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BuOH fraction of *Salix Babylonica* L. extract increases pancreatic beta-cell tumor death at lower doses without harming their function

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ABSTRACT

Salix babylonica L. is a species of the willow tree. Insulinoma is a tumor originating from pancreatic beta cells. This study aims to research the effect of different fractions of *Salix babylonica* L. leaf extract on INS-1 cells for treating pancreatic tumors. Cell death occurred at lower doses in the EtOAc fraction. The cells are functional in the BuOH fraction but not in EtOAc and H_2O fractions. The EtOAc fraction has a higher percentage of necrosis and ROS. *INS1, INS2*, and *AKT* gene expressions in the H_2O fraction, *GLUT2, IR*, *HSP70* gene expressions, and WNT4 protein levels increased in the BuOH fraction. *HSP90* gene expression, Beta-actin, GAPDH, insulin, HSP70, HSP90, HSF1, Beta-Catenin, and WNT7A protein levels were decreased, while IR immunolabelling intensity increased in both fractions. Ca^{+2}, K^{+}, Na^{+}, and CA-19-9 in the cell, Ca^{+2} and K^{+} in secretion increased. The secondary metabolites in the EtOAc fraction cause more damage in INS-1 cells. Since the water fraction also causes the cells to die in high doses, cell function is damaged. The secondary metabolites in the BuOH fraction kill INS-1 cells with less damage. This makes the BuOH fraction of *Salix babylonica* L. more valuable.

1. Introduction

Salix babylonica L., also known as Cluster Willow, belongs to the Salicaceae family (Argus, 1997). Plants belonging to the genus Salix are famous for their salicin content, the aspirin precursor. It also contains various flavonoids, terpenoids, lignin, and phenolic compounds. These compounds have versatile biological activities such as analgesic, antipyretic, and anti-inflammatory (Kim et al., 2015; Mahdi et al., 2006).

Insulinoma is a tumor originating from pancreatic beta cells and secreting insulin. Pancreatic beta cell tumor is characterized by hyperinsulinemia and hypoglycemia (Van Heerden and Edis, 1980). MIN6 and INS-1 cells are the cells that best mimic beta cells when stimulated with glucose (Skelin et al., 2010).

Ca^{+2}, Na^{+}, and K^{+} ions play very important roles in insulin secretion and hence, the survival of Insulinoma cells. The entry of glucose into the pancreatic beta cells leads to membrane depolarization, and an action potential occurs when the threshold value is exceeded. As a result of the destruction of glucose in the cells, the ATP/ADP ratio increases, and the increased ATP causes the inhibition of K_{ATP} channels (Velasco et al., 2012). The cell depolarizes with the accumulation of K^{+} in the cell, and Na^{+}-Ca^{+2} entries into the cell increase. With the rise in intracellular Ca^{+2} levels, insulin exocytosis is stimulated, and insulin is released from the cells (Hiriart and Aguilar-Bryan, 2008; Hiriart and Matteson, 1988; Yang et al., 2014). As a result of the stimulation of pancreatic beta cells with glucose, it was found that apart from K^{+} channels, Na—K $_{ATPase}$ pumps were also inhibited (Owada et al., 1999). In glucose-administered INS-1 cells, partial inhibition of Na channels increases insulin synthesis (Chen et al., 2018). Ca^{+2}, Na^{+}, and K^{+} ions form the pancreatic beta cell membrane potential, vital for insulin secretion.

The formation of reactive oxygen species (ROS) creates oxidative damage to various biomolecules (such as proteins, DNA, and lipids). As a result, oxidative stress-mediated dysfunctions such as cancer, coronary vascular disease, and diabetes occur (Ganguly et al., 2023; Miranda-Silva et al., 2021; Sies, 1997). Oxidative damage plays a role in tumor formation by different mechanisms. It has been determined that ROS levels and oxidative stress products increase in cancer (Prior, 2003). An increase in intracellular ROS, resulting in necrotic cell death, occurred in human colorectal cancer cells treated with rosemary (Rosemary) (*Rosmarinus officinalis*) extract (Pérez-Sánchez et al., 2019). *Dendrobium candidum* extract significantly increased intracellular ROS levels while

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inhibiting cell viability in liver cancer cells. It also inhibited the Wnt pathway by blocking the nuclear translocation of beta-catenin. *Dendrobium candidum* extract inhibited proliferation by inactivating the Wnt/beta-catenin signaling pathway in SMMC-7721, BEL-7404 liver cancer cell lines, and (primary) primary liver cancer cells (Guo et al., 2019).

Heat shock proteins (HSP) play several roles in many events, such as metabolism control of cells, protein aggregation, protein folding, recognition, and refolding of denatured proteins (Pratt and Toft, 2003; Schlesinger, 1994). In addition, HSP 70 and HSP 90 are cytoplasmic proteins involved in the survival and the prevention of cell death of tumor cells by various mechanisms (Beere, 2004; Ciocca and Calderwood, 2005). HSP 70 is overexpressed in pancreatic cancer cells compared to normal ones (Giri et al., 2017).

Therapeutic options against cancer include surgery, radiation, and chemotherapy treatments (Zadorozhna and Mangieri, 2021). But all of these treatments have some disadvantages (Khan et al., 2019). Since current therapies are often accompanied by severe toxicity and side effects, researchers are looking for new therapeutic solutions (Zadorozhna and Mangieri, 2021). Natural compounds are promising approaches as they reduce early tumorigenesis and cancer progression, with low toxicity and high safety (Romero et al., 2021). Natural compounds, including phytochemicals, are widely used for new anticancer drug discovery, and >60% of current anticancer drugs are derived from natural sources. Herbal medicines have become safe, non-toxic, and readily available cancer-curing compounds (Mirza et al., 2021). For the use of herbal drugs in standard treatment, further studies should be conducted on more plants, the secondary metabolites of these plants, and the discovery of their anticancer mechanism of action (Khan et al., 2019).

This study aims to study the effects and toxicity of different fractions of *Salix babylonica* L. leaf extract on pancreatic beta cell tumor cell line, INS-1, and research the molecular mechanism of cell death by studying the role of Ca^{+2}, Na^{+}, and K^{+} ions, reactive oxygen species and HSP ratios.

2. Materials and methods

2.1. Materials

HEPES (H3375), sodium pyruvate (S8636), trypsin-EDTA (T4049), 2,7-dichlorofluorescein diacetate (D6883) from Sigma Aldrich (St. Louis, Mo.); RPMI 1640 (21875–034), Fetal Bovine Serum (26140079), Penicillin-Streptomycin (10378016), low glucose DMEM (11880), high glucose DMEM (31053) from Gibco (Paisley, Scotland); 2-mercaptoethanol (#1610710) and Bradford Protein Assay (#500–0006), PVDF membrane (BR20180611) from Biorad (Hercules, California); Rat insulin ELISA Kit (201–02-0063) and CA19–9 ELISA Kit (201–02-0814) from Sunredbio (China); Phosphatase-Proteinase Inhibitor (A32959), UltraVision LP Kiti (TP-125-HL), A.E.C. Substrat Kit (TP-125-HA), Luminol reagent (34580) from Thermo Scientific (Rockford, IL), SYBR Green master mix (QPSY01) from GeneMark (Atlanta, Georgia, U.S.A.), Cytotoxicity Detection Kit (04744926001), Cell Proliferation Reagent WST1 (5015944001) from Roche (Basel, Switzerland), Albumin (A2134) from Biomatik Corporation (Ontario, Canada), Lysis buffer (#9803) from Cell Signaling (, Ponceau S (PON002) from BioShop was purchased. Auto-analyzer (Abbott ARCHITECT C16000) for the measurement of Ca^{+2}, Na^{+}, and K^{+} levels, Light microscope (Nikon Eclipse Ti—S) for microscopic examination, ChemiDoc XRS System Imaging Device for Western Blot analysis (Biorad), Quantstudio 7 real-time PCR device (Applied Biosystem) for gene expression analysis were used.

2.2. Cell culture

The rat insulinoma INS-1 cell line was a generous gift from Prof. Dr. Claes B. Wollheim (University Medical Center, Geneva). INS-1 cells were cultured in 5% CO_2, and 95% humidified air at 37 °C in RPMI 1640 containing 10 mM HEPES, 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol. 0.25% trypsin-EDTA was used for the passage of INS-1 cells. After that, the cells were incubated in an incubator with 5% CO_2/95% humidified air at 37 °C for 24 h.

2.3. Fractionation of Salix babylonica L. extract

Salix babylonica L. leaf extract was obtained from the leaves collected from the willow tree in the Beykoz district of Istanbul province in 2017 and 2019. After the *Salix babylonica* L. leaf was collected and identified by the Department of Pharmaceutical Botany, Bezmialem Vakif University, Istanbul- Turkey, it was dried in the shade and ground in a mortar. The aqueous extract of the powdered leaves was prepared with a 10% *w/v* (gr powder/ml water) ratio at 90 °C. When the water cooled down, the extract was separated from the leaf powder by filtration immediately. The obtained extract was dried under a vacuum. The dried extract was re-suspended in 30 ml of hot water, cooled down, and fractionated with Dichloromethane (DCM), Ethylacetate (EtOAc), and Butanol (BuOH) in a separation funnel. The organic phase was separated and evaporated to dryness on a rotary evaporator. The remaining aqueous extract in the separatory funnel was again evaporated to dryness.

