 2024). Its commercial

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demand has expanded in recent years due to its numerous pharmacological activities as well as its culinary and ornamental purposes, and it is currently employed as an important ingredient in various products in both the pharmaceutical and food fields (Zhang *et al*., 2024, Bommakanti *et al*., 2023).

Regarding plant polyphenols, many investigations revealed that carnosol has exerted anti-tumor action by preventing cell cycle division and triggering apoptosis in various forms of malignancy (Samarghandian *et al*., 2018). This is marked by the formation of characteristic ladder DNA fragments and their multiples on an agarose gel. On the other hand, random DNA breaking in necrotic cells causes a diffuse smear on DNA electrophoresis. Apoptosis is a complex process which is regulated by multiple factors, including PI3K-Akt and Ras-MAPK. Reduction of apoptosis, enhancement of proliferation, and enhanced survivability are the results of activating these intracellular signaling pathways (Alinaghi *et al*., 2024). In Previous study the authors found that polyphenol, which are products found in rosemary, decrease cell viability by downregulation of PI3K/AKT/mTOR/p70S6K mechanism (Mirza-Aghazadeh-Attari *et al*., 2020). Furthermore, y Heinzelmann-Schwarz *et al*, found that ESR1 was strongly expressed in breast cancer (Heinzelmann-Schwarz *et al*., 2018, Gird *et al*., 2021). In a recent interesting study, Lingling Wang *et al*. profiled the constituents in the seeds of *Rheum tanguticum*. The authors have used Swiss target prediction tools to recognize the potential targets of the plant constituents. They found ESR1, APP, and MAPK8 as the main targets (Wang *et al*., 2022, Bouammali *et al*., 2023). Low expression of estrogen receptor 1 (ESR1) cause G0/G1 cell cycle arrest and apoptosis via activating downstream pathways, which trigger TP53 leading to apoptosis (Ding *et al*., 2020).

The study examined the anticancer effects of rosemary aqueous extract as well as its effect on important signaling molecules such as ESR1, TP53, BAX and Bcl-2 as it has only recently been discovered that rosemary and its polyphenols, e.g., CA and RA, demonstrated powerful anticancer properties

MATERIALS AND METHODS

Materials

This study was conducted at Qassim University in Buraydah, Saudi Arabia in collaboration with the Umm Al-Qura University in Makkah. 250 gm of rosemary (ROSM) leaves was obtained from the Egyptian market (Cairo, Egypt). The plant sample was authenticated by local botanists, and a sample of the plant was stored under the voucher # PHQ-142 at the College of Pharmacy, Qassim University, Saudi Arabia. The MCF-7 cells were grown in DMEM supplemented with FBS (Hyclone, Logan, Utah, USA), insulin, and penicillin-streptomycin. The cells were of ATCC types (Manassas, VA, USA). Vials of MTT that have been reconstituted are supplied by Sigma (Sigma Aldrich/Merck KGaA, Darmstadt, Germany). Each and every chemical and analytical reagent was of the highest grade.

Aqueous extraction of ROSM

An aqueous extract of the herb, *Salvia rosmarinus*, was used as the test substance (ROSM). The following are the steps involved in the extraction process: With certain adjustments, extraction was done in this work similar to the methodology designated by Wang *et al*. (2021) (Wang *et al*., 2021). The plant leaves (100 gm) were air-dried at 40 °C and reduced in size. To extract the leaf powder, it was boiled in distilled water (300 mL) for 30 minutes. It was necessary to eliminate the particle debris using filtration; after that, the solutions were stored at 4ºC.

The filtrated extract was dried under vacuum using Rotavapor. The extraction value was calculated by determining the percentage of the dried extract relative to the original weight of the plant material used in the extraction process. The concentrated solution was freeze-dried using a freeze dryer. The dried powder was weighed, the amount was determined in mg/ml.

