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EVALUATING COMBINED EFFECT OF NARINGIN AND SALICYLIC ACID ON COLON CANCER CELL CULTURE

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ABSTRACT

Aims: Colorectal cancer is the 3rd most common cancer in the world. It affects more than a million people and causes the death of half million people annually. Flavonoids are natural products belonging to plantae and some fungal organisms that recently have started to be popular for cancer research for its strong antioxidant, anticarcinogen and anti-tumor properties. Naringin is a special chemical compound of flavonoid groups in grapefruit and useful for its antioxidant and immunostimulatory properties. Salicylic acid is a stress-specific hormone that also has an anti-tumor effects on colorectal cancer. In this study, it is aimed to evaluate the effect of naringin, salicylic acid and their combination on colon cancer cells via gene expression profiles of apoptosis genes and anti-proliferative properties.

Methods: HT29 colon cell culture was incubated in 37 C° and 5% CO_2. Salicylic acid, naringin and their combinations were applied seperetaly on 80% confluent cells in 11 different doses starting with 800 µM and going half of the previous. MTT survival test was performed at 24th and 48th hours after application. To see the effect on apoptosis and antioxidant pathway; apoptotic protease activating factor, B-cell lymphoma 2, B-cell lymphoma 2 associated X, B-cell lymphoma 2 - XL, Cytochrome C, Cellular inhibitor of apoptosis protein 1, Cellular inhibitor of apoptosis protein 2, Glyceraldehyde-3-phosphate dehydrogenas, Caspase 3, Livin, Survivin, p21, p27, p53 and X-linked inhibitor of apoptosis, Catalase, Glutathione peroxidase, Superoxide dismutase 1 and Superoxide dismutase 2 gene expressions were assayed on 24th and 48th hours by using real time PCR.

Results: Single and combined application of naringin and salicylic acid decreased cell proliferation at both 24th and 48th hours. Results in 48th hours were more obvious. None of the applications caused an increase in number of cells in any applied dose. In the real time PCR analysis, the expressions of apoptosis inhibitor genes that play a crucial role in antioxidant pathway were increased. The increase was more distinct in the combination of naringin and salicylic acid.

Conclusion: In this study, it is found that both salicylic acid and naringin cause a decrease in the number of colon cell culture. As for their combination it also worked well. The increase in apoptotic gene expression was exclusive. It can be said that naringin, salicylic acid and especially their combination can be a promising treatment as a supported option for colon cancer patients in the future.

Keywords: Colon cancer, salicylic acid, cell culture

INTRODUCTION

In 2013, colorectal cancer was the 3rd most common cancer and 4th leading cause of death by cancer in the world (1). It affects more than a million people and causes the death of half million people annually (2). In Europe, there are 250000 cases diagnosed annually and five-year relative survival rate is about 65 percent, however it may vary based on the state of the cancer (2). Therefore, some precautions should be taken in order to reduce the risk of colorectal cancer such as dietary modifications because it is suggested in many articles that dietary compounds may have a role in reducing the rate of colorectal cancer and additionally digestive tract is in direct interaction with dietary components (1, 3).

Flavonoids are compounds which have beneficial health properties such as anti-oxidant, anti-viral, anti-al-

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lergic, anti-inflammatory and antitumor activities by scavenging the free radicals. They are excessively found in vegetables and fruits such as citrus fruits (3, 4). Furthermore, some earlier animal model based experiments showed that fruits and vegetables have protective effects against colon cancer (4). Citrus fruits have abundant chemopreventive bioactive compounds such as flavonoids (4). Naringin is a citrus flavonoid found in grapefruit, lemon, orange and it is useful due to its anti-tumor, anti-inflammatory, anti-oxidant and anti-hypercholestrolemic activities (5).

Not only naringin is associated with reducing the risk of developing colon cancer, but also salicylic acid is found out to be effective in diminishing the risk of colon cancer (6). It has been suggested that salicylic acid plays a role in decreasing the synthesis of pro-inflammatory and potential neo-plastic prostaglandins, also increases the apoptosis (6).

As a conclusion, it is suggested that naringin and salicylic acid both have anti-carcinogenic effects in colon cancer. However, the effect of combination of naringin and salicylic acid is not investigated. In addition, the mechanisms behind such as gene expressions are not completely found out either. Therefore, the aim of this study is to evaluate the effect of naringin, salicylic acid and their combination on the number of colon cancer cells and examine gene expressions belonging to apoptosis pathway.