DCM is mainly used for separating volatile and essential oils, EtOAc isolates compounds with average polarity, and BuOH helps isolate compounds with higher polarity. However, polar glycosides and polyphenolic compounds remain in the aqueous phase. It is assumed that this method provided purification of glycosides from non-glycoside compounds.

2.4. Cell viability test

In the study, firstly, the extract was obtained from the leaf of *Salix babylonica* L. without separating into phases. 0 mg/ml, 0.25 mg/ml, 0.50 mg/ml, 1 mg/ml, 2 mg/ml, and 4 mg/ml of *Salix babylonica* L. extract dissolved freshly in RPMI medium and applied to the insulinoma INS-1 cells, and their effect on cell viability was analyzed using WST1 test. The process of the WST-1 assay involves the conversion of the tetrazolium salt WST-1 into formazan through the activity of mitochondrial dehydrogenases found within cells. If there are more viable cells, there will be more activity from the mitochondrial dehydrogenases, resulting in a greater amount of formazan dye produced. In another application, the extract was prepared from 25 mg/ml stock solution and 4 mg/ml stock solution one day before and stored at +4 °C. The extract was applied to the cells at the same concentrations. Then, the extracts were prepared fresh, one day before experiments, or one week and one month earlier. It was stored at 25 mg/ml and 4 mg/ml concentration at +4 °C and − 20 °C. The extract was used both in its stored form and by sonicating. It was applied to cells at a final 1 mg/ml concentration.

Salix babylonica L. leaf extract was separated into DCM, EtOAc, BuOH, H_2O, and $CHCl_3$ fractions. DCM and $CHCl_3$ extracts were not obtained, and this fractionation could not be done for this plant. For this reason, the analyses continued with EtOAc, BuOH, and H_2O fractions. After that, according to data, in 96-well plates, 5×10^4 cells were seeded in each well. *Salix babylonica* L. fractions were applied exogenously to the cells as 0.25–2 mg/ml for secondary metabolites in EtOAc, 0.25–4 mg/ml for secondary metabolites in BuOH, and 0.25–16 mg/ml for secondary metabolites in H_2O. The medium, which is the solvent of the fractions, was used as a control. They incubated for 24 h at 5% CO_2, 37 °C. After 24 h, the media was changed, and 10 μl WST1 was added to the fresh media. The cells were incubated for 3 h at 5% CO_2 and 37 °C. After 3 h, the absorption was recorded spectrophotometrically at 420–480 nm (620 nm reference wavelength). Cell viability was determined in dichloromethane, ethyl acetate, Butanol, and aqueous fractions of *Salix*

babylonica L. The viability of the control group was accepted as 100%, and that of the experimental groups was calculated accordingly. IC_{50} values were calculated.

2.5. Determination of insulin secretion by low and high dose glucose administration in INS-1 cells

5×10^4 INS-1 cells were seeded into 96-well plates and incubated at 5% CO_2 and 37 °C for 24 h to determine insulin release from low-dose (5.5 mM) and high-dose (15.5 mM) glucose administration. The *Salix babylonica* L. extract was applied to the cells in amounts of the IC50 value specified by the cell viability test. The medium, which is the solvent of the fractions, was used as a control. The cells were incubated for 24 h at 5% CO_2, 37 °C. After incubation, the cells were washed with PBS, and 5.5 mM low-dose glucose was applied. The cells were incubated for 2 h at the same conditions, after which cell secretions were collected, 15.5 mM high-dose glucose was applied to the cells, and the cells were incubated for two more hours. Cell secretions were collected at the end of the incubation period. The proteinase-phosphatase inhibitor was added to the secretions, and the secretions were stored at −86 °C until studied. Insulin secretion levels were analyzed with the ELISA kit.

2.6. Measurement of lactate dehydrogenase levels in INS-1 cells treated with Salix Babylonica L. extract

5×10^4 cells were seeded into 96-well plates and incubated at 5% CO_2 and 37 °C for 24 h. The IC_{50} value, determined by the cell viability test, was applied to the cells. The medium, which is the solvent of the fractions, was used as a control. The cells were incubated at 5% CO_2 and 37 °C for 24 h. The necrotic cell death levels were determined by ELISA using the LDH Kit. Low control groups were used to define the spontaneously released LDH activity from untreated normal cells, and high control groups were used to determine the maximum release of LDH activity from cells. The cell medium was applied only for the low control, while the lysis buffer was used for the high control. The following calculations were made as a result of incubations.

$$\%\text{cytotoxicity} = \frac{\text{sample absorbance–low control}}{\text{high control–low control}} \times 100$$

2.7. Reactive oxygen species analysis in INS-1 cells treated with Salix Babylonica L. extract

5×10^4 cells were seeded into 96-well plates and incubated at 5% CO_2 and 37 °C for 24 h. The *Salix Babylonica* L. extract fractions were applied in amounts above IC_{50} value. The medium, which is the solvent of the fractions, was used as a control. The cells were incubated at 5% CO_2 and 37 °C for 24 h. After 24 h, 10 mM 2,7-dichlorofluorescein diacetate (DCFA) was added to the cells and the start of the experiment was accepted as 0 h, measurements were taken every 30 min for 3 h, including 0 h. By subtracting the absorbance values at the 0th hour from the absorbance values at the 3rd hour, the difference was calculated, and the values of the experimental groups were calculated by accepting the control group values as 100% and the viability of the experimental group was calculated accordingly.

2.8. Gene expression analysis in INS-1 cells treated with Salix Babylonica L. extract

10^6 cells were seeded into 6-well plates and incubated for 24 h at 5% CO_2, 37 °C. The IC_{50} values of the fractions were applied to the cells. The medium, which is the solvent of the fractions, was used as a control. The cells were incubated for 24 h at 5% CO_2, 37 °C. Total RNA was isolated with a NucleoSpin® RNA II kit. RNA concentration and quality were measured with a Nanodrop spectrophotometer. 1 mg total RNA. was reverse-transcribed using the iScript™-cDNA-Synthesis kit. The PCR reaction mixture was prepared with SYBR Green master mix (Thermo Fisher Scientific) and PCR primers. Beta-actin was used as a control. The results were taken with Applied Biosystems 7500 Fast Instrument device. The primer is as follows: Beta Actin; forward: 5'–GGTCGGT GTGAACGGATTTGG-3, reverse: 5′- ATGTAGGCCATGAGGTCCACC-3; INS1; forward: GGGGAACGTGGTTTCTTCTAC, reverse: CCAGTTGG-TAGAGGGAGCAG, INS2; forward: CAGCACCTTTGTGGTTCTCA, reverse: CACCTCCAGTGCCAAGGT, GLUT2; forward: GCTGGAA-GAAGCGTATCAGG, reverse: AATCCTGATTGCCCAGAATG; IR; forward: CCTACTGCTATGGGCTCCG, reverse: AGGATCTGCAGATGGCCCTC, AKT; forward: AACGGACTTCGGGCTGTG, reverse: TTGTCCTCCAG-CACCTCAGG. The primers of HSP 70 and HSP 90 were procured from Applied Biosystems. Ct values were converted into arbitrary units using the $^{\Delta\Delta}$Ct method.

2.9. Preparing INS-1 cell secretions and lysates in INS-1 cells treated with Salix Babylonica L. extract

10^6 cells were seeded into 6-well plates and incubated for 24 h at 5% CO_2, 37 °C. The *Salix Babylonica* L. extract fractions were applied in amounts above the IC_{50} value. The medium, which is the solvent of the fractions, was used as a control. The cells were incubated at 5% CO_2 and 37 °C for 24 h. The cell media were collected after incubation. The supernatant obtained after 5 min centrifugation at 1500 rpm was used as cell secretion. Cells were washed with PBS and then removed from the plate. The Lysis buffer was added to the pellet obtained after centrifugation at 1500 rpm for 5 min. After five sonications for 10 s, the lysate was obtained by centrifuge at 14000 *g* for 10 min. The proteinase-phosphatase inhibitor was added to both secretions and lysates and stored at −86 °C until studied. The obtained secretions and lysates were used for Ca^{2+}, Na^{+}, K^{+}, CA19–9 levels, and western blotting analyses. The protein values of the obtained samples were measured by the Bradford method.

2.10. Western blotting analysis

Protein levels of cell lysates were measured, and equal amounts of protein were loaded onto 7.5% Sodium Dodecyl Sulphate Polyacrylamide Gels and run in Biorad. After the proteins were run, they were transferred to the PVDF membrane by wet transfer. After each loading and subsequent running of the proteins in the gel, it was checked with Ponceau S whether the transfer to the membrane was carried out correctly. Transfer of proteins was confirmed each time. After that, the dye was removed. The membrane was incubated with antibodies (insulin, p-AKT, AKT, HSP70, HSP90, HSF1, Beta-catenin, WNT4, WNT7A, Beta Actin, GAPDH) at +4 °C for one night after blocking in 5% skimmed milk powder at room temperature for 1 h. After incubation, all the membranes were washed with the washing buffer and incubated with secondary antibodies for 1 h at room temperature. Then, after washing again, the bands were treated with luminol reagent for 5 min at room temperature and visualized with Biorad Imaging Device.