Anticancer activity of ROSM

The MCF-7 cells were cultured and incubated at 37 °C and 5% CO_2. The MTT assay was used to test the effect of the plant extract. Briefly, cells were seeded in 96-well culture plates at 10,000 cells/well and incubated for 48h at 37 °C in 5% CO2 with the increased amount (3.125-100 µg/ml) of ROSM. As control, the cells were incubated with medium alone. After incubation, the medium was removed, the cells were washed with PBS, and we added freshly prepared MTT medium (10 µl MTT solution (5 mg/ml) in 100 µl medium) on the cells and incubated for 2-3 h. Next, violet crystals of formazon were dissolved with 100 ul DMSO and colorimetric absorbance was measured at 570 nm with 655 nm filter (Abdellatif and Alsharidah, 2023). The reduction in the cell viability was calculated as follows:

$$\% \text{ GI} = \text{ATC}/\text{ ACC} \times 100$$

Where GI is the growth inhibition, ATC is the absorbance of the treated cells, ACC is the absorbance of the untreated cells.

DNA fragmentation assay

The MCF7 were seeded at $4x10^6$ cells per 100 mm plate and cultured for 24 h with ROSM (12.5 µg/ml), DOX (2µg/ml) or only medium (control). Using lysis buffer (10.5 percent Triton X-100, 10 mM EDTA, and 10 mM Tris, pH 7.4) at 48°C for 10 minutes, low molecular weight genomic DNA was isolated following PBS rinsing. After incubating with 40 mg/ml of Proteinase K for an hour at 37°C, the DNA was treated with 20 mg/ml of RNase A for one hour at that temperature. After ethanol precipitation,

the DNA was separated in 0.8% agarose gel containing 0.1 µg/ml ethidium bromide and visualized under UV light. As previously mentioned (Abdellatif and Alsharidah, 2023), the diphenylamine test was also used to determine the DNA fragmentation. The percentage of total DNA (pellet and supernatant) recovered as low-molecular-weight DNA in the supernatant is used to quantify DNA fragmentation.

DNA damage using the comet assay (SCGE)

The cells were incubated for 24 h with ROSM (12.5 µg/ml), DOX (2µg/ml) or only medium (control). After that, cells were rinsed in 1 ml of PBS, centrifuged at 200 x g for 5 min at RT, and used with the comet assay kit from Abcam using standard protocol (Abdellatif *et al*., 2022) with slight modifications. The cell pellet was centrifuged and dispersed in 100 µl 0.5% low melting-point agarose (MPA). It was then spread out on glass microscope slides that had been covered with 1% normal- MPA beforehand and maintained at 4˚C for 20 minutes. Next, the cell-coated slides were incubated at 4°C for a whole night in lysis buffer (2.5 M NaCl, 10 mM Tris, 100 mM EDTA, and 1% Triton, pH 10).

Following that, slides were submerged for ten minutes at 4˚C and twice cleaned in distilled water. The electrophoresis was carried out for 20 minutes at 25 V while the slides were submerged in the electrophoresis buffer (200 mM EDTA and 10 M NaOH, pH >13). The slides were dried with ethyl alcohol (50, 75, and 100%; 5 minutes each) and stained with Vista Green DNA (Abcam, Cambridge, MA, USA) after being neutralized 3 times using 0.4 M Tris buffer adjusted at the pH 7.5. Cell images were captured using a fluorescent microscope IX71/DP72 (Olympus, Hamburg, Germany) at x200 magnification, and then examined using the CometScore V1.5 Software.

Analysis of combination Index (CI)

Doxorubicin is considered as the first agent for the treatment of breast cancer cells. The phenomenon of chemotherapy-resistant breast cancer cells develops in the cells. It is imperative to consistently create molecules capable of overcoming resistance to chemotherapy. For this reason, we tested the effect of ROSM alone (3.125-25ug/ml), doxorubicin (DOX) alone (0.25-3 ug/ml), and the combination of ROSM/DOX to show if there is a synergistic effect. After incubation for 48h, MTT was conducted as mentioned above.

The gene expression analysis

RNA isolation

Total cellular RNA has been isolated from treated and untreated cells utilizing the RNeasy Miniprep Kit (Qiagen, Hilden, Germany)24 hours after MCF7 cells were treated with ROSM (12.5µg/ml), DOX (2µg/ml), or only medium (control). The company's procedure was followed. To get rid of any DNA contamination, the RNA was processed with RNAse-free DNAse (Qiagen, Hilden, Germany) after isolation. Formaldehyde-containing agarose gel electrophoresis was utilized to verify the integrity of the RNA, and the 260/280 nm ratio was used to measure the quantity and purity, respectively. The isolated RNA aliquots were then kept in storage at -80°C (El-Readi *et al*., 2019).

