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# Epigallocatechin3gallate and Simvastatin Inhibit Myeloma Cancer Cells Proliferation by Apoptosis and Cell Cycle Arrest Upregulation

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**Abstract**

Myeloma cancer (MC) is the second most common hematologic malignancy. It represents a clonal disorder of plasma B-cells arising in the bone marrow. Despite the wide advance in treatment options over recent years, the disease remains incurable. The current study was designed to evaluate Epigallocatechin3gallate (ECG) and simvastatin (SVN) anticancer effect on MC cells. MC cells were divided into the control, MC+ECG, MC+SVN, and MC+ECG+SVN groups. MC cells proliferation in all groups was evaluated using cell morphological status, qRT-PCR was used to analyze apoptotic gene expression, the cell cycle was examined using flowcytometry, and biochemical analysis of oxidative and antioxidant mediators was determined. The doses of ECG (100µmol/L) and SVN (0.1µmol/L) induced cytotoxicity in about 60% of myeloma cells. ECG induced apoptotic genes upregulation (P53, poly (ADP-ribose) polymerase 1 (PARP-1), B-cell leukemia/lymphoma 2 (Bcl-2)-associated protein x (Bax), and caspase-3), and anti-apoptotic gene downregulation (Bcl-2). Moreover, the current regimens have a potent effect on cancer cell growth through G2/M arrest, as observed Graphical abstract figure. The current regimens induced oxidative stress downregulation and antioxidant enzymes activity upregulation. In **conclusion**, both ECG and/or simvastatin caused MC cell toxicity. Nevertheless, the synergistic influence of ECG and simvastatin possesses a more potent consequence on minimizing the resistance of tumor cell and killing tumor cells.

**Keywords:** Myeloma cancer cells, ECG, Simvastatin, apoptosis, cell cycle

## 1. Introduction:

The term "myeloma" is commonly used to refer to "multiple myeloma," which has been identified as the second most prevalent hematological malignancy following non-Hodgkin's lymphoma. This type of cancer accounts for 13% of all deaths associated with hematological malignancies **[1]**. It is characterized by the proliferation of malignant plasma cells within the bone marrow, accompanied by the secretion of an abnormal monoclonal immunoglobulin protein. This leads to a host of pathological consequences, including hypercalcemia, renal impairment, anemia, bone lesions characteristic, referred to collectively as CRAB, and immunological impairment **[2, 3]**.

For the last few decades, chemotherapy, using agents directed against symptoms of CRAB and immunological impairment, together with the use of ASCT, has considerably improved the perspective for patients with MM **[4]**. However, most patients treated with chemotherapy suffer a relapse after some time, and further repeated treatments lead to multiple recurrences that give an even greater fillip to the process of MM **[5, 6]**. Clinical trials indicate that ASCT significantly prolongs survival in younger patients. However, this approach is less effective in older individuals and, in some instances, may contribute to worse outcomes in elderly patients **[7]**. Therefore, long-term control of MM is still a big challenge, and the discovery of new agents that could suppress the growth of MM is a potential therapeutic option.

Simvastatin is primarily an anti-dyslipidemic agent that works through the inhibition of the cholesterol biosynthetic pathway (mevalonate) to reduce atherosclerosis of stroke, coronary artery disease, and ischemic heart conditions **[8].** Recently, simvastatin has been discovered as an anticancer agent against myeloma cells. The agent produced cell cycle arrest in G0/G1 and G2/M phases through a mitochondrion-dependent mechanic **[9]**.

Green tea can be considered a natural drink that contains a high amount of polyphenol in it. Polyphenols are known to be the pharmacological constituents of green tea for their strong antioxidant properties. Among all the polyphenols of green tea,

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epigallocatechin3gallate happens to be the most biologically active as well as potent constituent of green tea. In addition, it is also recommended for a wide range of health-promoting advantages and has extensively been studied in preventing and treating a wide range of chronic diseases. These include various forms of cancers, where compounds present within it have shown promise in inhibiting tumor growth and metastasis; cardiovascular diseases, as green tea improves heart health due to its antioxidant properties by way of a reduction in atherosclerosis and other cardiovascular ailments. Green tea has also been associated with benefits relating to obesity in that it enhances fat oxidation, improves sensitivity of insulin, and is of assistance with weight management. It also plays a role in the management of diabetes by regulating blood sugar and improving insulin activity. Neuroprotective and antimicrobial actions have also been reported for green tea, suggesting its potential use in both the prevention and treatment of various infectious diseases **[10-13]**. The main aim of our study is to investigate the combined or individual effects of simvastatin and Epigallocatchin3gallate on myeloma cell lines in vitro.

## 2. Materials and methods

Epigallocatchin3gallate (ECG) and Simvastatin (SVN) were purchased from Sigma Aldrich, USA Support Office.Tissue culture reagents (Fetal bovine serum, RPMI1640 media, Antibiotic-antimycotic mixture, L-glutamine, trypan blue) were bought from Sigma Company, Cairo, Egypt.

### 2.1 Cell lines

The myeloma cell line (MM) and P3NS1 (ATCC) were acquired from the VACSERA Tissue Culture Laboratory located in Cairo, Egypt.

### 2.2 Experimental design

Myeloma cancer (MC) cell lines were carefully cultured and maintained in triplicates across 24 tissue culture flasks to ensure they were adequately prepared for the experimental treatments. Following preparation, the MC cells were categorized into four distinct groups for the purpose of the study:

i. Control group: MC cells as control without any treatment
ii. ECG group: MC cells were treated with a single dose of ECG (100 µmol/l) for 24 hours [9].
iii. CVN group: MC cells were treated with a single dose of simvastatin (0.1 µmol/l) for 24 hours [12].
iv. ECG+SVN group: MC cells were treated with a single dose of ECG (100 µmol/l) combined with a single dose of simvastatin (0.1 µmol/l) for 24 hours.

### 2.3 Cell morphology analysis

Cell viability and death were assayed using a hemocytometer. Thus, 100 µl of the cell suspension was stained with 20 µl of Trypan blue solution (0.25 %), regarded as a vital dye that differentiates between live and dead cells. Following this staining, 10 µl of the resultant stained suspension was carefully withdrawn for cell counting. In the experimental treatment, the cells were exposed to ECG, SVN, and their combinations over a duration of 24 hours.

### 2.4 RNA Extraction and qRT PCR

Total RNA was extracted with great caution by Thermo Scientific RNA Purification Kits according to the detailed manual from the manufacturer (Thermo Scientific Co., USA). The extracted mRNA was then converted to cDNA using a First Strand cDNA Synthesis Kit based on random hexamer primers according to the instructions of the manufacturer (Thermo Scientific Co., USA). In the qRT-PCR analysis, RNA-direct SYBR Green Real-Time PCR master mix will be used: EXPRESS SYBR® GreenER™ qPCR Supermix, universal, Invitrogen™, which allows for amplification and detection of the specific gene sequence.

