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Sodium metabisulfite in dried plum and its cytotoxic effects on K-562 and L-929 *normal* cell lines

Ahmad Ghasemi[1,2] | **Amir Salari**[3] | **Mahboube Kalantarmahdavi**[3] | **Mohammad Reza Amiryousefi**[4]

[1] Department of Basic Medical Science, Neyshabur University of Medical Sciences, Neyshabur, Iran

[2] Healthy Ageing Research Centre, Neyshabur University of Medical Sciences, Neyshabur, Iran

[3] Department of Food Hygiene and Aquaculture, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad (FUM), Mashhad, Iran

[4] Department of Food Science and Technology, Neyshabur University of Medical Sciences, Neyshabur, Iran

Correspondence
Mohammad Reza Amiryousefi, Department of Food Science and Technology, Neyshabur University of Medical Sciences, Neyshabur, Iran.
Email: mramiryousefi@gmail.com

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Abstract: In the present study, the concentration of sodium metabisulfite (SMB) in dried plums and its toxicity effects on the cell lines of K-562 (human leukemia cell line) and L-929 (normal fibroblast cell line) were measured. Samples of dried plums were randomly collected from the shops located in Neyshabur and Mashhad (Iran). SMB residue was measured using iodometric titration and high-performance liquid chromatography. To analyze the cytotoxicity, the cells were treated with various concentrations of SMB, and cell viability was determined by the MTT and LDH methods. The average concentration of SMB in the samples of dried plums was selected to evaluate the apoptosis/necrosis by flow cytometer. The expression analysis of apoptosis marker genes (BAX, Bcl-2, and P53) was also assessed. Results indicated that the average concentration of SMB residue in 12 samples of dried plum was 516 ± 285.39 mg/kg. When K-562 cells were treated with 500 mg/L of SMB, apoptosis increased significantly ($p < 0.01$). The IC50 of SMB for K-562 and L-929 cells after a 48-h exposure was 200.31 and 257.82 mg/L, respectively. SMB-treated cells showed that cell viability in both cell lines decreased in a dose-dependent manner after 72 h ($p < 0.01$). The percentage of apoptotic but not necrotic cells was 69.49% for K-562 and 77.32% for L-929 cells, whereas apoptosis of untreated control cells was 0.17%. Our findings also showed an opposite mRNA expression of Bcl-2 (anti-apoptotic marker) and Bax2 (pro-apoptotic marker) when k-562 cells were treated with SMB. The results indicated that the concentration of sulfite residue in some dried plums poses a cell toxicity risk for normal cells.

KEYWORDS
cell apoptosis, cytotoxicity, dried plum, sodium metabisulfite

Practical Application: The results of current study provide important information concerning the toxicological effects of SMB, and give a warning that it needs to be replaced by natural products for fruit drying processes.

1 | INTRODUCTION

Additives are substances added in food or medicine to attain the particular chemical effects in the final product (Chan, 2015a, 2015b). Today, there are more than 3000 additives with different roles in the food industry which are classified according to their functions (Güngörmüş & Kılıç, 2012; Onyemaobi et al., 2012). Sulfite compounds are widely used in a variety of industries including, food, cosmetics, and pharmaceuticals, as preservatives and antioxidants (Vally & Misso, 2012). Sulfur salts and sulfur dioxide similar to sodium metabisulfite (SMB) as preservatives are added to a variety of foods, including concentrated soft drinks, some fruit juices, beer, wine, and dried fruits (Adebayo & Adenuga, 2012; Shekarforoush et al., 2015; Shoaei et al., 2019).

Plum (*Prunus salicina Lindell*) is one of the most important fruit crops in Iran. Perishability because of high moisture content and tender texture of fresh fruits such as plum causes difficulty in their preservation. Drying is one of the most popular preservation techniques for increasing the shelf life of fruits by decreasing the content of water to inhibit the cell growth and proliferation of microorganisms and to deactivate some of the moisture-realated deteriorative reactions (Amiryousefi & Mohebbi, 2010; Deng et al., 2019). Drying markedly decreases the weight and volume of fruits and minimizes the costs of their packing, storage, and transportation (Kamiloglu et al., 2016). The sulfuring process has been broadly used for fruit drying (Miranda et al., 2009). This process changes the cell membrane permeability of the fruits. This method reduces the drying time and improves the quality of dried fruits (Deng et al., 2019; Lewicki, 1998; Miranda et al., 2009).

Sulfidation for drying the fruits, depending on used forms (gas or solution), the amount of used solutions, sulfur processing time, and pH of the medium enhanced the sulfur residues in the final product (Deng et al., 2019; Mir et al., 2009). Before the 1990s, all studies, including those conducted by FDA-GRAS Commission, indicated that sulfiting agents could be considered "generally recognized as safe" compounds. Until several moderate-to-severe cases of skin and respiratory sensitivities were reported that caused the concerns regarding the sulfites usage in food. Successive research described many adverse reactions ascribable to sulfites ingestion. Today, the FAO/WHO lists sulfites as allergens (Grotheer et al., 2019). (Grotheer et al., 2019; FAO/WHO – CCFL, 2007). The presence of chemical residues in final products is the most important problem of sulfur processes that may result in many health problems (Deng et al., 2019; Kamiloglu et al., 2016). Due to asthmatic reactions in some sensitive individuals, the Food and Drug Administration (FDA) has required processors to declare the presence of sulfite agents in any food with sulfite concentration 10 mg/L and up since 1986 (Deng et al., 2019; Fernandes & Rodrigues, 2007). California's Proposition 65 sulfur dioxide is known as a chemical that causes reproductive toxicity (Barsa, 1997). In addition, the Ministry of Health of the People's Republic of China has raised the standards for use of sulfite agents in food processing (Deng et al., 2019). Some investigations have also reported toxicological effects of sulfite agents in different human body organs such as gonads (Adebayo & Adenuga, 2012), lungs (Vally & Misso, 2012), and the nervous system (Lai et al., 2018). Sulfite agents have also been reported to stimulate allergic reactions and lipid peroxidation (Borges et al., 2018; Ozturk et al., 2011) . Niknahad and O'Brien (2008) in an in vivo study indicated that sulfites may lead to oxidative stress by inducing glutathione oxidation (GSSG) and reducing glutathione in rat hepatocytes. In addition to increasing the formation of reactive oxygen species, this event may increase the toxicity of xenobiotics by forming glutathione sulfonate (GSSO3-) (Niknahad & O'Brien, 2008). Ozturk et al. (2011) found that the toxicological effects of sulfite agents are caused by the formation of free radicals oxidizing and the destruction of important lipids, proteins, and nucleic acids in cells (Ozturk et al., 2011).