Reverse - transcription (RT) reaction

For the RT, after the isolation of mRNA, RevertAidTM First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to generate first-strand cDNA from 1µg of total RNA that had been DNase-treated. The kit was maintained at -20°C until it was used in real-time PCR (qPCR)(Eid *et al*., 2020).

Quantitative qPCR

Using qPCR, the investigated genes' expression levels were measured in both treated and untreated cells in accordance with the manufacturer's instructions. 0.5µl of forward and reverse primer pairs (0.2µM each) that are specific for each gene, 6.5µl of dd H2O, 5µl of cDNA reaction, and 12.5µl of 1× SYBR® Premix Ex TaqTM were utilized in the reactions, which had an entire volume of 25µl.

The qPCR protocol was run with 40 cycles at 95°C for 15 s, 60°C for 30 s for annealing, and 75°C for 30 s of cycling, followed by three minutes of 95°C. A final elongation phase was performed at 72°C for 10 minutes following 40 cycles. Ultimately, a gradient dissociation protocol was employed to assess the qPCR primers' specificity and the presence of primer dimers, with increments of 0.5°C every 30 seconds from 65°C to 95°C. Water was used as a control in each trial. For data computations, normalization using the housekeeping gene beta-actin was used; the findings were expressed as 2-ΔΔCT expression. The StepOne TM qPCR System from Applied Biosystems (Thermo-scientific, Dreieich, Germany) was used for the quantification, and the StepOne TM Real-Time PCR System (v2.3) was used to determine the fluorescence threshold level (Abdellatif *et al*., 2021). All reactions were done in triplicates and the primers used for qPCR are listed in the supplementary file.

STATISTICAL ANALYSIS

The student's t-test was used, with $p < 0.05$ being statistically significant. The mean ± SEM was used to present data. For analysis, the IBM Statistic 22 program (SPSS, Chicago, USA) was utilized.

RESULT

Anticancer activity

Cell viability test (MTT) and DNA defragmentation

After addition of different concentration (3.125-100µg) of ROSM on MCF7 cells, the anti-proliferative and cytotoxic effect of ROSM were screened. We found that the IC50 of rosemary extract was at around 12.5 µg/ml (fig. 1A).