MATERIAL AND METHODS

Cell cultures and reagents

A cell line of colorectal adenocarcinoma HT-29 (ATCC® HTB-38™) were purchased from the American Type Culture Collection (ATCC, Rockville, Maryland, USA). The cell culture materials HAMS F 12, Dulbecco's modified Eagle's medium (DMEM) and L-glutamine, foetal bovine serum (FBS were supplied by Gibco (Thermo, USA). PBS, trypsin EDTA, dimethyl sulfoxide (DMSO), yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). The PureLink® RNA Mini Kit and High Capacity cDNA Reverse Transcription Kit were supplied from Life Technologies (USA). Ethanol, ultrapure water (LiChrosolv® Reag) were purchased from Merck-Millipore (Darmstadt, Germany).

HT29 colon cell culture (ATCC® HTB-38™) was incubated in 37 oC in a CO_2 incubator with a humidified atmosphere of 5% CO_2. When the cells grow enough salicylic acid, naringin and their combinations were applied on them in 11 different doses starting with 800 µM and going half of the previous. In 24th and 48th hours, cell viability was measured.

Cell viability (MTT assay) and treatments

In this study, the cell viability was measured by MTT assay. In this method, HT29 cells were sown in a 96-well sterile microplate at a density of approximately 5000-7500 cells/well in 180 µl of medium 12 h before treatment. The plates were incubated in a 5% CO_2 incubator at 37 °C for 12 h for the cells to attach well. After incubation, the cells were treated with 20 µl of naringin, salicylic acid and their combinations prepared at eleven concentration levels between 1.6 - 800 µg ml-1. After 24 and 48 h incubation MTT solutions (20 µl/200 µl per well of a 5 mg/ml solution) were pipetted in each well and the plates were incubated for 4 h at 37 °C. The blue formazan crystals were dissolved in DMSO (200 µl/well) and the absorbance was measured at 490 nm with a Thermo Multiscan Go Microplate Reader Spectrophotometer (Thermo Scientific, USA). The measurement of absorbance for each concentration of naringin, salicylic acid and their combinations were compared with the control.

Quantitative real-time PCR (qRT-PCR) analysis

Expression levels of the mitochondrial apoptosis genes: apoptotic protease activating factor 1 (*APAF 1*), B-cell lymphoma 2 (*BCL 2*), BCL2 associated X (*BAX*), BCL- XL, Cytochrome C (*CYC C*), cellular inhibitor of apoptosis protein 1 (*c-IAP 1*), cellular inhibitor of apoptosis protein 2 (*c-IAP 2*), Glyceraldehyde-3-Phosphate Dehydrogenas (*GAPDH*), Caspase 3 (*CASP3*), Livin, Survivin, p21, p27, p53 and X-Linked Inhibitor of Apoptosis (*XIAP*); and antioxidant genes: catalase (*CAT*), Glutathione Peroxidase (*GPX*), Superoxide Dismutase 1 (*SOD1*) and Superoxide Dismutase 2 (*SOD 2*), HT29 cells of control and 100 and 200 µM of naringin, salicylic acid and their combinations for 24 and 48 hours were analysed by qRT-PCR using the SYBR® Select Master Mix (Life Technologies, USA) on an ABI 7500 Real-Time PCR system with the primer pairs and PCR condition (Table 1). Gene expressions were determined as the relative fold change compared to the control and normalized with GADPH mRNA expressions. The comparative cycle threshold (2-ΔΔCt) method (User Bulletin 2, Applied Biosystems, CA) was performed to analyze the expression levels of mRNAs.

Table 1: Primer pairs of genes (7)

