Two Faces of Arbutine in Hepatocelluler Carcinoma (HepG2) Cells: Anticarcinogenic Effect in LD_{50} Dose and Protective Effect Against Cisplatin Toxication Through its Antioxidant and Anti-Inflammatory Activity in LD_0 Dose

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Abstract

Arbutine is one of the active substances used as a skin whitening agent in cosmetic products. Possible effects of arbutine on hepatocelluler carcinoma (HepG2) cells and cisplatin toxication in HepG2 cells were investigated in this study. Cytotoxicity, genotoxicity, oxidative stress, inflammation, apoptosis and proliferation levels were determined in experimental groups established for the purpose above. It was determined that when low dose of α-arbutine (in LD_0 dose) was administered to HepG2 cells alone, it had no genotoxic and cytotoxic effects and no effects on inflammation, apoptosis and proliferation. However, when low dose of arbutine was used with cisplatin, it was observed that oxidative stress, inflammation, and genotoxicity levels increased as a result of cisplatin toxicity, but caspase 3 levels were not affected by this situation. As a result of high dose (in LD_{50} dose) of α-arbutine administration to HepG2 cells, it was determined that it would have anticarcinogenic effects by increasing oxidative stress, genotoxicity, inflammation and apoptosis and by suppressing proliferation. In the presented study it was determined that as well as α-arbutine had an anticarcinogenic effect on HepG2 cells in high doses, it might be protective to reduce the side effects caused by low dose (LD_0 dose) of cisplatin treatment. In addition, it was concluded that α/β-arbutine-including cosmetic products were safe for cancer patients because α/β-arbutine had no effect on proliferation in HepG2 cells. In order to present the activity of arbutine isoforms more clearly it is recommended that studies should be conducted using healthy and different cell lines.

Introduction

Cancer is among the global problems affecting public health and economy. According to GLOBOCAN database data prepared by International Agency for Research on Cancer (IARC) using the data from 185 countries, 18.1 million people in the world were diagnosed as cancer and 9.6 million died of cancer in 2018. If cancer types are listed in terms of incidence, lung (11,6%), breast (11,6%), colon (10.2%), prostate (7,1%), stomach (5.7%) cancers can be uttered in the top five. Liver cancer is the 6th most common cancer type with its 4.7% of incidence rate [1].

From past to present Cisplatin [cis-diamminedichloroplatin (II)] has been one the most commonly used chemotherapeutics whose biological activity was first reported in 1965. Cisplatin is among the effective chemotherapeutics that can be also used for the treatment of hepatic cancer. Cisplatin is also effective against various types of cancer including carcinomas, germ cell tumors, lymphomas and sarcomas. However, side effects of cisplatin (nephropathy, allergic reactions, decreased immunity to infections, bleeding and hearing loss especially in young patients, etc.) and developing drug resistance may restrict the activity of cisplatin in the treatment [2–4].

Arbutine is used as a powerful skin whitening agent in cosmetic industry due to its strong inhibitory effect on human tyrosinase activity [5]. It can also cure cystitis and urinary tract infections as it expels kidney stones and has antibacterial properties [6]. Moreover, it is commonly used in pharmaceutic industry due to its antioxidant, antimicrobial and anti-inflammatory activities [7]. Many studies have shown that it can be particularly effective on skin cancer [8, 9].

Arbutine in glyloside form has two different isomers depending on the binding way of the hydroquinone bound to the anomeric carbon atom in the glucose molecule. These isomers are α-arbutine and β-arbutine. Natural arbutine isolated from plants is β isomer [10]. Alpha arbutine is usually produced through the transglucosylation of hydroquinone by microbial glycosyltransferases. Seven different microbial enzymes that can produce α-arbutine including alpha amylase, sucrose phosphorylase, cyclodextrin glycosyltransferase, α-glucosidase, dextransucrase, amylosucrose and sucrose isomerase were identified [5]. In addition, deoxycarbutin (4-[(tetrahydro-2H-pyran-2-yl) oxy] phenol) obtained by reducing arbutine (removing hydroxides from the pattern in glucose ring in the hydroquinone side chain) was also produced synthetically and used in the composition of cosmetic products [11]. The conducted studies indicate that cytotoxicity of arbutine types is lower than hydroquinone and therefore it can be regarded as a safer skin whitening agent [11–13].

Hydroquinones have been used for nearly half a century in the treatment of melasma, hyperpigmentation and especially facial freckles. The use of hydroquinone which is a derivative of benzene, a carcinogenic substance, has recently been forbidden especially in cosmetic products [14]. One of the most important reasons is that it has been shown by scientific studies

conducted in clinics, experimental animals and cell culture models that hydroquinones can induce formations such as oxidative stress and DNA damage in the organism [15–18]. Hydroquinones can easily be formed through arbutine hyrolysis (Fig. 1). Hydroquinone in low concentrations was found in human urine and plasma [19]. These hydroquinone amounts are lower than the amounts of arbutine taken with food. This means that arbutine isoforms release the hydroquinone in glycoslated form through hydrolysis only when they reach the tissues or cells they will affect. Therefore, arbutine isoforms can only have effects on some tissues and cells (including enzymes that can hydrolise the arbutine hydroquinone) without having toxic effects for the whole organism as hydroquinones do.

Concerning the reliability of α-arbutine and β-arbutine in cosmetics, Scientific Committee of Consumer Safety (SCCS) states that α-arbutine rate in cosmetics should have to be 2% in face creams and 0.5% in body lotions [20] and this rate can be increased to 7% in face creams for β-arbutine [21]. This information indicates us that β-arbutine isolated from natural sources is used in cosmetic products in higher rates than α-arbutine produced sythetically. In other words, it states that synthetically produced α-arbutine can have effects even in low concentrations. This can be due to the fact that α-arbutine inhibites the tyrosinose enzyme in 15 times lower concentrations than β-arbutine. Thus, the conducted studies indicate that while LD_{50} doses which are effective in inhibation of tyrosinose enzyme are 2 mM for α-arbutine, they are 30 mM for β-arbutine [22, 23].

New treatment protocols that are non-toxic because of their side effects, multi-targeted and do not cause drug resistance are needed in the treatment of liver cancer and other types of cancer. In this context, natural active substances with limited toxic effects continue to be alternative. The studies conducted in this field indicate that natural active substances may contribute to the development of new solutions for cancer treatment. The effects of α-arbutine and cisplatin in HepG2 cells were investigated separately and together in this study. Thus, the possible benefits/harms of arbutine against liver cancer and cisplatin intoxication in liver cancer were tried to be determined on an in-vitro model.

Materials And Methods

Cell lines and culture conditions

HepG2 cells were obtained from the American-Type Culture Collection (ATCC) (Manassas, VA, USA). The complete medium used for the reproduction of cells was prepared using low glucose (1000 mg/L) DMEM (Sigma) including 10% of Fetal bovine serum (Copricorn), 1% of penicillin streptomycin (Sigma), 1% of sodium pyruvate (Sigma), 1% of glutamin (Sigma). All incubations administered to the cells throughout the study were performed in a CO_2 incubator (Panasonic) at 37 °C and in a medium containing 5% of CO_2.

Thawing and Culturing Cells

HepG2 cells were brought to the laboratory in cryotubes in liquid nitrogen. The frozen cells in the cryotubes were first taken from liquid nitrogen to -80 °C. After waiting for 10 minutes, it was left to dissolve by incubating in a hot water bath at 37 °C for 1-2 minutes. In the meantime, 5 mL of medium was placed on a 15 mL of falcon tube. The cells in the cryotube which were about to dissolve completely in the water bath were taken to the prepared falcon tube (that medium was put into it). It was then gently pipetted and centrifuged immediately. At the end of the centrifuge, the medium was drawn with a pipette without contacting the cells accumulated on the falcon base. A second wash was performed by adding 2 mL of medium onto the cell palette again and gently pipetting. The medium remaining on the cells after the centrifuge was drawn again. HepG2 cells, which were dissolved as explained and purified from dimethyl sulfoxide (DMSO) in 2 washes, were dissolved in 1 mL of medium and then cultured to a 25 mL of flask. When the 25 mL of flask became capacious, the cells continued to be replicated by passaging to the 75 mL of flask.

Counting the Cells by Trypan Blue Method

When the cells are cultured to wellplates or flasks in order to use in analyses, it is essential to culture the same / close number of cells in each flask or wellplate. For that reason, before the cells were cultured in well plates, how many cells per mL were determined and the cells were counted. For this purpose, trypan blue cell counting method was used. Trypan blue is a paint that

enables live cells to be seen bright under the light. While live cells painted with trypan blue applied to the slide to be counted are seen as bright under the microscope, dead cells are seen as blue and flat. The cells in four counting zones (A-D) consisting of 16 squares each on the counting slide were counted and averaged and therefore the cell counting was completed. Trypan blue method used in live cell counting was tried to be outlined in Fig 2.

