Analytical Optimization of Extraction Parameters to Produce High-Yield Low Methoxy Pectin with Significant Bioactivity

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Research Article

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Abstract

The pectin isolation technique from sugar beet pulp was optimized to acquire high-quality bioactive pectin with the highest yield and low esterification degree. The influence of each isolation parameter on the success of pectin production was investigated by analytical characterization methods. With this step-by-step analytic investigation, we could be able to achieve ~ 45% yield with a ~ 10% low esterification degree. In addition to compositional quality, bioactivity and anticancer profile of the obtained sugar beet pulp pectin (SBPP) was investigated on SaOS-2 osteosarcoma cell line. In comparison with citrus pectin (CP), which is frequently applied pectin derivative as an anticancer agent, SBPP revealed prolonged antiproliferative profile for cancer cells. In addition to proliferation, intracellular reactive oxygen species (ROS) level and Bcl-2 anti-apoptotic gene expression were surveyed *in vitro*. Interestingly, the lower concentration of SBPP (0.25%) induced a significant rise in intracellular ROS level and anti-apoptotic gene Bcl-2 expression on the contrary to CP. All results demonstrated that different pectin types can lead different biological mechanism of action and the isolated SBPP by using optimized protocol exhibited great potential for biomedical applications without requirement of additional post-modification after isolation.

Statement Of Novelty

Diverting bio-waste applications from low value-added practices to the more practical applications, such as the medical field, have attracted great interest as a result of multiple advantages. Our effort focusses now on sugar beet pulp valorization to enhance the quality and the quantity of the pectin yield by optimizing each isolation parameter of commonly used techniques. In addition to chemical quality, we reported a new potential medical use for the obtained pectin isolates. In comparison to commonly used pectin types, without the requirement of post-treatment, sugar beet pectin significantly boosted reactive oxygen species production and inhibited the proliferation of aggressive osteosarcoma cells. The sugar beet pulp pectin has shown prolonged antiproliferative activity, these results indicated significant potential of pectin for future easy-to-access biomedical applications.

1. Introduction

Pectin is a natural plant-derived polysaccharide commonly used in the food industry as a thickening, gelling and stabilizing agent[1]. It is present in the cell wall of the plants and is made up of more than 60% of the homogalacturonan (HG) region[2]. The remaining contents are called Rhamnogalacturonan-I (RG-I) and Rhamnogalacturonan-II (RG-II) and give a brush-like structure to pectin with its galactan, arabinan, or arabinogalactan side chains. The pectin backbone is formed by the HG region with (α-1,4) linked D-galactopyranosyluronic acid (GalpA) residues. Carboxyl groups of this region can be esterified with methyl residues, and the number of methyl residues indicates the esterification degree of the pectin[2–4]. In general, low esterified pectin has higher water solubility than high esterified pectin due to the hydrophobicity of ester groups[1]. Due to the rich composition of pectin polysaccharides, these

biopolymers present great potential to develop niche applications for the biomedical field, as they are commonly used for various purposes[1].

Pectin can be extracted from various sources, and different isolation techniques were employed to obtain a high-quality yield. To this end, different methods are being used to reach optimum pectin yields, such as enzymatic extraction, microwave-assisted extraction, ultrasound extraction, high-pressure extraction, and conventional extraction via acid digestion. Most existing methods for pectin extraction are generally time-consuming, require complex/costly multistep procedures. The acid-based digestion technique is based on acidified hot water treatment, and this method is commonly utilized as a conventional industrial-scale process due to the ease of the application and relative yield potential[5]. However, yield and other physicochemical characteristics of pectin are highly dependent on isolation technique selection and plant source. For instance, low pH based isolation cause degradation of neutral sugars of the pectin chain[5]. Therefore, to improve the quality of the isolate, extraction techniques and parameters must be carefully selected by considering the plant source, and the isolation steps have to be investigated thoroughly.

Sugar beet pulp is the residue of sugar beet, which remains after sugar production, and this residual product has limited use in industry as animal feed. However, sugar beet pulp has a high content of carbohydrates, including pectin, and the rich content of sugar beet pulp can be exploited in a wide range of applications [6]. Therefore, the isolation of pectin from sugar beet pulp with the best yield and revealing its possible applications have the critical potential for recovering waste materials into valuable outputs.

In the literature examples, different isolation techniques yielded divergent esterification and physical profiles for the obtained pectin extracts (Supporting Table 1). So far, pectin could be isolated from sugar beet pulp with a maximum of 35.4% yield [7]. Although all those studies follow similar extraction steps (Supporting Table 1), the difference in the obtained yields possibly comes from the minor alterations of isolation parameters. In this study, to reveal the contribution level of each component/condition, we evaluated the extraction steps and successfully optimized the pectin yield. To prove and indicate the scope of applicability of pectin isolates, the success of the isolate was tested with a new biological model. Hence, we tested the bioactivity of the pectin isolate derived from different sources by using an optimized method on osteosarcoma cell line SaOS-2 since pectin is reported to have anticancer activities for various cancer types[8–10].

As already indicated, the isolation parameters significantly influence the yield; the composition of pectin biopolymer can also be modified with heat and pH treatments. The different extraction conditions may lead to fragmentation or depolymerization and de-esterification of the pectin[11]. Besides of product quality and quantity, the anticancer activity of pectin isolate varies among applications of these treatments. In a previous work[9], citrus pectin modified with heat treatment and the treated pectin induced apoptosis in a prostate cancer cell line while pH modified citrus pectin had little activity on this cell line. To modify the pectin with heat, it was autoclaved at 123.2°C and consequently fractionated. On

the other hand, in another approach, citrus pectin modified with pH treatment resulted in decreased tumor growth and metastasis in the *in vivo* model[12].