GENE	*PRIMERS*
APAF-1	F: 5'-GATATGGAATGTCTTCAGATGGCC-3' R: 5'-GGTCTGTGAGGACTCCCCA-3'
BCL 2	F: 5'-ATGTGTGTGGAGAGCGTCAA-3' R: 5'-ACAGTTCCACAAAGGCATCC-3'
BAX	F: 5'-TTCATCCAGGATCGAGCAGA-3' R: 5'-GCAAAGTAGAAGGCAACG-3'
BCL XL	F: 5'-GTAAACTGGGGTCGCATTGT-3' R: 5'-TGGATCCAAGGCTCTAGGTG-3'
CYC C	F: 5'-AGTGGCTAGAGTGGTCATTCATTTAC-3' R: 5'-TCATGATCTGAATTCTGGTGTATGAG-3'
c-IAP 1	F: 5'-GCATTTTCCCAACTGTCCAT-3' R: 5'-ATTCGAGCTGCATGTGTCTG-3'
c-IAP 2	F: 5'-GCATTTTCCCAACTGTCCAT-3' R: 5'-ATTTTCCACCACAGGCAAAG-3'
GAPDH	F: 5'-AATTCCGATCTTCGACATGG-3' R: 5'-GAAAAAGCGGCAGTCGTAAT-3'
CASP 3	F: 5'-GGTATTGAGACAGACAGTGG-3' R: 5'-CATGGGATCTGTTTCTTTGC-3'
Livin	F: 5'-TGGCCTCCTTCTATGACTGG-3' R: 5'-ACCTCACCTTGTCCTGATGG-3'
Survivin	F: 5'-GACGACCCCATAGAGGAACA-3' R: 5'-GACAGAAAGGAAAGCGCAAC-3'
p21	F: 5'-GGCGTTTGGAGTGGTAGAAA-3' R: 5'-GACTCTCAGGGTCGAAAACG-3'
p27	F: 5'-CCGGCTAACTCTGAGGACAC-3' R: 5'-TGGATCCAAGGCTCTAGGTG-3'
p53	F: 5'-CACGAGCGCTGCTCAGATAGC-3' R: 5'-ACAGGCACAAACACGCACAAA-3'
XIAP	F: 5'-GGGGTTCAGTTTCAAGGAC-3' R: 5'-TGCAACCAGAACCTCAAGTG-3'
CAT	F: 5'-TACGAGCAGGCCAAGAAGTT-3' R: 5'-ACCTTGTACGGGCAGTTCAC-3'
GPX	F: 5'-TGGGACCAGCAAGTAAAACC-3' R: 5'-TCGCGAATGTAGAACTCGTG-3'
SOD 1	F: 5'-GTTCGGTGACAACACCAATG-3' R: 5'-GGAGTCGGTGATGTTGACCT-3'
SOD 2	F: 5'-TCTGAAGAAGGCCATCGAGT-3' R: 5'-GCAGATAGTAGGCGTGCTCC-3'

RESULTS

Due to MTT analysis, the doses of 100 ml and 200 ml were chosen to be applied to tumor cells. Naringin, salicylic acid and their mixture had decreased the number of cancer cells on both 24th and 48th hours. Results in 48th hours were better. None of those caused an increase in number of cells in any applied dose.

In order to investigate the effects of naringin, salicylic acid and their mixture on antioxidants and apoptosis pathway, 100 and 200 µM of the molecules were studied in the real time PCR analysis. All of the results of gene expressions can be seen in Figure 1 and 2.

