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Preventive impact of Soybean and black seed extracts against colon cancer progression through stimulation of apoptosis

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Abstract

Colorectal cancer is considered one of the most occurring cancers leading to death. Apoptosis is defined as a programmed cell death controlled by different gene families via intrinsic and extrinsic pathways. Soybean and black seed have been found to possess anti-proliferative, anticancer, and anti-chemo-resistant properties. In this study, we assessed the preventive impact of soybean and black seed extracts different concentrations and their mixture against HT-29 colon cancer cell line through apoptosis using flow cytometry, DNA fragmentation assays and expression of six genes related to apoptosis. Flow cytometry analysis of the mixture of soybean and black seed extracts showed the highest total apoptosis (early and late apoptosis) and low necrosis percentages when compared to cisplatin and black seed extract at IC_{50}. DNA fragmentation increased significantly among the groups treated with the mixture of the extracts with the highest fragmentation percentage. Expression analysis revealed a significant downregulation of P53, BIRC5, BCL2L1 and XIAP genes and a significant upregulation of Bax genes. Whereas FAS gene was expressed without any significant difference in all groups compared to control. The mixture demonstrated the most significant difference in each gene among the groups. As a result, mixture exhibited pro-apoptotic effect through different analyses.

Keywords: Soybean (*Glycine max)*; black seed (*Nigella sativa)*; HT-29 colon cancer; Apoptosis; DNA fragmentation; apoptotic gene expression.

1. Introduction

The colorectal cancer is considered the second type of malignancy leading to death all over the world [1]. Apoptosis is a programmed cell death controlled by different genes which has an important role in maintaining normal function through cysteine protease activation and leading to morphological changes including nuclear concentration and membrane vesiculation [2]. If apoptotic signals do not reach to damaged cells, the disturbance between cell division and cell death occur leading to cancer incidence [3]. The critical disadvantage of cancer therapies including chemotherapy and radiotherapy is its side-effects including postoperative local recurrence and distant metastasis [4] in addition to a further damage for patient's health. Therefore, focusing on alternative treatments derived from natural resources is a globally top priority demand [5].

Soybean (*Glycine max*) contains highly valuable constitutes of proteins, vitamins in addition to many essential amino acids which have the biological impact and correlated with the decreasing frequency of different malignant disease [6]. Also, soybean supplementations were confirmed as agents to help in improving the efficacy and prevention of harmful side effects of cancer chemo- and radio therapies [7]. Isoflavones and genistein are the most active soybean components which have strong effects on malignant cells through epigenetic factors like DNA methylation, histone acetylation, and cell growth inhibition in addition to their effect as anti-metastasis, anti-inflammatory and antioxidant agents [8, 9].

Black seed (*Nigella sativa*) is an important nutrition agent used as a natural remedy for diseases in ancient systems of medicine [10]. Many active compounds having pharmacological, anti-inflammation and antioxidant effects are isolated from *N. sativa* seed including thymoquinones, nigellicines, phenolic acids and flavonoids [11, 12]. Anticancer properties of black seeds are mainly attributed to thymoquinone which is the major active

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constituent of the seed. The anti-proliferative, antioxidant and anti-mutagenic effects of thymoquinones on different cancer cell lines were reported [13].

n colon and blood cancer cell lines by 74% and 80%, respectively [14] while in HCT116 colon cancer cells, crude saponin extract of *Nigella sativa* and thymoquinone exhibited cell growth arrest [15, 16]. Moreover, soybean components showed an antiapoptotic effect through DNA fragmentation such as Genistein in colon cancer [17] and soybean lectin in HeLa cervical cancer cells [18]. Additionally, black seed components such as *Nigella sativa* extracts exhibited apoptotic DNA fragmentation effect on HeLa cell line [19].

This work aims to assess the preventive impact of soybean and black seed against colon cancer progression through apoptosis evaluation using flow cytometry, DNA fragmentation and expression of certain apoptotic genes in HT-29 colon cancer cell line.

2. Materials and Methods

2.1. Plant extraction

Black seed was purchased from local market, Egypt, while soybean (Giza-111 genotype) was purchased from Field Crops Research Institute (FCRI), Giza, Egypt. Both were crushed to be used for maceration and extraction by 70% ethanol. Identification of different constituents was determined by Gas Chromatography/Mass Spectrometry (GC/MS) (Agilent Technologies 7890B, USA), mass spectrometer detector (5977A, USA), using pyridine and *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide and carrier gas was Hydrogen and by High-Performance Liquid Chromatography (HPLC) (Agilent 1260 series, USA) using water and 0.05% trifluoroacetic acid in acetonitrile solution [20].

2.2. Cytotoxicity

To determine the half-maximal inhibitory concentration (IC_{50}) and the optimum mixture concentration, different concentrations of each plant extract were prepared by dissolving in DMSO. The concentrations used were 250, 500, 1000, 2000, 4000 µg/ml for soybean extract, and 60, 125, 250, 500, 1000 µg/ml for black seed extract as well as 0.01, 0.1, 1, 10, 100 µg/ml for cisplatin. Cisplatin (Sigma) was used as a positive control. HT-29 colon cancer and Human Skin Fibroblast (HSF) cell lines were obtained from Nawah Scientific Inc. (Cairo, Egypt). HT-29 cells were kept in RPMI-1640 media, whereas HSF were kept in DMEM with supplementation with streptomycin, penicillin and fetal bovine serum in humidified 5% CO_2 atmosphere at 37°C.