Although pectin from varied sources, like apple[13], ginseng[14], and sugar beet pulp[15], were also studied for investigation of anticancer activity, modified citrus pectin attracted most of the attention until now. Moreover, the anticancer activity of pectin is mainly investigated on colon cancer [14–16]. prostate cancer[9] or breast cancer [12] models. The anticancer effect of the isolated pectin on hard tissue tumors like osteosarcoma was not studied.

Osteosarcoma is a highly aggressive bone cancer mostly diagnosed in children and adolescents. This disease can be cured with heavy drug treatment and surgery, which causes serious toxicity and side effects[17]. Although many improvements are present for the treatment of osteosarcoma, there are still great possibilities for effective antiosteosarcoma agent development. Since pectin is a natural polymer and its anticancer activities were reported for other cancer types, it can be a promising candidate for osteosarcoma treatment as well.

In the recent study, at first the isolation of pectin from sugar beet pulp was optimized to get the highest yield and low degree of esterification (DE) and then anti-proliferative effects of sugar beet pulp pectin on the osteosarcoma model was investigated. The cancer cell inhibition activity was studied by tracing cell viability. Moreover, to get an insight into the mechanism of bioactivity of sugar beet pulp pectin, ROS formation upon pectin treatment was surveyed, and the expression level of apoptosis-related gene Bcl-2 was examined. As opposed to earlier studies, pectin was not modified, and this provided ease of implementation and prevented possible toxic by-products and related inconsistency of different treatments.

2. Methods

2.1. Isolation of pectin from sugar beet pulp

Pectin from sugar beet (SB) was isolated based on the literature examples[18], and modifications and optimizations were carried out to investigate the best isolation conditions. Parameters for isolation were changed as indicated in Table 1. SB pulp was dried at 60°C and pulverized into powder. An aqueous mixture was prepared by arranging the pH of the water at 1.2 with 6M H_2SO_4 and mixing SB pulp powder with this acidified water (solid: liquid ratio 1g:30ml). The mixture was stirred for a determined duration (4 or 5 hours) at a high temperature (80 or 90°C) with a magnetic stirrer (C-MAG HS 7 IKAMAG, IKA-Werke GmbH & Co. KG). The hot mixture was cooled and filtered with different techniques mentioned in Table 1. Then, the filtrate was collected, and the pH of the filtrate was not changed or increased either with 1M NaOH, 0.2M K_2HPO_4, or 0.2M Na_2HPO_4. The resulting filtrate precipitated in absolute ethanol (ethanol to filtrate volume ratio is 4, 3 or 2) for 1 hour. Precipitates were collected through centrifugation (Beckman Coulter® Allegra X-30R) for 20 minutes at 17.700g (or 10.000 rpm). Then, the pectin pellets were washed with 70% ethanol and centrifuged again for 20 minutes at 17.700 g (or at 10.000 rpm). Supernatants were discarded, and wet pectin was prepared for drying. For drying, pectin was placed on a 60°C heater

for a night, or wet pectin pellet was dissolved in enough distilled water, frozen at 80°C, and freeze-dried in a lyophilizer (Buchi Lyovapor™ L-200) (Fig. 1).

For isolating citrus pectin, the orange peel's white layer was collected, sun-dried and then pulverized. An aqueous mixture was prepared by arranging the pH of the water at 1.2 with 6M H_2SO_4 and mixing orange peel powder with this acidified water (solid: liquid ratio 1g:30ml). The mixture was stirred for 5 hours at 90°C with a magnetic stirrer. Since the slurry became very dense after stirring, instead of filtration, centrifugation (Beckman Coulter® Allegra X-30R) was performed for 20 minutes at 10.000 rpm to separate peel residues from pectin containing supernatant. The supernatant was collected and precipitated in absolute ethanol (ethanol to supernatant volume ratio is 2.5) for 1 hour. Pectin precipitates were collected through centrifugation (Beckman Coulter®Avanti™ J-HC) at 3.300g for 1 hour. The pectin yield was washed with 70% ethanol, and second centrifugation was performed with the same conditions. The resulting pellets (pectin) dissolved in enough distilled water, frozen at -80°C, and freeze-dried in a lyophilizer (Buchi Lyovapor™ L-200).

2.2. Calculation yield of pectin

The yield of the isolated pectin is calculated according to the equation below;

mass of dry pectin/mass of dry sugar beet pulp) X 100) %

2.3. Determination esterification degree (DE) of pectin

The degree of esterification is a parameter that shows the percentage of carboxyl groups esterified with methyl residues in pectin structure. This parameter affects special characteristics of pectin, like solubility[1], and it was calculated with a titration-based method with additional modification[19].

CO_2 free water was prepared by boiling distilled water for at least five minutes and then cooling in a closed bottle for further use. 25 mg of dry pectin is placed in a 50 ml beaker and moistened with 500 µl 65% ethanol. The wet pectin was completely dissolved with 5 ml of CO_2 free water and then transferred into a 250 ml Erlenmeyer flask. Three drops of phenolphthalein were added to the pectin solution as an indicator. The solution was titrated with 0.1 M NaOH until the faint pink color was observed and retained. The added volume of NaOH was recorded (NaOH 1), and it represented the unesterified carboxyl units. 10 ml 0.1 M NaOH was added to the solution, and the solution was stirred for 30 minutes with a magnetic stirrer. This step allows for removing methyl esters from the esterified carboxyl units of pectin[19]. After stirring, at least 10 ml of 0.1 M HCl was added to the solution. More HCl is watchfully added to the solution until the pink color completely disappears. The solution is titrated again with 0.1 M NaOH until the faint pink color is observed and retained (Fig. S1). The volume of added NaOH was recorded (NaOH 2). Esterification degree was calculated according to the equation below;

(NaOH 2 / NaOH 1 + NaOH 2) × 100%

1.3. Determination of protein residues in pectin

Protein residues in pectin structure were quantified with Qubit Protein Assay Kit (Invitrogen™) according to manufacturer indications. Briefly, 2 µl of Qubit™ protein-dye was mixed with 398 µl of Qubit™ Buffer. 0.25% pectin solution was prepared with double distilled water, and 20 µl of pectin solution was mixed with 180 µl previously prepared dye solution. The resulting sample was inserted into a fluorometer (Qubit 4 Fluorometer, Thermofisher, US) and read.