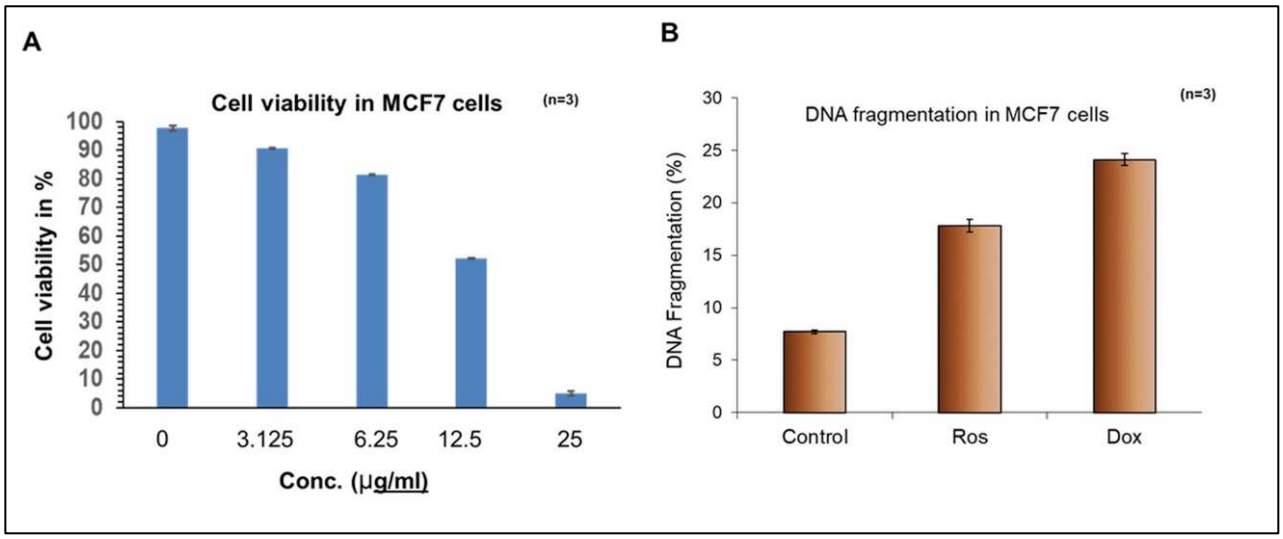


Fig. 1: Cell viability and DNA fragmentation.
A) The MCF7 cells were exposed to 3.125-100 µg/ml of ROSM for 48 h and MTT assay was applied. The IC50 values was calculated from the best fitting curves (using value 3.125-50g/ml).
B) MCF7 cells were incubated with 12 µg/ml ROSM, 2 µg /ml DOX, or only medium (control); after that, the DNA fragmentation was analyzed. The data are from triplicate and are expressed as mean ± SD and *** and ** is indicating for the significant difference of P<0.001 and P<0.01, respectively.

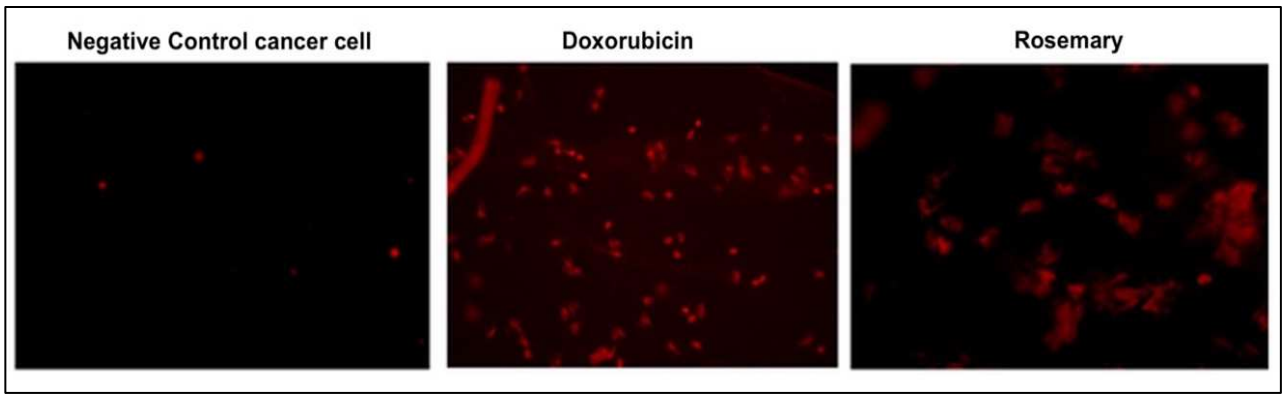


Fig. 2: Comet assay visualization scores for intact DNA (class 0) and damaged DNA (Class 1, 2, and 3) in MCF7 incubated with ROS, DOX, or only medium (control).

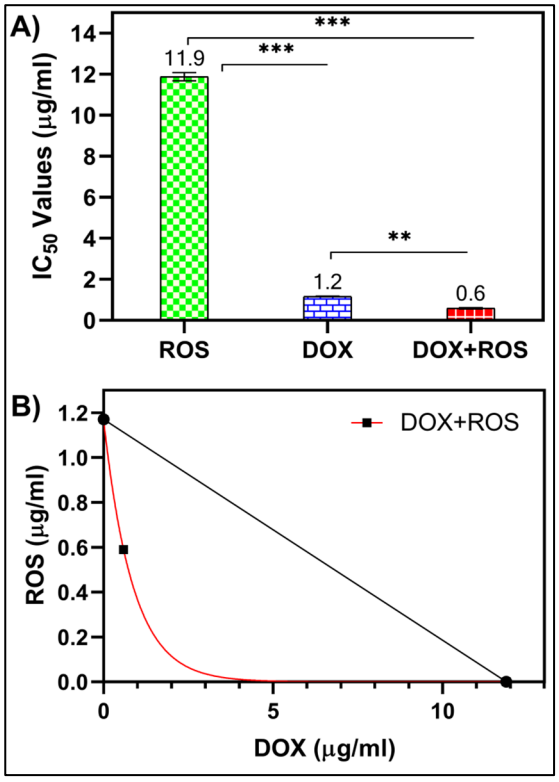
In the next step, we test the effect of ROSM on the DNA to confirm the apoptotic effect of ROS. For this reason, we add 12.5 µg/ml ROSM on MCF7 cells and incubate the cells for 24h. As positive control, we compare the ROSM treated cells, with cells treated with 2µg/ml DOX, as negative control we incubated the MCF7 cells with medium only. As shown in fig 1B, in the MCF7 cells treated with ROSM, the rate of DNA fragmentation was significantly raised (+++) compared to control cells, however the DNA fragmentation in cells treated with DOX as positive control was higher (++++).