Sulfite agents are still used as a plum drying process in the Khorasan province, Iran. Sulfidation to dry plums is usually done on-farm and off-industry, so there is no strict monitoring of the amount and duration of plums exposure to sulfur. This has raised concerns over the health communities due to increasing the permitted amount of SMB in dried fruits and its harmful effects on consumers' health. In 2016, the EFSA panel on food additives and nutrient sources added to food reported that there were only quantitative and non-specific studies on the uptake, distribution, metabolism, and excretion of SO_2 additives, and the exact mechanism of sulfite sensitivity was not known (D'Amore et al., 2020). Despite the sulfite additives are now used widely in the food industry, there are limited studies regarding their toxic effects and related mechanism. Toxicological study of chemical additives gives a lot of information about the harmful effects of these compounds and warns us about the need to replace these chemical compounds with similar natural products (Atanasov et al., 2021). Therefore, we have performed a basic toxicological study to investigate the toxic effects of sulfite agents according to concentrations detected at collected dried plums.

The current study is the first to examine the cytotoxic effects of residual SMB on Neyshabour dried plums in vitro. Accordingly, the concentration of SMB in the dried plums was measured, and its toxicity effects on the K-562

(human leukemia cell lines) and L-929 (normal fibroblast cell lines) were investigated.

2 | MATERIALS AND METHODS

2.1 | Materials

Cell-culture reagents such as Roswell Park Memorial Institute Medium (RPMI 1640) were purchased from Sigma-Aldrich (St Louis, MO, USA), and fetal bovine serum (FBS). Furthermore, phosphate-buffered saline (PBS), and antibiotics (penicillin-streptomycin) were purchased from Gibco, Germany. The K-562 and L-929 cells were purchased from the National Cell Bank of Iran (NCBI, Pasteur Institute of Iran, Tehran). The chemicals and plastic labware were obtained from Sigma-Aldrich Chemical Co. and Falcon Labware (ExtraGene, Taichung, Taiwan), respectively.

2.2 | Sample collection

In the present study, a sample of dried plums (*Prunus salicina Lindell*) available in the market of non-brand manufacturing was collected from supermarkets and stores in Neyshabur and Mashhad from April to July 2019. The collected plums were all traditionally dried by peeling the very ripe plums and then placing them on wooden shelves. Some sulfur was burned, and the plums were exposed to sulfur fumigation for 30 min. In the next step, the plums were exposed to sunlight to dry completely. The production time of all dried plums was at the same time of sampling. The sampling was done by a completely randomized design (CRD). Accordingly, the treatments were assigned completely at random so that each supermarket or store had the same chance of receiving any one sample. The collected samples (12 samples) were immediately transferred to the laboratory and then packed in plastic bags and stored at 4°C.

2.3 | Analyses of sulfite

The SO_2 contents of dried fruits in twelve samples were measured according to Iran's standard method (ISIRI, 2017). This method is according to the Monier-Williams method for determining sulfites in foods which is confirmed by the Association of Official Agricultural Chemists (AOAC) (Moinier-Williams, 1927). Briefly, 25 ml of 0.1 N iodine solution was poured separately into Erlenmeyer flakes and placed under a digestion balloon collection tube. Then about 32 g of each sample and 250 ml of distilled water were poured into the digestion balloon and heated to a boil. Then, 10 ml of hydrochloric acid (37%) was added to the balloon, and acidified sample solution was refluxed for 55 min. Finally, sulfur dioxide was collected in Erlenmeyer flakes containing 25 ml of 0.1 N iodine solution. After that, the solution was titrated with sodium thiosulfate 0.1 N until the solution turned bright yellow. The emitted sulfur dioxide acted on a certain amount of iodine solution. The difference between the amount of iodine consumed and the remaining iodine indicated the amount of sulfur dioxide that calculated by the following equation:

$$C_{SO_2} = \frac{(A - B) \times N \times M}{2 \times M}$$

where C_{SO_2} = concentration of sulfur dioxide (mg/kg); A = the volume of sodium thiosulfate 0.1 Normal consumed for 25 ml of iodine solution (ml); B = the volume of sodium thiosulfate 0.1 Normal remained for 25 ml of iodine solution (ml); N = Sodium thiosulfate normality; M = molecular mass of SO_2 (64.066); m = weight of sample (g). This method was validated with various concentrations of sulfite standard solutions (20, 40, and 80 mg/L). The standard solutions of sulfite were tested five times to determine procedure repeatability data. Working standards of various concentrations were prepared of an 80 mg/L sulfite standard solution (CPAchem Company, France). The standard solutions were filtered through a 0.45 μm PVDF filter.