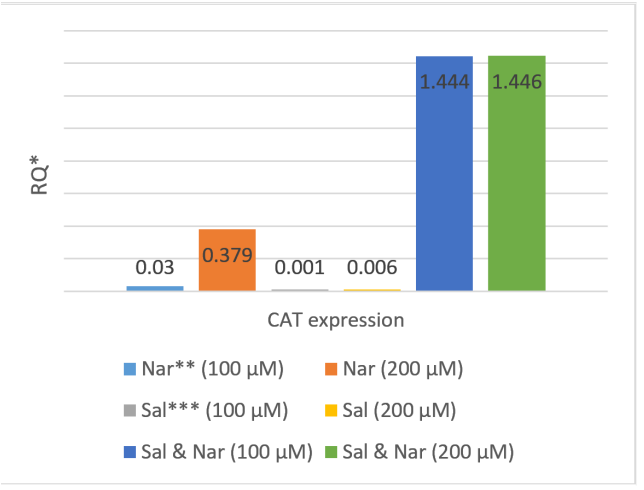

Figure 1.1: CAT expression

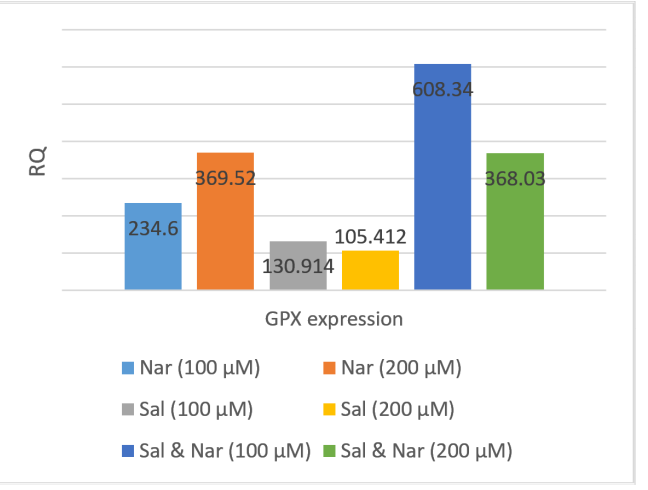

Figure 1.2: GPX expression

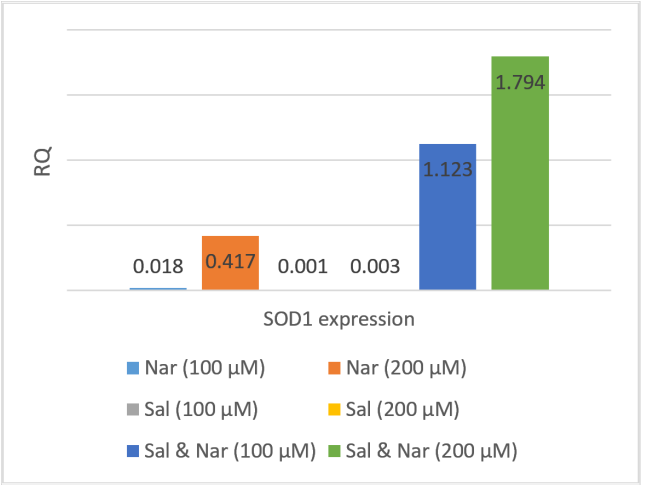

Figure 1.3: SOD1 expression

Figure 1.4: SOD2 expression

Figure 1: Expression levels of the mitochondrial apoptosis genes
(*Relative quantity unit)
(**Naringin)
(Salicylic acid)***