The cell viability was measured by Sulforhodamine B (SRB) test [21]. An aliquot of cell suspension ($5x10^3$ cells) was used as a negative control whereas the other aliquots were cultured in media containing various tested concentrations of soybean, black seed extracts or cisplatin for 72 hours. The absorbance was measured at 540 nm [21, 22]. The dose response curve was prepared, and IC_{50} was calculated by Graph pad prism (ver. 8.4.2 for windows) using log inhibition vs normalized response method in both extracts whereas inhibition vs normalized response method was used in cisplatin. IC_{50} is the concentration that causes 50% growth reduction of the cells, IC_{25} is calculated as half the value of IC_{50}. The Ns_{50} and Ns_{25} are the concentrations of black seed at IC_{50} and IC_{25}, respectively whereas, Gm_{50} and Gm_{25} are the concentrations of soybean at IC_{50} and IC_{25}, respectively. The mix is a mixture of Ns_{25} and Gm_{25}. The cell line HSF was used to determine the cytotoxic effect of the mix, Ns_{25} and Gm_{25} on non-cancerous cell line.

Several studies have recognized the antiproliferative activity of Soybean and black seed in different cancer cell lines. Soybean isolated peptide inhibited cell proliferation i

2.3. Apoptosis assessment using Flow cytometry

Apoptosis and necrosis cell populations were evaluated using the Annexin V-FITC apoptosis detection kit (Abcam Inc.). HT-29 cell line (10^5 cells) were treated for 48 hours with soybean extract at 1233 µg/ml (Gm_{50}), 616 µg/ml (Gm_{25}); black seed (*Nigella sativa*) extract at 928 µg/ml (Ns50), 465 µg/ml (Ns25); mixture of both extracts (Gm_{25}+Ns_{25}) and cisplatin at 20 µg/ml as positive control. After treatment with the test compounds, cells were harvested, washed and stained - with Annexin V-FITC/PI solution -, injected - via ACEA NovoExpress™ flow cytometer -, analyzed and quantified via quadrant analysis and calculated using ACEA NovoExpress™ software (ACEA Biosciences Inc.) according to the protocol by Mohamed *et al* [23]. The quadrants Q1, Q2, Q3 and Q4 represent necrosis phase, late apoptosis, normal intact cells and early apoptosis, respectively.

2.4. DNA fragmentation

DNA fragmentation is used to determine the apoptotic effect through the DNA gel electrophoresis laddering assay which shows multiples of 180-200 bp of DNA fragments due to caspase-activated DNase enzyme activity. The diphenyl reaction procedure is used for quantitation of the DNA fragmentation.

2.4.1. DNA gel electrophoresis laddering assay

Approximately, 1×10^6 cells were plated and treated with various treatments. HT-29 cell line samples, treated with different treatments, were lysed in extraction buffer overnight at 37˚C then

lysate was incubated with DNase-free RNase A for 2 hours at 37˚C, and extracted three times with phenol/chloroform followed by chloroform-extraction of the aqueous phase by centrifugation at 4°C. The extracted DNA was precipitated by absolute ethanol with sodium acetate for 1 hour at -20°C. After washing with ethanol, the DNA pellet was air-dried and dissolved in Tris–HCl/1 mM EDTA. The DNA was electrophoresed on ethidium bromide (EtBr)-stained 1.5% agarose gel in TAE buffer. A 50-bp DNA ladder was used as a marker and DNA fragments were photographed using UV transillumination [24].

2.4.2. Diphenylamine reaction procedure (Quantitation of DNA fragmentation)

After treatment of the HT-29 cells with the tested substances in different Petri-dishes, the cells were trypsinized, suspended, homogenized in 1 ml of medium and centrifuged. Samples of the colon cancer cell line were lysed in 0.5 ml lysis buffer, centrifuged and the pellets were re-suspended in 0.5 ml of lysis buffer. To the pellets (P) and the supernatants (S), 25% tri-chloroacetic acid (TCA) was added and incubated at 4°C for 24 h. The samples were centrifuged, and the pellets were suspended in 5% TCA, followed by incubation at 83°C for 20 min. Subsequently, freshly prepared Diphenyl Amine (DPA) solution was added, to each sample, and incubated at room temperature for 24 h [25]. The percentage of the fragmented DNA was calculated from the following equation at 600 nm absorbance:

$$\%\, Fragmented\ DNA = \frac{OD_S}{OD_S + OD_P} \times 100$$

(OD: optical density, S: supernatants, P: pellets)

2.5. Gene expression analysis:

2.5.1. RNA extraction and cDNA synthesis:

RNA was extracted from cells using Genezol reagent (Geneaid), according to manufacturer's instructions. RNA concentration was determined using NanoDrop spectrophotometer and the purity of RNA was assessed. Moreover, RNA integrity was assured with the presence of 28S and 18S bands by gel electrophoresis on 1% EtBr-stained agarose gel. cDNA synthesis was performed on extracted DNase I treated RNA. One µl of DNase and 1 µl buffer were added to 1 µg/ml RNA concentration and the volume was completed to 10 µl by DEPC-treated water and incubated at 37°C for 30 min, then 1 µl of EDTA was added and incubated at 65°C for 10 min. The DNase-treated RNA was reverse transcribed into first strand cDNA using "H- cDNA synthesis kit" according to the manufacturer's instructions.

2.5.2. Real-time PCR:

Gene expressions were detected by real-time PCR, which was performed using Rotor-Gene Q system. A 20 µl reaction mixture included 10 µl SYBR Green PCR Master-Mix, 0.5 µl of each primer (10 pmole) (**Table 1)**, 1 µl cDNA and 8 µl RNase free water. The optimal amplification conditions were determined through empirical observation according to each tested gene. The program was 2 minutes at 95°C, 40 x (95°C for 10 seconds, annealing temp. for 45 seconds) and final extension at 72°C for 30 seconds. The mean cycle threshold (Ct) values from triplicate samples are used for analysis. The Ct value signifies the fractional cycle number at which the amplified target reaches a fixed threshold.

Table 1: Primer sequences, annealing temperature and product size of genes used in qRT-PCR analysis.
(*): Housekeeping gene.