The number of cells per milliliter of medium-cell suspension is calculated using the formula following the cell count. The amount of dilution is important in terms of the reliability of counting. The average number of cells between 50 and 150 is an indicator that the cell suspension was processed to ideal dilution. If the average number of cells is very high, volume of the cells in trypan blue-cell mixture is reduced and more diluted cell suspension is obtained and the count is repeated. In this case dilution rate will also increase. The counting can be performed using the mixture obtained after the volume is completed to 100 µL with trypan blue (dilution rate is 10 or 5) by taking 10 or 20 µL of cell suspension in cases where the number of cells is very high. The following formula is used in order to determine the number of cell per mL:

Number of live cells per mL = (Average Number of Cells) x (10^4) x (Dilution Factor)

Cells were counted in the presented study as the following. Cells removed from the flask base by trypsinization and purified from trypsin by detripinization were dissolved in 4 mL of medium. 50 µL of cell suspension was taken and mixed with 50 µL of trypan blue (dilution rate is 2). Slides and lamellas (Neubauer counting chamber) to be used in counting were prepared. 10 µL of trypan-blue suspension mixture was taken by pipetting and mounted to the neubauer slide properly. Taking the averages of the cells counted in counting zones, the amount of cells per mL of cell suspension was found with the help of the formula. Afterwards, how many mL of the cell suspension should be taken according to the number of cells to be used in the applications was calculated and manipulations with the cells were initiated.

Cytotoxicity Analyses (MTT analyses)

Cytotoxicity levels of the arbutine isoforms, α-arbutine and β-arbutine, in HepG2 cells were determined by MTT (3-4,5-dimethyl-thiazolyl-2,5-diphenyltetrazolium bromide) method. Cancer cells were cultured in 96 piece-well plates as 5000 cells per 200 µL of medium in each well. Cells were incubated for 24 hours so that the cultured cells could take roots to the flask base. Following the incubation mediums of cells were exchanged with the mediums prepared by dissolving α-arbutine and β-arbutine in appropriate concentrations. The mediums with α/β-arbutines used in MTT analyses were prepared in 7 different concentrations between 0,5 and 200 mM. Each dose application was studied as at least 3 replications. Mediums without arbutines were added into the control wells in the same volume. Then, cells were again left to incubation for 24 hours.

The MTT solution was prepared as in 5 mg / mL of concentration by dissolving the MTT salt (Sigma) in phosphate buffer (PBS) with pH 7.4. At the end of the incubation, MTT solution in a volume of 10% of the well volume was added to each well. Cells were incubated in this way for 2-4 hours more. Incubation may be limited to 2 hours if there is excessive formation of formazan crystals (purple) in the wells. Because excessive increase in colour density in wells may lead to higher optical densities to be obtained from spectrophotometers. In this case it is possible to get no results from MTT analyses. For that reason, it is important to monitor the colouration in wells after the first two hours as the cells are incubated with MTT solution. If the formation of formazan crystals in the wells in MTT analysis is low, duration of the incubation can be extended to four hours. However, duration of incubation should not be exceed to 4 hours. Because long incubation durations may lead to coloration in dead cells due to the coloration in mediums by formazan crystals occuring in wells. This may cause to get false results from MTT analysis.

The medium in the wells was pipetted without damaging the formazan crystals formed after incubation with the MTT solution. Following this operation, 200 µL of DMSO was added to each well and formazan crystals were dissolved. Finally, optical density of the samples were almost immediately determined at 540 nm using ELISA microtype reader (Biotek, ELx800). Cell viability of the control group without any arbutine was regarded as 100 % and the effect of each dose on cell viability was calculated using the given formula below [24,10].

Cell viability (%) = [(100 * $Absorbance_{sample}$)/ ($Absorbance_{control}$)]

Lethal doses (such as LD_0, LD_{50}) belonging to arbutine isoforms in HepG2 cells were determined using % viability rates that each dose created in cells. Lethal doses were calculated using Epa Probit Analysis Program (Version 1.5). After the lethal doses were determined in the study, experimental groups were created and administrations were initiated. The experimental groups created within the scope of the study were presented in Table 1.

Genotoxicity (Comet Assay and Micronucleus Test) Analyses

In order to determine how high and low doses of β-arbutin affect genotoxicity when administered to HepG2 cells, DNA damage and micronucleus frequencies were determined in the samples. 24 hours after the necessary operations in experimental groups indicated in Table 1, the cells were collected from the flask base by trypsinisation and detrypsinisation. Cell suspensions belonging to the experimental groups created in 1 mL of PBS (phosphate buffer seline) were used in the analyses.

Comet assay was used in order to determine DNA damage. 20 µL was taken from the cell suspension prepared for this purpose and mixed with 100 µL of LMA (low melting agarose) in eppendorf. All of the cell-LMA mixture in eppendorf was taken and mounted to slides prepared by treating with NMA (normal melting agarose) the day before. Each prepared material was painted following the lysis and electrophoresis stages. Painted preparations were scored by counting as 100 cells under fluorescence microscope [25].

Numbers (frequencies) of micronucleus as a result of arbutine administrations in samples were determined using micronucleus test. For this purpose, the cells belonging to the samples in each group were spread on clean slides. Dried slides were fixed with pure ethanol for 20 minutes. Then, slides were air dried. Cell nuclei were painted by waiting the dried slides for 20 minutes in 5 % of Giemsa solution. Three slides were prepared from each sample in the analyses. 1000 cells from each preparation were counted at 100X [26]. Micronucleus index values were evaluated over an average of 1000 cells.

Biochemical Analyses

Biochemical analyzes were performed on cell lysates obtained from cells. Cell lysates were prepared as the following. 24 hours after the mentioned administrations to the experimental groups (Table 1) cells were collected from falcon base following trypsinisation and detrypsinisation. Cells were finally washed with 1 mL of PBS. After the wash, 500 µL of lysis buffer (PBS solution containing 1 % of triton-X-100 and 8 % of protease inhibitor cocktail) was added onto the cells. They were pipetted. Then, they were sonicated (Binder) for 20 seconds in ice. It was waited for 40 seconds. Sonication was repeated for 10 times in this way. As a result of sonication, intracellular / organelle fluids were allowed to pass into the lysis buffer. Insoluble proteins in lysis buffer despite all administrations were precipitated by centrifugation at 8500 rpm at 4 ^{0}C for 10 minutes. The supernatants obtained in this way were used as cell lyzates in chemical analyses. Lysis buffer was used as a blank in analyses. Total protein, oxidative stress and proinflammatory cytokine levels were determined through biochemical analyses in the obtained cell lyzates.

Total antioxidant status (TAS) and total oxidant status (TOS) analyzes, which were analyzed as oxidative stress parameters, were spectrophotometrically conducted using commercial kits (Rell Assays). In addition, OSI levels were calculated by considering TAS levels determined as a general expression of antioxidants in the samples and TOS levels determined as a general expression of oxidants in the samples. OSI levels were calculated according to the following formula in accordance with the kit protocols [10].

Oxidative Stress Index (OSI) = [(TOS/TAS) x 100]

Proinflammatory cytokine (TNF-α, IL-6 and TGF-β) levels were determined at 540 nm in a microplate reader (Biotek ELx800) using human specific ELISA kits (Sun Red). The data obtained from the biochemical analysis were normalized by dividing each sample's own total protein levels. Protein levels of cell lyzates used in biochemical analyses were determined by spectrophotometrical method at 595 nm with the help of a commercial ELISA kit (Fluka) in which Coommassi Brilliant Blue reactive was used.

Determination of RNA Isolation, cDNA Synthesis and mRNA Expression Levels

Bcl-2 and Caspase 3 mRNA expression levels were analyzed by Reverse Transcription-Polymerase Chain Reaction (RT-PCR) method using total RNA isolated from control and treatment groups in HepG2 cells and cDNAs synthesized afterwards. In the analyses total RNAs belonging to HepG2 cells were isolated by using commercial kit (Gene Matrix). Concentration and quality of the obtained RNAs were determined in nanodrop (BioTek, Epoch 2). Among the isolated RNAs the ones with optical densities ($OD_{260/280}$) between 1.7 and 2.2 obtained at 260 nm and 280 nm were used in the study.

In order to be used as a template in the PCR reaction, 2 µg of total RNA belonging to each sample was taken and complementary DNA (cDNA) synthesis was first performed through reverse transcriptase (RT). For this purpose, cDNA synthesis kit was used (Thermo). Sybr Green PCR Master Mix (12.5 µL) and primer pair (oligonucleotide) were added in accordance with the protocols using 1.5 µL of the cDNA belonging to each sample. Primers are specific for each transcription analysis and were determined using studies in the literatüre [24,28]. The primers presented in Table 2 were used as 100 ng in each RT-PCR reaction.