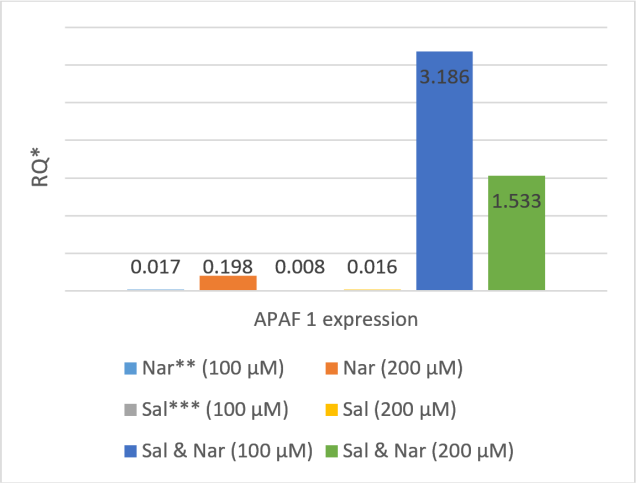

Figure 2.1: APAF 1 expression

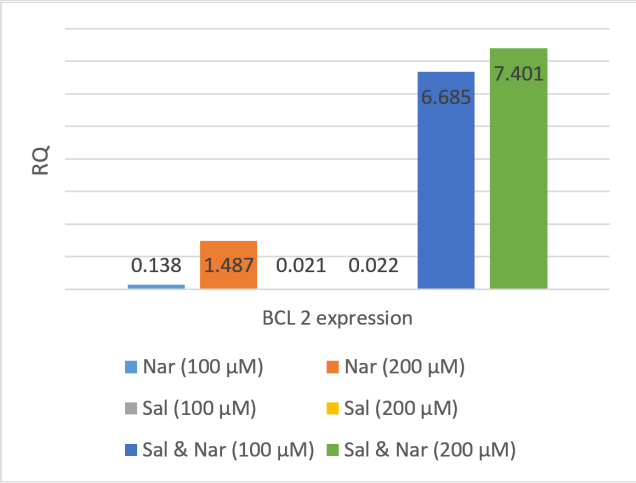

Figure 2.2: BCL2 expression

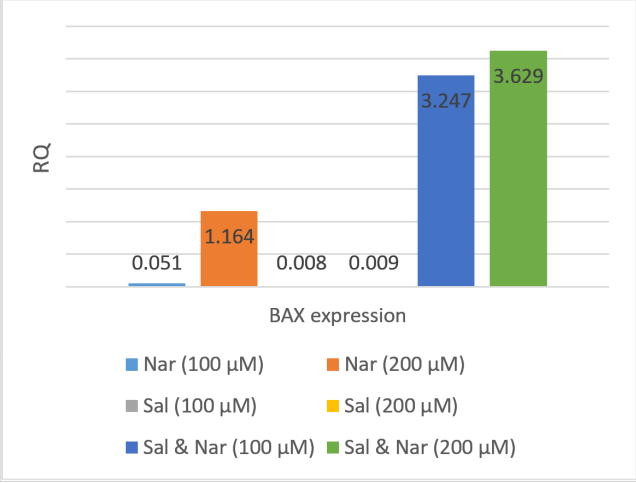

Figure 2.3: BAX expression

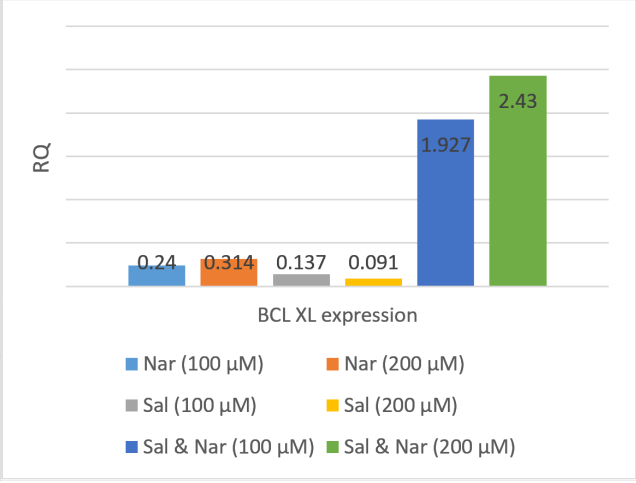

Figure 2.4: BCL XL expression

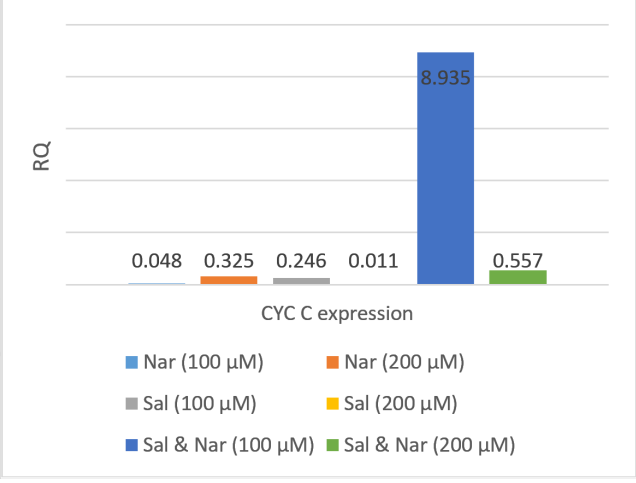

Figure 2.5: CYC C expression