The sequences of the primers used to target genes such as P53, poly (ADP-ribose) polymerase 1 (PARP-1), B-cell leukemia/lymphoma 2 (Bcl-2)-associated protein x (Bax), and Bcl-2 in mouse models are shown in Table 1 [9]. The major steps in the entire qRT-PCR procedure are an initial denaturation at 95°C for 15 s where the DNA strands are completely separated; followed by annealing at 60°C for 30 s where the primers bind to the sequences that are complementary to them; and finally, extension at 72°C for 30 s where the new DNA strand is synthesized. This process was carried out for 40 cycles to ensure sample amplification occurred. Care was taken to determine the CT values and normalize them against the reference gene GAPDH. Then, the fold changes in the gene expression were calculated based on the $2^{-\Delta\Delta CT}$ method, which gave the relative expression degree of the target gene under different experimental conditions.

**Table 1:** Primer sequences for the under-study genes

| **1** Gene | **2** Forward | **3** Reverse |
|---|---|---|
| **4** P53 | **5** 5'GGCAACTATGGCTTCCACCT3' | **6** 5'AACTGCACAGGGCACGTCTT3' |
| **7** PARP-1 | **8** 5'GTCCAACAGGAGCATGTGCA3' | **9** 5'CCAGCGGTCAATCATACCCA3' |
| **10** Bax | **11** 5'CCAGGATGCGTCCACCAAGA3' | **12** 5'GGTGAGGACTCCAGCCACAA3' |
| **13** Caspase-3 | **14** 5'CAAGTCAGTGGACTCTGGGA3' | **15** 5'CGAGATGACATTCCAGTGCT3' |
| **16** Bcl-2 | **17** 5'TTC GCAGAG ATG TCC AGTCA3' | **18** 5'TTC AGAGAC AGC CAG GAG AA3' |
| **19** GAPDH | **20** 5'CAGGAGCGAGACCCCACTAACAT3' | **21** 5'GTCAGATCCACGACGGACACATT3' |

### 2.5 Cell Cycle Analysis by Flow Cytometry

The detailed instructions of the manufacturer of the Cycle TESTTM PLUS DNA reagent kit were followed to stain the cells with propidium iodide (PI). Cells were analyzed by flow cytometry using a Becton Dickinson FACSCalibur instrument to analyze phases of the cell cycle: treated cells with ECG, SVN, and their combinations for 24 hours. The obtained data were then subjected to the Modfit software, which determined the distribution of cell populations across

different phases of the cell cycle [14]. The cells were then incubated for a duration of 24 hours, trypsinized at a density of $3x10^5$ per well, and washed twice with ice-cold PBS. The cells were fixed in 70% ethanol at a 4°C and left overnight for all experimental groups of MC cell lines. On the next day, the cells were resuspended in PBS, centrifuged, and then stained further with propidium iodide at a final concentration of 50 µg/mL for evaluating the detailed cell cycle. Finally, the FACSCanto-II flow cytometer analyzed stained cells, after which the BD Accuri-C6 Plus software was used to process the data obtained with Biosciences, CA, USA [15].

**2.6 Estimation of oxidative and antioxidant enzyme activity**

The treated cells were collected and washed in ice-cold PBS. The cell pellets were homogenized in 1ml of PBS. The homogenate was centrifuged at 12,000 rpm for 10 minutes at 4°C to separate the supernatant. The supernatant was collected very carefully and stored at -80°C until further analysis. In this study, the levels of the markers of oxidative stress were determined; these include nitric oxide (NO), $H_2O_2$, and lipid peroxidation/malondialdehyde (MDA), while the activities of some vital antioxidant enzymes, such as glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT), were carried out using the specific protocol details provided by the Scientific Company, Biodiagnostic Co., Egypt.

**2.7 Statistical analysis**

Data was processed using SPSS, version 22. For cell viability, the experimental data mean of three parallels at each dose for both cell lines at 24 hours are presented as mean ± standard error of the mean (SEM). The statistical significance was determined as follows: $^{a1}$ $p < 0.001$, $^{a2}$ $p < 0.01$, $^{a3}$ $p < 0.05$ vs. control; $^{b1}$ $p < 0.001$, $^{b2}$ $p < 0.01$, $^{b3}$ $p < 0.05$ vs. ECG group; $^{c1}$ $p < 0.001$, $^{c2}$ $p < 0.01$, $^{c3}$ $p < 0.05$ vs. SVN group by one-way analysis of variance (*ANOVA*) with post hoc multiple comparisons. The mean of in vitro studies represents three replicates ± standard error.

**3. Results**

**3.1 Effect of ECG and/or SVN on morphological status of MC cells**

After treatment with ECG for 24 hours, the viable cell count was decreased. There is a significant difference in viable cell count (TC) in doses 100µmol/l ($P<0.01$), in comparison with the control (Fig. 1). The viability Percentage was significantly changed in 100µmol/l dose ($P<0.05$) and reached 42.52%, as shown in Fig. 1.

Moreover, the 100µmol/l dose of ECG after 24 hours was killing 50.96% of myeloma cells. There was a significant decrease in total cells in SVN and ECG+SVN groups, where the Percentage change in total cell count reached -27% and -55% respectively. It was noted that the effect of SVN, dual therapy group had a significant difference regarding total cell count in comparison to the ECG group ($P<0.05$) (Fig. 1). There were significant differences in the viability Percentage of all groups in comparison to control ($P<0.05$), and the Percent change in viability Percentage reached -43%. -41%, and -71% in ECG, SVN, and Dual therapy groups, respectively, as shown in Fig. 1. The combination therapy of ECG and SVN (D) (dual therapy) showed a more potent effect than a single dose of ECG (B) or Simvastatin (C) ($P<0.01$) as this combination killed 71% of myeloma cells (A).