With reference to the cycling threshold (Ct) values of the amplification curves of each sample in the analysis performed on the RT-PCR device (Biorad CFX-96), the relative changes in the mRNA expression levels of the target genes were calculated by 2^{-DDCt} method [29]. Calculations were performed using REST 2009 software (Qiagen, Germany). mRNA expression levels were determined as fungible decrease or increase. Beta actin gene was used as the endogenous control. Expression levels of other genes were emended (normalized) according to the beta actin gene level of each sample.

Immunocytochemical Analyses

The number of caspase 3 positive ($Cas3^+$) cells and the percentage of the number of p53 ($p53^+$) positive cells in experimental groups were determined in the study through immunocytochemical analyses. For this purpose, the cells detected in 4 % of buffered neutral paraformaldehyde solution for 24 hours were washed 3 times with distilled water at the end of incubation. Routine tissues were monitored using palettes through cytoblock administration. Cells were blocked in paraffine and sections with 4 µm of thickness were cut using a microtome. The samples taken onto the adhesive coated slides were stained using immunocytochemical technique. For this purpose, rabbit anti-caspase 3 (1/200 of dilution, Abcam, ab13847, Cambridge, UK) and Mouse anti-p53 (1/25 of dilution, DAKO, M7001, CA, US) antibodies were instilled on the samples. Avidin Biotin Complex (ABC) method kit (TA-125-UDX, UltraVision Polyvalent HRP Kit, LabVision/ThermoScientific-US) administration was initiated. First, biotinylated igG was dropped. It was incubated for an hour at room temperature. Then, horse radish peroxidase enzyme conjugated avidin was instilled and left to the reaction for 30 minutes at 37 ºC. Slides were washed and tissues were treated with 3-amino-9-ethylcarbazole (AEC) peroxidase substrate (TA-060-HA, AEC Substrate System, LabVision/ThermoScientific-US). Finally, Gill's (III) hematoxylin was used for the background staining and the slides were covered using an aqueous mounting medium. All samples were examined under light microscopes. They were analyzed by using Zeiss Axio Lab.A1 Microscope- AxioCam ICc 5 Camera (ZEN 2, Carl Zeiss Microscopy GmbH) and image J software [10]. The number of caspase 3 positive ($Cas3^+$) cells per mm^2 was used when evaluating caspase 3. The percentage of the number of $p53^+$ cells per unit squares (10 largest magnification zones) was used when evaluating p53.

Statistical Analyses

The obtained data were identified as average ± standard deviation (SH) and SPSS 18 package program was used in data evaluation. Whether the data were normally distributed or not was initially tested. One-way analysis of variance (ANOVA), one of the parametrical tests, and Duncan test as post hoc test were applied to the data with normal distribution. Kruskal-Wallis test, one of the non-parametrical tests, was applied to the analysis results without normal distribution to determine whether there was a statistical difference or not. Which groups from the control groups indicated statistical difference in the parameters in which there was a statistical difference between each other was determined by the Mann-Whitney U test.

Results

Determination of cytotoxicity levels of arbutine isoforms and cisplatin in HepG2 cells

Data of the cells applied arbutine in different doses were compared with control group and the obtained % cell viability rates were presented in Fig 3.

The effect of seven different concentrations (0,5mM, 1 mM, 10 mM, 25 mM, 50 mM, 100 mM ve 200 mM) of α/β-arbutines on HepG2 cell viabilities were determined in the conducted analyses. The obtained findings indicate that α/β-arbutines have cytotoxic effect in all used doses. It was determined that cytotoxicity increased in direct proportional to used α/β-arbutine doses.

Cytotoxicity of the cisplatin in HepG2 cells was also determined through MTT analysis. The effect of seven different concentrations (1µg, 2µg, 5µg, 10µg, 20µg, 40µg, 60µg, 80µg, and 100µg) of cisplatin on HepG2 cell viability was determined in the conducted analyses. It was concluded that cisplatin had cytotoxic effect in all used doses. Toxicity of cisplatin in HepG2 cells were indicated in Fig 4.

Lethal doses of both α-arbutine and β-arbutine and cisplatin in HepG2 cells were calculated using the data obtained from MTT analyses (Tablo 3). When the calculated lethal doses of α-arbutine and β-arbutine are compared, it is observed that LD_{50} dose of α-arbutine is lower. This means that the same rate of cytotoxicity in HepG2 cells can be achieved with lower doses of α-arbutine in LD_{50} dose. In other words, toxicity of α-arbutine is higher than toxicity of β-arbutine. Therefore, α-arbutine was used for the rest of the study.

The Effect of α-Arbutine in HepG2 Cells on Genotoxicity, Oxidative Stress and Inflammation

Conducted genotoxicity tests indicate that α-arbutine in HepG2 cells in LD_0 dose has no genotoxic effect. However, as a result of the administration of α-arbutine and cisplatin in LD_{50} doses, it was determined that both DNA damage (Comet scores) and micronucleus formation were statistically higher than the control group (Table 4) and therefore genotoxicity occurred. With the administration of cisplatin, it was determined that α-arbutine in LD_0 dose reduced the DNA damage (comet scores) and decreased the genotoxicity induced by cisplatin.

When the effect of arbutine in HepG2 cells on oxidative stress was examined, it was determined that arbutine did not affect TAS, TOS, OSI, MDA, GSH, NO parameters in LD_0 dose as compared to control group. It was observed that oxidative stress increased as levels TOS, OSI, MDA, NO went up when α-arbutine was administered in LD_{50} doses. It was determined that oxidative stress increased as OSI, NO levels went up when cisplatin was administered. However, it was observed that oxidative stress index decreased in the cells (Group 5) administered α-arbutine in LD_0 dose with cisplatin (Table 4).

In this studies conducted in order to identify the effect of α-arbutine on inflammation it was determined that arbutine in LD_0 dose did not affect the inflammation as compared to control group. It can be stated that acute inflammation occurs as IL-6 and TGF-β levels increase when α-arbutine is administered in LD_{50} dose and TNF-α, IL-6 and TGF-β levels increase when cisplatin is administered. It was identified that in the group (Group 5) in which α-arbutine was administered in LD_{50} dose along with cisplatin only TNF-α levels decreased as compared to the group in which cisplatin was administered.

The effect of cisplatin and α-arbutine on expression levels of caspase 3 and Bcl-2 mRNA in HepG2 cells

mRNA expression levels of Bcl-2, a proliferative gene and caspase 3, an apoptotic gene, were determined when α-arbutine and cisplatin were administered to HepG2 cells in different doses (Fig. 5). While Cas3 and Bcl-2 gene expression levels did not change compared to control group as α-arbutine was administered to the cells in LD_0 dose, Bcl-2 levels were suppressed as α-arbutine was administered to the cells in LD_{50} dose (Fig5A and Fig5B). However, it was found that when cisplatin in LD_{50} dose was administered to the cells, it suppressed the proliferation (Bcl-2) as expected from a chemotherapeutic agent, but induced apoptosis (Cas 3) (Fig5C). When the data of the group in which α-arbutine was administered to the cells in LD_0 dose along with cisplatin, it was observed that Bcl-2 levels were suppressed (Fig5D).

The numbers of Cas3 and p53 positive cells belonging to experimental groups

As a result of immunocytological analyses, the numbers/rates of $Cas3^+$ and $p53^+$ cells in experimental groups were calculated and presented in Table 5. The obtained data refer that number of $Cas3^+$ cells statistically increased in the groups in which α-arbutine or cisplatin was administered in LD_{50} dose. It was determined that α-arbutine in LD_0 dose did not affect the number of $Cas3^+$ cells. When $p53^+$ cell rates % were examined, it was found out that α-arbutine in LD_0 dose decreased the number of $p53^+$ cells; however, α-arbutine in LD_{50} dose could noticeably induce p53-mediated apoptosis. The microscope images obtained from the slides used in the statistical evaluation of the experimental groups mentioned in Table 5 were presented in Fig 6.

Discussion

New treatment protocols that are non-toxic because of their side effects, multi-targeted and do not cause drug resistance are needed in the treatment of liver cancer and other types of cancer. In this context, natural active substances with limited toxic effects continue to be alternative. The studies conducted in this field indicate that natural active substances may contribute to the development of new solutions for cancer treatment. It was aimed to determine the potential effects of α-arbutine in HepG2 cells, liver cancer cell lines, in this study. In addition, the effects of α-arbutine on inflammation, oxidative stress and genotoxicity occuring as a result of cisplatin toxication in HepG2 cells were tried to be determined. Thus, the question of “Are there any possible benefits/ harms of α-arbutine in reducing side effects by cisplatin in liver?” was sought to be answered.