2.4 | High-performance liquid chromatography

A mix of 12 dried plum samples was prepared and SO_2 residue was measured by high-performance liquid chromatography (HPLC) to select appropriate concentrations in vitro experiments. Sample injections of 50 μl were operated from a YL9100 HPLC system (Young Lin Bldg, Hogye-dong, Anyang, Korea) equipped with an auto sampler, and UV detector (YL9120UVD A). The used column was C18 with size particle 0.4 μm and L x I.D. 25 cm x 5 mm. The mobile phase consisted of water and acetonitrile (4/6 v/v). The flow rate was 1 ml/min, and detection was performed at 275 nm. The concentration of SMB in the dried plum samples was measured according to the standard curve and related equation generated of three standard samples (S1:5 mg/L, S2: 10 mg/L, and S3: 20 mg/L).

To prepare standard samples, 5, 10, and 20 mg of SMB was dissolved in 1000 ml mobile phase solution and sonicated 5 min with handshaking. In addition, 3 g of dried plums samples (mixture of 12 samples) were diluted with

300 ml of mobile phase solution, sonicated 5 min with handshaking until particles disintegrated completely, and filtered through 0.45 μm PVDF Filter. The 50 μl volume of each standard sample or dried plum samples were separately injected into the HPLC system.

2.5 | Cell culture

L-929 fibroblast and K-562 human blood cell lines were purchased from the National Cell Bank of Iran (NCBI, Pasteur Institute of Iran). The cells were cultured in RPMI 1640 (Sigma-Aldrich) supplemented with 10% FBS (Gibco, Germany) and 1% penicillin-streptomycin in a humidified incubator with 95% air and 5% CO2 at 37°C.

2.6 | Preparation of SMB different concentrations

For the preparation of different concentrations of SMB, the powder of SMB (Sigma-Aldrich) was dissolved in dimethyl sulfide (DMSO) (Sigma D 8779 ACS) followed by the RPMI medium to obtain 20 mg/ml and mixed for 20 min at 37°C. This solution was centrifuged to remove insoluble ingredients, and then the supernatant was passed through 0.22 mm filters for sterilization. The solution was diluted with the medium and prepared at different concentrations (50, 115, 230, 518, and 800 mg/L), these concentrations were selected according to sulfite residue analysis in collected samples.

2.7 | MTT assay

Cytotoxicity exerted by SMB on cells was assessed by 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (Ghasemi et al., 2019). The L-959 and K-562 cell lines were cultured separately on 96-well plates in a density of 1×10^5 cells/well for 24 h. Then, the cells were treated with various concentrations of the SMB for 48 h. Subsequently, 50 μl MTT solution (2 μg/ml in culture medium) was added to each well, and samples were incubated for a further 4 h at 37°C. After forming the formazan crystals, the culture medium supernatant was aspirated from the wells without disturbing the formazan precipitate. The formazan crystals were dissolved in 100 μl of DMSO. Microplates were read at 570 nm by a microplate reader (Bio-Rad, Hercules, California, USA). Finally, the percentage of cell viability was measured by the following equation after subtracting OD treated and non-treated groups from the OD blank sample. (Mean OD of treated group/mean OD of non-treated group × 100)(Ghasemi et al., 2019).

The half-maximal inhibitory concentration (IC50) was calculated using free online IC50 calculator software (https://www.aatbio.com/tools/ic50-calculator).

2.8 | LDH assay

Lactate Dehydrogenase (LDH) assay quantitatively measured the activity of LDH, a stable cytoplasmic enzyme, released into the cell culture supernatant upon damage of the plasma membrane. LDH colorimetric assay kit (Sigma, Roedermark, Germany) was used to measure LDH in cells in the presence or absence of SMB according to the manufacturer's instructions. Briefly, the cells (5×10^4 cells/well) were cultured in 96-well plates overnight and then exposed to various concentrations of SMB for 48 h. Subsequently, the supernatant was obtained by centrifugation (12,000 rpm for 3 min), and 50 μl of supernatant was transferred into a new plate and mixed with 50 μl of the Master Reaction Mix and incubated for 15 min at 37°C. The reaction was stopped by adding 0.4 mol/l of NaOH. LDH activity was quantified by reading the optical absorbance of samples by the plate reader (Bio-Rad, Hercules, CA, USA) at 450 nm after subtracting sample absorbance from background absorbance (Yang et al., 2017).

2.9 | Quantification of cell apoptosis/necrosis by flow cytometry

Cells were cultured in a density of 1×10^6 cells per well and placed at 37°C (5% CO2) for 24 h. After 18 h, the medium was replaced by fresh RPMI containing various concentrations of SMB and incubated for 48 h. Subsequently, the cells were harvested by centrifugation, and washed twice with PBS, and stained with Annexin V-FITC/ PI (Annexin-V-FLUOS Staining kit, Mannheim, Germany) for 15 min at room temperature. The sample was run on a FACS Calibur flow cytometer (Becton Dickinson, Mountain View, CA, USA) to measure forward and side scatter of light. The cells were analyzed using a Becton Dickinson FACScan instrument. The data were collected, stored, and analyzed using Cell Quest software (Becton Dickinson).