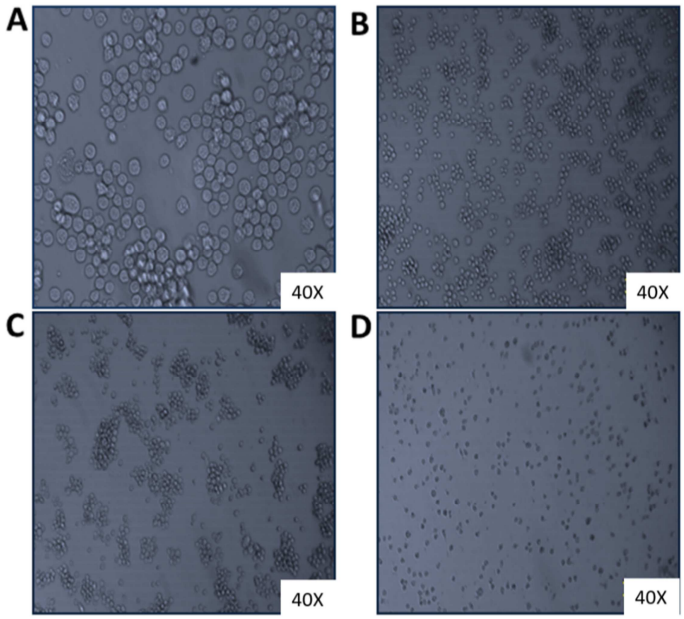


**Figure 1:** The Effect of ECG and/or SVN on MC cells population, total count, and viability (x40); A) MC, B) MC treated with ECG, C) MC treated with SVN, D) MC treated with ECG and SVN (combination therapy). All cells appear without any kind of stains, and the viability was assessed using hemocytometer. ECG: Epigallocatchin3gallate; MC: Myeloma cancer cell line and SVN: Simvastatin.

### 3.2 Effect of ECG and/or SVN on apoptotic gene expression in MC cell line

Relative gene expression was performed in the control and treated groups, and its calculations were done regarding the housekeeping gene (GAPDH-2). The gene expression revealed that the regulated genes of apoptosis mediators P53 and PARP-1 were upregulated in all treated groups, which led to stimuli of the apoptotic genes Bax and Bcl-2 in comparison with the control group. In contrast, the apoptotic genes Bax and Caspase-3 were upregulated; the Bax gene expression was increased up to 15286 fold in the combination therapy group compared to the control. All results of different gene expression and their fold change are shown in (Table 2) and illustrated in (Fig. 2). The gene expression revealed that the Bcl-2 gene expression as the anti-apoptotic gene was downregulated in all treated groups. The cell status and its prognosis toward different regimens of treatment were evaluated through the Bax/Bcl-2 ratio (Table 3), which indicated the cell resistance in control and increased the rate of apoptosis with different regimens of treatment. The direction to apoptosis is very high in combination therapy compared to the single therapy with 10.97, 6.77, and 5 folds in comparison with control, SVN, and ECG groups respectively.

**Table 2:** Fold change of P53, PARP-1, Bax, Caspase-3, and Bcl-2 gene expression in different groups.

| Groups | Relative expression 2-ΔΔCT | | | | | Log relative expression | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | P53 | PARP | Bax | Caspase-3 | Bcl-2 | P53 | PARP | Bax | Caspase-3 | Bcl-2 |
| Control | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 |
| MC+ECG | 5.10 | 1.56 | 781.44 | 5.90 | 0.03 | 0.71 | 0.19 | 2.38 | 0.77 | -1.54 |
| MC+SVN | 7.31 | 5.78 | 242.19 | 2.75 | 0.09 | 0.86 | 0.76 | 2.89 | 0.44 | -1.06 |
| MC+ ECG+SVN | 16.22 | 0.07 | 8659.09 | 38.85 | 0.23 | 1.21 | -1.13 | 3.94 | 1.59 | -0.63 |

Bax: B-cell leukemia/lymphoma 2 (Bcl-2)-associated protein x; ECG: Epigallocatchin3gallate; PARP-1: poly (ADP-ribose) polymerase 1 and SVN: Simvastatin.

**Table 3:** The Bax/Bcl-2 ratio, and the cell status ever resistance to treatment in MC.

| Groups | Bax CT values | Conc. | Bcl-2 CT Values | Conc. | Bax/Bcl-2 Ratio | Cell Status | |
|---|---|---|---|---|---|---|---|
| | | | | | | Resistance | Apoptosis |
| Control | 38.5 | 0.000343 | 32.8 | 0.056314 | 0.00608317 | √ | |
| MC+ECG | 31.7 | 0.043044 | 33.7 | 0.031159 | 1.38143355 | | √ |
| MC+SVN | 28.8 | 0.338174 | 32.9 | 0.05273 | 6.41327703 | | √ |
| MC+ ECG+SVN | 29.2 | 0.254484 | 38.1 | 0.001726 | 147.475542 | | √ |

Bax: B-cell leukemia/lymphoma 2 (Bcl-2)-associated protein x; ECG: Epigallocatchin3gallate; PARP-1: poly (ADP-ribose) polymerase 1 and SVN: Simvastatin.

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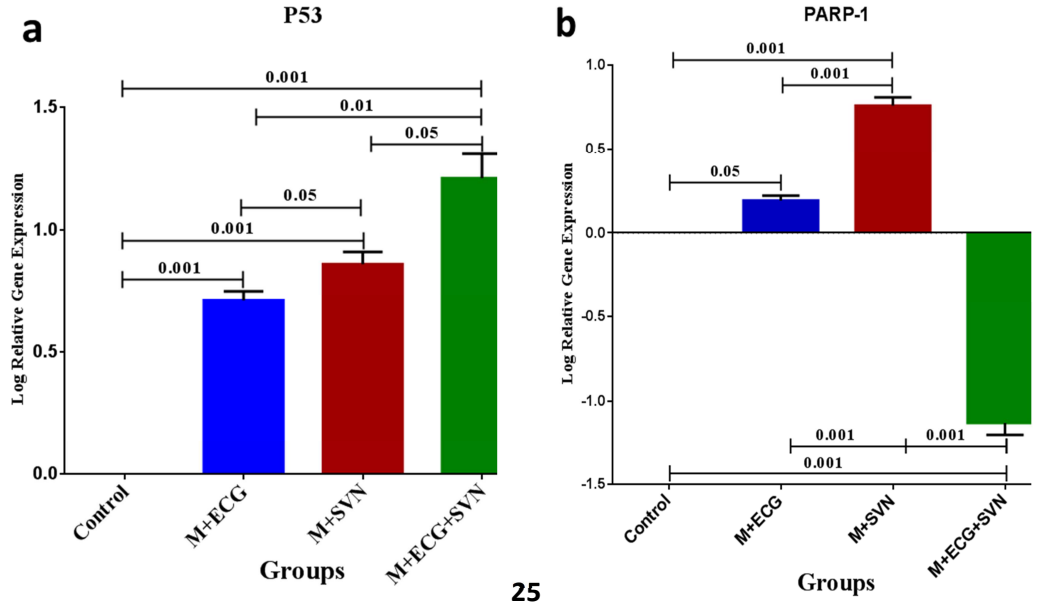