2.11. Measurement of immunolabelling intensity by immunohistochemical analysis

2×10^5 cells were seeded into 24-well plates and incubated at 5% CO_2 and 37 °C for 24 h. The *Salix Babylonica L* extract fractions were applied in amounts above the IC_{50} value. The medium, which is the solvent of the fractions, was used as a control. The cells were incubated at 5% CO_2 and 37 °C for 24 h. After incubation, cells were fixed with 4% paraformaldehyde for 15 min at room temperature. And then, cells were washed with PBS. It was stored at +4 °C until the experimental time. Immunolabelling was performed using the UltraVision LP Large Volume Detection System Kit and AEC Substrate Kit. At least ten random fields were photographed in a Nikon light microscope to measure the intensity of the positive staining at the immunohistochemical reaction. The

computer-aided image analysis program (NIS-Element D 3.1) was used for intensity analysis. Photographs were evaluated at ×40 magnification. Staining intensity in immunohistochemistry experiments is shown as the mean intensity value.

2.12. Measurement of calcium, potassium, sodium, and CA19–9 levels in cell secretion and in INS-1 cells treated with Salix Babylonica L. extract

The measurement of Ca^{+2}, Na^{+}, and K^{+} levels was made by taking measurements on an auto-analyzer using the ion-selective electrode method in cell lysate and culture media. CA19–9 levels were measured spectrophotometrically using the ELISA kit in cell lysate and culture media. During the analysis, the standards included in the kit were worked. The graph has been obtained. The equation created from the graph obtained was used in the calculation.

2.13. Statistical analysis

All experiments were repeated in triplicate on different days. The results were expressed by ±SEM. Multiple comparisons were made with the two-way ANOVA variance analysis test and non-parametric Tukey's test using GraphPad Prism 5 computer software. The normality of variances was checked. The value of $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. When Salix babylonica L. leaf extract is not freshly prepared, it loses its lethal effect on insulinoma INS-1 cells

According to the results, freshly prepared extract resulted in better toxicity, while the toxicity of the extracts decreased with the duration of storage. This observation may be due to two reasons. Either the compounds contained in the extract deteriorate when stored, or they lose their solubility and form aggregates and collapse. Furthermore, the cell viability of insulinoma INS-1 cells treated with the extracts stored at +4 °C and − 20 °C differed significantly. While a significant variation in the effect of extracts stored at +4 °C or − 20 °C on cell viability was observed, no change was observed as a result of applying these stored extracts to the cells by sonicating according to unsonic conditions (Fig. 1). This situation suggested that there may be a loss in the activity of *Salix babylonica* L. Thus, the fractions of the raw extract was prepared to determine the secondary metabolites, which deteriorated rapidly by the storage conditions. Therefore, Dichloromethane (DCM), Ethylacetate (EtOAc), Butanol (BuOH), Water (H2O), and Chloroform (CHCl3) fractions of *Salix babylonica* L. were obtained, and cell viability was determined in these fractions.

3.2. Different fractions of Salix babylonica L leaf extract are effective on INS-1 cell viability at various concentrations

IC_{50} values were determined in EtOAc, BuOH, and H_2O fractions. IC50 values were calculated for EtOAc fraction as 1.413 mg/ml, BuOH fraction as 1.770 mg/ml, and H_2O fraction as 5.2419 mg/ml as a result of at least 3 independent experiments. As a result, it was observed that the EtOAc fraction was more effective than the other fractions, and the BuOH fraction was more effective than the H_2O fraction (Fig. 2).

3.3. Different fractions of Salix babylonica L. extract have versatile effects on pancreatic beta cell function according to glucose-stimulated insulin secretion analysis

The level of insulin secretion in the control group stimulated with high glucose increased significantly compared to the level of insulin secretion in the cells of the control group stimulated with low glucose (p<0,001). This evidence indicates that insulinoma INS-1 beta cells are functional. A decrease was observed in insulin secretions of cells treated with EtOAc and BuOH fractions as a result of stimulation of cells with low-dose glucose compared to the control group (p<0,001). In contrast, an increase was observed in H_2O fractions as a result of the stimulation of cells with high-dose glucose (p<0,001). A decrease was observed in the insulin secretions of the cells treated with all fractions as a result of the stimulation of cells with high-dose glucose compared to the control group (p<0,001). In EtOAc and H_2O fractions, the amount of insulin secreted due to stimulating cells with low-dose glucose is higher than that of insulin in cells stimulated with high-dose glucose (p<0,001). This data suggests that the active component in these fractions impairs beta cell function. However, in the BuOH fraction, the amount of insulin secreted by the administration of high-dose glucose to the cells is higher than the amount of insulin in the cells stimulated with low-dose glucose (p<0,001). This situation indicates that the beta cells are functional (Fig. 3).

3.4. Application of different fractions of Salix babylonica L. extract to INS-1 cells results in necrotic cell death at different rates

While the highest level of necrotic cell death was observed in the EtOAc fraction of *Salix babylonica* L. leaf extract, this rate was the lowest in the water fraction. The percentage of necrotic cell death in the EtOAc fraction increased significantly compared to BuOH (p<0,01) and water fraction (p<0,001). The rate of necrotic cell death in the water fraction was also significantly decreased compared to both groups (p<0,001) (Fig. 4).

3.5. An increase in different levels of reactive oxygen species occurs by the application of different fractions of Salix babylonica L. extract to INS-1 cells

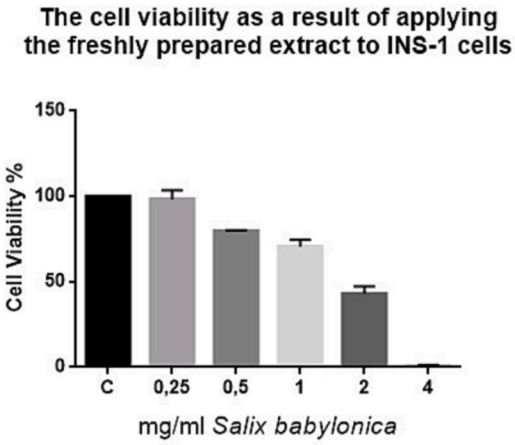
According to the experiment's results, reactive oxygen species increased significantly in the presence of EtOAc, BuOH, and H_2O fractions of *Salix babylonica* L. leaf extract compared to the control group (p<0,001). The highest increase was seen in the EtOAc fraction compared to the BuOH and H_2O fractions (p<0,001). The ROS level in the H_2O fraction was significantly decreased compared to the EtOAc and BuOH fractions (p<0,001) (Fig. 4).

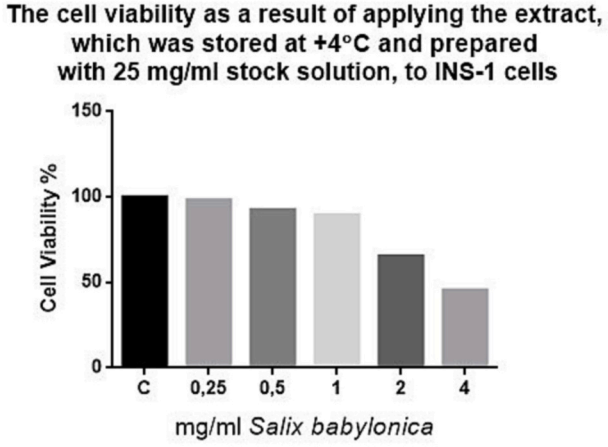
3.6. While INS1, INS2, GLUT2, IR, AKT, and HSP70 gene expressions increase, HSP90 gene expression decreases by the application of different fractions of Salix babylonica L. extract to INS-1 cells

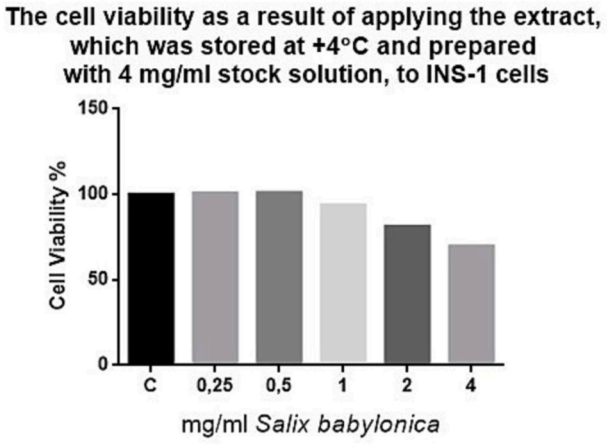
While *INS1* (p<0,01, p<0,05), *GLUT2* (p<0,001, p<0,05), and *AKT* (p<0,001, p<0,05) gene expressions increased, *HSP90* (p<0,001, p<0,001) gene expressions decreased in BuOH and H_2O fractions compared to the control group. *INS2* gene expression increased in only the H_2O fraction (p<0,001), while *IR* (p<0,05) and *HSP70* (p<0,05) gene expressions increased in the BuOH fraction compared to the control group (Fig. 5).