2.10 | Real-time PCR experiments

In order to determine the mRNA levels of BAX, Bcl-2, and P53 in K-562 human cells, the SYBR Green Quantitative

TABLE 1 Primer sequences used for the analysis of expression of apoptotic markers

Gene	Forward (5′–3′)	reverse (5′–3′)
BAX	AAACTGGTGCTCAAGGCCC	AAAGATGGTCACGGTCTGCC
Bcl-2	TCTTTGAGTTCGGTGGGGTC	GTTCCACAAAGGCATCCCAGC
P53	AGGAAATTTGCGTGTGGAGTAT	TCCGTCCCAGTAGATTACCACT
NFkβ	GCAGCACTACTTCTTGACCACC	TCTGCTCCTGAGCATTGACGTC
GAPDH	CTCCCGCTTCGCTCTCTG	TCCGTTGACTCCGACCTTC

Real-Time RT-PCR was performed on an ABI 7500 detection system (Applied Biosystems, Foster City, CA, USA). For this, total RNA was extracted from cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA), then the cells were treated with 500 mg/L of MBS for 12 and 24 h. A total RNA of 2 μg was reverse-transcribed using the Excel RT Reverse-transcriptase kit (RP1300, SMOBIO, Hsinchu City, Taiwan). PCR Reactions were done in 96-well plates under the following conditions: denaturation at 95°C for 15 min, annealing at 59°C for 5 s, and elongation at 72°C for 5 s. GAPDH was used as the endogenous control gene. The gene expression alterations were calculated using the $2^{-\Delta\Delta Ct}$ method according to the Ct of target and control genes in treated and non-related samples. The primer sequences are indicated in Table 1.

2.11 | Statistical analysis

All of the tests were carried out in triplicate, and data were reported as the mean ± standard deviation. Statistical analysis of differences was performed by one-way analysis of variance (ANOVA) followed by Dunnett's test, and the means were compared using Duncan's multiple range test at $p < 0.05$. The SPSS Inc. (Chicago, IL, USA) Ver. 21 was used to perform statistical analysis.

3 | RESULTS AND DISCUSSION

3.1 | Measurement of sulfite residue in dried plum

Sulfite agents are still used as a fruit drying process in Iran. The plum drying with sulfite agents is usually done on-farm and off-industry, so there is no strict monitoring of the amount and duration of plums exposure to sulfur. In the current study, we analyzed the contents of sulfite in dried plums using two different methods including Iran's standard method to measure SO2 contents of dried fruit and HPLC. Iran's standard method to measure SO2 contents of dried fruit was validated with different internal standards prepared from 80 mg/kg. Method repeatability data for area calculation (%CV) for all of the solutions were good (standard concentration = 20 mg/kg, %CV = 21; standard concentration = 20 mg/kg, %CV = 16; standard concentration = 80 mg/kg, %CV = 14). A total of 12 dried plum samples were analyzed for the concentration measurement of sulfite residue. The data shows that 50% of samples had more than 700 mg/kg of sulfite residue. The average sulfite residue in all samples of dried plum was 516 ± 285.39 mg/kg. Consistent with this, HPLC results showed that the concentration of sulfite residue in the collected dried plum samples was 492.42 ± 120 mg/kg.

According to our results, the concentration of sulfite residue was variable in different dried plum samples, and the lowest and the highest concentrations were 190 mg/kg and 765 mg/kg, respectively. The mean concentration value (516 ± 285.39 mg/kg) measured of our samples was in accordance with that reported by Shoaei et al. (2019). In that study, the mean sulfite residue concentration in 18 samples of dried plums collected from Hamadan (Iran) was 597.32 ± 401.82 mg/kg (Shoaei et al., 2019). In addition, Vandevijvere et al. reported that the average concentration of sulfite residue in dried apricots collected in Belgian was 533 mg/kg (Vandevijvere et al., 2010). Many discrepancies were observed between the mean sulfite residue concentrations values in our plum samples compared with values reported by others worldwide. For example, the mean concentration values of sulfite residue in dried fruits in Austria (339.5 mg/kg) (Mischek & Krapfenbauer-Cermak, 2012) and Belgian (179 mg/kg) (Vandevijvere et al., 2010) were lower, whereas values in France (1005.9 mg/kg) (Bemrah et al., 2008) and Korea (1070 mg/L) (Suh et al., 2007) were higher than our reports. These discrepancies may be related to some factors such as the initial concentration sulfite amount used in the product, the difference in the sulfuring process and drying method, the delay time between sulfidation, and analysis time. For SMB, the Joint Expert Committee of Food and Agriculture (JECFA—WHO/FAO Commission) and the Scientific Committee on Food (SCF—European Commission) established the

acceptable daily intake (ADI). It was expresses as SO_2 and fixed at 0.7 mg/kg/day body weight. This ADI was reassessed and reconfirmed in 2007 (D'Amore et al., 2020). Accordingly, an adult person with 70 kg weight is allowed to consume 49 mg per day. According to the data obtained in this study, the maximum and minimum amount of SO_2 remaining in dried prunes were 765 and 190 mg/kg, respectively. As a result, based on the maximum amount of sulfite remaining 64 g and based on the minimum amount of residual 258 g from these plums can be consumed per day. More than this amount, the liver is not able to metabolize sulfite, and its concentration in the blood increases and causes adverse health effects. It should be noted that the amount of plums consumed per day based on body weight in children will be less (18 and 73 g of plums based on the maximum and minimum amount of residual sulfite, respectively).