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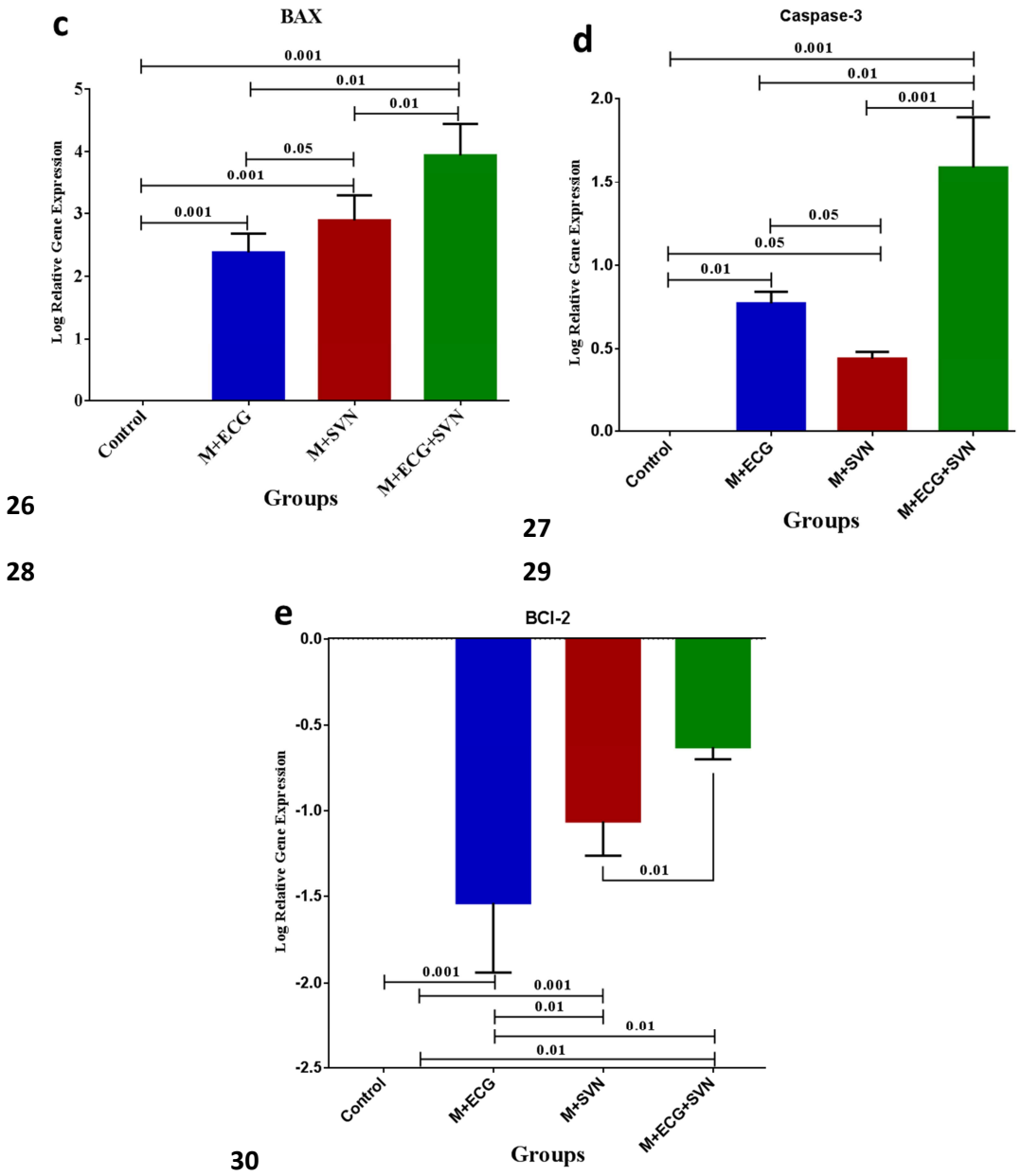


**Figure 2:** The fold of change in P53, PARP-1, Bax, Caspase-3, Bcl-2 and gene expression in control and treated groups. qRT-PCR was used to quantify gene expression in early and late apoptotic cells. The data are expressed as mean ± SEM, significant at $p < 0.001$, $p < 0.01$, $p < 0.05$, using one-way *ANOVA* with post hoc multiple comparisons. *ANOVA:* analysis of variance; Bax: B-cell leukemia/lymphoma 2 (Bcl-2)-associated protein x; ECG: Epigallocatchin3gallate; MC: Myeloma cancer cell line; PARP-1: poly (ADP-ribose) polymerase 1; SEM: standard error of the mean and SVN: Simvastatin.

### 3.3 ECG and/or SVN-induced cell cycle arrest and DNA fragmentation in human MC cells

Detailed flow cytometry analysis, investigating both cell cycle progression and DNA fragmentation, SubG1 phase, was performed in an attempt to elucidate further the mechanism through which ECG exerts its anticancer effects and enhances the response to SVN in MC cells. From the obtained results presented in Fig. 3a and 3b, untreated control cells were located mainly at the S phase. However, after the treatment with either ECG or SVN alone, a remarkable cell cycle distribution change appeared, with lots of cells accumulating in both the G2/M phase, indicative of cytotoxicity through cell cycle arrest, and in the sub-G1 phase, associated with apoptotic DNA fragmentation. It suggests that the single treatment of either of them leads to the arrest of cell cycle and apoptosis induction in the cells. More noticeably, with the combined treatments of ECG and SVN, the accumulation of cells in the G2/M phase was even larger, including a considerable increase in the sub-G1 population. These not only indicate that such agents arrest the cell cycle more effectively at the G2/M phase but also increase the degree of DNA fragmentation and apoptosis to a far higher degree than either treatment alone. These findings point toward a possible synergistic effect of ECG and SVN in initial inhibition of cell division and induction of cancer cell death in human MC cells.