DNA damage using the comet assay

To confirm that the increased toxicity is caused by apoptosis, we used another assay, and we further studied this effect using a comet assay. This method allowed the confirmation that breast cancer cells treated with rosemary for 24h experienced an increase in the level of cellular DNA damage. Images obtained using fluorescent microscopy showing the formation of comets in MCF7 cells treated with ROSM showed that DNA double-strand breaks were being induced in the cells (fig. 2). When compared to the non-treated cells, there was a notable rise in comet-positive cells after the ROSM treatment, whereas in DOX treated cells, the comet-positive cells higher.

ROSM/DOX synergistical effect

Chemotherapy-resistant tumor cells are a consequence of anticancer drug treatment, particularly for breast cancer-related tumor cells. Therefore, the creation of substances that can defeat chemotherapy's drug resistance has very high importance.



A) The IC50 values were calculated from the best-fitting curves, and B) The isobologram was drawn to indicate the synergism. The data are from triplicate assay and are expressed as mean ± SD, *** and **, indicating the significance difference of P<0.001 and P<0.01, respectively.

Fig. 3: ROSM/DOX synergistic effect, The MCF7 cells were incubated with increased doses of ROSM, DOX, or both (DOX+ROSM).

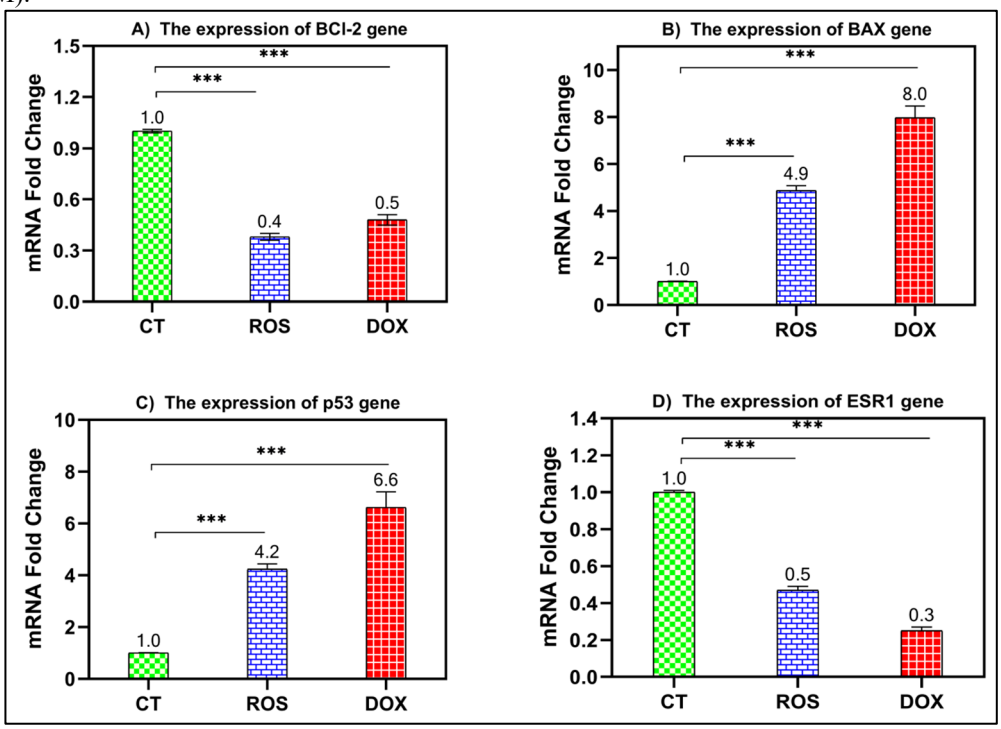


Fig. 4: Fold change of the mRNA expression of Bcl-2, Bax, TP53, and ESR1 genes in MCF7 breast cancer cell line after treatment with ROSM, DOX or control cells (CT). The data are from triplicate assay and are expressed as mean ± SD of fold change, *** for P<0.001.