Oxidative stress and inflammation are also regarded among the significant reasons of cellular damage occuring in cancer etiology. An increase in oxidative stress and inflammation with weakening of the immune system in the organism in advanced stages is observed during the cancer development. Therefore, antioxidant supplements may be important to increase the body resistance. However, considering that supplements can contribute to the growth of cancer cells, the use of antioxidant supplements in right doses (LD_0 dose) and in conscious way is important. For that reason, analyzing the effects of antioxidant substances on cancer cells and sharing those effects with the science world may contribute to the development of new approaches in cancer treatment.

While several antioxidants such as arbutine have a protective role for organisms and cells in low doses, they can be cytotoxic by inducing apoptosis and suppressing proliferation in high doses. Bioactive substances having protective effect in low doses and cytotoxic effect in high doses have been the subject of researches for many years due to their anticarcinogenic effects in cancer cells. In order to determine by which mechanisms α-arbutine in high doses induces apoptosis in HepG2 cells, inflammation, oxidative stress and genotoxicity levels were analyzed in experimental groups as well as apoptosis level. In the light of the findings, it was tried to be outlined that by which mechanisms α-arbutine applied to HepG2 cells especially in LD_{50} dose might have anticarcinogenic effect.

As a result of MTT analyses, it was determined that high doses of arbutine isoforms in HepG2 cells might have a cytotoxic effect. It was observed that as the doses of both α-arbutine and β-arbutine increased in HepG2 cells, cell viability decreased, but their cytotoxicity increased. No similar study was found in literature on HepG2 cells and arbutine. However, it is stated in the literature that arbutine does not indicate cytotoxicity in low doses [1], but it may have cytotoxic effects in high doses, therefore we can mention about the anticarcinogenic activity [10].

As a result of MTT analyses, it was determined that the cytotoxicity of α-arbutine, one of the isoforms of arbutine, was higher than β-arbutine in HepG2 cells. Different cytotoxicities of arbutine isomers may be related to the different pharmacokinetics of both isomers. It is stated also in a study conducted with a different cell line in literature that the cytotoxicities of α-arbutine and β-arbutine in MCF-7 cells are different. It is stated in the related study that β-arbutine has more cytotoxic effects than α-arbutine in MCF-7 cells [10].

Since it was also aimed to determine the effects of arbutine on cisplatin toxication in this study, LD_{50} dose of cisplatin that might have cytotoxic effect in HepG2 cells were determined through MTT analyses. It was determined that cytotoxicity of cisplatin in HepG2 cells rapidly increased as of 2 μg/mL of concentration. LD_{50} dose of cisplatin for HepG2 cells was found as

11,06 µg/mL in the calculations. As the studies on HepG2 cells in literature are examined, it is stated that cytotoxic doses of cisplatin are higher. For instance, in a study using cisplatin doses in the 5-80 µg/mL concentration range, it is stated that cisplatin can only reduce proliferation in HepG2 cells by 30-40 % in high doses (40-80 µg / mL) [3]. Such different cytotoxicities of cisplatin in HepG2 cells can be related to the used cisplatin solution. Because additional buffering agents such as mannitol can be added to cisplatin solutions in injectable form used for cancer patients in order to reduce toxic effects and side effects. Not the injectable cisplatin solution prepared for the treatment of cancer in humans, but the active substance cisplatin itself was used in the presented study. For that reason, toxication rate of cisplatin might have been found higher than the literature.

After the lethal doses of α-arbutine, β-arbutine and cisplatin were determined in this study, the second part of the study was initiated to determine the effects of arbutine on genotoxicity, oxidative stress, inflammation, apoptosis and proliferation in HepG2 cells. As previously mentioned, only α-arbutine was administered to the cells since the cytotoxicity of α-arbutine was found higher in this stages of studies. Possible therapeutic (toxication decreasing) effect of arbutine was tried to be determined by using LD_0 dose (0,003 µM) of α-arbutine against cisplatin toxication.

It can be said that cisplatin in LD_{50} dose has a genotoxic effect in HepG2 cells by increasing both DNA damage and micronucleus development. In literature it was stated with different studies that cisplatin caused genotoxicity by increasing DNA damage and micronucleus development [31,32]. It was identified that when α-arbutine was administered to HepG2 cells in LD_{50} dose, α-arbutine had statistically more significant and higher genotoxic effects not as much as cisplatin as compared to control group. No study has been found in the literature on arbutine and HepG2 cells. However, in an in-vitro study it is stated that orally administered arbutine did not cause DNA damage in rat hepatocytes [33]. However, in a study on MCF-7 cells, a cell line out of HepG2, it is stated that β-arbutine, another isomer of arbutine, might have genotoxic effects by increasing both DNA damage and micronucleus development in high doses [10].

It was determined in this study that when α-arbutine was administered in LD_0 dose (group 5) after cisplatin toxication was induced in HepG2 cells, DNA damage decreased as compared to cisplatin group (Group 4). These data bring to mind that α-arbutine in LD_0 dose may be beneficial in reducing possible side effects of cisplatin in healthy cells by reducing toxicity caused by cisplatin toxication. In addition, the reason why low doses of α-arbutine reduce DNA damage may be related to the antioxidant activity of arbutine administered in low doses. In fact, in a study conducted by using U937 macrophage cells [34], it is stated that arbutine has antioxidant activity by reducing the oxidative stress caused by ionizing radiation in U937 cells. It is stated that arbutine shows this activity by neutralizing (scavenging) hydroxyl radicals.

It is seen in the studies conducted using arbutine and different cell lines that the use of arbutine for therapeutic purposes is in low doses. Studies indicate that arbutine in low doses may create antioxidant and anti-inflammatory effects without having any cytotoxicity. The effects of arbutine on oxidative stress and inflammation increasing as a result of toxication created with lipopolisaccharide in rat BV2 microglia cells were investigated in a study. The findings indicate that arbutine reduces the oxidative stress and inflammation caused by the lipopolisaccharide without any cellular toxicity [35]. Protective effects of arbutine in low doses (50-100 ve 150 µM) determined as non-cytotoxic against the toxication created with tert-butyl hydroperoxide (t-BHP) in HepG2 cells were investigated in another study [36]. It is stated that the administration of arbutine in low doses before toxication increases the antioxidant power in HepG2 cells and therefore might reduce the cell deaths as a result of toxication. In this study cisplatin toxication was created and it was determined that both oxidative stress and inflammation increased in HepG2 cells as a result of toxication. It was also determined that α-arbutine treatment in low doses following the toxication with cisplatin in HepG2 cells reduced the oxidative stress by decreasing OSİ levels and the inflammation by decreasing TNF-α levels. (Table 4). Probably, these effects of arbutine in low doses may be related to strong antioxidant activity of Arbutine [37,34].

When a high dose of α-arbutine (57,471 mM) was administered to HepG2 cells, it was determined that oxidative stress was induced by the increase in MDA, NO and OSI levels and acute inflammation was induced by the increase in IL-6 and TGF-1β levels (Table 4). No source studies have been found in the literature regarding the administration of arbutine in high doses in

HepG2 cells. However, it is stated in a study with MCF-7 cells, another cell line, that β-arbutine used in a high dose may increase the oxidative stress and inflammation in cells [10].

The effects of arbutine, cisplatin and arbutine following the cisplatin toxication on apoptosis and proliferation were also aimed to be determined in our study. As a result of the analyses through RT-PCR and immunocytochemical methods it was determined that cisplatin caused apoptosis by inducing Cas3 expression in HepG2 cells. These data are parallel with the information in literature. Because it is stated that when cisplatin is administered to HepG2 cells, Cas3 can be induced and it can lead to apoptosis [38]. According to the obtained results, it can be said that cisplatin inducing the apoptotic Cas3 is an effective anticarcinogenic drug in HepG2 cells considering that it also supresses Bcl-2 mRNA gene expression levels as well (Fig5).

In the immunostochemical analyses, it was indicated that the number of p53 positive cells in the cisplatin group did not change compared to the control group. P53 is a highly important gene which plays a role in regulation of apoptosis and cellular cycle. Because cellular cycle can be deactivated by p53 effect for DNA repair in case of DNA damage. If DNA repair is not possible, apoptosis can be induced by p53 effect. Therefore, excessive inhibition or loss in function of p53 is considered among the reasons of cancer diseases. For that reason, if there is an induction / suppression of the p53 gene in HepG2 cells as a result of cisplatin toxication, it is important to clearly reveal it. As previously mentioned, although it was determined in the presented study that cisplatin did not cause any change in the number of $p53^+$ cells in HepG2 cells, it is stated in the literature that p53 can be induced via cisplatin and deactivate the cell cycle [39,40]. Information in literature partly contradicts with our findings. To determine and clarify the possible effects of cisplatin on p53 in hepatocellular carcinoma is important for explaining cisplatin activity in treatment. For that reason, it would be beneficial to carry out further studies on this subject.