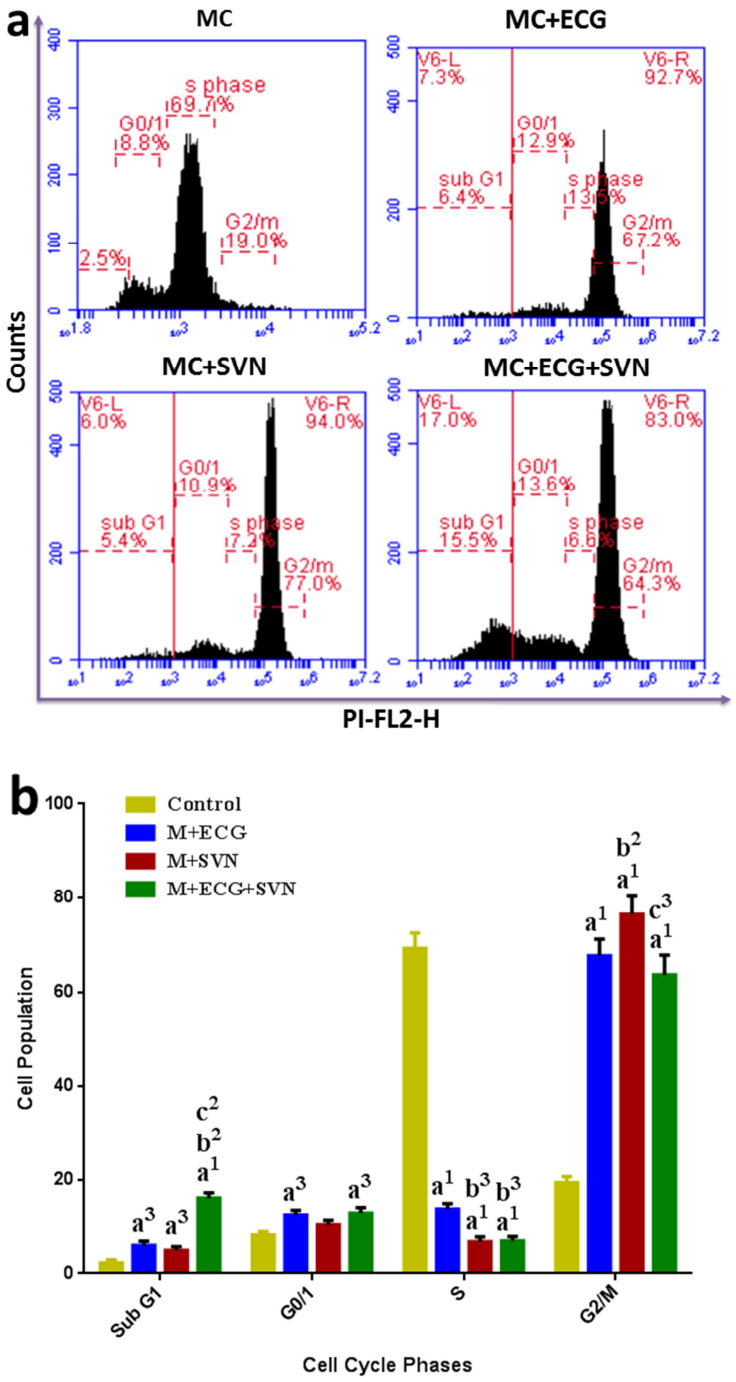


**Figure 3:** ECG and/or SVN-induced cell cycle arrest and DNA fragmentation in MC cells. Representative images (a and b) showing MC cell cycle distribution, emphasizing G2/M phase arrest following 24-hour treatment with ECG and/or SVN formula compared to the negative control. The data are expressed as mean ± SEM, with statistical significance indicated as follows: a1p < 0.001, a2p < 0.01, a3p < 0.05 vs. Control group; b1p < 0.001, b2p < 0.01, b3p < 0.05 vs. ECG group; c1p < 0.001, c2p < 0.01, c3p < 0.05 vs. SVN group, using one-way *ANOVA* with post hoc multiple comparisons. *ANOVA:* analysis of variance; ECG: Epigallocatchin3gallate; MC: Myeloma cancer cell line; SEM: standard error of the mean and SVN: Simvastatin.

### 3.4 Effect of ECG and/or simvastatin on oxidative stress (OS) markers

Malondialdehyde, nitric oxide, and hydrogen peroxide are some of the most important components of OS assessed and calculated in different groups under study in myeloma cells. There are different changes in OS levels depending on the type of treatment (Fig. 4a-c). To verify the mechanism of ECG and /or SVN inhibitory effect on OS, we tested the expression of MDA, NO, and $H_2O_2$, after treatment with ECG and /or SVN for 24 h in human MC cells. Accordingly, the treatment either with ECG or SVN remarkably decreased the MDA, NO, and $H_2O_2$ levels compared to controls. Importantly, the inhibition of oxidative stress was much higher in SVN-treated cells compared to the ECG-treated cells. To

further elucidate the mechanisms by which ECG enhances the sensitivity of MC cells to SVN, the comparative combined effects of ECG and SVN on MC cells were estimated (Fig. 4a-c). Treatment with ECG + SVN powerfully down-regulated the levels of MDA, NO, and $H_2O_2$ than all treatments.

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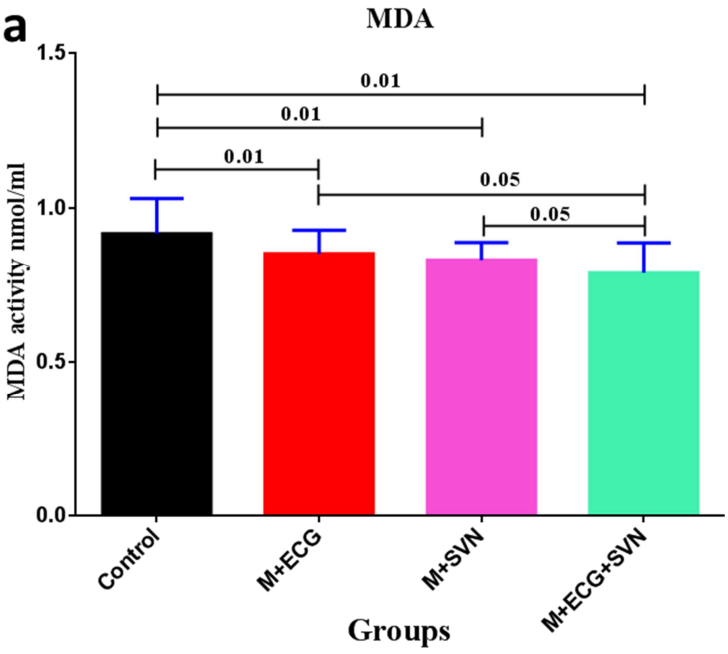


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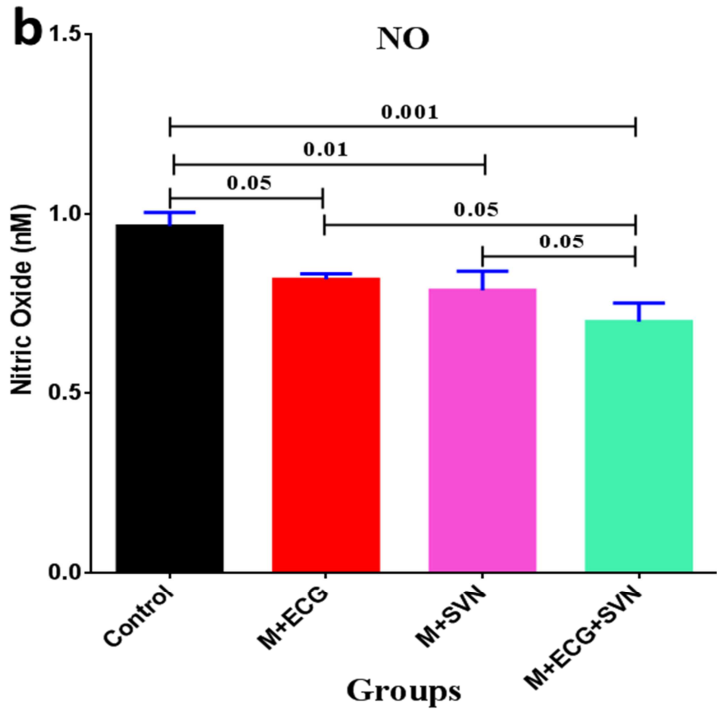