3.7. Beta-actin, GAPDH, insulin, AKT, HSP70, and HSP90 protein levels decrease by the application of different fractions of Salix babylonica L. extract to INS-1 cells

Though the levels of loaded protein concentrations were equal, Beta-actin and GAPDH protein levels were not equal in the western blot assay. There was a significant decrease in levels of beta-actin (p<0,05) and GAPDH (p<0,05), especially in the H_2O fraction. Beta-actin and GAPDH are often used for normalization in western blotting applications. However, we think that the extracts applied exogenously in the current study also have an effect on these proteins. Therefore, in western blotting analysis, significant changes were observed in the levels of these two proteins. The gel was checked with Ponceau S staining and confirmed the loading of proteins at equal concentrations. Therefore







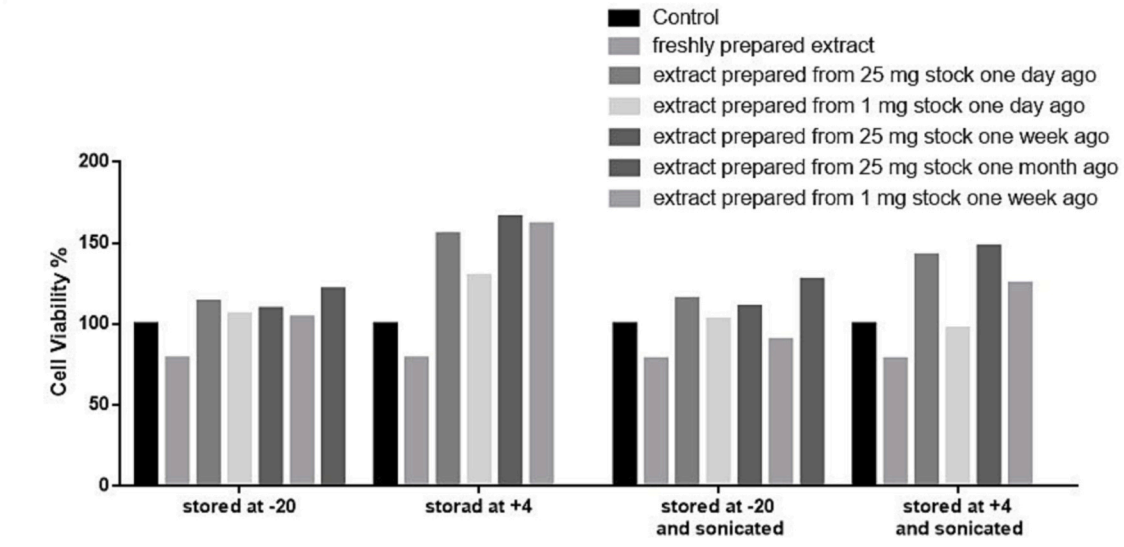


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Fig. 1. The effect of *Salix babylonica* L leaf extract on cell viability according to the preparation and storage method.
Freshly prepared; control group %100 ± 0; 0,25 mg/ml *Salix babylonica* L extract % 98,26,114 ± 3775; 0,5 mg/ml *Salix babylonica* L extract % 79,68,263 ± 0,2306; 1 mg/ml *Salix babylonica* L extract % 70,50,837 ± 2867; 2 mg/ml *Salix babylonica* L extract % 42,98 ± 3104; 4 mg/ml *Salix babylonica* L extract % 0,7322 ± 0,3781.
Stored at +4 °C and prepared with 25 mg/ml stock solution; control group %100 ± 0; 0,25 mg/ml *Salix babylonica* L extract % 98,17,964; 0,5 mg/ml *Salix babylonica* L extract % 92,47,575; 1 mg/ml *Salix babylonica* L extract % 89,47,211; 2 mg/ml *Salix babylonica* L extract % 65,29,127; 4 mg/ml *Salix babylonica* L extract % 45,47,937.
Stored at +4 °C and prepared with 4 mg/ml stock solution; control group %100 ± 0; 0,25 mg/ml *Salix babylonica* L extract % 100,7922; 0,5 mg/ml *Salix babylonica* L extract % 100,8836; 1 mg/ml *Salix babylonica* L extract % 93,72,331; 2 mg/ml *Salix babylonica* L extract % 81,20,046; 4 mg/ml *Salix babylonica* L extract % 69,62,215.
Stored at −20 °C and applied to the cells without any treatment; control group %100; Freshly prepared %79; 25 mg/ml stock *Salix babylonica* L extract (prepared 1 day ago) % 113,8614; 1 mg/ml stock *Salix babylonica* L extract (prepared 1 day ago) % 106,0726; 25 mg/ml stock *Salix babylonica* L extract (prepared 1 week ago) % 109,505; 1 mg/ml stock *Salix babylonica* L extract (prepared 1 week ago) % 104,0924; 25 mg/ml stock *Salix babylonica* L extract (prepared 1 month ago) % 121,7162.
Stored at +4 °C and applied to the cells without any treatment; control group %100; Freshly prepared %79,01; 25 mg/ml stock *Salix babylonica* L extract (prepared 1 day ago) % 155,5776; 1 mg/ml stock *Salix babylonica* L extract (prepared 1 day ago) % 129,901; 25 mg/ml stock *Salix babylonica* L extract (prepared 1 week ago) % 166,0726; 1 mg/ml stock *Salix babylonica* L extract (prepared 1 week ago) % 161,7822.
Stored at −20 °C, sonicated, and applied to the cells; control group %100; Freshly prepared %78,41,584; 25 mg/ml stock *Salix babylonica* L extract (prepared 1 day ago) % 115,7756; 1 mg/ml stock *Salix babylonica* L extract (prepared 1 day ago) % 102,7723; 25 mg/ml stock *Salix babylonica* L extract (prepared 1 week ago) % 110,6931; 1 mg/ml stock *Salix babylonica* L extract (prepared 1 week ago) % 90,36,304; 25 mg/ml stock *Salix babylonica* L extract (prepared 1 month ago) % 127,6568.
Stored at −20 °C, sonicated, and applied to the cells; control group %100; Freshly prepared %78,41,584; 25 mg/ml stock *Salix babylonica* L extract (prepared 1 day ago) % 142,4422; 1 mg/ml stock *Salix babylonica* L extract (prepared 1 day ago) % 97,29,373; 25 mg/ml stock *Salix babylonica* L extract (prepared 1 week ago) % 148,1188; 1 mg/ml stock *Salix babylonica* L extract (prepared 1 week ago) % 125,0825.

bands of analyzed proteins were not normalized with Beta-Actin or GAPDH. There are studies on the fact that beta-actin and GAPDH cannot be used for normalization in western blotting analyses due to different reasons (Dittmer and Dittmer, 2006; Vigelsø et al., 2015).

Insulin level was significantly decreased in both cell lysate (p<0,001, p<0,01) and secretion (p<0,01, p<0,01) BuOH and H2O fractions compared to the control group. While the pAKT/AKT ratio did not change in both fractions compared to the control group (p>0,05), it decreased significantly in the H_2O fraction compared to the BuOH fraction (p<0,05). HSP 70 (p>0,05, p<0,05), HSP90 (p<0,001, p<0,01), HSF1 (p<0,001, p<0,001), Beta-catenin (p<0,05, p<0,05), and WNT7A (p<0,05, p<0,05) levels were significantly decreased in BuOH and H_2O fractions compared to the control group. WNT4 levels also were increased in BuOH fractions compared to the control group (p<0,05) (Fig. 6).

3.8. GLUT2 immunolabelling intensity did not change, while IR immunolabelling intensity increased by the application of different fractions of Salix babylonica L. extract to INS-1 cells

GLUT2 immunolabelling intensity did not change in any group, while IR immunolabelling intensity increased in the cells treated with H_2O fraction compared to the control group (p<0,01) and BuOH fraction (p<0,001) (Fig. 7).

3.9. Ca^{+2}, K^+, and Na^+ levels increase by the application of different fractions of Salix babylonica L. extract to INS-1 cells

Ca^{+2} level increased in both cell lysate (p<0,05) and secretion (p<0,05) in the BuOH fraction, while it increased only in the secretion (p<0,001) of H_2O fraction compared to the control group. While Na^+ levels increased in both BuOH (p<0,01) and H_2O (p<0,001) fractions in cell lysate compared to the control group, there was no difference in secretion between the groups (p>0,05). K^+ levels increased in the BuOH fraction (p<0,05) and decreased in the H_2O fraction groups (p<0,01) of the cell lysate. In secretion, K^+ levels increased in both BuOH and H_2O fractions (p<0,01, p<0,01) (Fig. 8).

3.10. CA19–9 levels increase by the application of different fractions of Salix babylonica L. extract to INS-1 cells

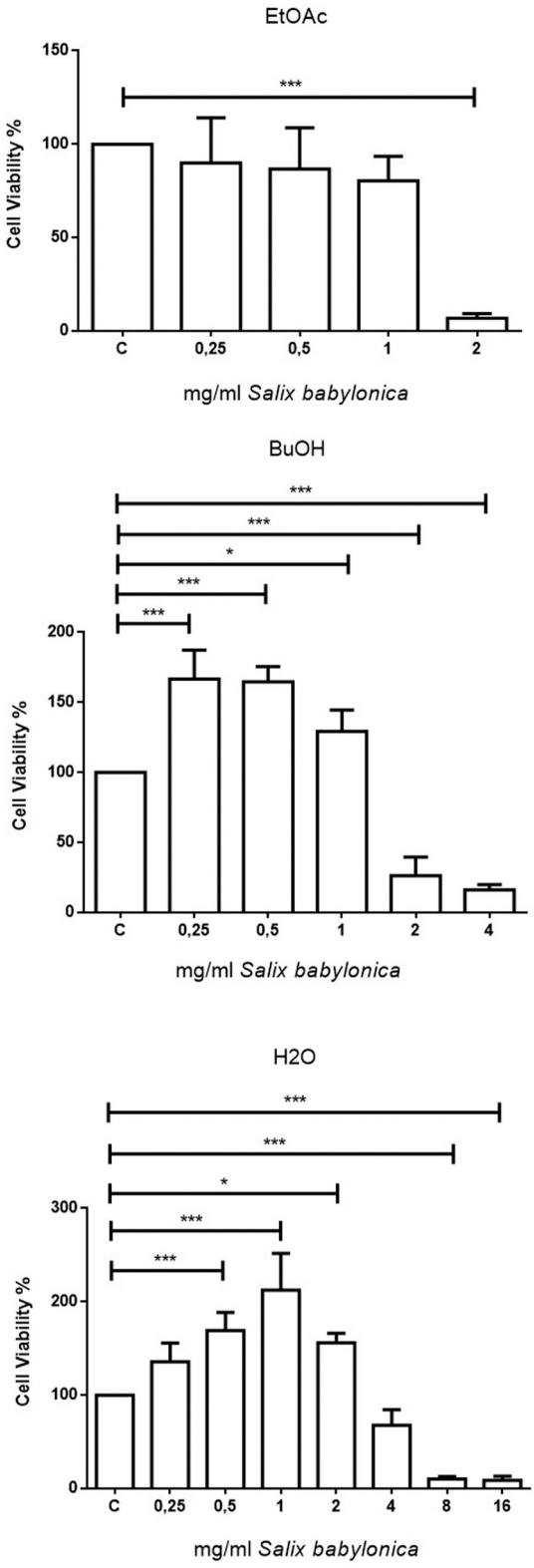
CA19–9 levels increased in both BuOH (p<0,001) and H_2O (p<0,001) fractions treated cell lysates compared to the control group but did not show any change in secretions (p>0,05) (Fig. 9).