3.2 | Effect of SMB on cell viability in K654 and L929 normal cells

Based on the results of SMB residue in dried plum, four concentrations (50, 115, 230, 518, and 800 mg/L) of SMB were prepared for cytotoxicity analysis against cells (K-562 and L-929) by MTT, and LDH assays. As seen in Figure 1, treatment with SMB decreased cell viability of K-562 and L-929 in a dose and time-dependent manner. The significant decrease of cell viability in both cell lines started in presence 115 mg/L of SMB (% cell viability for K-562 cells: 78.2 ± 0.63; $p < 0.05$ and for L-929 cells: 87.5.2 ± 13.2; $p < 0.05$) and reached to maximum in 800 mg/L (% cell viability for K-562 cells: 30.5 ± 3.2; $p < 0.01$ and for L-929 cells: 42.5.2 ± 8.4; $p < 0.01$). The cell viability percentage values for concentrations 230 and 518 mg/L in K-562 cells was 47.6 ± 8.5 and 63.4 ± 8.1, and in L-929 cells was 56.2 ± 8.1 and 77.32 ± 8.9, respectively. In addition, no significant reduction of cell viability was observed when cells were treated with 50 mg/L of SMB in both cell lines. The IC50 of SMB for K-562 and L-929 cells after a 48 h exposure was 200.31 and 257.82 mg/L, respectively (Figure 1).

The results of LDH assays have also shown that SMB in concentrations observed in dried plum samples has cytotoxicity for normal cells. As seen in Figure 2, cell treatment with 115 mg/L of SMB significantly increased LDH activity when compared with non-treated control in both cell lines (% LDH activity for K-562 cells: 123.3 ± 10.9; $p < 0.05$ and for L929 cells: 120.4 ± 8.3; $p < 0.05$), and maximum effect was seen in 800 mg/L (% LDH activity for K-562 cells: 464.95 ± 41.6; $p < 0.01$ and for L929 cells: 391.7 ± 47.1; $p < 0.01$).

Many studies have demonstrated SMB cytotoxicity for normal human cells in these concentrations. For example, Yoo et al. (2018) reported that the treatment of the A-549 cell with SMB caused a dose-dependent reduction in cell viability at concentrations above 100 μg/ml. The IC50 value was determined as 281.5 mg/L (Yoo et al., 2018). In addition, Alimohammadi et al. (2021) revealed that SMB as a cytotoxic food additive promotes apoptosis in human fetal foreskin fibroblasts (HFFF2) cells. Various concentrations of SMB markedly reduced cell viability of HFFF2 cells in a dose-dependent manner, and the concentration of 25 μm of SMB was determined as IC50 value (Alimohammadi et al., 2021). Qu et al. (2017) reported that sodium sulfite had IC50 values 0.30 g/L at 24 h (Qu et al., 2017). In animal model studies, SMB increased oxidative stress, the number of apoptotic cells, caspase activation in the liver (Ercan et al., 2015), and gastric mucosal tissues (Ercan et al., 2013). In addition, genotoxic analysis in the presence of SMB has shown that some doses of SMB may pose genotoxic risks such as an increase in mean micronucleus frequencies in peripheral blood and bone marrow cells, a significant reduction in the ratio of polychromatic to normochromic (Carvalho et al., 2011), and chromosome aberrations and sister chromatid exchanges in cultured human lymphocytes (Rencüzoğullari et al., 2001).

According to toxicity data of SMB reported by mentioned studies, we decided to cytotoxicity analysis of SMB on the normal cell in the presence of concentrations detected in the dried plum samples. Our results in agreement with previous studies showed that SMB decreased the cell viability of normal cells in a dose-dependent manner. In addition, the IC-50 value of the SMB for k562 and L929 cells were approximately similar to the value reported for A549 cells (Yoo et al., 2018). However, the SMB IC50 value revealed for HFFF2 (4.75 mg/L) (Alimohammadi et al., 2021) was remarkably lower than the values found in the current study and work performed by Yoo et al., (Yoo et al., 2018). As our results indicated that k-562 and L-929 cells did not show any cytotoxic effect at concentrations less than 50 mg/L. The differences in IC50 values can be related to cell-specific responses in different cell lines, because of their unique and individual biological properties even when they were established from the same tissue, species, and histological type of tumor.

3.3 | Effect of SMB on cell apoptosis/necrosis by flow cytometry and real-time PCR

Previously, it was indicated that SMB could exert its cytotoxic effects on normal human cells via activation of cell apoptosis (Alimohammadi et al., 2021). Therefore, to find the underlying mechanism involved in the cytotoxicity of SMB on normal human cells, the number of apoptotic cells, as well as the mRNA expression of apoptosis-related genes in the presence of SMB was analyzed. To