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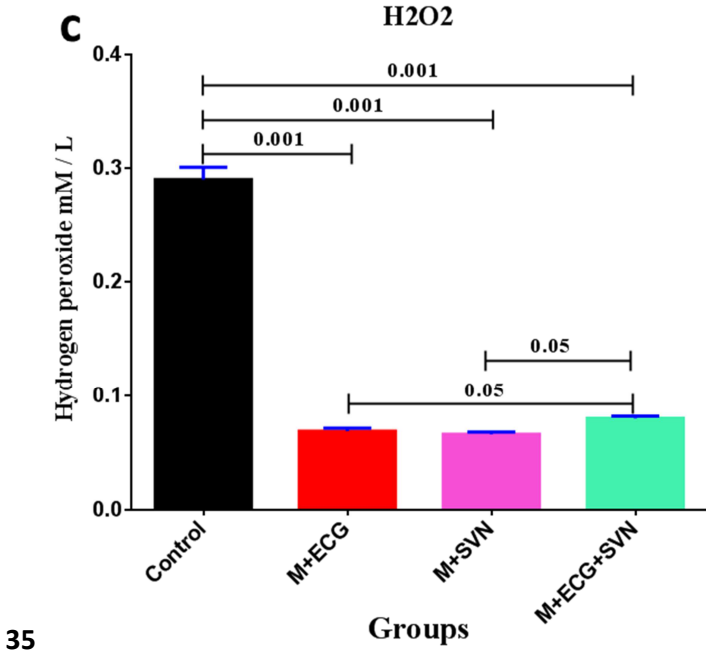


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**Figure 4:** Expression of MDA, NO, and $H_2O_2$ in MC cells upon treatment with ECG and/or SVN. **(a)** The expression level of MDA in MC cells. (b) Level of NO in MC cells. The expression ratio of $H_2O_2$ in MC cells. The data are expressed as mean ± SEM, significant at $p < 0.001$, $p < 0.01$, $p < 0.05$, using one-way *ANOVA* with post hoc multiple comparisons. *ANOVA:* analysis of variance; ECG: Epigallocatchin3gallate; MDA: malondialdehyde; MC: Myeloma cancer cell line NO: nitric oxide; SEM: standard error of the mean and SVN: Simvastatin.

### 3.5 Modulatory effect of ECG and/or SVN on antioxidant parameters

To gain deeper insights into how the combined treatment of MC cells with ECG and SVN promotes anticancer effects and enhances chemosensitivity, the alternation of expression of key antioxidant enzymes, namely SOD, GPx, and CAT, was examined. Both untreated and treated cells were collected after 24 hours of treatment, followed by mediator analysis. As shown in (Fig. 5a-c), SOD, GPx, and CAT expressions have improved significantly in ECG-treated cells as compared to all groups except for the level of GPx in SVN-only treated group. Increased expression is also noticed when ECG-treated cells were compared to the untreated group.

Moreover, the combined treatment with ECG and SVN significantly raised the expression of SOD, GPx, and CAT compared with the control group. However, while comparing with the combination treatment group, no significant change in the SOD levels from the SVN-alone group were observed. Moreover, a very significant decrease in GPx expression and an enhanced increase in CAT expression has been found in the combination treatment groups comparing to the SVN-alone group.

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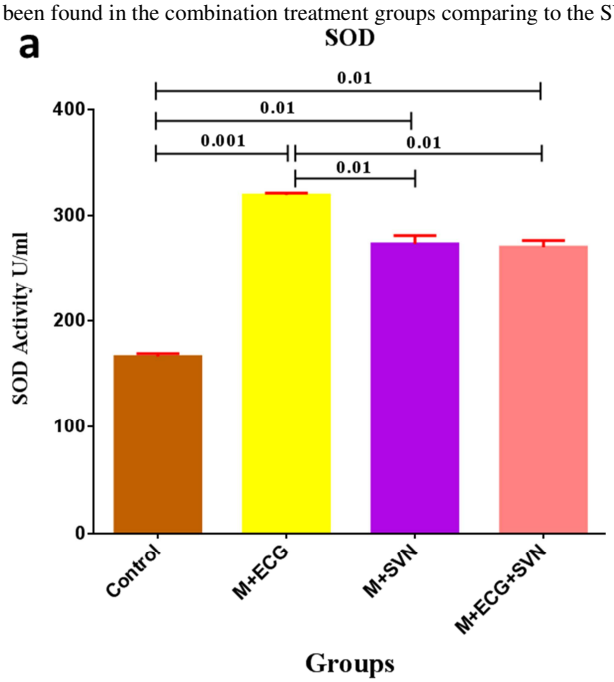