4. Discussion

In a previous study, the ethanol fraction of *Salix babylonica* L. leaf extract was given to a group of mice fed a high-fat diet. It has been shown that the extract can be used for anti-obesity, observing that it can accelerate noradrenaline-induced lipolysis and inhibit fatty acid absorption in small intestinal cells (Liu, 2012). Fifty-nine active components were determined in the leaf extract of *Salix babylonica* L. using Gas Chromatography-Mass Spectrometry. Among them, the main components were tritetracontane, octadecenoic acid-1,2,3-propanetriol ester, hexadecanoic acid-methyl ester, 1,3 dioxane-4-(hexadecycloxy)-2-pentadactyl (Salem et al., 2011). However, this analysis with Gas Chromatography-Mass Spectrometer detected only the compounds belonging to the oil phase. In another study on lambs, lambs' growth and gas production performances were evaluated by administering the oil phase to lambs (Cedillo et al., 2014). Another study with *Salix babylonica* L. was carried out by administering the leaf extract to cows mixed with their feed and analyzing the gas production kinetics in the cow digestive system (Salem et al., 2014).

In a study by Zhou et al. in 2017, a mixture of *Salix babylonica* L. bark extract was used to study the mechanism of cancer-related fibroblasts. They revealed that it did not affect proliferation and apoptotic potential after administration. Still, they inhibited the activation of cancer-related fibroblasts and the inflammation process by applying an extract of normal cervical cells, Cervicitis (cervical inflammation), Cervical Intraepithelial Neoplasia I, Cervical Intraepithelial Neoplasia III, and Cervical Squamous Cell Carcinoma cells. Previously the effect of extract obtained from the leaf of *Salix babylonica* L. on pancreatic cancer has not been studied *in vitro* or *in vivo*. In addition, the DCM, EtOAc, BuOH, and H_2O phases of this extract were not handled separately in any study.

In our study, we observed that *Salix babylonica* L. extract reduced the viability of pancreatic beta tumor cells. We found that *Salix babylonica* L. extract had different effects on cell viability when prepared beforehand and stored at +4 or − 20. We hypothesized that this could be caused by several secondary metabolites in different fractions of *Salix babylonica* L. extract. Therefore, we obtained different fractions of *Salix babylonica* L. and freshly prepared the samples before applying them to the cells. We continued the experiment with the EtOAc, BuOH, and H_2O fractions. Since these fractions contain different secondary metabolites, the effective doses that kill pancreatic beta cell tumors were found to be different. EtOAc fraction had the lowest IC_{50} value, suggesting that the most secondary metabolites-killing cells were in this fraction. On the other hand, the H_2O fraction had the highest IC_{50} value, with secondary metabolites less active for cell death.



(caption on next column)

Fig. 2. Effect of different fractions of *Salix babylonica* L leaf extract on cell viability.

For EtOAc Fraction; control group %100 ± 0; 0,25 mg/ml *Salix babylonica* L extract %89,94 ± 12,08; 0,5 mg/ml *Salix babylonica* L extract %86,73 ± 11,01; 1 mg/ml *Salix babylonica* L extract %80,44 ± 7520; 2 mg/ml *Salix babylonica* L extract %6791 ± 1422. IC50: 1413 mg/ml.

For BuOH Fraction; control group %100 ± 0; 0,25 mg/ml *Salix babylonica* L extract %166,6 ± 14,62; 0,5 mg/ml *Salix babylonica* L extract %164,7 ± 6348; 1 mg/ml *Salix babylonica* L extract %129,3 ± 8845; 2 mg/ml *Salix babylonica* L extract 26,31 ± 7623; 4 mg/ml *Salix babylonica* L extract %16,07 ± 2255. IC50: 1770 mg/ml.

For H_2O Fraction; control group %100 ± 0; 0,25 mg/ml *Salix babylonica* L extract %135,9 ± 9990; 0,5 mg/ml *Salix babylonica* L extract %169,2 ± 9669; 1 mg/ml *Salix babylonica* L extract %212,5 ± 19,71; 2 mg/ml *Salix babylonica* L extract 156,0 ± 5779; 4 mg/ml *Salix babylonica* L extract %67,94 ± 8219; 8 mg/ml *Salix babylonica* L extract %10,16 ± 1603; 16 mg/ml *Salix babylonica* L extract %8670 ± 2267. IC50: 1770 mg/ml.

Viscothionine obtained from mistletoe (mistletoe) has shown an increasing effect on insulin secretion in pancreatic beta cells (Park et al., 2019). Previously *Vernicia fordii* methanolic extract exhibited potent insulin secretion function *via* intracellular Ca^{2+} influx of pancreatic beta cells without toxicity (Hyun et al., 2021). Alkaloid extract from *Zanthoxylum zanthoxyloides* stimulated insulin secretion in diabetic rats (Kyei-Barffour et al., 2021). In a high-content screening method, myricetin showed an insulin-inhibiting effect. In contrast, gracillin showed a stimulatory impact on an 1100-sample screening of bioactive compounds that stimulate or inhibit insulin secretion in MIN6 beta cells (Hager et al., 2021). In INS-1E cells stimulated with *Leonurus sibiricus* extract in different solvents, 24-h insulin release was significantly increased in water and methanolic extracts but remained unchanged in butanol extract. Extracts from *Leonurus sibiricus* increased insulin secretion by causing an increase in intracellular calcium concentration in rat INS-1E insulinoma cells and stimulated INS 1-E cell proliferation (Schmidt et al., 2013).

In our study, as a result of the analysis of glucose-induced insulin secretion in the control group, cells released more insulin when stimulated with high-dose glucose compared to those stimulated with low-dose glucose. This evidence indicates that pancreatic beta cells are functional. The same is true for the BuOH fraction, except that the amount of insulin released is reduced. In other words, when the BuOH fraction is applied to the cells, the functionality of the cells is not impaired. However, when EtOAc and H_2O fractions are applied to cells, the function of beta cells is impaired. According to the experimental results, the insulin level released from the cells is lower when high-dose glucose is applied to cells treated with EtOAc and H_2O fractions, according to low-dose glucose is administered.

Enterolobium cyclocarpum extract induced necrosis in Hep G2 carcinoma cells and showed a tumorigenicity inhibitory effect in rats with liver cancer models (Gamal-Eldeen et al., 2021). *Angiopteris evecta* extract increases dose-dependent necrotic cell death in HT-29 colon cancer cells (Sara and Ruby, 2022). In our study, necrotic cell death occurred due to applying different fractions of *Salix babylonica* L. leaf extract to INS-1 cells. The difference between the fractions, from the highest necrotic cell death to the lowest, was observed in EtOAc, BuOH, and H_2O fractions. It has been reported that the ROS level decreased, and apoptosis was inhibited due to the antioxidants contained in the plant extract in the MIN6 insulinoma cell line that was applied with *Ficus carica* leaf extract (Zhang et al., 2020).

In line with our results, cell viability either remained unchanged or increased when cells were incubated with low doses of plant extract. Cell viability decreased when higher doses of plant extract were applied to kill beta-cell tumors; meanwhile, the ROS levels were increased. The increase in the level of ROS at these doses, in which cell death also occurs, suggests the relationship between cell death with ROS.