Gene	Primer Sequence	Annealing Temperature (˚C)	Product Size (bp)	Accession number
GAPDH* (HKG)	F: AAGGTGAAGGTCGGAGTCAAC R: GGGGTCATTGATGGCAACAATA	60	102	NM_001357943.2
P53	F: TAACAGTTCCTGCATGGGCGGC R: AGGACAGGCACAAACACGCACC	60	121	NM_001126118.2
BAX	F: CCCGAGAGGTCTTTTTCCGAG R: CCAGCCCATGATGGTTCTGAT	60	155	NM_001291430.2
FAS	F: CACCCGGACCCAGAATACC R: TGTTGCTGGTGAGTGTGCATT	61	131	NM_152872.4
BIRC5	F: AGGACCACCGCATCTCTACAT R: AAGTCTGGCTCGTTCTCAGTG	62	118	NM_001012270.2
BCL2L1	F: GAGCTGGTGGTTGACTTTCTC R: TCCATCTCCGATTCAGTCCCT	60	119	NM_001317919.2
XIAP	F: AATAGTGCCACGCAGTCTACA R: CAGATGGCCTGTCTAAGGCAA	60	103	NM_001378592.1

2.6. Data and statistical analysis:

The student's *t* test was used to evaluate the significant differences in gene expression of the tested genes. A one-way ANOVA test was used to assess the significant difference among the groups. An *F*-test was performed to determine whether the groups being tested have equal or unequal variances needed for the *t*-test calculations. Data from real-time PCR were analyzed using the $2^{-\Delta\Delta Ct}$ method [26]. Data were represented as the fold change in target gene expression normalized to GAPDH as a house-keeping gene.

3. Results

3.1. Plant Extraction

Black seed and soybean extracts were analyzed by GC-MS, resulting in 13 compounds for black seed and 24 compounds for Soybean. The major compounds of black seed found were Propanoic acid ethyl ester (30.87%), Glycerol (25.54%) and Linoleic acid ethyl ester (13.62%) whereas for Soybean, the D-Pinitol, pentakis (trimethylsilyl) ether (51.54%) was the principal compound beside myo-inositol (0.61%). Moreover, these extracts were analyzed for polyphenols by HPLC. The major polyphenols for black seed found were Gallic acid, Catechin, Syringic acid, methyl gallate and chlorogenic acid, where the highest polyphenols in soybean found were Kaempferol, Syringic acid, Gallic acid and Taxifolin. Black seed extract was missing Coffeic acid whereas Soybean extract was missing Chlorogenic acid, Catechin, Coffeic acid, Pyro Catechol, Vanillin and Cinnamic acid.

3.2. Cytotoxicity

The SRB cytotoxicity test was performed on HT-29 cell line to determine IC_{50} of each extract as well as the positive control (cisplatin) to choose an optimum mixture concentration and to determine the effect of each treatment. IC_{50} was calculated by Graph pad prism using log inhibition vs normalized response in both extracts whereas inhibition vs normalized response was used in cisplatin **(Fig.1)**. The resulting IC_{50} was 1233 µg/ml, 928.3 µg/ml and 20.05 µg/ml for Soybean (Gm_{50}), black seed (Ns_{50}) extracts and the positive control (Cisplatin), respectively. Also, IC_{25} was calculated as 616 µg/ml for Soybean (Gm_{25}) and 465 µg/ml for black seed (Ns_{25}) as well as the mixture of Gm_{25} + Ns_{25}. The cytotoxicity test SRB was also done on human skin fibroblast (HSF) for IC_{50} of each extract as well as the mixture of Gm_{25} + Ns_{25} to test the cytotoxic effect of these concentrations on HSF normal cell line compared to HT-29 colon cancer cell line. The cytotoxicity test SRB showed a mild toxic effect on HSF at 37.096 %, 69.67 % and 66.58 % cell viability for Soybean, black seed extract and their mixture (Gm_{25} + Ns_{25}), respectively.

3.3. Flow cytometry analysis for apoptosis assessment

Flow cytometry was used to determine early and late apoptosis and necrosis to assess apoptosis as an effect of the different treatments. The toxic effect of the extracts was determined at 48 hours as the optimum time between three different time intervals (24, 48 and 72 hours), which had toxic effects on the HT-29 cell line with the largest apoptotic population. **Fig. 2** shows the flow cytometry charts of different treatment groups in HT-29 cell line. The mixture of IC_{25} of both extracts (Gm_{25} + Ns_{25}) showed a synergistic effect compared to IC_{25} of each extract alone. Total apoptosis percentages (i.e. early apoptosis (EA) and late apoptosis (LA)) were shown in a descending manner as 20.65, 14.93, 9.4, 7.45, 5.71 and 3.55% for Mix, Ns_{50}, cisplatin, Ns_{25}, Gm_{50} and Gm_{25}, respectively. On the other hand, Necrosis percentages were 9.13, 17.17, 7.8, 5.73, 3.68 and 1.62% for Mix, Ns_{50}, cisplatin, Ns_{25}, Gm_{50} and Gm_{25}, respectively (**Table 2**). Thus, these findings indicate that the mixture treatment showed the highest total apoptosis percentage (20.65%) and low necrosis (9.13%) compared to cisplatin (9.4% and 7.8%) and Ns_{50} (14.93% and 17.7%), respectively.

The one-way ANOVA test indicated a significant difference among the different treatment groups for necrosis, Normal, late apoptosis, and early apoptosis with p-values of 1.76×10^{-12}, 2.86×10^{-17}, 2.97×10^{-14} and 3.96×10^{-4}, respectively. The significant difference between the groups with respect to the control was calculated using Student's t-test.