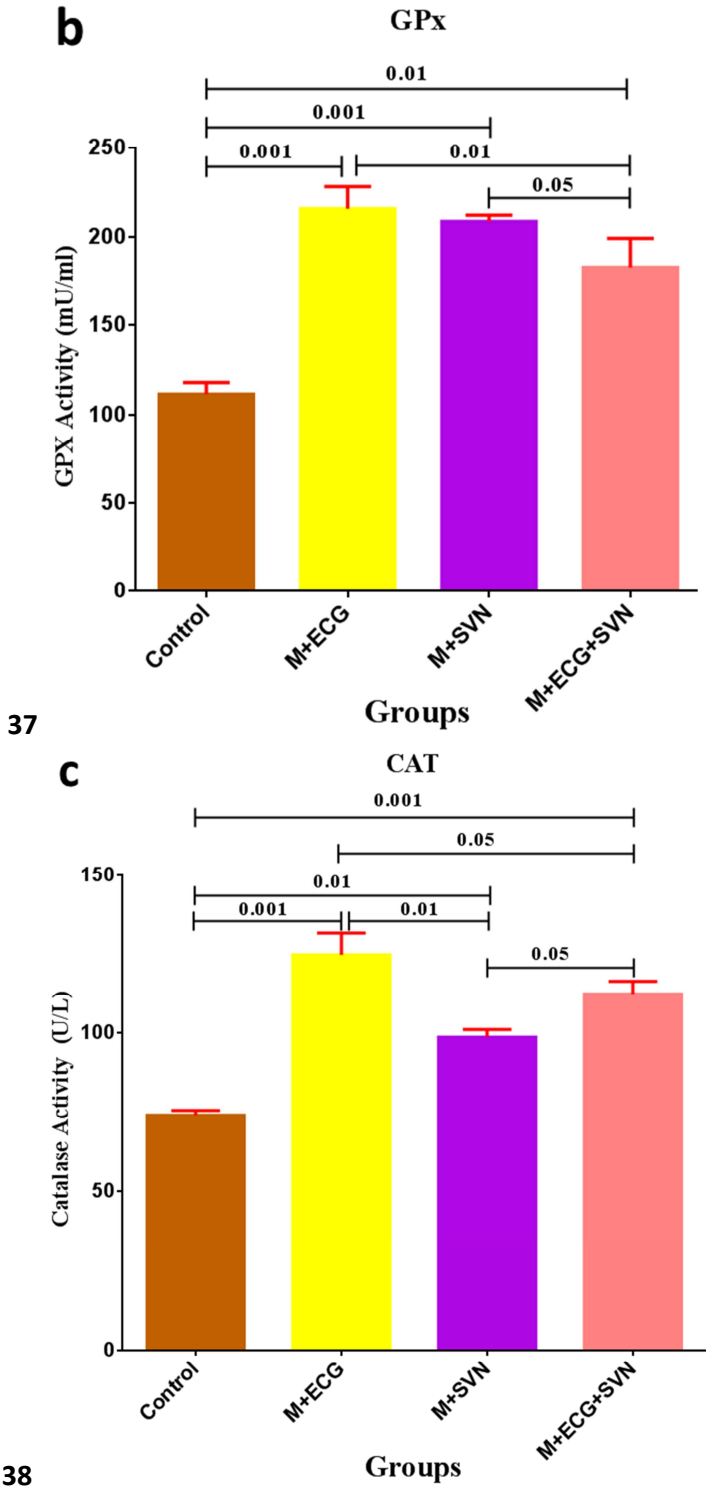


**Figure 5:** The impact of ECG and/or SVN on antioxidant levels in MC cells. **(a)** The enzyme expression of SOD in MC cells. (b) Enzyme level of GPx in MC cells. The enzyme expression ratio of CAT in MC cells. The data are expressed as mean ± SEM, significant at $p < 0.001$, $p < 0.01$, $p < 0.05$, using one-way *ANOVA* with post hoc multiple comparisons. *ANOVA:* analysis of variance; CAT: catalase; ECG: Epigallocatchin3gallate; GPx: glutathione peroxidase; MC: Myeloma cancer cell line; SEM: standard error of the mean; SOD: superoxide dismutase and SVN: Simvastatin.

**4. Discussion**

Cancer, among other diseases, is said to be one of the major life-threatening diseases in the world globally**[16]**; its global burden has been estimated to rise by year 2030. It was estimated that there would be about 21.6 million new cases of cancers and 13 million cancer-related deaths in the year 2030 **[17]**. Increasing cancer mortality and morbidity are due to many factors; as individual's genetic variation, increasing many carcinogenesis factors, poor quality of life and loss of healthy awareness about healthy nutrition besides anticancer drug resistance **[18, 19]**.

The drug resistance development is the main reason for cancer treatment failure by using different chemotherapy regimens **[20]**. Therefore, the current goals are to find a drug or a treatment regimen that can attack cancer cells directly and kill them as a cytotoxic drug without affecting the healthy tissues. The cancer cell resistance towards some anticancer regimens can be concluded in many factors as individuals genetic variations, drug metabolism and absorption, changes that may occur during drugs migration from the oral cavity to the tumor area, targeting of the drug, barriers that prevent the drug from arrival to its target, and drug accessibility and linkage on the tumor cell membrane to start its action mechanism **[21, 22]**. Combination therapy between two anticancer agents is more powerful and reduces the cancer cell resistance, but its side effects on the normal cells remains, especially with combination by high doses **[23, 24]**.

In the current study, the cell line was used as an example for cancer models; P3NS1 myeloma cell lines. Two treatment types were used; ECG and simvastatin. Green tea has a powerful antioxidant polyphenol (30% of dry weight) **[25, 26]**, and the most polyphenols content is ECG which represents about 10% of leaves' dry weight **[27]**. ECG was used with a selected dose according to a previous study by *Ibrahim Abdelrahman et al.* (100µmol/l) against myeloma cells after 24 hours. Simvastatin was used with different doses in the previous studies on the myeloma cell line, and it was very effective and induced programmed cell death by arresting cell growth at G0/G1 and G2/M phases when treated with 0.1µM of simvastatin for 24 hours **[9]**.

Total cell count was reduced in the primary studies with a selected dose of ECG for 24 hours. This dose of ECG killed around 60% of myeloma cells confirms the antitumor activities of ECG. These results agreed with many research articles findings where they used ECG (10 µM) against MC cells for 48 hours, and their findings approved the antitumor activity of ECG, and they stated that the reduction in cell count and viability is associated with the modulation of cytokine mainly for TNF-α and IL-6 **[28, 29]**. Others used ECG (20 mg/kg b.wt.) in combination with approved anti-cancer, doxorubicin, to decrease their side effect and increase their killing tumor cells potency **[30]**.

Regarding the mechanism of ECG as an antitumor through gene expression, the current results approved apoptotic genes upregulation; P53, PARP, Bax and Caspase-3 with log relative expressions and anti-apoptotic gene downregulation; Bcl-2 in both myeloma and after the calculation of Bax/Bcl-2 ratio confirming the direction of apoptosis under the ECG effect in myeloma cells. These agreed with previous studies findings; Caspase-3 and PARP genes upregulation on prostate cancer **[31]**, and it induced Bcl-2 downregulation and Bax gene expression upregulation in breast cancer **[32]**.

Simvastatin is one of statins different types which act as an anti-hyperlipidemia through its effect on the cholesterol synthesis process rate-limiting enzymes, hydroxymethylglutaryl CoA reductase (HMGCR) (mevalonate pathway) **[33]**. Many in vitro preclinical studies used simvastatin as an anticancer drug against different cell lines; myeloma cell line **[9]**, colon cancer (HCT-116, KM-12), ovarian cancer (IGROV1, OVCAR3), breast cancer (HS-578T, T47D), lung cancer (HOP-92, NCI-H322 M), prostate cancer (PC-3, DU-145) **[34]**, moreover simvastatin exert anti-proliferative and pro-apoptotic effects in different cancer cell lines with varying sensitivity. Our results showed that simvastatin upregulates pro-apoptotic Bax gene expression and downregulates Bcl-2 gene expression. These observations are consistent with actions of simvastatin described in other cell types such as MCF7 human breast cancer cells, SAEC human normal small airway epithelial cells, HepG2 human hepatocellular carcinoma cells and NCI-N87 human gastric cancer **[35, 36]** but these are on the contrary with its effect on normal guinea pig brain cells, so they recommended it as a neuroprotective in normal cases **[37]**.