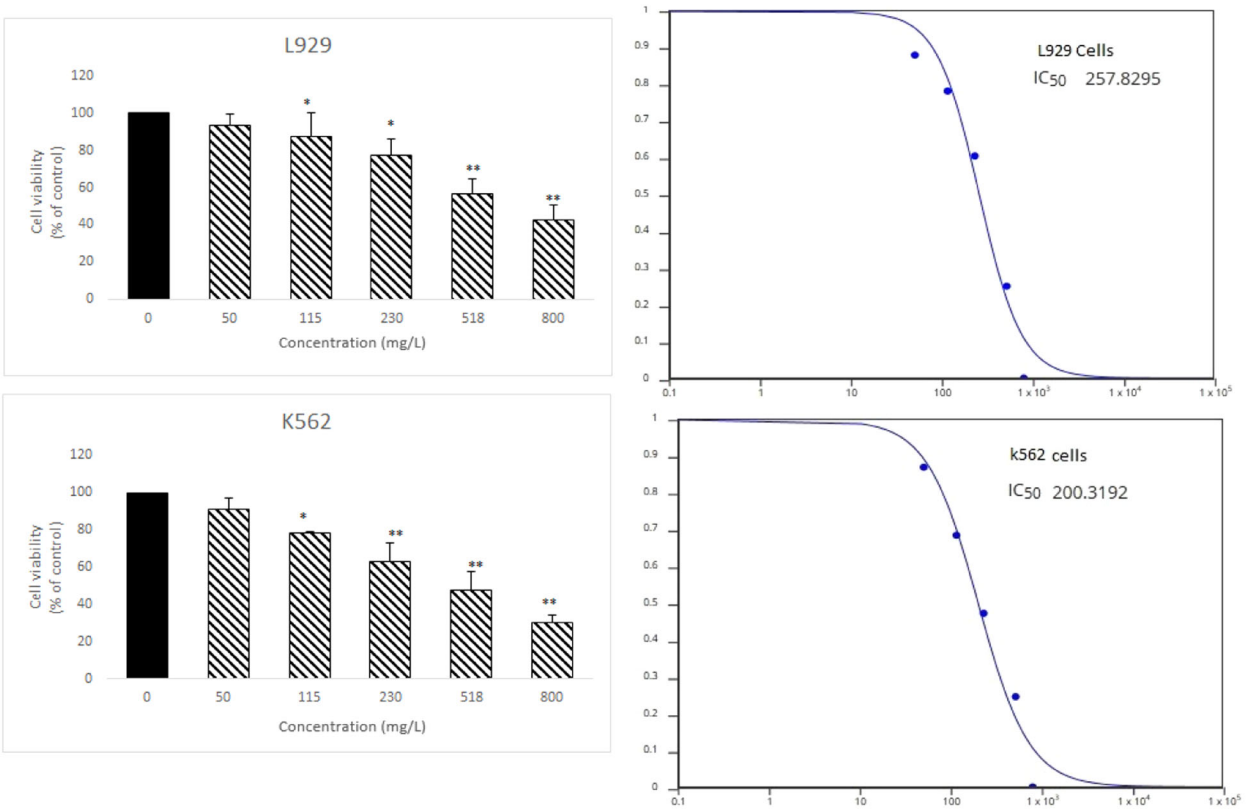

FIGURE 1 Effect of SMB on K-562 and L929 cell growth. The cells were cultured in 10% FBS medium and treated with 50, 115, and 250, 515, and 800 mg/L SMB for 48 h by MTT assay. The percent of viable cells was calculated in comparison to untreated cells. The number of cells in the control was taken as 100%. Values were expressed as mean ± SD of three independent experiments, each performed in triplicate ($^{*}p < 0{:}05$ and $^{**}p < 0{:}01$). IC50 for each experiment was calculated using free online IC50 calculator software (https://www.aatbio.com/tools/ic50-calculator)

reveal the effects of SMB on cell apoptosis, the K-562 cells were treated with a selected concentration (500 mg/L) of SMB for 48 h, and the percentage of alive, necrotic, and apoptotic cells were detected using Annexin V-FITC/ PI staining in conjunction with flow cytometry. We found that the percentage of apoptotic cells (29.1% alive, 2.61% necrotic, 21.29% early apoptotic and 48.2% late apoptotic) increased in compression with non-treated controls (96.48% alive, 3.35% necrotic, 0.00% early apoptotic and 0.17% late apoptotic) after SMB treatment for 48 h ($p < 0.01$; Figure 3).

Similar to our findings, Han et al. (2020) used human hepatocyte L02 cells as a model cell line to evaluate the toxicity of sodium sulfite (Han et al., 2020). They reported that with an increase of sodium sulfite concentration, the morphology of L02 changed, cell proliferation and activity were inhibited, and sodium sulfite caused apoptosis in a concentration- and time-dependent manner (Han et al., 2020). Another study investigated the toxicity of Sodium sulfite and mechanisms of damage in HepG2 cells using High Content Analysis (HCA). Sodium sulfite was observed to decrease the growth of HepG2 cells in a dose-dependent manner. Sodium sulfite had effects on cell number, membrane permeability, mitochondrial membrane potential, intracellular calcium level, oxidative stress, and high dose group DNA damage (Qu et al., 2017).

In order to confirm this notion, we decided to measure the mRNA expression of anti-apoptotic (Bcl-2) and apoptotic (P53 and BAX) markers in K-562 cells treated with SMB. We found cell treatment with SMB significantly reduced the mRNA expression of Bcl-2 and potentiates the expression of P53 and BAX in a time-dependent manner. As shown in Figure 4, after MBS treatment for 12 h and 24 h, the expression of Bcl-2 decreased by 72 ± 16% ($p < 0.01$) and 61 ± 15% ($p < 0.01$) compared with control values, in K-562 cells, respectively. Whereas, we observed the increase in the expression of BAX (in 12 h: 2.2 ± 0.14 fold and in 24 h: 3.0.3 ± 0.14-fold vs non-treated control values, $p < 0.01$) and p53 (in 12 h: 1.9 ± 0.12 fold and in 24 h: 2.2 ± 0.13-fold vs non-treated control values, $p < 0.01$) in presence of SMB.

Previously, it was indicated that SMB could exert its cytotoxic effects on normal human cells via activation of cell apoptosis (Alimohammadi et al., 2021). Therefore, to