In a study, when plant flavonoids quercetin and routine were applied to INS-1 cells, insulin secretion was stimulated by KATP channel inhibition, and insulin secretion was increased. Flavonoids caused cell death by inhibiting the PI3K/Akt pathway in a study by Kittl et al. (Kittl et al., 2016). Insulin secretion was analyzed by applying *Artemisia dracunculus* L. plant extract to beta cells isolated from NIT-1 and mouse Langerhans islands. In both cell lines, the extract has been shown to stimulate insulin

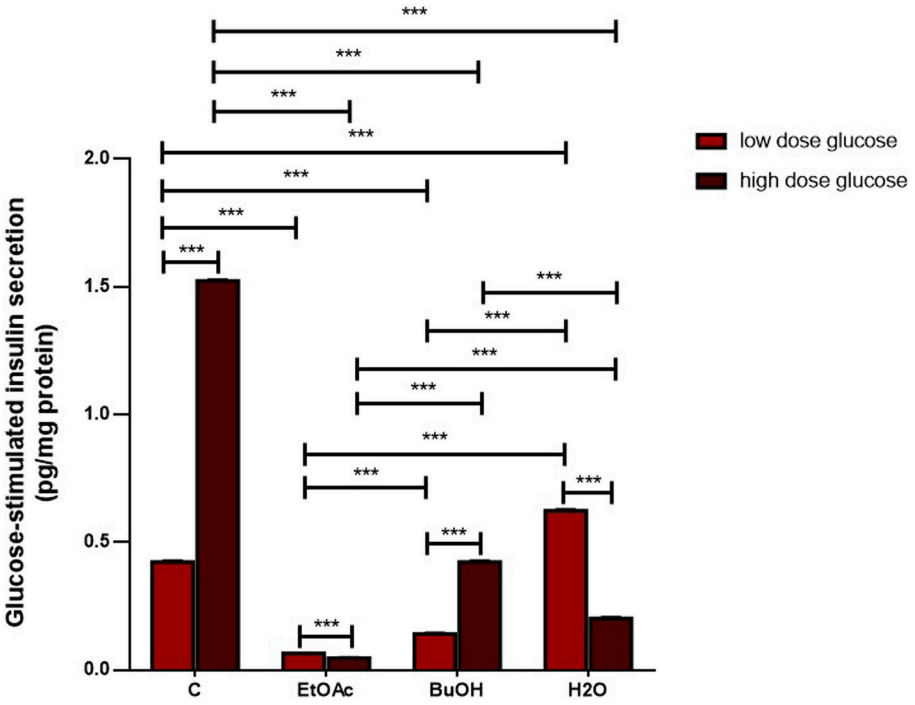


Fig. 3. The effect of different fractions of *Salix babylonica* L on glucose-stimulated insulin secretion. C: Control, EtOAc: Etilasetat, BuOH: Bütanol, H_2O: Water, LG: low-dose glucose, HG: high-dose glucose. C group: LG, 0,4232 ± 0,003186; HG, 1523 ± 0,002935; EtOAc group: LG, 0,06524 ± 0.000236; HG, 0,04732 ± 0,0003185; BuOH group: LG, 0,1419 ± 0,001874; HG, 0,4233 ± 0,003325; H_2O group: LG, 0,6236 ± 0,003626; HG, 0,2028 ± 0,002795.

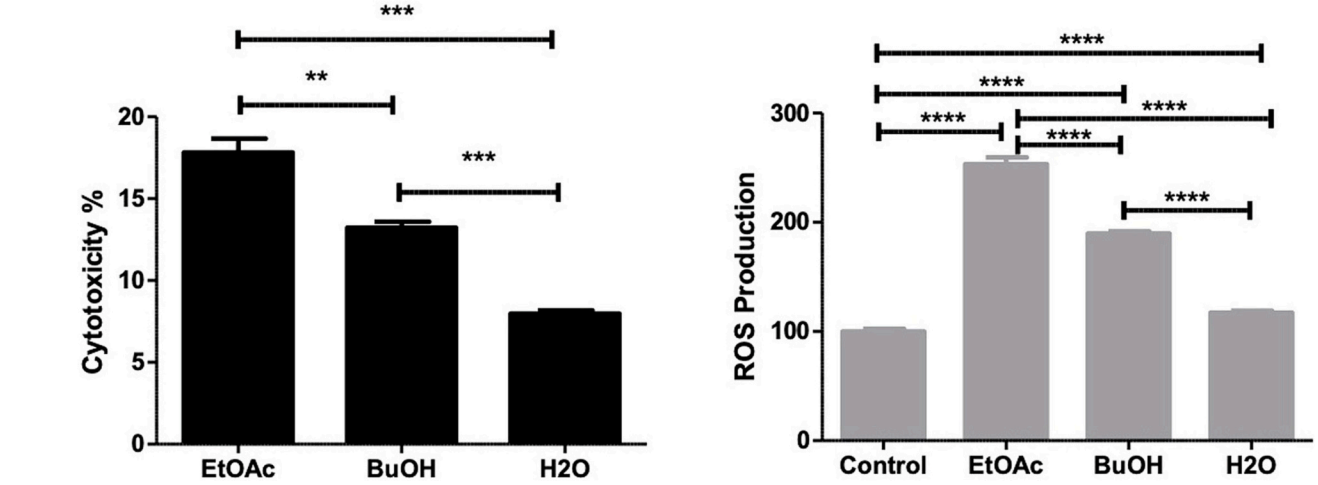


Fig. 4. A. Effect of different fractions of *Salix babylonica* L on necrotic cell death. B. Effect of different fractions of *Salix babylonica* L on reactive oxygen species. C: Control, EtOAc: Etilasetat, BuOH: Bütanol, H_2O: Water. For LDH; EtOAc group 17,83 ± 0,8399; BuOH group 13,22 ± 0,3790; H_2O group 7981 ± 0,1867. For ROS; C group: 100,0 ± 2302; EtOAc group 253,2 ± 6200; BuOH group 189,6 ± 2164; H_2O group 117,1 ± 1903.

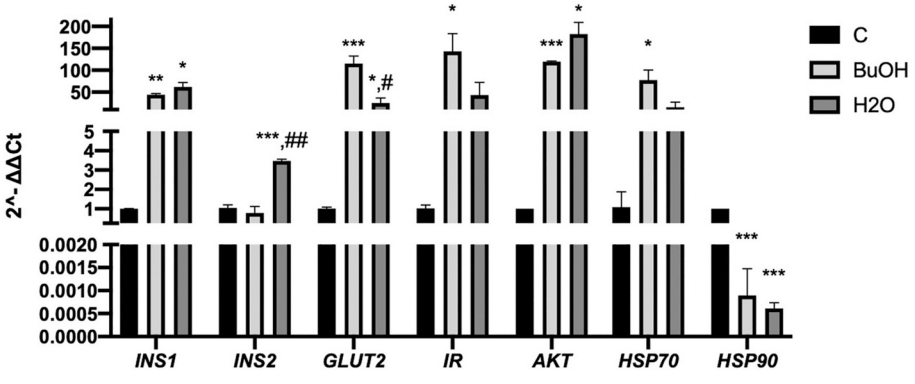


Fig. 5. Effect of different fractions of *Salix babylonica* L on INS1, INS2, GLUT2, IR, AKT, HSP70, and HSP90 gene expressions. C: Control, BuOH: Bütanol, H_2O: Water. For INS1; C group 1,00 ± 0,009576; BuOH group 44,09 ± 2,3; H_2O group 61,62 ± 10,04. For INS2; C group 1,00 ± 0,1650; BuOH group 0,7828 ± 0,3329; H_2O group 3469 ± 0,09076. For GLUT2; C group 1,00 ± 0,07701; BuOH group 115,4 ± 17,15; H_2O group 24,93 ± 11,67. For IR; C group 1026 ± 0,1647; BuOH group 143,0 ± 40,61; H_2O group 43,02 ± 29,21. For AKT; C group 1,00 ± 0,00; BuOH group 119,5 ± 1375; H_2O group 182,5 ± 26,96. For HSP70; C group 1083 ± 0,7942; BuOH group 77,34 ± 22,94; H_2O group 15,46 ± 11,30. For HSP90; C group 1,00 ± 0,00; BuOH group 0,0008934 ± 0,0005809; H_2O group 0,0006107 ± 0,0001217.