Various treatment regimens induce their effects on programmed cell death through the impact on the cell cycle, concomitantly causing cell-cycle arrest at the G2/M checkpoint. This dual effect is quite important because it arrests the cancer cells at a phase of the cell cycle which prevents the advancement of cells into mitosis, inhibiting growth and inducing apoptosis. Several approved chemotherapeutic agents induce G2/M arrest in glioma cells, which is associated with the activation of Chk1 and the phosphorylation of cdc25; such agents include temozolomide **[38, 39]**. Similarly, silibinin-derivative of milk thistle has been recently reported to enhance doxorubicin mediated G2/M arrest modulating key G2/M cell cycle regulators **[40]**.

As for the mechanisms by which ECG and simvastatin reduce tumor aggressiveness, including that of prostate and breast carcinomas, ECG acts through antioxidant activity **[26, 41, 42]**, as well through 20S proteasome inhibition. The result is an increased expression of proapoptotic proteins, including IκB and Bax, each of which act to inhibit NF-κB **[43]**. Further, ECG has antiangiogenic **[44, 45]** and anti-inflammatory action **[46]**. On the contrary, simvastatin inhibits cholesterol synthesis and coenzyme Q, also known as ubiquinone, an enzyme that is necessary in tumor growth **[9, 47].**

According to our results regarding the cell viability, ECG killed myeloma and EC cells through its potency in induction the OS in vitro where NO and hydrogen peroxide percent change was increased in cells; 33% and 134%, respectively in myeloma cells. Together, the change in the level of antioxidant enzyme activities was detected where the GPx was downregulated to reach -52%, in myeloma cells. Our results are agreed with many research articles and review articles findings that assure the role of blocking tumor progression and suppressing metastasis through increasing reactive species concentration in tumor area **[48]**. The change in the intracellular reactive species level even high or low, causes cancer cell survival rate reduction **[49]**.

The current results clearly indicated that cell cycle arrest of myeloma cells was induced by ECG at the G2/M phase effectively, which corroborated the findings from various other studies across different cancer types. In this regard, the growth inhibitory action of ECG has been documented on gastric cancer cells, SGC-7901, where cell cycle arrest was induced at G0/G1 phase besides inducing apoptosis **[50]**. Similarly, it was stated that ECG has been found to affect the cell cycle to cause G0/G1 phase arrest in A549 non-small-cell lung cancer cells **[51]**, as well as in the case of prostate cancer cells **[52]**. However, in other studies, the cell cycle arrest for ECG was seen at the G2/M phase in breast cancer cells **[53]**.

This will indicate the varied cell cycle arrest in different types of cancer and also illustrate the diverse mechanisms of ECG's anticancer action. Its influence may even vary with the type of cancer cell line and tumor kind under investigation.
Related to the in vitro effect of simvastatin, it is clear that its anticancer properties were through increasing OS, decreasing antioxidant enzyme activities, upregulating pro-apoptotic gene expression, inhibiting anti-apoptotic gene expression and finally induced cell cycle arrest at G2\M phase. After showing the results of each type of treatment under study singly, the effect of the combination of each regimen will be illustrated on each type of cell lines under study. Regarding the combination therapy was recorded significant decrease in cell viability that confirmed the excellent synergism between ECG and simvastatin. The mechanism of action of this synergism was confirmed with some measurements. The first was the detection of OS intracellularly of MC; the percent change of NO and $H_2O_2$ increased in dual therapy in comparison to the single treatment of each. On the contrary, the antioxidant enzyme activity of SOD, GPx, and CAT recorded significant elevation compared with a single treatment. Moreover, OS production under the effect of the current regimens confirmed their role in killing MC cells in vitro where increasing OS through increasing reactive oxygen species (NO and $H_2O_2$) play a very important role in various physiological functions in the cells and help in some changes in signal transduction, cell cycle regulation, regulation of gene expression of apoptotic and antiapoptotic genes. For instance, it has been noted that the $H_2O_2$ free radical, since it maintains no ionic charge, can thus freely diffuse across cellular membranes, inducing possible intracellular macromolecular injury **[54].** In addition, $H_2O_2$ reacts with transition metal ions, particularly iron, to form the highly reactive hydroxyl radical (HO•) via the Fenton reaction, which further enhances oxidative stress and injury in cells **[55]**. The previous findings were agreed with others who reported that the role of simvastatin in increasing OS in breast cancer cell lines **[56]**, lung cancer **[57]**, prostate cancer **[58]** and squamous cell carcinoma **[59]**. $HO^{\bullet}$ toxic effects include DNA base damage and strand breaks, lipid peroxidation, and amino acid residues modification of numerous proteins **[60]**. Any changes at the level of cells or body take place under the control of genes, the effect of dual on the gene expression where they increase apoptotic genes expression (P53, Bax, and Caspase-3), increasing PARP gene expression which is a first responder that detects DNA damage **[61]** and increases tumor suppressor gene P53 expression preventing tumor cells from growth **[62].** Simultaneously, the dual therapy regimes downregulate Bcl-2 gene expression, which has the main role of anti-apoptotic regulation in cancer cells. In myeloma cells, the Bax/Bcl-2 ratio represents the high efficacy of dual therapies in comparison to untreated control and a single treatment with ECG, simvastatin. These results showed the highest efficacy and role of combination therapy to decrease cell resistance toward anticancer treatment.

## 5. Conclusions

In conclusion, each ECG and/or simvastatin induced MC cell toxicity. However, the synergistic effect between ECG with simvastatin has a more powerful effect in killing tumor cells and reducing tumor cell resistance through increasing the Bax/Bcl-2 ratio, caspase-3 and cell cycle arrest through $P53_{high}$, $PARP\text{-}1_{high}$ without the combination, $Antioxidants_{high}$, and oxidative stressors in vitro, as observed in graphical abstract figure. More investigation will be needed to be performed on the in vivo model to detect the safety of combination therapy on the animal model and its role if it is used before tumor incidence (graphical abstract).

## Conflicts of interests

Authors declare that there are no known conflicts of interest, financial or personal, under the terms which might be regarded as having influenced the interpretations and conclusions presented in this paper. No personal relationships or associations exist that might have appeared to influence the impartiality, neutrality, and integrity of the research and its results. All means were taken to ensure that the work was performed individually and free of any potential influences.

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