For this reason, we tested if the combination of ROSM with doxorubicin (DOX) shows a synergistic effect. As shown in fig 3A, the combination of DOX with ROSM synergistically enhanced the DOX cytotoxicity; the IC50 value for DOX was significantly decreased to be 0.59 µg/ml ($P<0.01$). The synergistic interaction between ROSM and DOX was illustrated by isobolgram, whereas the combination value showed down deviation of curve from the straight line of additive to indicate the synergism. Combination index calculation confirm the synergism of the combination CI= 0.63, where CI=1 additive, CI>1 antagonism, and CI<1 synergism

Gene expression analysis

The gene TP53, BAX, Bcl2 and ESR1 are important players in apoptosis to validate the apoptotic impact of ROSM on gene expression. After treatment as described in material and methods, we investigate how the treatment with ROSM influences the proportional expression of the genes in comparison to DOX exposed cells. Using real-time PCR. As we can see in fig 4, exposing cells to ROSM extracts resulted in a marked reduction in the levels of ESR1 and the anti-apoptotic Bcl-2 gene, For ESR1, the expression reached (0.5±0.025) and (0.3±0.004) for ROSM and DOX respectively, whereas for Bcl2 the level reached (0.4±0.03) for ROSM and (0.5±0.021) for DOX. Whereas the expression was increased for the pro-apoptotic genes. For TP53 the expression reached (4.2±0.375) for ROSM and (6.6±0.066) for DOX. Also, the BAX gene was increased; it reached (4.9±0.01) for ROSM and (8±0.014) for DOX.

DISCUSSION

Natural compounds are considered the best alternative for improving anticancer therapy efficacy while causing minimal or no side effects. Plant compounds have long been used to cure diseases, and more than half of the medications in current clinical trials come from natural sources. In early publications, different groups showed anticancer properties of phenolic acids, flavonoids, and diterpenoids such as carnosol and carnosic acid (Abdellatif and Alsharidah, 2023). Rosemary has demonstrated anticancer properties in multiple *in vitro* studies employing colon cancer cell lines (Abdellatif *et al*., 2024, Gird *et al*., 2021). In previous studies on rosemary phytochemicals, properties were conducted to determine their anti-carcinogenic potency. The research groups conducted an *in vivo* investigation in which oleanolic acid and carnosic acid (OL, CA) were found to have this property against 7,12-dimethylbenz[a]anthracene (DMBA)-induced cancer induction (Hosny *et al*., 2021). In our results we also found a significant effect of the ROSM extract on the MCF7 cells; the IC_{50} reached 12.5µg/ml (fig 1). This result is in line with previous studies showing IC_{50} values between 8.82-90µg/ml (Moore *et al*., 2016). In one study, the authors used MCF7 ER+, MDA-MB-468 cells revealed that rosemary has an IC_{50} between 26.8 µg/mL and 90 µg/mL (Cheung and Tai, 2007), whereas in other study using MCF7 cells, the authors found an IC_{50} of 24 µg/mL and generally, the published IC_{50} values show a disparity, which could be due to the different extraction procedures utilized to prepare rosemary extract (Yesil-Celiktas *et al*., 2010, Cheung and Tai, 2007).

Based on early research, cancer cells in general and breast cancer particular develop resistance to chemotherapy. For this reason, it's imperative to keep looking for substances that can combat chemotherapy-induced medication resistance. Previous research demonstrated that natural substances can either enhance the effects of other chemotherapeutic medications or operate independently by attacking oncogenes (Jaglanian and Tsiani, 2020, Jaglanian *et al*., 2020). This result was also confirmed in our results as we showed that the application of ROSM extract in combination with DOX led to a synergistic effect (fig. 3).