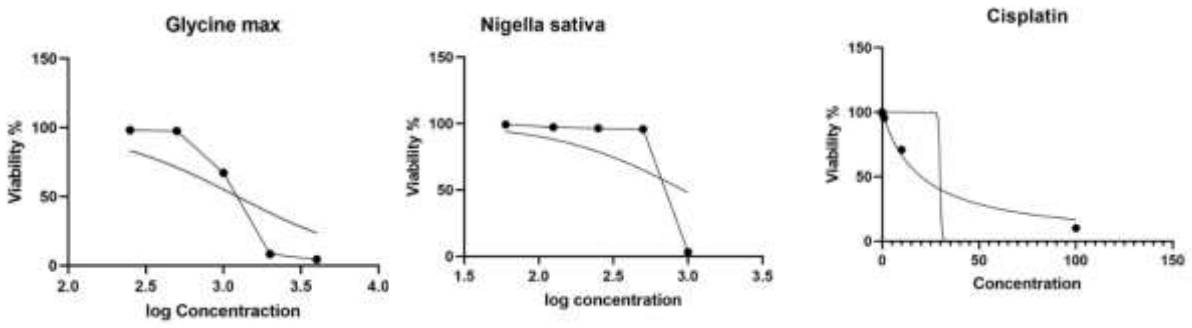

Figure 1: Cytotoxicity curve plotting viability percentage against concentration to determine IC50. (a) Glycine max (Soybean), (b) Nigella sativa (black seed) extracts and (c) Cisplatin (positive control). In (a), (b) and (c) the dots represent the concentrations used from soybean, black seed and cisplatin. For soybean and black seed, the analysis used in Graph pad prism was log inhibition vs normalized response whereas inhibition vs normalized response was used for cisplatin. X-axis represents viability percentage while Y-axis represents the concentration (log concentration in soybean and black seed and concentration in cisplatin). From these curves, IC50 was determined by Graph pad prism calculations.

Table 2: Flow cytometry findings for different treatments on the HT-29 colon cell line (Mean ± SEM)

	Control	Gm_{25}	Ns_{25}	Mix	Gm_{50}	Ns_{50}	Cisplatin
Necrosis (Q1)	0.44±0.14	1.62±0.23*	5.73±0.99*	9.13±0.13***	3.68±0.14***	17.17±0.35***	7.8±0.07***
LA (Q2)	0.76±0.21	2.91±0.23**	7.22±0.63**	20.13±0.22***	5.19±0.07***	14.74±0.34***	9.23±0.56***
Normal (Q3)	98.54±0.18	94.82±0.11***	86.82±0.53***	70.23±0.37**	90.59±0.13***	67.9±0.59***	82.80±0.50***
EA (Q4)	0.26 ± 0.09	0.64 ± 0.11	0.23 ± 0.01	0.52 ± 0.04	0.52 ± 0.06	0.19 ± 0.05	0.17 ± 0.04

LA: Late apoptosis, EA: Early apoptosis. The significant difference using Student's t-test is represented as follows: (*) p≤0.05, (**) p≤0.01, (***) p≤0.001. Error bars represent Standard Error of Mean (SEM).

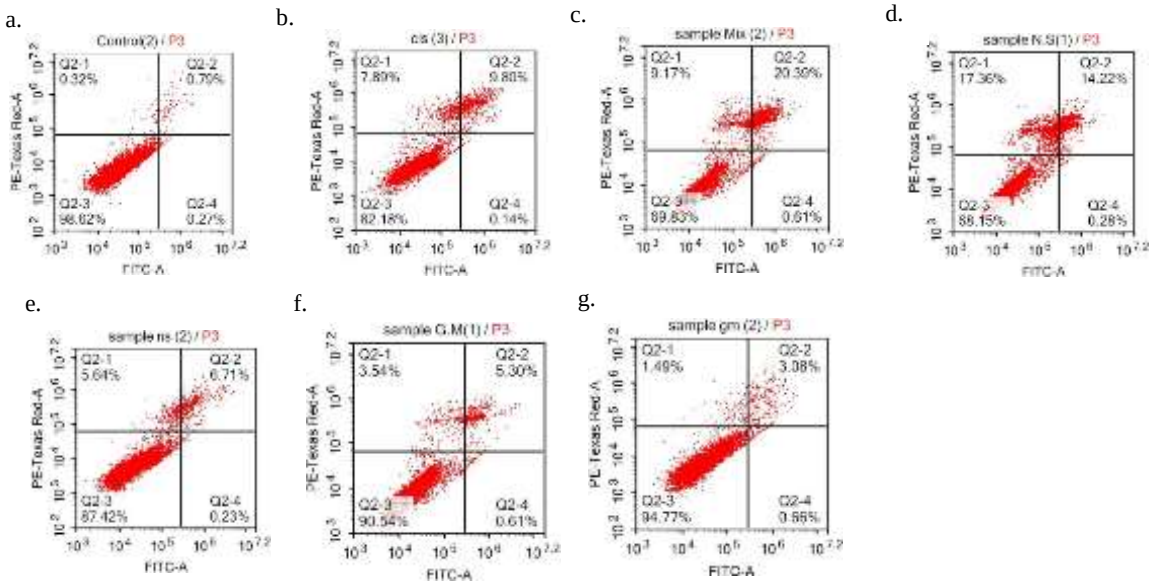

Figure 2: The Flow cytometry charts of different treatment groups in HT-29 colon cancer cell line; a. Control, b. Cisplatin, c. Mixture (IC_{25} of each extract). d. Black seed IC_{50}, e. Black seed IC_{25}, f. Soybean IC_{50}, g. Soybean IC_{25}. The charts shown are one of the replicates of each sample. Quadratnts (Q) represent Normal intact cells (Q2-3), early apoptosis (Q2-4), Late apoptosis (Q2-2) and necrosis (Q2-1). The mixture sample showed the highest early and late apoptosis followed by Ns_{50}, cisplatin, Ns_{25}, Gm_{50} and Gm_{25} in a descending order. The mixture showed the highest early and late apoptosis combined with less necrosis when compared to cisplatin and Ns_{50}.