1.4. Fourier Transform Infrared Spectroscopy (FTIR) Analysis

FTIR analysis of the lyophilized pectin samples was performed using a Nicolet iS10 FTIR Spectrometer (Thermo Fisher Scientific, USA) in the 550–4000 cm^{-1}. The wavenumbers were accumulated for 64 scans at room temperature at 4 cm^{-1} resolution. Two independent replicates with two different pectin extracts were acquired for FTIR analysis. A pressure applicator was used to immobilize the samples against the ATR crystal during spectra measurement.

1.5. Cell culture

Human osteosarcoma cell line SaOS-2 was cultured in a humidified incubator at 37°C with 5% CO_2 in complete low glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco) containing %10 Fetal Bovine Serum, 2 mM L-Glutamine and 1% penicillin/streptomycin.

Human Umbilical Vein Endothelial Cells (HUVEC) was cultured in a humidified incubator at 37°C with 5% CO_2 in complete high glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco) containing 10% Fetal Bovine Serum, 2 mM L-Glutamine and % 1 penicillin/streptomycin.

For subculturing or experimental usage, cells in flasks were washed with phosphate-buffered solution (PBS) and then trypsinized with trypsin-EDTA (Sigma).

1.5.1. Alamar Blue viability assay

To investigate the anticancer activity of pectin, we chose the pectin that is isolated with a procedure giving the highest yield and low esterification degree.

Pectins were UV sterilized at least for 15 minutes before using in cell culture experiments. Pectin solutions were prepared with cell culture media with the concentrations of %2, %1, %0.5 and %0.25 for sugar beet pulp pectin (SBPP) and %0.66, %0.33, %0.16 and %0.083 for citrus (orange peel) pectin (CP). 100 µl of SaOS-2 cells were seeded to 96 well plate (1×10^4 cells per well) (4 replicas for each group). Cells were treated with 100 µl of different concentrations of pectin and incubated for 24, 48 and 72 hours. 100 µl cell culture media was added for control cells instead of pectin solution. After incubation, culture media was removed from wells, and wells were washed with PBS. Later, 100 µl of 10% Alamar blue cell viability reagent (Invitrogen™) prepared with FBS free cell culture media was added to each well and incubated for 1 hour at dark. The active molecule of this reagent is the blue colored oxidized resazurin, and it is reduced with the metabolic activity of living cells and becomes pink-colored resorufin enabling

the quantification of living cells in the sample. To quantify the resorufin molecules, the plate was read with excitation at 560 nm and emission at 590 nm in a plate reader (BioTek Synergy™ H1), and the same procedure was applied to the HUVEC cell line.

1.5.2. Intracellular Reactive Oxygen Species (ROS) measurement

Reactive oxygen species produced in the cell upon pectin treatment was measured with the DCFDA assay (Abcam). In this assay, DCFDA diffuses into the cell, deacetylated with cellular enzyme, and then oxidized to highly fluorescent DCF (2', 7'–dichlorofluorescein) by ROS.

For this assay, 1% and 0.25% sugar beet pulp pectin (SBPP) and 0.33% and 0.083% citrus (orange peel) pectin (CP) were selected and prepared with cell culture media and UV sterilized for 1 hour before using. As in the viability test, 100 µl of SaOS-2 cells were seeded to 96 well plate (1×10^4 cells per well) (4 replicas for each group). Cells were treated with 100 µl of different concentrations of pectin and incubated for 24 and 72 hours. 100 µl cell culture media was added for control cells instead of pectin solutions. After incubation, cell culture media was removed from the wells and wells were washed with 100 µl 1X buffer provided in the kit (Abcam). 100 µl 20 µM DCFDA solution was added to each well and incubated for 45 minutes at 37°C in a dark incubator. After incubation, DCFDA solution was removed from wells, 100 µl PBS was added to the wells, and fluorescence was read in a plate reader with excitation at 485 nm and emission at 535 nm. The same procedure was applied to the HUVEC cell line.

1.6. Gene expression analysis

1.6.1. RNA isolation from cells

For RNA isolation, 1 ml of SaOS-2 and HUVEC cells were seeded in 24 well plate (8×10^4 cells per well) (4 replicas for each group) and treated with 1 ml of pectin solutions (1% and 0.25% SBPP, and 0.33% and 0.083% CP) and incubated in a humidified incubator at 37°C for 24 and 72 hours. Then, the RNA was isolated from cells with Universal RNA Purification Kit (EURx), as the manufacturer indicates. The purity and yield of RNA were calculated using Nanodrop 2000 (Thermo scientific).

1.6.2. Real Time Semi-Quantitative PCR

For gene expression analysis, cDNA was synthesized from extracted RNA using the OneScript Plus cDNA synthesis kit (abm) according to the manufacturer's instructions. Semi-quantitative real-time PCR was performed using BlasTaq 2X qPCR MasterMix (abm) kit. Primers for the Bcl-2 gene were selected as 5'-TGTGTGTGGAGAGCGTCAAC-3' for forward, 5' -TGCTTGGCAATTAGTGGTCG-3' for reverse from the literature. Bcl-2 expressions were normalized to the expression level of the housekeeping gene GAPDH. Primers for GAPDH were selected as 5'-TCCCATCACCATCTTCCAGG- 3' for forward, 5'-CCATCACGCCACAGTTTCC – 3' for reverse.