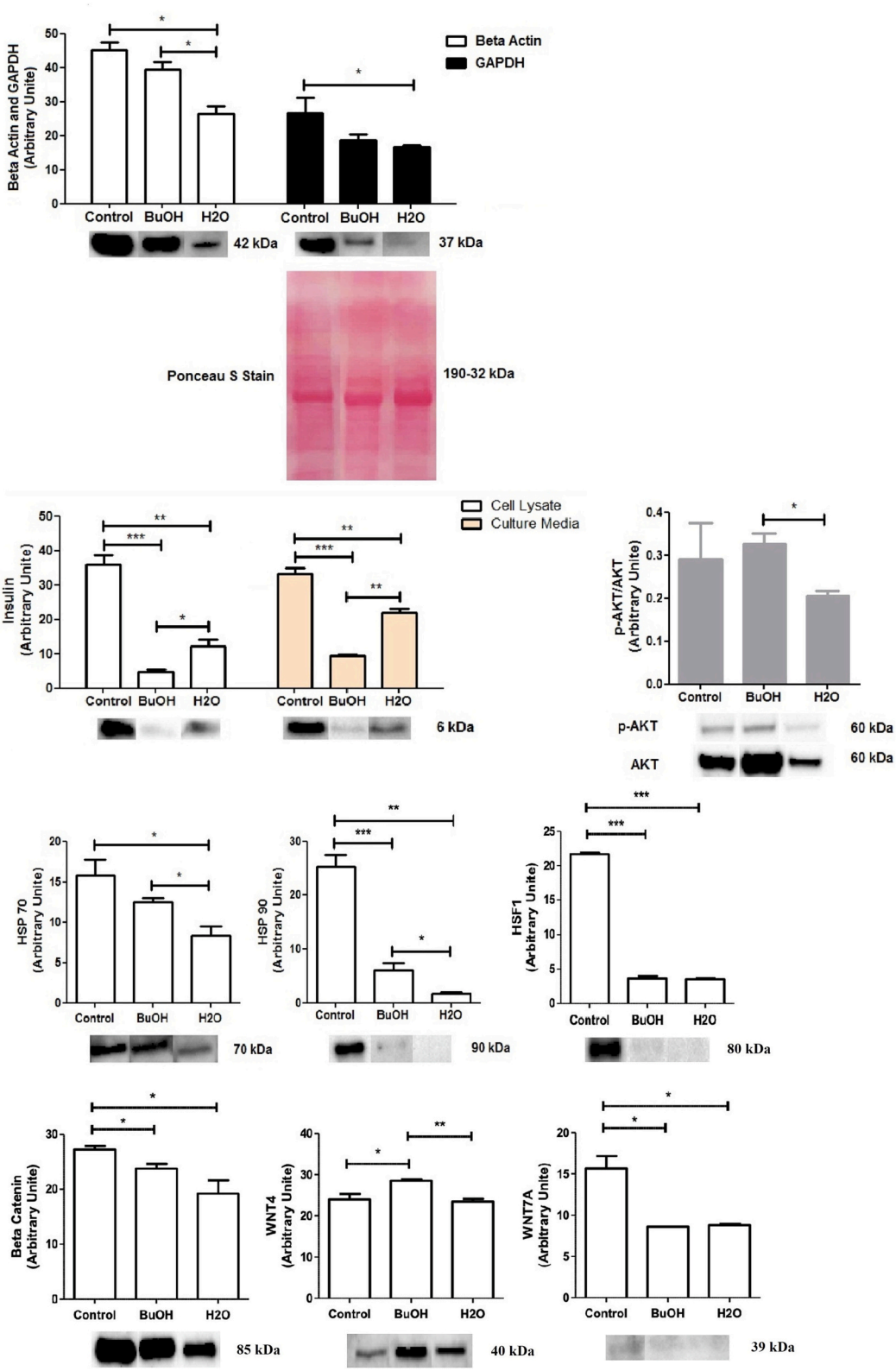


Fig. 6. Effect of different fractions of *Salix babylonica* L on Beta-Actin, GAPDH, insulin, AKT, HSP70, HSP90, HSF1, Beta Catenin, WNT4, and WNT7A protein levels. C: Control, BuOH: Bütanol, H_2O: Water. For Beta-Actin; C group 45,27 ± 2110; BuOH group 39,49 ± 2113; H_2O group 26,39 ± 2154. For GAPDH; C group 26,77 ± 4271; BuOH group 18,62 ± 1811; H_2O group 16,60 ± 0,5075. For insulin lysate; C group 36,03 ± 2694; BuOH group 4597 ± 0,8658; H_2O group 13,74 ± 1878. For insulin secretion; C group 33,17 ± 1732; BuOH group 9392 ± 0,3062; H_2O group 22,00 ± 1165. For p-AKT/AKT; C group 0,2906 ± 0,08453; BuOH group 0,3270 ± 0,02342; H_2O group 0,2057 ± 0,01229. For HSP70; C group 15,76 ± 1989; BuOH group 12,47 ± 0,5369; H_2O group 8355 ± 1141. For HSP90; C group 25,33 ± 2214; BuOH group 6113 ± 1402; H_2O group 1680 ± 0,3865. For HSF1; C group 21,70 ± 0,2300; BuOH group 3640 ± 0,3400; H_2O group 3,50 ± 0,15. For Beta Catenin; C group 27,26 ± 0,6041; BuOH group 23,76 ± 0,8637; H_2O group 19,22 ± 2386. For WNT4; C group 23,99 ± 1343; BuOH group 28,54 ± 0,3711; H_2O group 23,48 ± 0,6447. For WNT7A; C group 15,67 ± 1528; BuOH group 8608 ± 0,007895; H_2O group 8791 ± 0,2050.

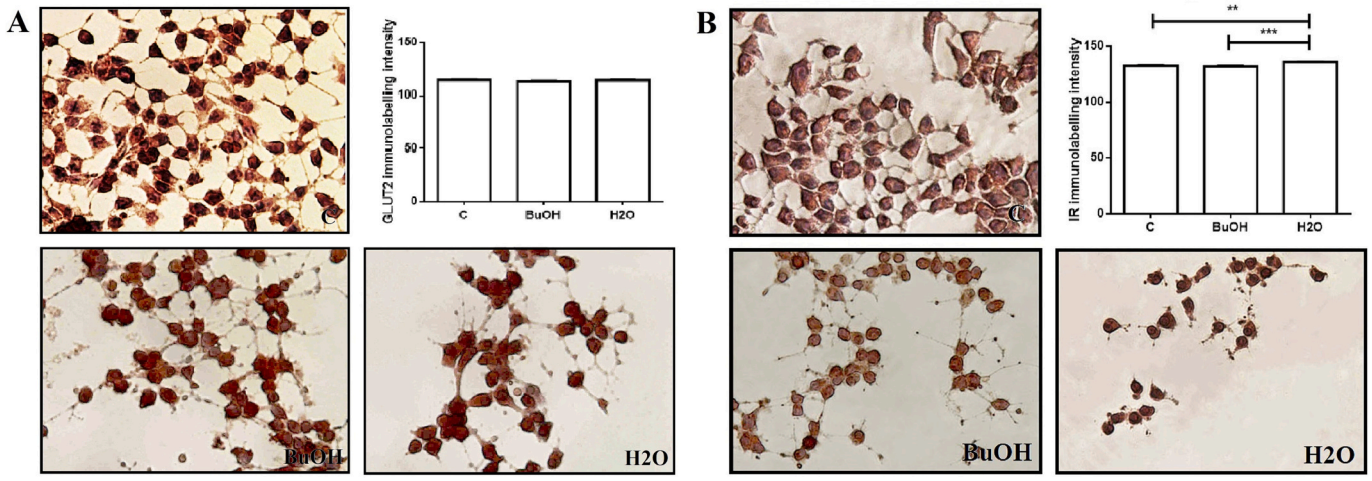


Fig. 7. Effect of different fractions of *Salix babylonica* L of GLUT2 and IR immunolabelling intensity. C: Control, BuOH: Bütanol, H_2O: Water. For GLUT2; C group 114,3 ± 1558; BuOH group 112,9 ± 0,7368; H_2O group 114,1 ± 0,8839. For IR; C group 132,7 ± 0,6096; BuOH group 131,9 ± 0,9404; H_2O group 135,9 ± 0,5009.

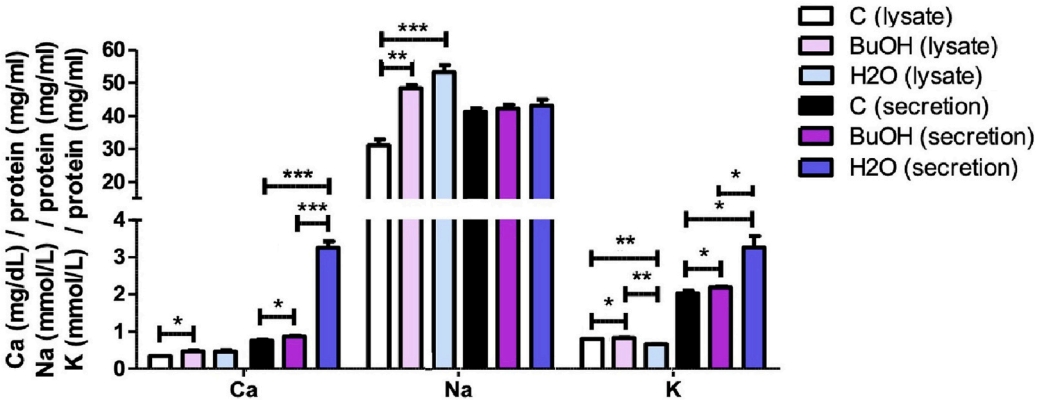


Fig. 8. Effect of different fractions of *Salix babylonica* L on Ca, K, and Na levels. C: Control, BuOH: Bütanol, H_2O: Water. For Ca in cell lysate; C group 0,3397 ± 0,0003475; BuOH group 0,4635 ± 0,02784; H_2O group 0,4528 ± 0,04992. For Ca in cell secretion; C group 0,7544 ± 0,03014; BuOH group 0,8643 ± 0,02531; H_2O group 3256 ± 0,1752. For Na in cell lysate; C group 31,09 ± 1854; BuOH group 48,37 ± 1067; H_2O group 53,21 ± 2215. For Na in cell secretion; C group 41,27 ± 1119; BuOH group 42,20 ± 1163; H_2O group 43,13 ± 1890. For K in cell lysate; C group 0,8042 ± 0,002579; BuOH group 0,8314 ± 0,004371; H_2O group 0,6536 ± 0,01140. For K in cell secretion; C group 2024 ± 0,07281; BuOH group 2190 ± 0,03629; H_2O group 3258 ± 0,3112.

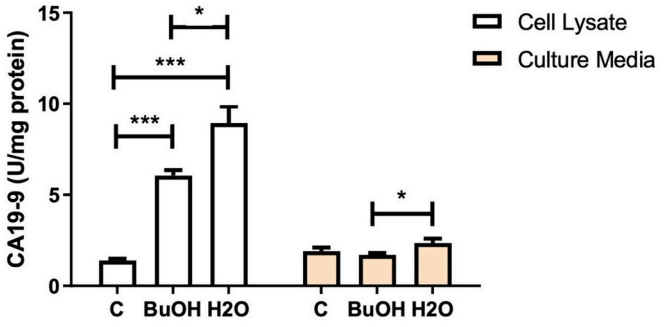


Fig. 9. Effect of different fractions of *Salix babylonica* L on CA19–9 levels. C: Control, BuOH: Bütanol, H_2O: Water. For cell lysate; C group 1394 ± 0,09462; BuOH group 6061 ± 0,2950; H_2O group 8948 ± 0,9120. For cell secretion; C group 1906 ± 0,2038; BuOH group 1707 ± 0,1044; H_2O group 2359 ± 0,2321.

secretion (Aggarwal et al., 2015). In addition, it was stated that the amount of insulin and the level of *INS1* and *INS2* genes increased when phenolic compounds obtained from the Ocimum plant were given to diabetic mice (Casanova et al., 2017).