It was determined that the number of $p53^+$ cells increased (Table 5, Fig 6) when HepG2 cells were treated with α-arbutine in LD_0 dose after administration of cisplatin. The fact that an agent used in combination with cisplatin induces apoptotic p53 means that anticarcinogenic activity is increased. Therefore, it can be considered that a low dose of arbutine treatment along with cisplatin in liver cancer may contribute to the current treatments. Hence, the studies with arbutine and HepG2 indicate that the number of apoptotic cells may increase as a result of the increase in Bax/Bcl-2 rate and the induction of Cas3 as the used arbutine dose increases [41]. In this context, it was determined in this study that while the number of p53 positive cells significanty increased when α-arbutine was administered to HepG2 cells in LD_{50} dose, Bcl-2 mRNA expression levels were suppressed. These data indicate that while high doses of arbutine induce apoptosis through p53, it may have anticarcinogenic effect (Fig 7) by suppressing the proliferation through Bcl-2. Similarly, in a study conducted with B16 murine melanoma cells, a different cell line, it was indicated that arbutine induced apoptosis [42] while it reduced the proliferation of cancer cells by suppressing the genes such as Bcl-xL and Bcl-2.

In conclusion, it was determined that when a low dose of α-arbutine (in LD_0 dose) alone was administered to hepatocellular carcinoma (HepG2) cells, it had no genotoxic and cytotoxic activity and did not affect inflammation, apoptosis and proliferation. In addition, it was determined that when a low dose of arbutine was used for therapeutic purpose following the cisplatin toxication, decreases in oxidative stress, inflammation and genotoxicity levels increasing as a result of cisplatin oxidation were observed; however, caspase 3 levels were not affected by this situation. In other words, arbutine administered in LD_0 dose reduces the severity of symptoms accompanying toxication without impairing the anticarcinogenic activity of cisplatin created by apoptosis in HepG2 cells (Fig 8).

These data bring to mind that when low doses of arbutine is used combined with cisplatin, it may be beneficial to reduce the side effects that may occur in other healthy cells. In order to determine this precisely, it may be useful to conduct similar studies using in-vitro models with healthy cell lines or in-vivo models using experimental animals.

As previously mentioned and the mechanism of activity is indicated in Fig. 7, it was determined that α-arbutine may have anticarcinogenic effects on hepatocellular carcinoma cells by increasing oxidative stress, genotoxicity, inflammation, apoptosis and suppressing proliferation as a result of arbutine administration in high doses (LD_{50} dose) to HepG2 cells. It may be recommended to determine what kind of positive/negative effects the arbutine in high dose may have on cells in other

tissues and organs while the activity of α-arbutine, which is determined in the cellular dimension, occurs. For this purpose, by creating a hepatocellular carcinoma model in experimental animals, as well as determining the therapeutic effects on liver cancer with high dose of arbutine administrations, the possible effects on other tissues and organs can be determined.

Conclusıon

It is important to determine the effects of active substances such as arbutine on different disease models primarily in cell culture medium. Afterwards, if there are possible beneficial effects of the active substance whose effects are investigated, it is necessary to carry out further studies using modelings to be created with experimental animals and then phase studies in clinics. When the presented study is regarded in this context, the possible effects of arbutine as an active substance in liver cancer (HepG2) cells were investigated. It was determined that low doses of arbutine might be beneficial to remove the side effects of chemotherapeutics (cisplatin) used in cancer treatment; however, in high doses it might be used as an active substance with anticarcinogenic effect in hepatocellular carcinoma (HepG2) cells. In the light of the obtained findings, it is thought that it might be beneficial to carry out further studies in order to present the possible effects of arbutine on liver cancer and metabolism in detail.

Declarations

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Ethical approval: This article does not contain any studies with animals performed by any of the authors.

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Authors'contributions: Ömer Hazman: Methodology, Investigation, Writing-Reviewing and Editing; Hatice Evin: Investigation, Writing- Original draft preparation; Mehmet Fatih Bozkurt: Pathological analyses, Visualization, Data curation, İbrahim Hakkı Ciğerci: Software, genotoxicity analysis, Validation.

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Tables

Table 1. Experimental groups created in the study and the administrations to cells

Groups	Administrations to the cells
Group 1 : **Control**	The medium used as a solvent to dissolve α-arbutine and saline solution used as a solvent for cisplatin were administered in the same volume.
Group 2 : **α-arbutin (LD_0)**	α-arbutine dissolving in the medium was administered to cells in LD_0 dose.
Group 3: **α-arbutin (LD_{50})**	α-arbutine dissolving in the medium was administered to cells in LD_{50} dose.
Group 4: **Cisplatin (LD_{50})**	Cisplatin dissolving in the sadline solution was administered to cells in LD_{50} dose.
Group 5: **Cisplatin (LD_{50})+α-Arbutin(LD_0)**	Arbutine in LD_0 dose was administered to the cells with cisplatin in LD_{50} dose simultaneously.

Table 2. Oligonucleotide primer series and RT-PCR programs

Transcripts	Primer Sequences	RT-PCR programs	Cycles
β-Aktin	F-5¢CACCCCAGCCATGTACGTTGC R-5¢CCGGAGTCCATCACGATGCCA	93º-15 s / 61º-30 s / 68º-1 dk	35
Caspase 3	F-5¢GGAAGCGAATCAATGGACTCTGGA R-5¢CCTGAGGTTTGCTGCATCGAC	94º-1 dk / 59º-1 dk / 72º-1 dk	35
Bcl-2	F-5¢GACGGGCTACGAGTGGGATGC R-5¢GGAGGAGAAGATGCCCGGTGC	94º-1 dk / 58º-1 dk / 72º-1 dk	35

Table 3. Lethal doses of cisplatin, α-Arbutine and β-Arbutine (LDs)

Lethal Dozlar	Cisplatine ait Lethal Dozlar (µg/mL)	α-Arbutine ait Lethal Dozlar (mM)	β-Arbutine ait Lethal Dozlar (mM)
LD_0	0,006	0,003	0,022
LD_5	0,055	0,057	0,226
LD_{10}	0,178	0,261	0,779
LD_{15}	0,393	0,733	1,795
LD_{50}	11,060	57,471	61,270

Table 4. The effect of α-arbutine administered to experimental groups on genotoxicity, oxidative stress and inflammation

Analyzed parameters	Experimental Groups					P
	Group 1 Control	Group 2 α-arbutin (LD_0)	Group 3 α-arbutin (LD_{50})	Group 4 Cisplatin (LD_{50})	Group 5 Cisplatin (LD_{50}) + α-arbutin (LD_0)	
Comet Scores (Arbitrary Unit)	3,33±0,82	4,67±1,21	7,67±0,52 [a]	13,67±2,07[a]	10,00±3,95[b]	0,000**
Micronucleus frequencies (%)	0,0117±0,0041	0,0183±0,0041	0,0433±0,0052 [a]	0,0533±0,0163 [a]	0,0467±0,0082	0,000**
TAS (mmolTroloxEquiv/g-protein)	0,297 ± 0,079	0,152 ± 0,037	0,226 ± 0,041	0,136 ± 0,060	0,192 ± 0,032	0,253*
TOS ($\mu molH_2O_2Equiv./g$-protein)	2,66 ± 1,16	2,34 ± 1,18	7,24 ± 2,45 [a]	4,74 ± 0,96	2,52 ± 1,26	1,26*
OSI (Arbitrary Unit)	862,8 ± 256,1[b]	1635,4 ±550,6 [b]	3400,7 ± 977,3 [a,b]	4695,3±1089,8 [a]	1289,6 ±493,9 [b]	0,006*
MDA (nmol/g-protein)	1,81± 0,94	3,51 ± 1,62	5,29 ± 2,78 [a]	3,53 ± 2,46	2,32 ± 0,49	0,138*
GSH (µmol/g-protein)	45,65 ±31,76	33,34 ± 1,86	49,67 ±28,65	38,69 ±26,90	29,36 ± 7,64	0,225*
NO (µmol/g-protein)	3,74 ±0,45 [b]	7,03 ± 3,36	10,52 ±2,88 [a]	11,93 ±5,29 [a]	7,54 ± 2,58	0,026*
TNF-α (pg/mg-protein)	39,94 ± 14,86 [b]	43,05 ± 16,30 [b]	27,71 ± 15,88 [b]	85,42 ± 15,88 [a]	51,16 ± 29,78 [b]	0,008*
IL-6 (pg/mg-protein)	55,75 ± 17,42	48,80 ± 5,84	97,17 ± 42,72 [a]	82,72 ± 15,73 [a]	73,72 ± 25,93	0,085*
TGF-1β (pg/mg-protein)	22,53± 7,06	17,79± 2,90	45,47±16,20 [a]	24,94 ± 5,95	26,87± 10,52	0,011*
Total Protein (mg/L)	2,12 ±0,72	2,35 ± 0,20	1,36 ± 0,49 [a]	1,51± 0,18	1,54 ± 0,36	0,025*

Data are given as mean ± standard deviation (n = 4-7). * Shows the P value of the data showing normal distribution and applying the ANOVA test and Duncan posttest. ** It shows the P value of the data that do not show normal distribution and which are applied Kruskal-Wallis test, which is one of the non-parametric tests. Statistical differences between data belonging to a single parameter in the same row are expressed as superscript ([a, b]) with letters. LD_0: Lethal dose that does not cause any death or proliferation in cancer cells, LD_{50}: Lethal dose that causes the death of half of cancer cells,

[a] p 0.05; refers to the groups that are statistically different from the control group.