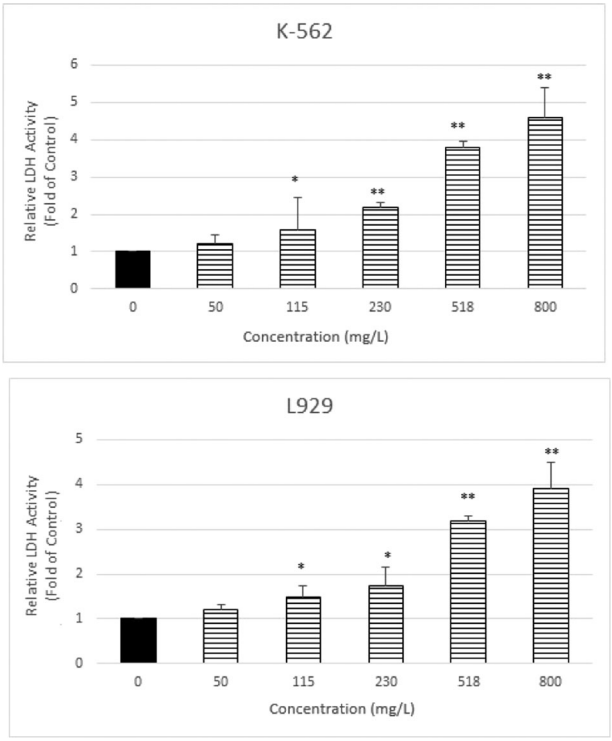

FIGURE 2 Effect of SMB on LDH activity in K-562 and L929 cells. The cells were cultured in 10% FBS medium and treated with 50, 115, and 250, 515, and 800 mg/L SMB for 48 h by LDH analysis assay kit. The percent of LDH activity was calculated in comparison to untreated cells. The number of cells in the control was taken as 100%. Values were expressed as mean ± SD of three independent experiments, each performed in triplicate ($^{*}p < 0{:}05$ and $^{**}p < 0{:}01$)

find the underlying mechanism involved in the cytotoxicity of SMB on k-562 and the normal human cells, the number of apoptotic cells and the mRNA expression of some apoptosis-related genes in the presence of SMB was analyzed. Our findings demonstrated that increased cell toxicity by SMB was associated with a significant increase in early and late apoptosis, but not cell necrosis. To confirm the stimulatory effect of SMB on cell apoptosis, we measured the gene expression alterations of the P53, Bax, and Bcl-2 as apoptosis markers in the presence or absence of SMB. P53 is an important tumor suppressor gene that has a crucial role in the induction of apoptosis-mediated cell death (Borrero & El-Deiry, 2021; Lim et al., 2021). Our findings showed a significant increase in the expression of P53 in k-562 cells following treatment with SMB. The Bax2 (apoptotic protein) and Bcl-2 (anti-apoptotic protein) are apoptosis-related markers that are known downstream targets of the P53 transcription factor (Hemann & Lowe, 2006; Toshiyuki & Reed, 1995). After activation by various signals, the P53 induces expression of Bax2 and inhibits Bcl-2 expression, which ultimately leads to a promotion of cell apoptosis (Aloni-Grinstein et al., 2018; Dewson & Kluck, 2010). Our findings also showed an opposite expression pattern of Bcl-2 and Bax2 when k-562 cells were treated with SMB. Our results regarding the effects of SMB on cell apoptosis agree with findings reported by Alimohammadi et al. They also reported a significant upregulation of Bax, caspase 8, and caspase 9 pro-apoptotic genes and downregulation of Bcl-2 expression after cell treatment with SMB (Alimohammadi et al., 2021). Our results also showed that SMB suppressed the gene expression of nuclear factor kappa B (NF-κB) (Figure 4d), a pro-survival and

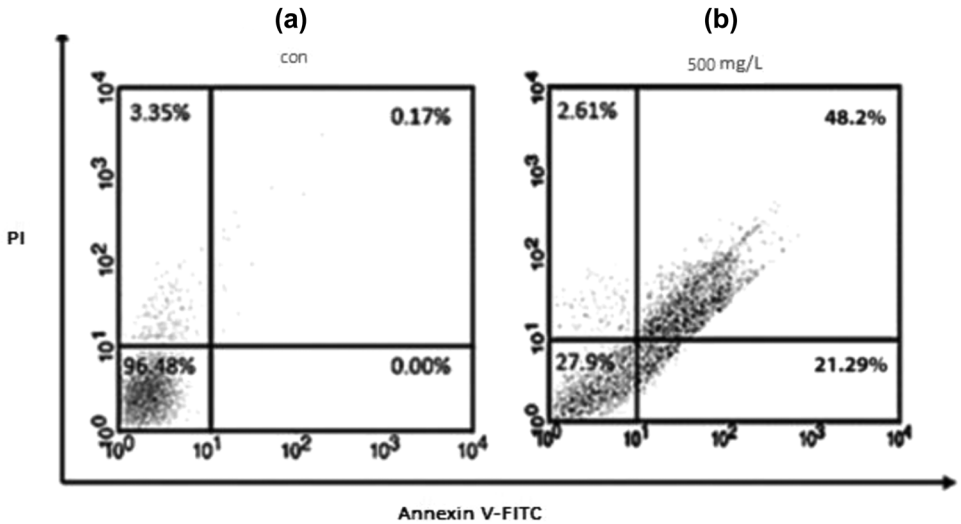

FIGURE 3 Effect of SMB on K-562 cell apoptosis. (a) Non-treated control cells; (b) The K-562 cells ($1{\times}10^6$ cells per well) were treated with 500 mg/L SMB for 48 h. Then, the cells were harvested by centrifugation, and washed twice with PBS and stained with Annexin V-FITC/ PI the treated cells for 15 min. Percentage of alive, apoptotic and necrotic cells was detected by Annexin V-FITC/ PI staining in conjunction with flow cytometry. The data analysis was performed by Cell Quest software