1.7. Statistical Analysis

All experiments were performed with 4 replicas in each group. Results were expressed quantitatively with mean and standard deviation (SD) values. One-way analysis of variance (ANOVA) was used for statistical analysis of ROS measurement experiments. Differences were determined as statistically significant at $p < 0.05$. Graph-pad Prism v5.0 was used for this statistical analysis.

3. Results And Discussion

3.1. Investigation of the role of filtration method on pectin yield

The acid digestion technique is the most common procedure of pectin isolation. In this method, sugar beet pulp was mixed in hot acidified water for several hours, and the pulp released its pectin content to water. Following this process, pectin solution is separated from residual pulp via filtration. In previous studies, filtration step was performed with nylon cloth[18], centrifugation [18-20], vacuum filtration[21]. The pectin product efficiency is known to be mainly reaction condition-dependent, whereas the filtering method is one of the critical contributors of the biopolymer isolation process. In this study, the potential effects of the filtering method on such isolation processes were investigated by comparing various separation applications. According to current investigation, the filtration method has a critical influence on the yield as it is anticipated. Filtration of digested pulp solution through cellulose filter paper (Whatman No.1 Cat.No: 1001-125) (11 µm) resulted in a low amount of filtrate since some of the pectin content is absorbed by filter paper. Therefore, a faster and better way of filtering through a vacuum filter (0.2 µM Polyethersulfone-PES-membrane) was applied for the next isolations. Despite the smaller pore size of PES filtration, the pectin yield increased almost two-fold from 18% to 33% (Table 1. trials 3 and 5). Although both cellulose and PES are hydrophilic membranes, PES get wet quickly and had superior flow rate compared medium flow cellulose filter paper. These results indicated that the material of the filtration apparatus and vacuum has a significant role in the biopolymer isolation process, the filtration method has to be carefully selected by considering the content of the extract.

3.2 Investigation of importance of pH arrangements on pectin yield and DE

Soluble pectin in filtrated isolate is recovered via ethanol precipitation. Different treatments can be applied before pectin retrieval; according to previous methods, the isolate's pH was either increased to 3.5[18] for kept constant [22]. To understand the pH dependency of the resulting extract, the effect of pH adjustments on pectin yield and esterification degree were investigated. Firstly, the pectin sample was alkalized to pH 3.5, and this pH treatment was found to result in a significant improvement in product yield (up to 3-fold) compared to non-treated groups (Table 1. trials 4, 5 and trials 7, 9). Increasing pH to 2.5 has also improved the yield, whereas optimum pectin yield was acquired from pH 3.5 treated groups (Table 1. trials 7,9,10). The pH arrangements affected to esterification degree of pectin as well. In addition to the higher yields, increasing pH to 2.5 or 3.5 results in a higher esterification degree (DE) but the resulting pectin is still in the low-ester pectin range. It is also valid that pectin isolated in all trials is in low DE since the highest DE was 20%. It was clearly observed that in all trials, increasing the pH of filtrate to

2.5 or to 3.5 after the vacuum filtration, enabled improved precipitation in ethanol (Fig. S2) and better pectin yield than the groups in which pH was not changed (Table 1).

3.3 Investigation of the relationship between the type of agent used for pH arrangements and pectin yield

After observing the importance of pH for pectin yield, it has been questioned whether the type of agent used to adjust pH conditions of isolate influences the yield as well. In previous studies, before alcohol precipitation, pH adjustments were made with salts like K_2HPO_4 [18] or bases like $NH_3 \cdot H_2O$ [20, 21]. In this study, the possible effects of salt or base use on pectin isolation, K_2HPO_4 and NaOH were separately investigated. Using salt solution (K_2HPO_4) resulted in higher pectin yield than base solution (NaOH) (Table 1. trials 10 and 11).

3.4 Investigation ethanol precipitation step for pectin yield and DE

Following the treatments indicated above, pectin can be extracted from the solution via alcohol precipitation, and this step can have a possible influence on the yield quantity. The administered volume of alcohol (absolute ethanol) varies among the studies. Previous studies reported that two volumes (2X) of ethanol to one volume of extract [18, 22] or four volumes (4X) of ethanol [18, 23]can be applied to precipitate pectin. To optimize the required ethanol volume for precipitation, three different volumes of ethanol (4X, 3X and 2X) were applied (Table 1. trials 6, 7, 8). When the filtrate was precipitated in ethanol, pectin yield became 47.6%, 35.8% and 38.89%, respectively, with 4X, 3X and 2X ethanol volumes. Esterification degree of pectin was 11%, 9.1% and 14.3%, respectively. There is no direct correlation between ethanol volume and pectin yield, and the DE of pectin was obtained; the increasing volume of ethanol led to a slight increase in the quantity of obtained yield.

3.5 Investigation of the effect of drying method on pectin properties

In the last step of pectin isolation, the precipitated pectin is retrieved from the solution and dried. According to our observations and literature examples [24], drying conditions directly influence the physical characteristics of pectin. In this study, pectin samples were dried with the freeze-drying method or at 60 °C in a heater overnight. The most demonstrable characteristic of the resulting pectin was its dark brown color which is dried at 60°C. The quality and quantity of resulting pectin yield were poor, the brown-colored product is comparably different from commercial pectin, and the yield was insufficient to disable any further analysis (Fig. S3 A). The quantity of the isolated pectin was not sufficient to calculate the esterification degree. Therefore, wet pectin was dried with the freeze-drying method and this technique considerable increased the quality and the quantity of the obtained pectin. As indicated in previous work [24] on soy pectin, drying conditions affected the appearance of isolated pectin. It has been shown that pectin produced by oven drying has the lowest Hunter values (lower Hunter L values indicate dark color) for color compared to freeze-dried and spray dried pectin. It was also mentioned in another work [24]that dehydration and drying with hot air dryer results in degradation of pectin backbone because of β-elimination reaction. Temperatures above 50 °C can also increase the activity of enzymes that degrade pectin[24].