According to our results, both intracellular and secretory insulin levels decreased. It is thought that there may be disruptions in insulin synthesis and release due to damage to the cells by associating with cell death. However, *INS1* and *INS2* gene expression levels increased in our study. This contradiction could be attributed to the beta cells' demand to remain under control or protection by the gene expression to produce insulin which is vital for these cells. While gene expressions increase, intracellular insulin levels decrease, indicating that insulin is affected during the synthesis phase, and its synthesis is not observed. According to the results, the exogenous application of BuOH and aqueous fractions of *Salix babylonica* L. leaf extract to insulinoma INS-1 cells cause increased insulin expression. However, insulin levels decreased due to possible negativity in insulin synthesis. Thus, it could be concluded that cell death occurs due to the lack of insulin synthesis.

In a study by Cheng et al., Aluminum (Al) caused a significant increase in the level of LDH activity, Na^+, and Ca^{2+}, in the heart tissue of rats. Mung bean extract treatment significantly improved cardiac tissue integrity and ion levels and reduced oxidative damage and Al accumulation in Al-administered rats (Cheng et al., 2017). Erianine obtained from *Dendrobium* extract significantly increased the intracellular Ca^+ level compared to the control group. It inhibits the growth and migration of lung cancer cells through calcium/calmodulin-dependent

ferroptosis (Chen et al., 2020). Voltage-gated Na^+ channels mediated intracellular Ca^{2+} release in INS-1 cells treated with *Portulaca oleracea* (Hu et al., 2019). In our study, excess Ca^{2+} outflow of cells decreased insulin secretion.

High levels of HSP 70 were detected in pancreatic cancer patients whose intracellular, and plasma HSP 70 levels were measured (Dutta et al., 2012). Inhibition of HSP 70 in pancreatic cancer cell lines MiaPaCa-2 and PANC-1 cells increased cytosolic Ca^{2+} level and lysosomal membrane permeability. As a result of these events, apoptotic cell death pathways were activated, and apoptotic death was observed in cells (Dudeja et al., 2009). Combined inhibition of HSP 90-proteosome with HPAC, MiaPaCa-2 cells caused increased autophagy and apoptosis by reducing cell viability. In animal experiments, a reduction in tumor size was observed in HSP 90-proteosome inhibition (Belalcazar et al., 2017). In a study on insulinoma cells, the MIN6 porosome complex containing HSP 90, which is an essential factor in insulin secretion, was isolated. In this study, insulin secretion was reduced by inhibition of HSP 90 in a ratio of 50%. HSP 90 in porosomes is thought to have a function and play a critical role in insulin secretion (Rajagopal et al., 2015). Inhibition of HSP 90 interacting with various proteins in INS-1 cells leads to endoplasmic reticulum stress and impaired insulin secretion. In these glucose-stimulated cells, inhibition of HSP 90 impaired insulin synthesis and secretion. (Ota and Wang, 2012).

HSP 70, HSP 90, and HSF1 protein levels were decreased in INS-1 cells treated with different fractions of *Salix babylonica* L. extract. While the *HSP70* gene expression level increased, the *HSP90* gene expression level decreased. Considering the high HSP levels in cancer patients and occurred cell death as a result of HSP inhibition, it can be said that *Salix babylonica* L. leaf extract is curative for insulinoma INS-1 cells. It can be noted that this plant extract has more effect on HSP90. It seems more likely that the resulting cell death was due to HSP90.

In a study by Qi et al., it was shown that Piperine modulates antitumor effects by inhibiting the Wnt/beta-catenin signaling pathway on U2OS and 143B human osteosarcoma cells (Qi et al., 2020). Korean Red ginseng extract has also been shown to reduce cell viability and self-renewal capacity of glioblastoma cells by blocking the Wnt signaling pathway (Ham et al., 2019). Macleayins isolated from *Macleaya cordata* and *Macleaya microcarpa* attenuated Wnt/beta-catenin signaling cascade activation in HeLa cells in a dose-dependent manner. Macleayins inhibits proliferation, migration, and invasion by promoting cell apoptosis through the inhibition of the Wnt/beta-Catenin Signaling Pathway (Sai et al., 2021).

In our study, as a result of some proteins studied concerning the WNT signaling pathway, there was no change in WNT4, while WNT7A and beta-catenin levels decreased. This evidence brings the possibility of occurring necrotic cell death *via* WNT7A.

Silybum marianum extract significantly suppressed proliferation, Wnt/beta-catenin, and PI3K/Akt/mTOR signaling pathways in hepatocarcinoma cell lines. It reduced serum CA19–9 levels in rats with hepatocarcinogenesis compared to control rats (Yassin et al., 2022). It has been shown in a clinical study that serum CA 19–9 levels in pre-diabetic individuals are significantly correlated with early-phase insulin secretion, and an increase in serum CA 19–9 levels may reflect the extent of insulin secretion disorders in pre-diabetic individuals (Shimodaira et al., 2015). It has been stated that pancreatic cancer exosomes containing CA19–9 cause dysfunction in INS-1 beta cells by inhibiting insulin secretion (Javeed et al., 2015).

While no change was observed in CA19–9 levels in cell secretion in our study, BuOH and H_2O fractions increased in cell lysate compared to the control group. In this study, considering the relationship between the increase in CA19–9 levels and pancreatic beta cell function, it can be hypothesized that the CA19–9 levels are also higher because of dysfunction observed in the H_2O fraction. Table 1 summarises all *in vitro* activity of different fractions of *Salix babylonica* L. extract studied in the current article.

According to studies, it is stated that pancreatic cancer may take second place among cancer-related deaths in 2030. The annual survival rate of pancreatic cancer patients is below 5%. The most important reason the survival rate is low and the most visible among cancers is that it is difficult to diagnose early. (Canto et al., 2013; Rahib et al., 2014). Treatment options for this disease, which can be diagnosed at a late stage, are also limited. The situation is very similar for insulinoma, which is a pancreatic tumor. All metabolism is adversely affected in insulinoma, which causes excessive insulin secretion. It is important to target relevant cells and develop treatment strategies without harming the organism, especially in cancer treatments. *Salix babylonica* L. extract was applied to the cells and investigated to treat insulinoma without disturbing the cell function of insulin, which is excessively released from pancreatic beta cells. This extract has not been studied in the pancreas before and its effects are unknown. As a result, different fractions of *Salix babylonica* L. leaf extraction have various effects on insulinoma INS-1 beta-cell tumors. Different fractions cause different degrees of cell damage and cell death. Accordingly, insulin synthesis and secretion, which is essential for insulinoma cells, varies. Since the BuOH fraction of the subjected plant extract causes a higher level of cell death in lower doses without harming the function of the cells, it could be concluded that the BuOH fraction is the most effective fraction of *Salix babylonica* extract.

Table 1
The summary of the *in vitro* activity of different fractions of *Salix babylonica* L. extract.

In vitro activity	Fractions		
	EtOH	BuOH	H_2O
IC50 against insulinoma INS-1 pancreatic beta cell tumor (mg/ml)	1.413	1.770	5.2419
The functionality of insulinoma INS-1 pancreatic beta cell tumor for insulin secretion	Impaired	Not impaired	Impaired
Induction of the necrotic cell death of insulinoma INS-1 pancreatic beta cell tumor	Highest necrosis level	Medium necrosis level	Lowest necrosis level
ROS production level/ Cell viability	Highest/ lowest	Medium/ medium	Lowest/ highest
INS1, GLUT2, and AKT gene expressions	–	Increased	Increased
HSP90 gene expressions	–	Decreased	Decreased
INS2 gene expression	–	–	Increased
IR and HSP70 gene expressions	–	Increased	–
Insulin, HSP90, HSF1, Beta Catenin, and WNT7A levels in cell lysate/ secretion	–	Decreased/ Decreased	Decreased/ Decreased
pAKT/AKT level in cell lysate and GLUT2 level in cell	–	–	–
HSP70 level in cell lysate	–	–	Decreased
WNT4 level in cell lysate	–	Increased	–
IR level in cell	–	–	Increased
Ca^{+2} level in cell lysate/ secretion	–/–	Increased/ Increased	–/ Increased
Na^+ levels in cell lysate/ secretion	–/–	Increased/–	Increased/–
K+ levels in cell lysate/ secretion	–/–	Increased/Increased	Decreased/Increased
CA19–9 levels in cell lysate/ secretion	–/–	Increased/–	Increased/–

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Author contributions

Ayse Karatug Kacar created the main idea, experimental design, all cell culture experiments, cell viability, insulin secretion, LDH, and ROS analysis, determinate of gene expression by qRT-PCR, determinate of proteins by western blot, immunohistochemical analysis, determination of Ca, K, Na, CA-19-9 levels, evaluation of all experimental results, statistics, written manuscript. Dilara Aylar, helps to cell culture experiments, insulin secretion analysis, determination of proteins by western blot, and immunohistochemical analysis. Fatma Kazdal, *Salix babylonica* L. leaf extraction, and fractionation. Fatemeh Bahadori, *Salix babylonica* L. leaf extraction, and fractionation, edited manuscript.

Declaration of Competing Interest

None.

Data availability

Data will be made available on request.

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