3.4. DNA fragmentation

DNA laddering assay was used to determine apoptosis based on concept of the activation of the endonuclease Caspase-activated DNase (CAD) that cleaves the DNA into fragments of 180-200 bp and its multiples which indicates the activation of caspase-3 due to apoptosis activation. The smear represents necrotic activity (completely damaged DNA) while the bands/ladder pattern represent the DNA fragments resulted from endonuclease activity (apoptosis). In **fig. 3**, the control group showed no DNA fragmentation whereas in Gm_{50}, a band around 360 bp was observed indicating an endonuclease activity. Moreover, Ns_{50} and Mix showed bands at approximately 360 and 540 bp represented the endonuclease activity denoting apoptosis

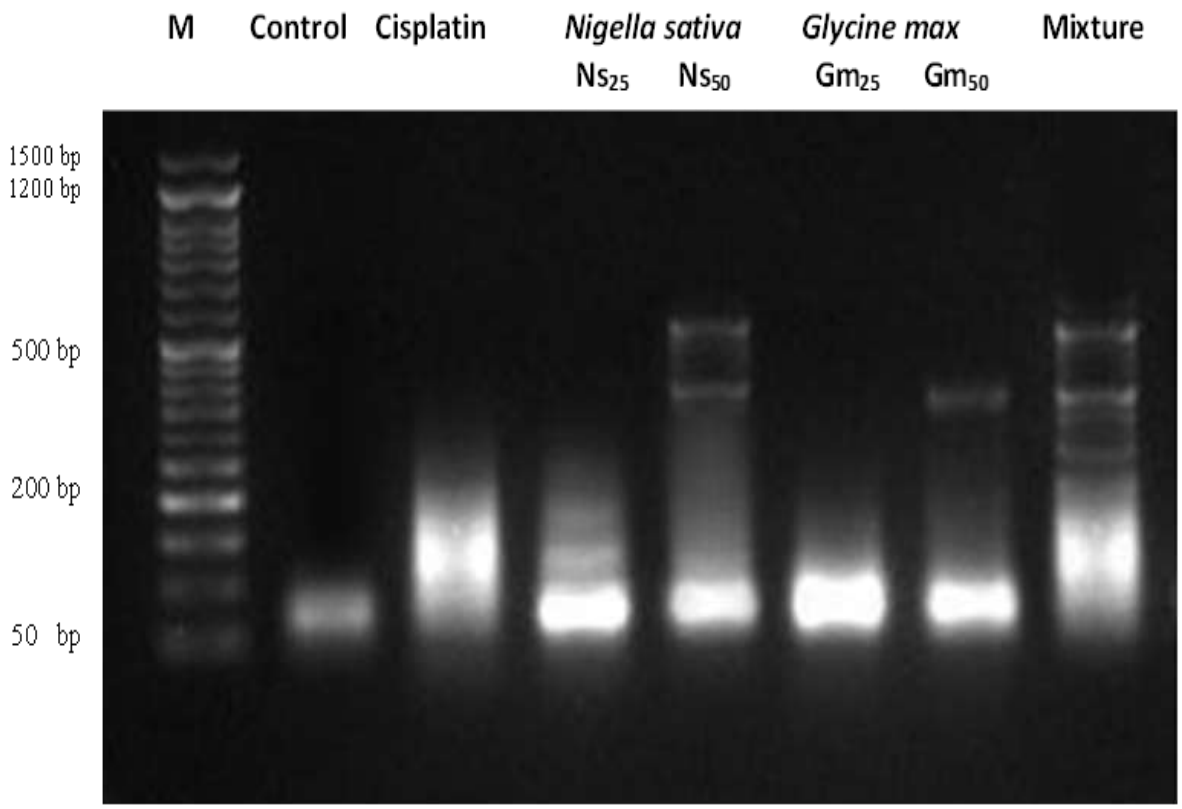

Figure 3: DNA fragmentation in HT-29 colon cancer cell line with different treatments detected with agarose gel electrophoresis. (M) represents the marker; $Ns_{25}$and Ns_{50} represent *Nigella sativa* (Black seed) at IC_{25} and IC_{50}, respectively; Gm_{25} and Gm_{50} represent *Glycine max* (Soybean) at IC_{25} and IC_{50}, respectively. The mixture showed the highest apoptotic effect represented by DNA fragmentation laddering effect. Ns_{50}, Ns_{25}, Gm_{50}, cisplatin and Gm_{25} followed in DNA fragmentation.

Quantitation of DNA fragmentation via colorimetric diphenylamine reaction data results were represented in **table 3,** where the fragmentation percentage - represented as mean ± SEM - of Mix, Ns_{50}, cisplatin, Ns_{25}, Gm_{50} and Gm_{25} were 31.82 ± 0.83, 26.62 ± 0.92, 21.93 ± 0.8, 17.8 ± 0.62, 13.91 ± 0.53 and 9.54 ± 0.56, respectively. Compared to the control, data analysis showed significantly increasing difference using Student's t-test ($p \leq 0.05$) in Gm_{25}, Gm_{50}, Ns_{25}, cisplatin, Ns_{50} and Mix with p-values of 0.039, 8.8×10^{-5}, 8.1×10^{-6}, 3.9×10^{-6}, 1.4×10^{-6} and 2.09×10^{-7}, respectively. The one-way ANOVA test resulted in a significant difference of 1.82×10^{-16} among the groups.

Table 3: DNA fragmentation using diphenylamine reaction.

Treatment	DNA Fragmentation % (Mean ± SEM)
Control	7.61 ± 0.41
Cisplatin	21.93 ± 0.80**
Ns_{25}	17.80 ± 0.62**
Ns_{50}	26.62 ± 0.92**
Gm_{25}	9.54 ± 0.56*
Gm_{50}	13.91 ± 0.53**
Mix	31.82 ± 0.83**

(*) $p \leq 0.05$, (**) $p \leq 0.001$.

3.5. Gene expression

The gene expression of 6 genes; P53, FAS, BAX, BIRC5, BCL2L1 and XIAP were evaluated in this study to detect their performance during apoptosis declaring effect of the two tested extracts. Data for each treatment were represented as Mean ± SEM from three independent experiments. Student's t-test was used to declare the significant difference between each group compared to the control. One-way ANOVA test (at $p \leq 0.05$) showed a significant difference among the groups in five of the tested genes; P53, BAX, BIRC5, BCL2L1 and XIAP with p-values 9.82×10^{-11}, 4.4×10^{-4}, 6.02×10^{-12}, 6.2×10^{-4} and 5.2×10^{-5}, respectively whereas p-value in FAS was 0.81 without significant difference (**Table 4**).