3.6 Fourier Transform Infrared Spectrophotometer (FTIR) Analysis

Figure 2 shows the FT-IR spectra of pectin extracted from sugar beet pulp, using the various conditions presented in Table 1. Carbohydrate specific spectral regions of four selected samples were compared to elucidate the relationship between the isolation method and the quality of the product. In most cases, improvement in the yield quantity results in a decrease in quality [25, 26]. Therefore, two high yield and two low yield extracts were selected for comparison.

In comparison with previous works on the infrared spectra of pectin extracts [27, 28] for the four samples that were analyzed in this study, v (O-H) peaks were found in the region of 3600–3000 cm^{-1}. The peak at ~1620 cm^{-1} was attributed to carboxylate; since the esterification degree of all samples is low, strong carboxylate ion stretching was observed in each spectrum. For the trials 7, 9, 10 and 11 carboxylate specific absorption peaks were observed at 1606 cm^{-1}, 1618 cm^{-1}, 1618 cm^{-1}, 1624 cm^{-1} and 1640 cm^{-1}, respectively (Fig.2).

The strong asymmetrical stretching at 1620 cm^{-1} is accompanied by another carbohydrate-specific weak symmetric stretching band around 1441 cm^{-1}, followed by strong absorption patterns between 1300 and 800 cm^{-1}. This set of absorption peaks enables identifying the major chemical moieties in polysaccharides, and this 'fingerprint 'region is unique to the carbohydrates [29]. In this region, galacturonic acid in pectin molecules was identified using spectral region between 1120–990 cm^{-1} (Fig.2). The band at 1019 cm^{-1} indicated that pectin extracts contain pyranose. The week peaks characterized the presence of D-glucopyranosyl and α-D-mannopyranose at 917 cm^{-1} and 825 cm^{-1}, respectively[30, 31]. In addition to this, the most characteristic bands of L-arabinose were also observed at 1445 and 1260 cm^{-1} [32]. Trials 7 and 10 have shown stronger carbohydrate specific peak profiles indicating better preservation of saccharide content during isolation. This result can be attributed to use of salt (K_2HPO_4) during pectin isolation in trial 7 and 10. Salt might have acted as stabilizer to prevent saccharide degradation from pectin chain.

3.7 Protein content of pectin

Like low DE low protein content is also indicates the success of the pectin isolation procedure and low protein content is desirable for biomedical applications. Therefore, protein content of the pectin isolates was measured for each trial. It was found that protein content ranged from 1.02- 3.17% among trials. As it was already declared by Li et al. [22] and shown in the study of Yapo et al. [18] longer extraction time and higher temperatures assist to breakage of covalent linkage between protein residues and pectin chain. Hence, low protein content of pectins isolated in all trials seems to be associated with these harsh isolation conditions used in this study.

3.8 Investigating toxicity of pectin in vitro

Anti-proliferative and antimetastatic [10] activity of pectin extracted from various sources became the subject of previous studies. For instance, pectin was shown to have an anticancer activity on prostate cancer [9, 10]and colon cancer[8]. However, these studies mainly focused on citrus pectin, modified citrus pectin and apple pectin. There are very few studies investigating the anticancer activity of sugar beet pectin[15, 19]. Pectin isolated from various sources with different methods can result in specific metabolic responses on different cancer derivatives. This potential activity is possibly gained after pectin modifications like heat and pH treatments, resulting in depolymerization and de-esterification of pectin[11]. In this study, by optimizing the isolation technique, it was aimed to isolate pectin from sugar beet pulp not just with improved yield and quality but also with already acquired bioactivity, which dismiss the requirement of post isolation treatments.

Hence, we investigated the toxic effect of pectin on cancerous SaOS-2 osteosarcoma. The osteosarcoma cell line was selected as being relatively aggressive [17], which is advantageous for tracing the anticancer activity of pectin. In addition, the effect of pectin isolates from different sources on cell lines was also investigated by isolating citrus (orange peel) pectin (CP). The isolates obtained from high yield methods with low esterification degree (Table 1, trial 6) were used for cell culture analysis.

SaOS-2 cells were incubated with different concentrations of sugar beet pulp pectin (SBPP) and CP for up to three days. 2%, 1%, 0.5% 0.25% of SBPP [8, 15] and %0.66, %0.33, %0.16 and %0.083 CP were applied to investigate toxicity profile of pectin isolates. According to our preliminary studies, the viscosity of the CP solution were much higher than the same concentrations of SBPP; therefore, 3-fold lower concentrations of CP was used, which have similar activity level with 1-fold SBPP. Together with an obvious decrease in cell viability after 24 and 48 hours of treatment with 1% SBPP, the viability of SaOS-2 cells decreased considerably (69.3%) for 72 hours of treatment with 2% SBPP. The highest anti-proliferative activity of CP was observed after 24 hours of treatment with the highest concentration (%0.66). Lower concentrations of SBPP and CP were not effective to disable cell growth.

Although after 24 hours of treatment activity of SBPP and CP on cell viability rather close to each other, for longer durations SBPP was more effective to hinder cell viability (Fig. 3). This outstanding property of SBPP was observed in HUVEC cells as well. SBPP revealed better antiproliferative profile compared to CP, which can be critical parameter to suppress metastasis of aggressive cancer lines. Therefore, for longer treatments SBPP could be selected as the first choice over CP.