[b] p 0.05; refers to the groups that are statistically different from the cisplatin group.

Table 5. Numbers of caspase 3 and p53 positive cells

Experimental Groups	Caspase 3 (Number of Cas3 $^{+}$ cells in mm^2)	p53 ($p53^{+}$ cell percentage per unit area)
Grup 1: Kontrol	60,45 ± 6,03[b]	48,82 ± 12,38[b]
Grup 2 : α-Arbutın (LD_0)	75,59 ± 30,92	31,28 ± 9,81[a,b]
Grup 3: α-Arbutın(LD_{50})	104,93 ± 45,16[a]	73,20 ± 8,63[a,b]
Grup 4: Cisplatin LD_{50}	121,35 ± 41,02[a]	49,97 ± 10,81
Grup 5: Cisplatin LD_{50} + α-Arbutın (LD_0)	107,81 ± 43,41[a]	59,68 ± 15,76[a,b]
P	0,004	0,000

The data were presented as average ± standard deviation (n=6). Statistical differences between experimental groups are expressed in letters as superscripts ([a, b]).

[a] p 0.05; refers to the groups that are statistically different from the control group.

[b] p 0.05; refers to the groups that are statistically different from the cisplatin group.

Figures

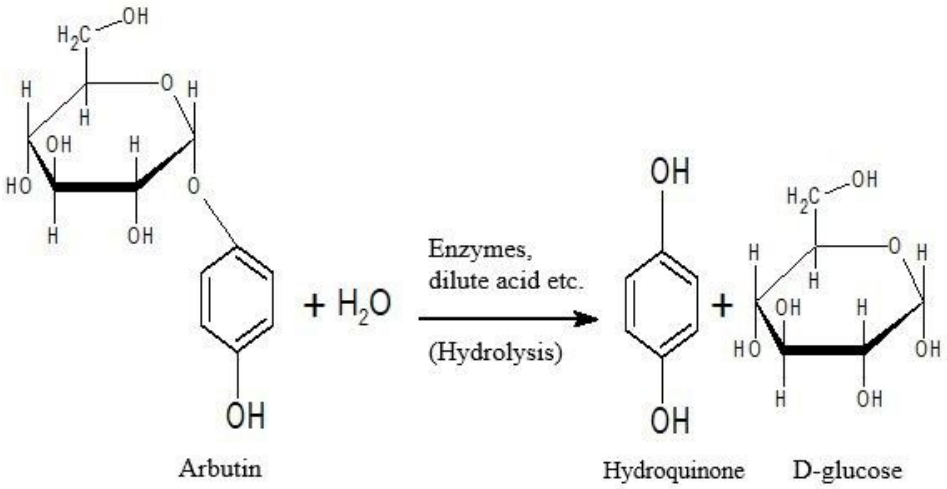

Figure 1

Hydrolise of Arbutine

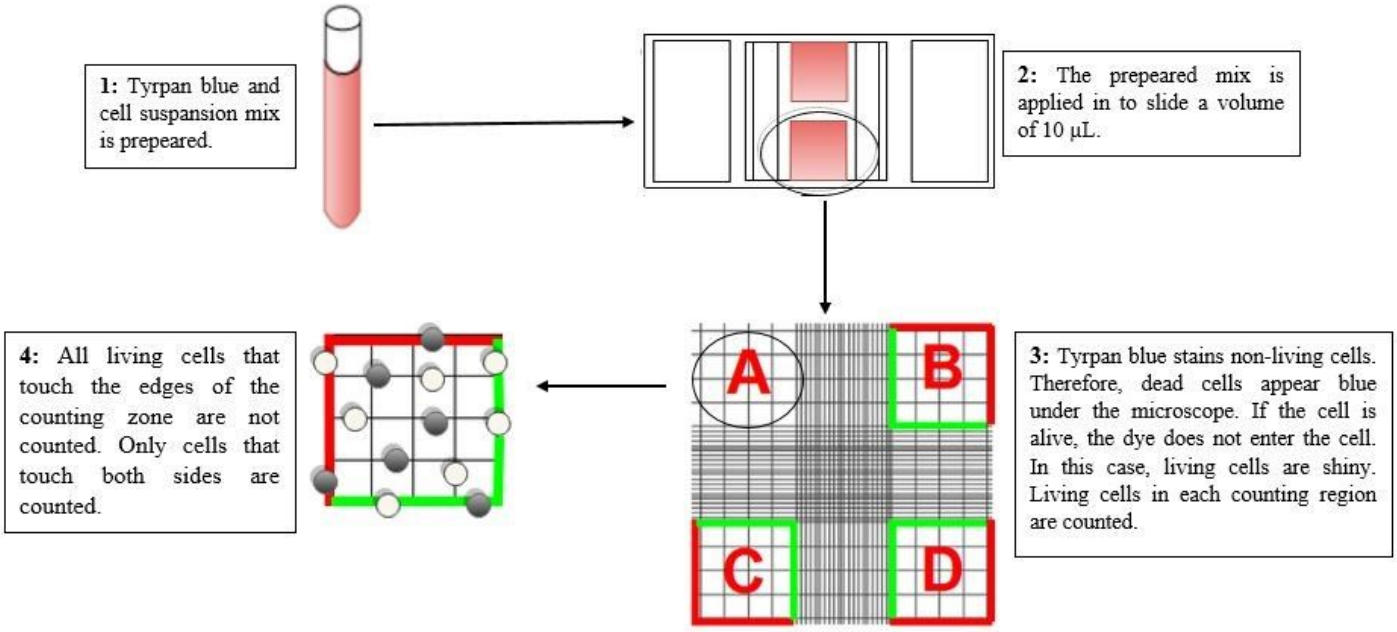

Figure 2

The methodology used in counting the cells by trypan blue method

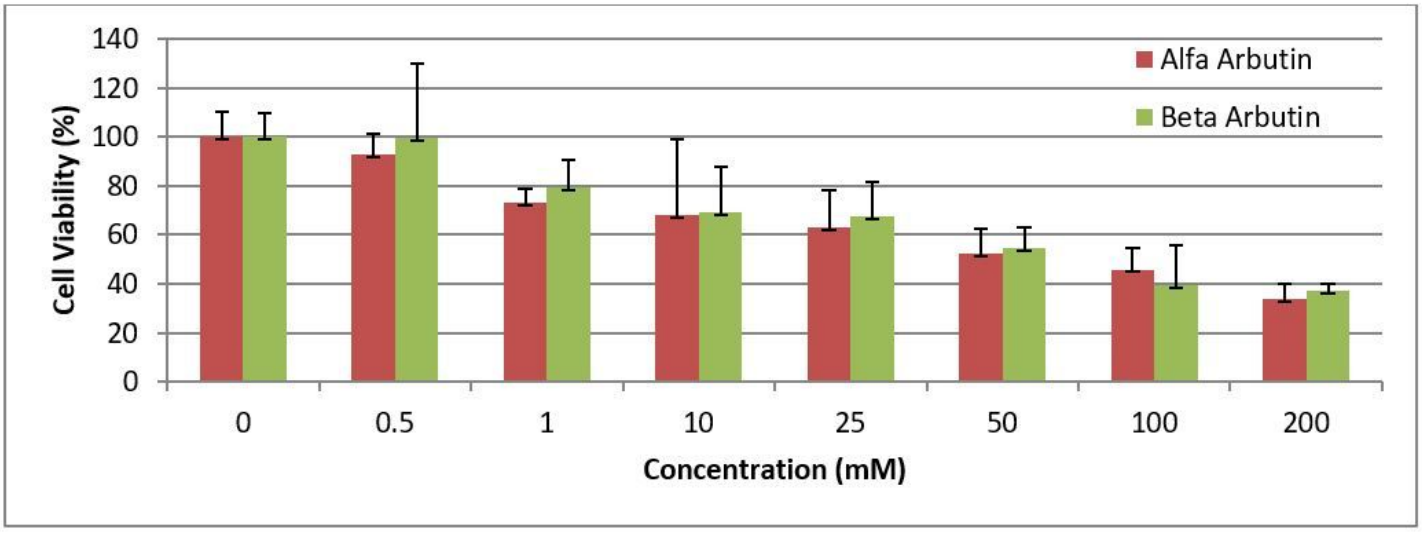

Figure 3

Cytotoxicity levels of α-arbutine ve β-arbutine in different doses in HepG2 cells