Table 4: The gene expression (fold) of the apoptotic genes in control, Gm_{25}, Ns_{25}, Mix, Gm_{50}, Ns_{50} and cisplatin in terms of Mean ± SEM

Groups	Mean ± SEM					
	P53	**FAS**	**BAX**	**BIRC5**	**BCL2L1**	**XIAP**
C1	1 ± 0.16	1 ± 0.46	1 ± 0.31	1 ± 0.06	1.00 ± 0.15	1 ± 0.09
Gm_{25}	0.90 ± 0.16	1.58 ± 0.32	2.20 ± 0.20	0.98 ± 0.06	0.90 ± 017	0.88 ± 0.03*
Ns_{25}	0.50 ± 0.31*	1.07 ± 0.21	4.61 ± 0.29**	0.55 ± 0.06***	0.62 ± 0.04*	0.64 ± 0.07**
Mix	0.15 ± 0.04***	1.67 ± 0.37	6.13 ± 0.31**	0.44 ± 0.09***	0.48 ± 0.10**	0.49 ± 0.16**
Gm_{50}	0.51 ± 0.07***	1.61 ± 0.26	2.92 ± 0.14*	0.87 ± 0.06*	0.79 ± 0.08	0.74 ± 0.13*
Ns50	0.21 ± 0.09***	1.64 ± 0.29	5.89 ± 0.34**	0.48 ± 0.06***	0.55 ± 0.24*	0.58 ± 0.18*
Cisplatin	0.34 ± 0.03***	1.44 ± 0.33	5.19 ± 0.48*	0.49 ± 0.06***	0.63 ± 0.05*	0.61 ± 0.06**

* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

Data showed a significant downregulation of P53 (P53 in HT-29 cell line is mutated, thus an oncogene) between control and Ns_{25}, Mix, Gm_{50}, Ns_{50} and cisplatin groups with p-values of 0.04, 1.99×10^{-4}, 9.63×10^{-4}, 1.18×10^{-5} and 1.48×10^{-3} respectively (**Fig. 4a**). FAS gene expression data analysis showed no significant difference between control and Gm_{25}, Ns_{25}, Mix, Gm_{50}, Ns_{50} and cisplatin groups in Student's t-test at $p \leq 0.05$. Also, one-way ANOVA showed no significant difference among the groups with a p-value of 0.81 (**Fig. 4b**). BAX gene expression data analysis showed an upregulation and a significant difference in the groups Ns_{25}, Mix, Gm_{50}, Ns_{50} and cisplatin with respect to the control group with p-values 0.006, 0.005, 0.012, 0.006 and 0.016, respectively. On the other hand, one-way ANOVA showed a significant difference with a p-value of 4.4×10^{-4} at $p \leq 0.05$ (**Fig. 4c**). BIRC5 gene expression data analysis showed a downregulation and a significant difference in the groups Ns_{25}, Mix, Gm_{50}, Ns_{50} and cisplatin with respect to the control group with p-values 7.2×10^{-4}, 1.8×10^{-4}, 0.03, 1.2×10^{-4} and 1.6×10^{-4}, respectively. Moreover, one-way ANOVA showed a significant difference (at $p \leq 0.05$) among groups with a p-value of 6.02×10^{-12} (**Fig. 4d**). BCL2L1 gene expression data analysis showed a downregulation and a significant difference in the groups Ns_{25}, Mix, Ns_{50} and cisplatin with respect to the control group with p-values 0.018, 0.0027, 0.024 and 0.03, respectively. Moreover, one-way ANOVA showed a significant difference (at $p \leq 0.05$) among groups with a p-value of 6.2×10^{-4} (**Fig. 4e**). XIAP gene expression data analysis showed a downregulation and a significant difference in the groups Gm_{25}, Ns_{25}, Mix, Gm_{50}, Ns_{50} and cisplatin with respect to the control group with p-values 0.03, 0.0085, 0.002, 0.03, 0.01 and 0.002, respectively. Moreover, one-way ANOVA showed a significant difference (at $P \leq 0.05$) among groups with a p-value of 5.2×10^{-5} (**Fig. 4f**).