3.9 Measuring increase in Reactive Oxygen Species (ROS) Level in cells treated with pectin

The viability tests have shown that SBPP and CP have toxic activity for osteosarcoma and healthy HUVEC cell lines in determined concentrations. At this point, to get an insight into the cellular death mechanism and understand whether/how cells undergo apoptosis, we measured cellular reactive oxygen species (ROS) and level of anti-apoptotic gene (Bcl-2) expression upon pectin treatment.

Although low levels of ROS are essential for the maintenance of biological functions, excess amount of ROS production upon toxic signals may induce apoptosis [33]. ROS regulates apoptosis via the intrinsic

mitochondrial pathway and by regulating pro-apoptotic or anti-apoptotic genes' expression, including Bcl-2[34]. However, in some cases, excessive accumulation of ROS can provoke other cell death mechanisms. Since most toxic concentrations of pectin disable proper cell growth, for ROS measurement, second-most and least toxic concentrations of pectin were selected for comparison. SaOS-2 and HUVEC cells were incubated with %1 and %0.25 SBPP and %0.33 and % 0.083 CP for 24 hours and 72 hours. After 24 hours of treatment, ROS level increased more than 4-fold (407%) in 0.25% SBPP treated SaOS-2 cells. The increase in ROS level was comparatively low (39.86%) in the higher concentration of SBPP. 0.33% CP treated SaOS-2 cells experience an 84.3% rise in the ROS level after 24 hours (Fig. 4A). These results exhibited that SBPP can be used as ROS inducing agent, and the effective dose was determined as 0.25%.

Cellular ROS level decreased after 72 hours, compared to 24 hours of treatment in all concentrations. SBPP is more effective in boosting cellular ROS levels than CP in shorter durations. 0.25% SBPP and 0.083% CP were nontoxic for SaOS-2 cells. However, these concentrations were the most influential ones in ROS rise in SaOS-2 cells (Fig. 4A). Intracellular ROS inducing function of citrus pectin was previously shown in the study of [35], our results have found consistent with previous studies and SBPP induced significant enhancement in ROS production compared to CP.

SBPP boosted ROS formation in cancer cells compared to normal cells. Since cancer cells are believed to exhibit elevated steady-state fluxes of ROS relative to healthy cells due to the impairment in oxidative metabolism[35], pectin treatment was caused predominant ROS production in cancer cells. After 24 hours of incubation of HUVEC cells with %1 SBPP pectin resulted in a 34.32% increase in ROS formation while only a 6.65% increase was observed for 0.25% SBPP treated cells compared to the control group. For the 0.33% and 0.083%, CP treated group, 57% and 48.28% increase was observed respectively (Fig. 4B). According to these results, even though an anti-proliferative effect was observed in both cancerous and healthy cell lines, SBPP induced different cellular mechanisms in cancerous SaOS-2 cells, which have found promising to develop different treatment methods.

3.10 Investigating apoptosis-related gene Bcl-2 expression level in pectin treated SaOS-2 and HUVEC cells

Bcl-2 is a member of a group of proteins that regulates cell survival but the mechanisms whereby Bcl-2 functions are not well characterized. It is an anti-apoptotic oncogene whose imperative expression causes cell proliferation by surpassing the effect of oncogenic stress[36]. Bcl-2 prevents apoptosis by regulating various mechanisms [34] and in one reported mechanism Bcl-2 regulates the protection of the cells against oxidative injury resulting from post-hypoxic reoxygenation[37]. Thus, if pectin exposure leads to apoptosis in cancer cells, it can be because of the decrease in Bcl-2 anti-apoptotic gene expression. To investigate the efficacy of pectin as an anticancer agent, PCR analysis were performed with the reference of GAPDH housekeeping gene. Similar to ROS measurements, cells were treated with 1% and 0.25% SBPP and 0.33% and 0.083% CP for 24 and 72 hours. Since most toxic concentrations of pectin prevent cell growth and thereby disable RNA isolation from cells, second most toxic and non-toxic concentrations of pectin were selected for PCR analysis.

In SaOS-2 cells, Bcl-2 expression decreased compared to control cells only when cells were incubated with 0.33% and 0.083% CP for 24 hours (Fig. 5A) and 72 hours (Fig. 5B) of exposure. In the viability test, 0.083% CP have found almost non-toxic (Fig. 2). However, there is an increase in ROS and a decrease in Bcl-2 expression level in cells treated with 0.083% and 0.33% CP. 24 h treatment of 0.33% CP induced a rise in the cellular ROS level, a decrease in cell viability and in the Bcl-2 expression. These results indicate that CP may induce cellular death via apoptosis[35].

For the SBPP treatment, even though ROS level increases in 0.25% SBPP, the elevated ROS production did not decrease Bcl-2 expression, unlike CP. It was also suggested that under certain circumstances higher ROS level and high Bcl-2 expression eventuate simultaneously. These results mean that CP and SBPP activate different pathways in the SaOS-2 cell line. Moreover, the cell death induced by SBPP does not directly cause apoptosis, or other factors might overcome the Bcl-2 effect. The observed prolonged cell death and better antiproliferative activity of SBPP could be caused by this action mechanism.

Interestingly, 24 hours of treatment did not cause a difference in Bcl-2 expression for HUVEC cells both for SBPP and CP (Fig. 5C). These results are meaningful since the cellular ROS level also did not change at this concentration in HUVEC cells. Blc-2 expression decreased after 72 hours of treatment for all tested concentrations of SBPP and CP (the highest decrease was observed in 1% SBPP) (Fig. 5D). Regarding the viability results, the proliferation of HUVEC cells was not negatively influenced by these concentrations of pectin after 72 hours (Fig. S4). Even though the expression of Bcl-2 declined, ROS levels did not change, and the viability of cells was not affected by pectin treatments.