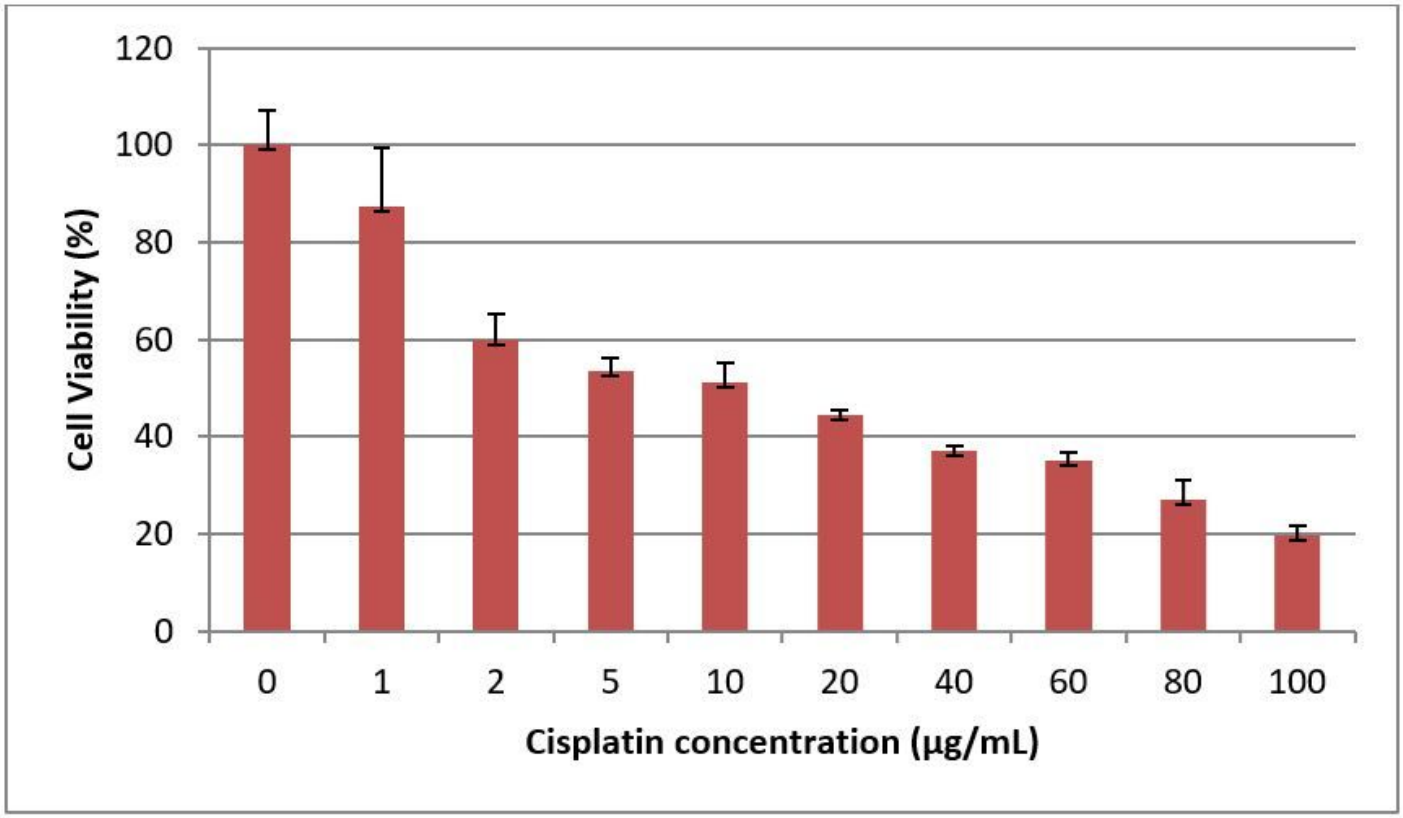

Figure 4

Cytotoxicity of cisplatin in HepG2 cells

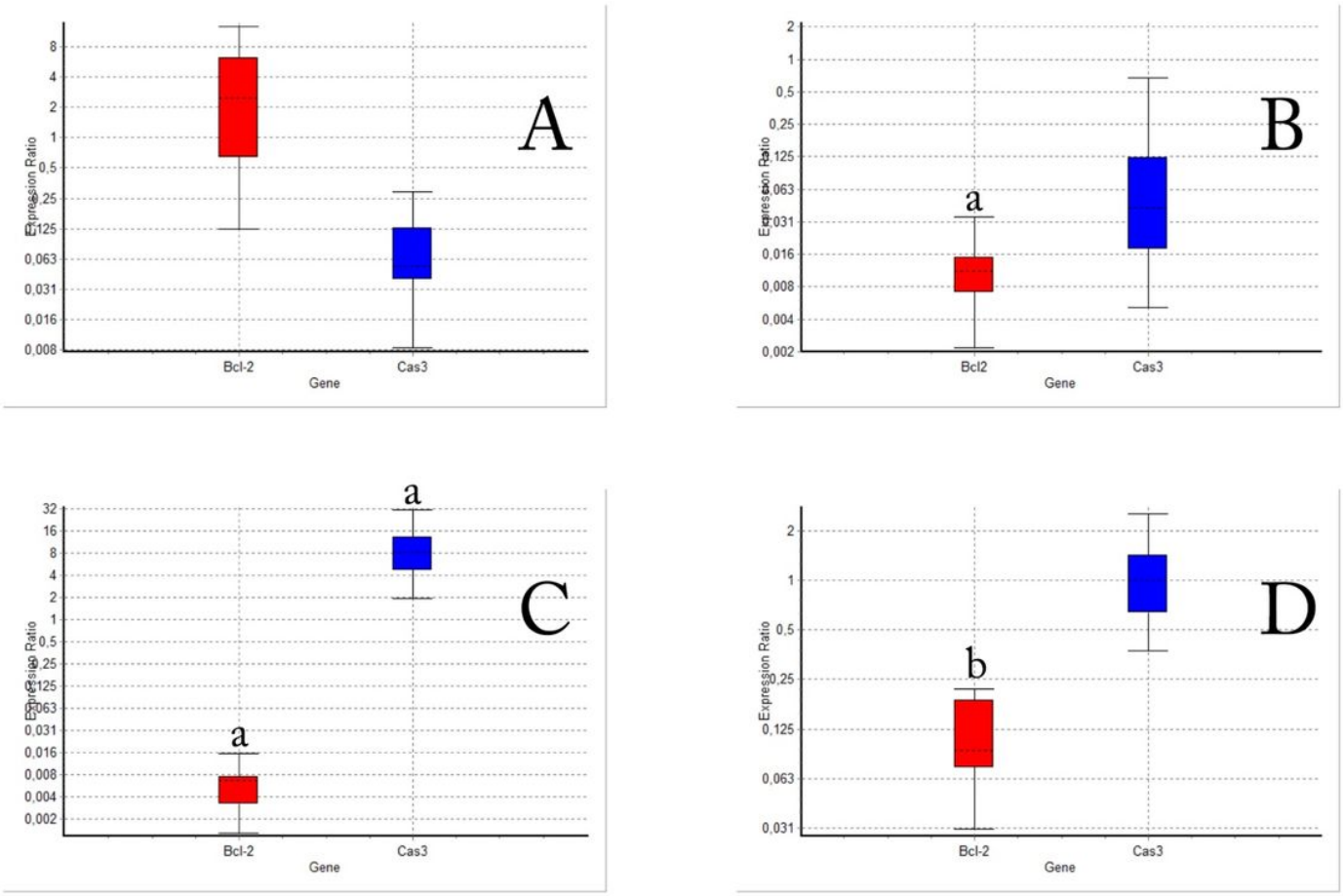

Figure 5

The effect of α-arbutine on apoptasis (Cas3) and proliferation (Bcl-2) in HepG2 cells (A; the effect of α-arbutine administered in LD0 dose, B; the effect of α-arbutine administered in LD50 dose LD50, C; the effect of cisplatin administered in LD50 dose, D; the effect of α-arbutine administered to the cells in LD0 dose along with cisplatin in LD50 dose). a ;refers to the groups that are statistically different from control group (p 0.05). b ; refers to the groups that are statistically different from cisplatin group (p 0.05).