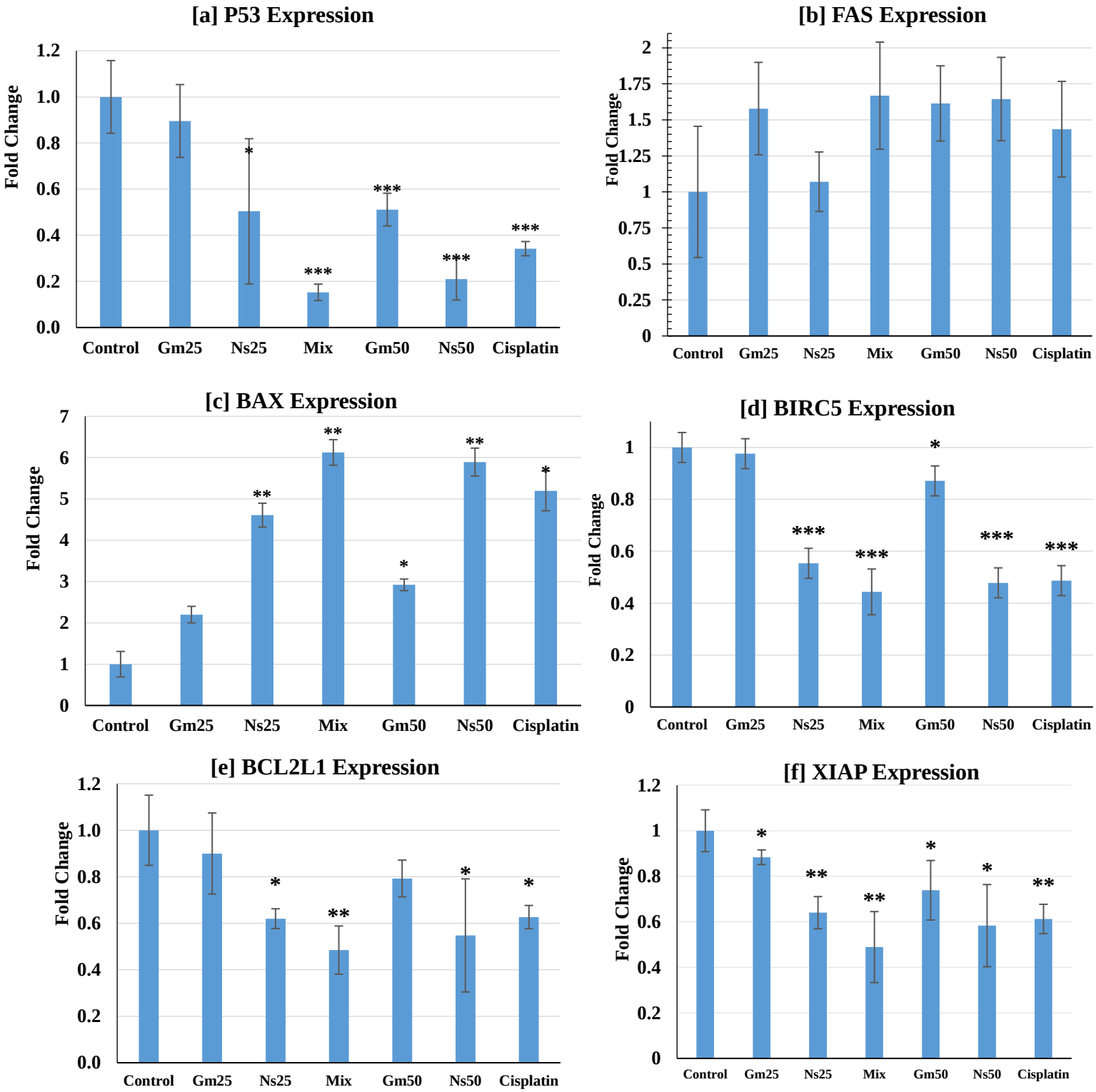

Figure 4: Relative expression of the tested genes, a: P53, b: FAS, c: BAX, d: BIRC5, e: BCL2L1, f: XIAP in HT-29 colon cancer cell line treated with soybean and/or black seed extracts. (*) $p \leq 0.05$, (**) $p \leq 0.01$, (***) $p \leq 0.001$. Error bars denote Standard Error of Mean (SEM).

4. Discussion

Flow cytometric assay resulted in a high total apoptosis (20.65%) and low necrosis (9.13%) in the mixture treatment compared to cisplatin (9.4% and 7.8%) and Ns_{50} (14.93% and 17.7%), respectively. Soybean and its molecules such as protein, small RNA and flavonoids were substantially effective in colon cancer [27, 28, 29]. Genistein, an isoflavone of Soybean, significantly increased apoptotic cells ratio in HCT-116 colon cancer cell line in a dose-dependent manner [28]. Lunasin, a soybean protein, induced apoptosis in a dose-dependent manner of 14.6% and 18.7% of tumor cells treated with 10 and 50 uM lunasin in HT-29 cell line [27] and 21.7%, 24.7% and 27.7% in 5, 10 and 25 mM lunasin treatment in KM12L4 colon cancer cell line as well as inducing cell cycle arrest at G2/M checkpoint and activating the mitochondrial pathway evidenced by downregulation of BCL-2 and upregulation of Bax, cytochrome-c and caspase-3 expression levels [30]. In HCT-116 stem-like cell (CSC) and bulk tumor (non-CSC) subpopulations, Lunasin increased apoptosis by up to 1.8-fold and 2-fold of total apoptotic cells in Non-CSC and CSC in a dose-dependent manner, respectively [31]. In a study on mouse colon cancer cell line (Coca), soy saponin exhibited higher effect compared to cisplatin confirmed by 84% and 91% early apoptotic cells at 48 and 72 hours, respectively [32]. Furthermore, small RNA derived from soybean significantly inhibited the proliferation and induced apoptosis in Caco-2 cells indicated by a reduction in cell growth to 3.6 ± 2.7%, 9.6 ± 7.1% and 21.5 ± 8.1%, compared to negative controls at 24, 48 and 72 h, respectively without any drop in NCM460 non-cancerous colon cell line [29]. In black seed, crude saponin extract of *Nigella sativa* (CSENS) and TQ-rich fraction induced apoptosis in HCT-116 and HT-29, respectively in a dose- and time-dependent manner [15, 33]. Moreover, herbal melanin (HM) exhibited an apoptotic effect in HT-29 cell line with early apoptosis of 42% of cells while in a combination treatment of N-Acetylcysteine (NAC) + 100 ug/ml HM it was 30% [34].