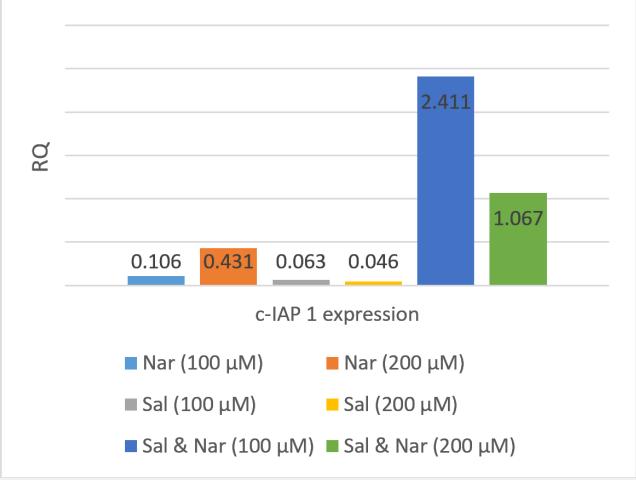

Figure 2.6: c- IAp1 expression

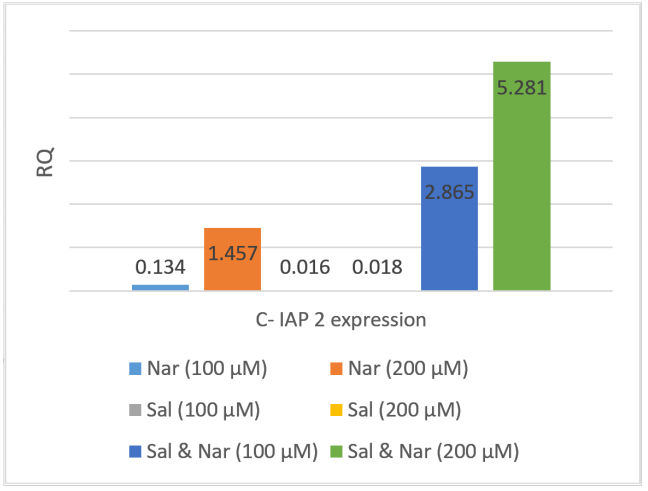

Figure 2.7: c- IAP 2 expression

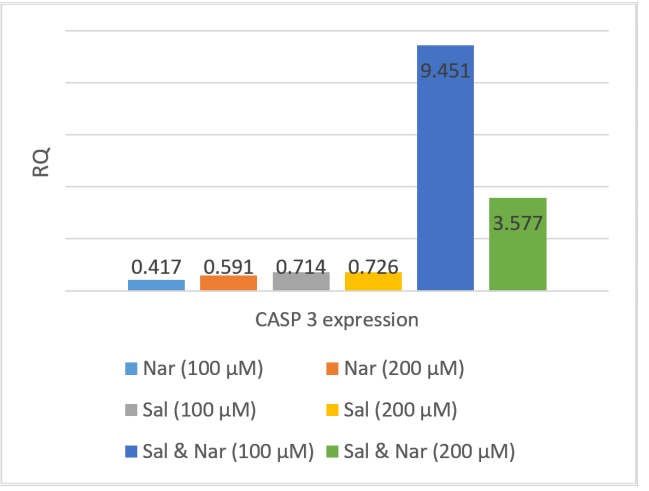

Figure 2.8: CASP 3 expression

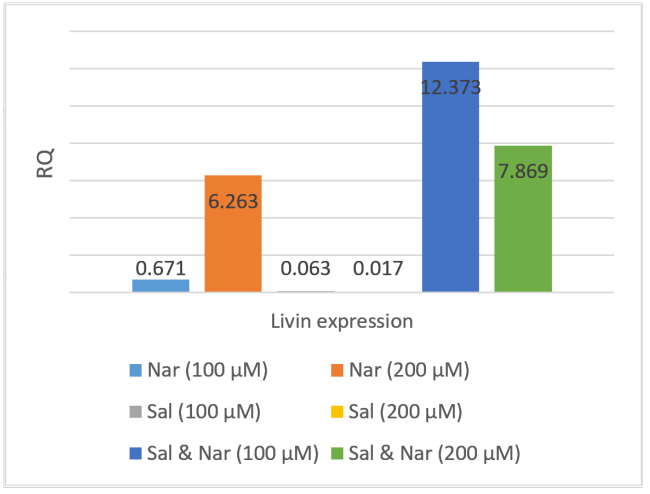

Figure 2.9: Livin expression

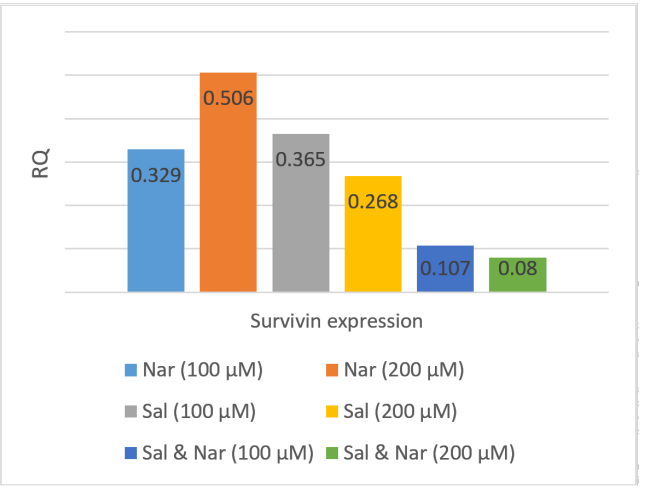

Figure 2.10: Survivin expression

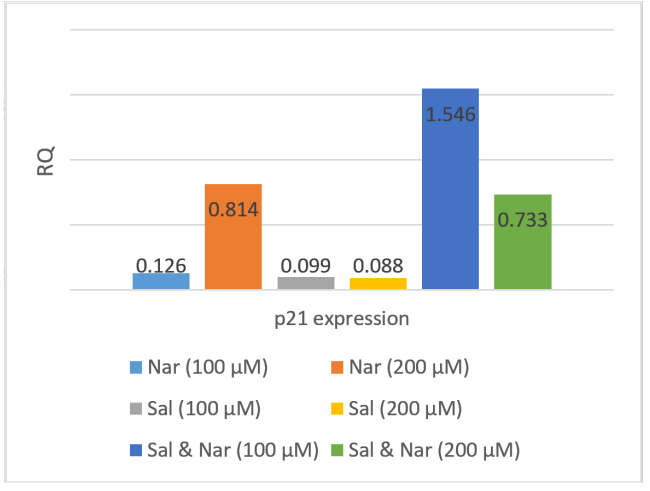