All these results indicate that SBPP and CP induce different pathways in different cell lines, and an increase in the ROS level does not always cause apoptosis and a decrease in the Bcl-2 expression. Since many other genes are also responsible for cellular death, further analysis is necessary to better understand the mechanism of anticancer activity of SBPP.

4. Conclusion

In the recent study, the isolation protocol of pectin was optimized to increase production efficiency up to 47%. Filtration via vacuum filter with the pore size 0.2 µM, increasing pH of isolate after filtration and collecting pectin with freeze-drying are found to be the most significant steps for high-quality pectin extraction from sugar beet pulp with high yield. With the intent of determining whether optimized yield quality is also adequate for bioactivity, the pectin cell interactions were investigated. In the scope of this study, the anticancer activity of isolated sugar beet pectin and citrus pectin was investigated on aggressive osteosarcoma cell line SaOS-2. A higher concentration of sugar beet pulp pectin (2%) and orange peel pectin (0.66%) results in a considerable decrease in cell viability.

Contrary to the cytotoxicity results, SBPP has shown a better antioxidative profile, even lower concentration (%0.25) of SBPP induces a significant increase in cellular reactive oxygen species level in SaOS-2 cells within a short period. In addition to this approach, apoptosis-related gene profiles of moderate toxic concentrations of pectin groups were investigated. Gene expression analysis showed that

SBPP did not trigger a decrease in anti-apoptotic gene Bcl-2 expression while CP triggers. These results indicate that SBPP and CP reveal different bioactivity profiles for osteosarcoma treatment. SBPP presents strong antiproliferative features while CP exhibiting strong apoptotic activity. Future investigations will shed new light on natural biomolecule-based cancer treatment for developing bio-based functional future formulations.

Declarations

ACKNOWLEDGMENT

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Declaration of Interest

The authors declare no conflict of interest.

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Tables

Table 1 is available in the Supplementary Files section.

Figures

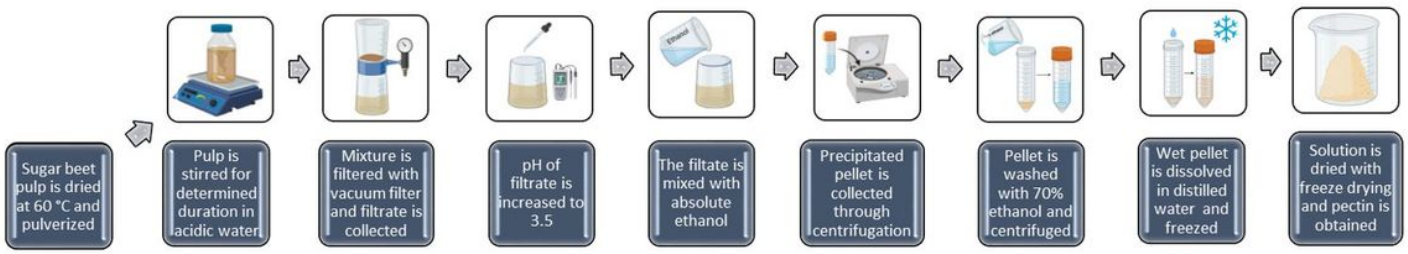

Figure 1

Schematic representation of pectin isolation process. Summary of optimized pectin isolation method is depicted.