P53 was significantly downregulated by the treatments Mix, Gm_{50}, Ns_{50} and cisplatin which might be due to the mutated P53 gene in HT-29 cell line, which was proved by previous investigation [35]. Also, syringic acid, a soybean and black seed component, induced apoptosis via downregulation of mutant P53 in hamster [36]. Even though soy saponin prevented colon cancer (WiDr; an HT-29 cell line derivative), it had no significant difference on P53 expression [37]. TQ and TP (topoisomerase I inhibitor) treatment induced apoptosis via P53-independent mechanism, however this synergism was absent in apoptotic and anti-proliferative protein expression [38]. The present treatments showed no significant change in the expression of FAS gene. In the same line, [39] found that FAS/CD95 expression was unaffected by 24-hour treatment of Kaempferol, a black seed constituent, on HCT-116 [39]. FasR/FasL expression in colorectal cancer was associated with cancer recurrence and metastasis [40, 41]. Expression of BAX gene was significantly upregulated in Ns_{25}, Mix, Gm_{50}, Ns_{50} and cisplatin which was in concordance with other studies on the effect of soybean or black seed extract or specific components of either plant. Expression of Bax gene was upregulated in TQ- treated HT-29 by 15-fold [42]. In addition, Bax protein and BAX/Bcl-2 ratio were upregulated by soybean-derived sRNAs in Caco-2 cell line [29] while Lunasin, soybean protein, upregulated BAX/Bcl-2 ratio in HT-29 [27]. BCL2L1 was downregulated in our study with a significant difference in the groups Ns_{25}, Mix, Ns_{50} and cisplatin which was consistent with different studies. TQ downregulated BCL2L1 in HCT-116 [16] and HT-29 [42], while herbal melanin of black seed inhibited BCL2L1 expression in HT-29 but had no effect in SW620 colon cancer cell line [34]. Interestingly, targeting and inhibiting BCL2L1 could be effective in treating colorectal cancer [43]. Furthermore, a combination treatment of Daidzein (a soybean isoflavone) and Gifitinib (a cancer drug) significantly activated mitochondrial pathway by downregulating BCL2L1 in lung adenocarcinoma [44]. BIRC5 (survivin) expression was significantly downregulated in Ns_{25}, Mix, Gm_{50}, Ns_{50} and cisplatin. Soybean isoflavones; genistein and daidzein had no significant effect on BIRC5 regardless of the reduction in HT-29 cell line proliferation [45]. TQ downregulated BIRC5 expression in HCT-116 [16]. Taxifolin, a polyphenol of soybean and black seed, was found to reduce BIRC5 gene and protein expression *in vitro* and *in vivo* while causing cell growth arrest, affecting G2-phase of cell cycle and apoptosis in HCT-116 and HT-29 [46]. A combination treatment of genistein and Indol-3-carbinol downregulated XIAP protein expression in HT-29 [47]. XIAP protein expression was reduced in a combination treatment of TQ with doxorubicin in breast cancer xenograft mouse model [48].

The significant DNA fragmentation rates shown in the present study indicated a potent antiapoptotic effect of the mixture (Gm_{25} + Ns_{25}) as well as soybean and black seed extracts. Similarly, colon cancer showed comparable DNA fragmentation after treatment with soybean components such as Daidzein-rich fraction in breast cancer and leukemia which both increased caspase-3 protein [49]. Moreover, black seed components exhibited apoptotic DNA fragmentation effect such as *Nigella sativa* extracts on Human lymphoma U937 cells [50] and DNA degradation but not apoptotic DNA fragmentation in crude saponin extract of *Nigella sativa* (CSNS) in colon cancer [15]. According to Elkady *et al* [15], CSNS also had no effect on caspase-3 activity which might explain the lack of DNA fragmentation

indicating an apoptotic effect of CSNS with a caspase-independent pathway. Interestingly, when the intrinsic pathway of apoptosis is activated, the mitochondrial membrane permeability is altered releasing cytochrome-c into cytosol forming a complex with Apaf-1 activating caspase-9 consequently activating caspase-3. Since apoptotic DNA fragmentation is dependent on the endonuclease caspase-activated DNase (CAD), the increased DNA fragmentation could be considered as an indication of an upregulation of caspase-3 which activates CAD by cleaving inhibitor of CAD (ICAD) [51, 52].

In various research, some major components found in soybean and black seed extracts exhibited anti-proliferative and proapoptotic properties. Syringic acid exhibited significant reduction in tumor volume and incidence, cellular proliferation inhibition and apoptosis induction in *in vivo* and SW-480 colon cancer cell line studies [53]. Interestingly, Kaempferol, a polyphenol in soybean, overcame 5-FU resistance in LS174-R resistant colon cancer cell line [54] and oxaliplatin-resistant HT-29OxR colon cancer cell line [55]. Moreover, D-pinitol, showed antioxidant effect and reduced free radicals Azoxymethane (AOM)-induced colon cancer in rats [56] while gallic acid inhibited the growth of HCT-116 by downregulating calcium channel, upregulating P53-pathway via caspase activation thus activating mitochondrial pathway [57]. Methyl gallate, gallic acid and cisplatin combination treatment exhibited synergistic and enhanced effect on cervical cancer [58].

Furthermore, Taxifolin downregulated BIRC5 gene and protein expression *in vitro* and *in vivo* (Athymic male mice) in HCT-116 and HT-29 cell lines [46], inhibited Non-Small-Cell Lung Cancer (NSCLC) growth by downregulating genes associated with immune evasion tactics [59]. Interestingly, in resistant cervical cancer cells an adjuvant treatment with chemotherapeutic drugs significantly re-sensitized multidrug resistance [60].

Most of the components of both soybean and black seed extracts such as taxifolin, D-pinitol, methyl gallate, chlorogenic acid, gallic acid and kaempferol were found to exhibit apoptotic effect. Also, these components increased the expression levels of the apoptotic genes such as caspase-3, cytochrome-c, P53, Bax. Furthermore, several studies proved that such compounds reduced the expression levels of anti-apoptotic genes (Bcl-2, BCL2L1, BIRC5 and XIAP) in colon, breast and lung cancer, leukemia, hepatocellular carcinoma, and oral squamous carcinoma [39, 61, 62, 63, 64, 65, 66, 67].

5. Conclusion

Finally, the anticancer effect of black seed and soybean extracts and their mixture (Gm_{25} + Ns_{25}) was shown through induction of apoptosis. This action was confirmed through significant early and late apoptosis cell percentage, highly significant DNA fragmentation percentage suggesting an increased activity of caspases in the form of endonuclease caspase-activated DNase (CAD/DFF40). Additionally, over-expression of Bax and downregulation of BIRC5, BCL2L1, XIAP and mutated-P53 genes and the nonsignificant FAS gene expression suggest the prevalence of intrinsic pathway of apoptosis. Thus, these findings suggest that the apoptotic effect of soybean and black seed extracts and their mixture might occur through intrinsic pathway and caspase-dependent pathway.

6. Conflict of Interest

There is no conflict of interest.

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9. Ethics Approval

National Research Centre, ethics committee approval number **20-030**

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