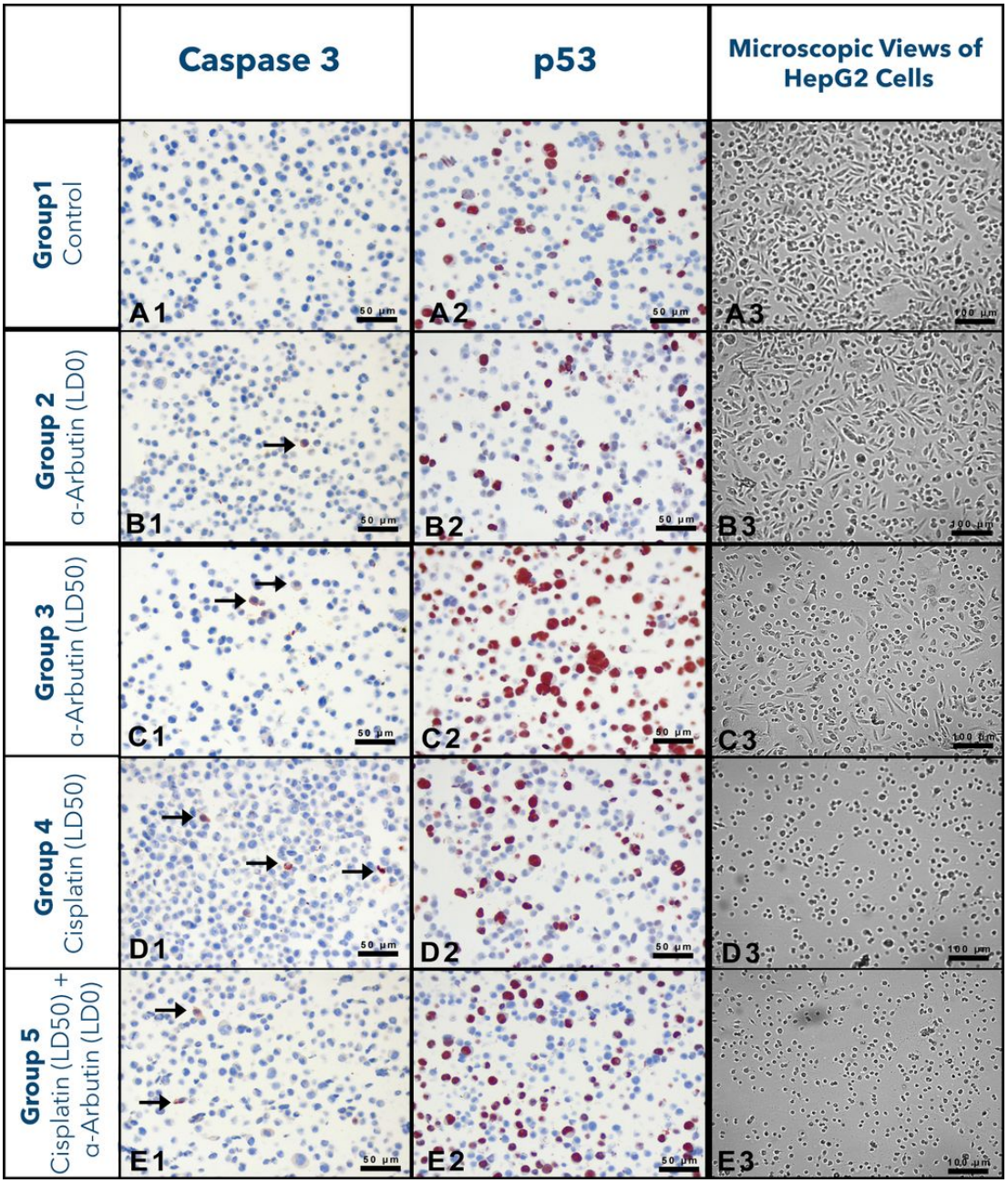

Figure 6

Microscope images of the cells used in immunocytochemical evaluation. The microscope image of only one of the slides used in the evaluation of each experimental group was included. The microscope images in the 1st column (A1-E1) belong to Cas 3 and arrows (→) indicate Cas3+ cells. The microscope images in the 2nd column (A2-E2) belong to p53 parameters and the cells painted to red indicate p53+ cells. The images of HepG2 cells formed in flasks after the administrations in experimental groups are presented in column 3. It was observed that the number of Cas3+ cells was highest in α-arbutin (C1) and cisplatin (D1) administered groups in LD50 doses and the number of p53+ cells was the highest in α-arbutin applied group (C2) in LD50 dose.

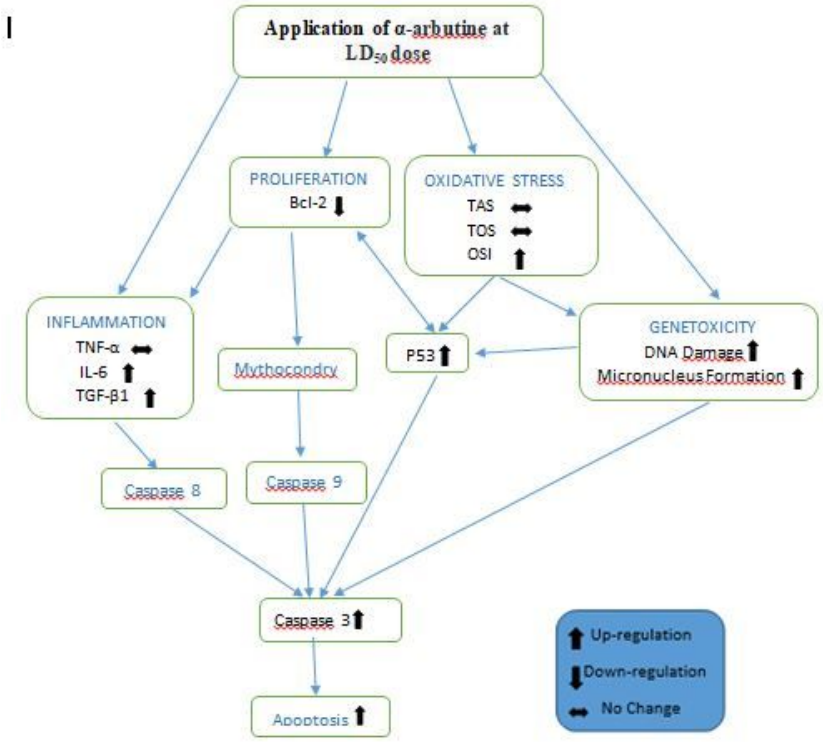

Figure 7

Mechanism that mediates the anticarcinogenic effects of α-arbutine in HepG2 cells (obtained by comparing the data of control group and the experimental group administered α-arbutine in LD50 dose.)

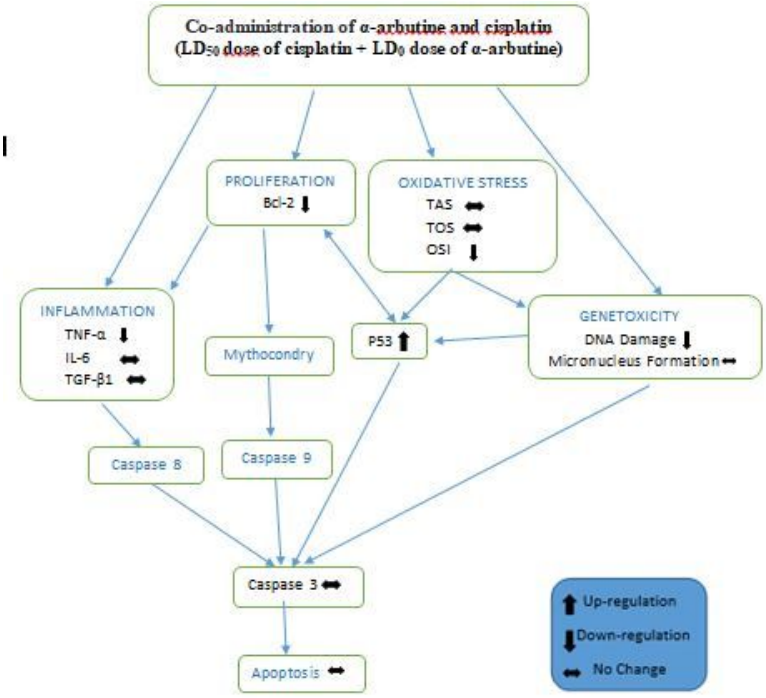

Figure 8

Mechanism that mediates the cisplatin toxication reducing effects of α-arbutine in HepG2 cells (obtained by comparing the data of cisplatin and the experimental group administered LD0 dose of α-arbutine following cisplatin toxication).