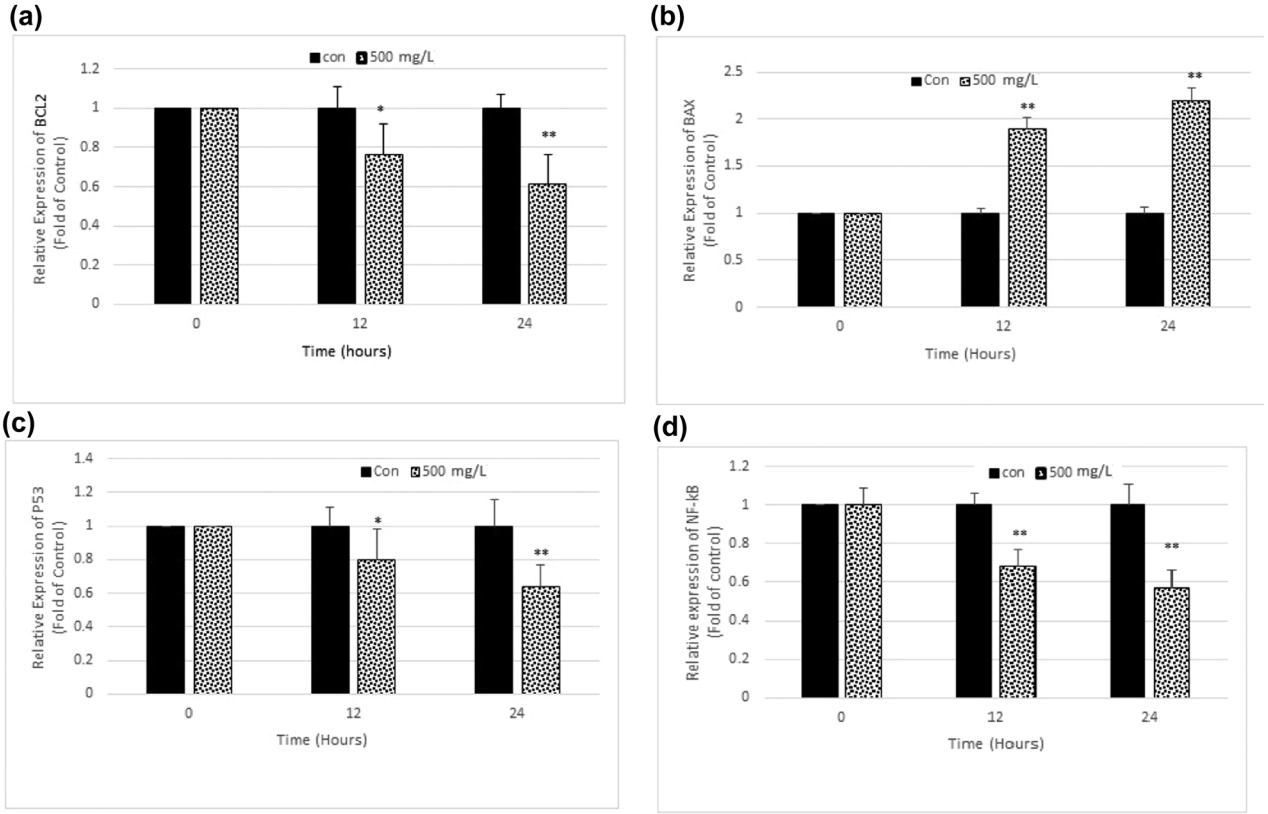

FIGURE 4 Effect of SMB on mRNA gene expression of apoptosis markers. The cells were treated with 500 mg/L SMB for 12 and 24 h. mRNA were extracted from treated cells, cDNA were synthesized and subjected to Real-time PCR and gene expression was detected by Real Time PCR. The histogram represents the quantitative analysis of mRNA expression of (a) BCL2; (b) BAX; (c) P53; and (d) NF-kB in the presence and/or absence of SMB. Values were normalized by GAPDH. The mRNA levels in control cells were taken as 1 fold. Values were expressed as mean ± SD of three independent experiments, each performed in triplicate ($^{*}p < 0{:}05$ and $^{**}p < 0{:}01$)

anti-apoptotic marker (Li et al., 2013). NF-κB plays a critical role in modulating cell apoptosis and is generally regarded as an anti-apoptotic factor (Puar et al., 2018). This anti-apoptotic protein generally is inhibited in the cytoplasm by the I-kappaB (IkB) protein (Ahn et al., 2006). Degradation of IκB is important for activation and nuclear translocation of NF-κB (Ahn et al., 2006; Li et al., 2013). Once NF-kB is translocated into the nucleus, the NF-κB binds promoter regions of its target genes to activate transcription (Ahn et al., 2006; Puar et al., 2018).

4 | CONCLUSION

The present findings reveal that SMB decreases the cell viability of normal cells in a dose-dependent manner in the concentrations that are present in some dried fruits. SMB induces cell apoptosis and apoptosis-related genes as an important mechanism responsible for its cytotoxicity. Thus, its widespread usage as a way for drying, and preserving dried fruit, such as plum, should be reconsidered. It is essential to be careful when using it, and it is necessary to find new safe substances alternative to SMB.

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AUTHOR CONTRIBUTIONS

Ahmad Ghasemi: Data curation; Funding acquisition; Methodology; Project administration; Software; Supervision; Writing – review and editing. Amir Salari: Conceptualization; Funding acquisition; Supervision. Mahboube Kalantarmahdavi: Data curation; Investigation; Methodology; Writing – original draft. Mohammad Reza Amiryousefi: Data curation; Funding acquisition; Project administration; Supervision.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ORCID

Ahmad Ghasemi https://orcid.org/0000-0002-4912-9396
Amir Salari https://orcid.org/0000-0001-7815-0496
Mahboube Kalantarmahdavi https://orcid.org/0000-0002-1179-1376
Mohammad Reza Amiryousefi https://orcid.org/0000-0002-3832-9325

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