Figure 2.11: p21 expression

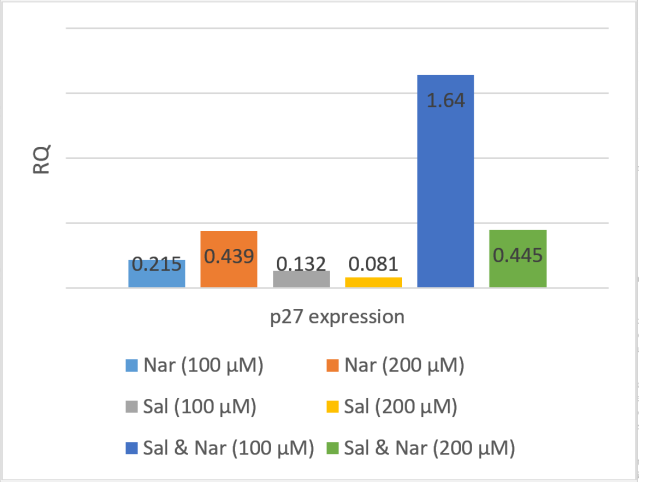

Figure 2.12: p27 expression

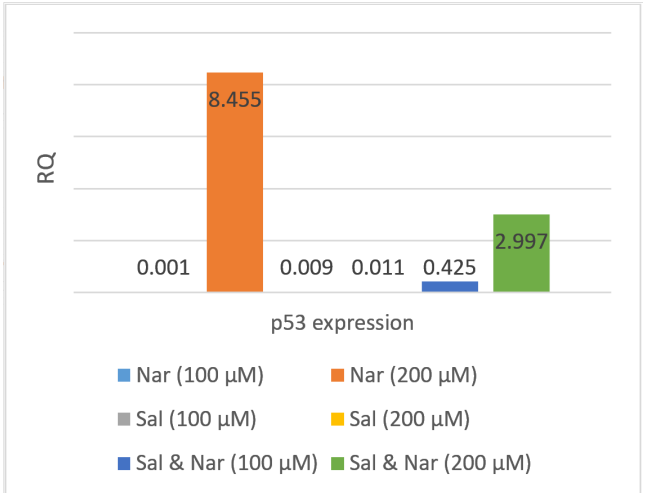

Figure 2.13: p53 expression

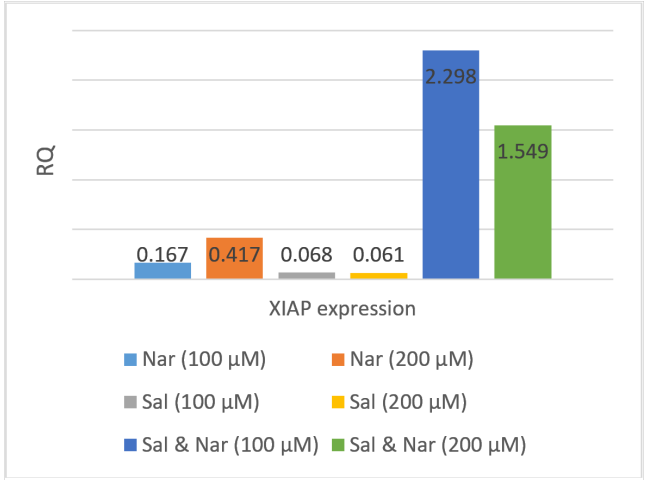

Figure 2.14: XIAP expression

Figure 2: Expression levels of the mitochondrial apoptosis genes
(*Relative quantity unit)
(**Naringin)
(Salicylic acid)***