Nuclear DNA fragmentation, characterized by the development of distinctive ladder segments of 180–200 base pairs and numerous thereof on an agarose gel, constitutes one of the molecular signs of apoptosis (Abdellatif *et al*., 2022). On the other hand, DNA electrophoresis reveals a widespread smear due to random DNA breakage in necrotic cells. Therefore, in order to verify that rosemary-induced cell death was a viable explanation, the DNA gel electrophoresis method was utilized. DNA fragmentation and nuclear condensation are characteristics of late apoptosis (Abdellatif *et al*., 2023). In our results, we found that the toxicity of ROSM extract was mediated via apoptotic effect, which was demonstrated by DNA damage using DNA fragmentation and comet assay (figs. 1 and 2). This is also supported by early studies showing that the use of rosemary decreases the DNA adduct generation through apoptosis and cell cycle arrest (Alexandrov *et al*., 2006). In addition, rosmarinic acid has decreased the amount of DOX-induced strand breaks and the recurrence of micronuclei without inducing genotoxic effects (Furtado *et al*., 2010).

Apoptosis is a very complicated process, and there are different pathways inducing this programmed cell death. Therefore, knowing the cause of apoptosis and anti-cancer effects after treatment with ROSM extract is important. In previous studies, the authors found that polyphenols, which are found in rosemary, decrease cell viability by controlling the PI3K/AKT/mTOR/p70S6K signaling pathway (Mirza-Aghazadeh-Attari *et al*., 2020). This was linked to a marked reduction in cell viability and decreased cell transformation by increasing apoptosis (Cheung and Tai, 2007). In a leukemic cell line, rosemary was demonstrated to reduce serine/threonine-protein kinases1 (AKT1) mRNA and protein expression; this is involved in the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway (Okumura *et al*., 2012), but no Akt activity measurement was given. The levels of ERK2 protein, which are essential for cell division and proliferation, were

unaffected by these cells. Cell cycle arrest inhibits proliferating cells from dividing. In this context, a previous study showed that rosemary has been proven to cause cell cycle arrest in a variety of cancer cell lines (Petiwala *et al*., 2014a) as well as enhance retinoblastoma-related gene 2 (Okumura *et al*., 2012), which governs cell proliferation.

As described above, different studies showed the important role of other genes, including cytochrome c (Tai *et al*., 2012), enhanced expression of Bax and cleaved-caspase 3 (Yan *et al*., 2015, Petiwala *et al*., 2014b), enhanced TP53 and BAX (Kim *et al*., 2016), decreased c-FLIP, and Bcl-2 (Kim *et al*., 2016), and many other mechanisms (Moore *et al*., 2016) as key factors in apoptosis. Another key factor in the breast cancer apoptosis is the ESR1 gene(Heinzelmann-Schwarz *et al*., 2018). Moreover, in recent study from Wang *et al*. 2023, the author defined ESR1 as key target for the active compounds in the seeds of Rheum (Wang *et al*., 2022). In our results, we found that the ROSM extract resulted in a significant reduction of ESR1 compared to control cells (fig 4). Taking into consideration the fact that lower expression of estrogen receptor 1 (ESR1) causes G0/G1 cell cycle arrest and apoptosis via activating downstream pathways, which trigger TP53 leading to apoptosis (Ding *et al*., 2020), we can suggest that ESR1 could also be a key target for the active compounds in the ROSM extract. TP53 and BAX were considerably elevated in ROSM extract-treated cells compared to control cells (fig. 4), supporting this idea. Previous investigations have shown increased expression of TP53 and BAX and reduced Bcl2. fig. 5 summarizes rosemary's anticancer mechanism.

CONCLUSION

Modern research concentrated on developing innovative cancer drugs that target and change cancer-mutated pathways. In vitro research in MCF-7 suggests rosemary polyphenols CA and RA could target signaling molecules and pathways, promoting apoptosis and decreasing cell viability, and enhancing the anticancer effects of another chemotherapeutics. At the molecular level, rosemary reduced MCF-7 ESR-1 expression. Upregulation of Bax and TP53 and downregulation of Bcl-2 were also observed. These molecular data may help find novel anticancer drug proliferation inhibitory targets, reducing dosages and healthy tissue toxicity. In vivo trials in animals and people may be necessary to determine dose levels, delivery routes, active metabolites, and potentially detrimental long-term effects of plant anticancer treatment.

ACKNOWLEDGEMENTS

The authors extend their appreciation to the Deputyship for Research and Innovation, Ministry of Education, Saudi Arabia, for funding this research (project number QU-IF-1-2-1).

Conflict of interest

All authors declare that no conflict of interest

Availability of data and materials

All data generated and/or analyzed during this research study are available from the corresponding author on reasonable request.

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