DISCUSSION

It is known that both naringin and salicylic acid have anti-carcinogenic effects on colon cancer (5, 6). However, the effect of two materials combined had not been investigated. In this study, it is found that both salicylic acid and naringin cause a decrease in the number of colorectal adenocarcinoma cells. The combination of naringin and salicylic acid also caused a decrease.

In addition, the results of MTT assay were better in 48th hour than in 24th hour. That shows that naringin and salicylic acid affect cancer cells more efficiently in this time period. However, 48 hours are also not enough to make a conclusion which supports the idea that naringin and salicylic acid affect more efficiently in longer periods.

The increase in apoptotic gene expression was exclusive. The expressions of *APAF 1*, *BAX*, *BCL2*, *BCL XL*, *CASP3*, *p21*, *p27* and *p53* increased. *p53* have a major importance in maintaining genome stability and integrity. It can lead cell cycle arrest and provide DNA repair. In addition, if the repair of the damage is not possible, then *p53* causes cell death via apoptosis (8). *BAX* is a cell death promoter protein (9, 10). As it has been suggested in the previous studies, the expressions of *p53* and *BAX* are higher in cancer cells than normal cells (8, 9, 10). It is also showed in this study. Therefore, these results are compatible with the literature. *APAF 1*, *CASP3*, *p21* and *p27* are also apoptosis inducer genes (11). Their expressions were increased especially in 200 µM concentration of naringin and salicylic acid combination. Therefore, the mixture of the molecules affects better. The increase in CASP3 is also an indicator of damaged mitochondrial potential. *CYC C* is an important component of electron transport chain (11). The expression of *CYC C* increased especially in 100 µM concentration of naringin and salicylic acid combination. This indicates that mitochondrial membrane potential is damaged. The ratio of *BAX*/ *BCL2* is important while evaluating the mitochondrial membrane potential. The increase of these genes is distinct in the combination of naringin and salicylic acid. In addition, they do not increase proportionally. Therefore, it also indicates a damaged mitochondrial membrane potential.

Survivin is an apoptosis inhibitor gene (11). The expression of *survivin* especially decreased when treated with the combination of naringin and salicylic acid. When the concentration is increased from 100 µM to 200 µM, the decrease became more distinct. This shows that not only the combination of the molecules causes an increase in apoptosis inducer genes, it also causes a decrease in apoptosis inhibitor genes such as *survivin*.

The expression of genes which are members of antioxidant pathway such as *CAT*, *GPX*, *SOD* and *SOD2* were also studied. *CAT* plays a role in protection against oxidative stress (11). The increase in the expression of *CAT* indicates oxidative stress. The increase in *GPX* is also an indicator of oxidative stress, because *GPX* is a protector of cell against oxidative damage. The increase in *SOD2* is more than *SOD1*. This shows that naringin and salicylic acid are more effective on the expression of *SOD2*. *SOD1* and *SOD2* both destroy free superoxide radicals. *SOD1* converts free radicals to molecular oxygen and hydrogen peroxide whereas *SOD2* converts to diatomic oxygen and free radicals (11). *SOD1* is cytosolic whereas *SOD2* is mitochondrial (11). Therefore, it can be concluded that naringin and salicylic acid are more effective on mitochondrial antioxidant pathway. However, the expression of *SOD2* decreased when it is treated with 200 μM of the combination. This is due to the mitochondrial damage which causes distraction and dysregulation of protein damage.

Although the study has reached its aims, time is a limitation factor because the results after 48th hour were not investigated. It needs to be examined in the further studies in order to explain the long period effects of naringin and salicylic acid.

As a result, it can be said that naringin, salicylic acid and especially their combination can be a promising treatment as a supported option for colon cancer patients in the future.

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Informed Consent: N/A
Conflict of Interest: The authors declared no conflict of interest.
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