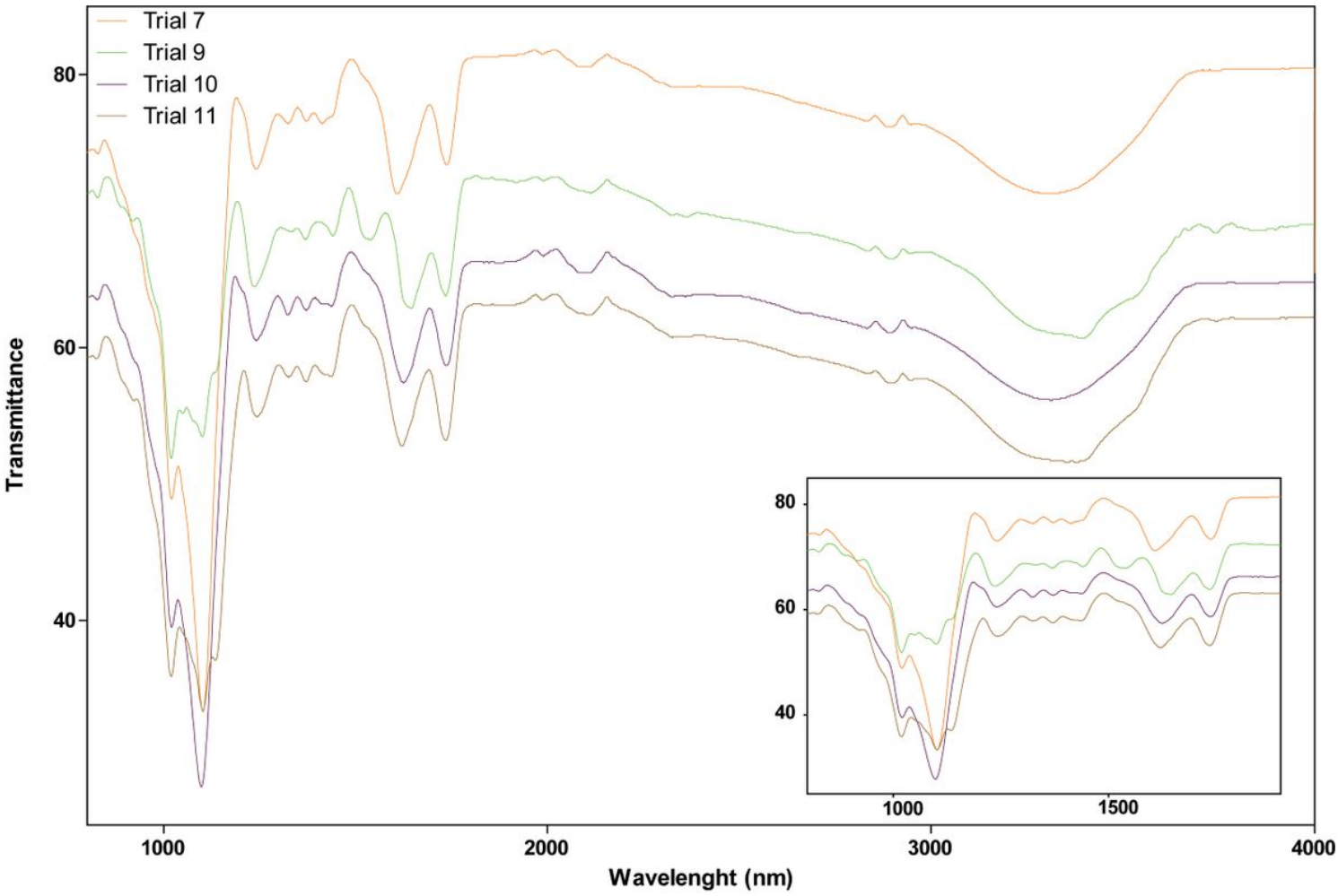

Figure 2

Spectral representation of Fourier Transform Infrared Spectroscopy analysis of isolated pectin from trials 7,9,10 and 11, biomolecular finger printing region was magnified.

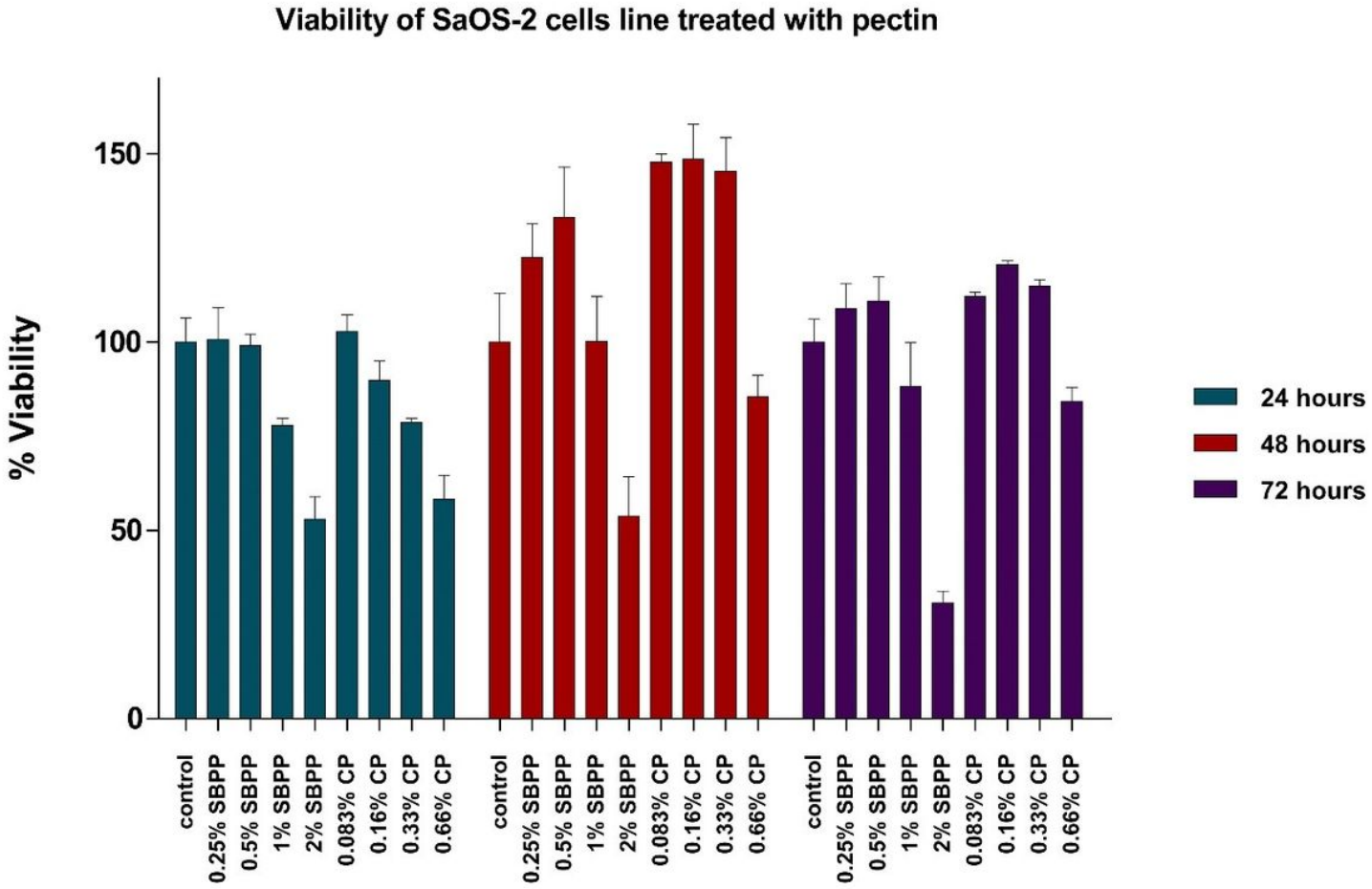

Figure 3

Viability of SaOS-2 cells after treatment with different concentrations of SBPP and CP for 24, 48 and 72 hours. Blue colored bars demonstrate viability of SaOS-2 cells after 24 hours, red colored bars demonstrate after 48 hours, purple-colored bars demonstrate after 72 hours of pectin treatment. Error bars represent standard deviation (SD) of replicas of each group.

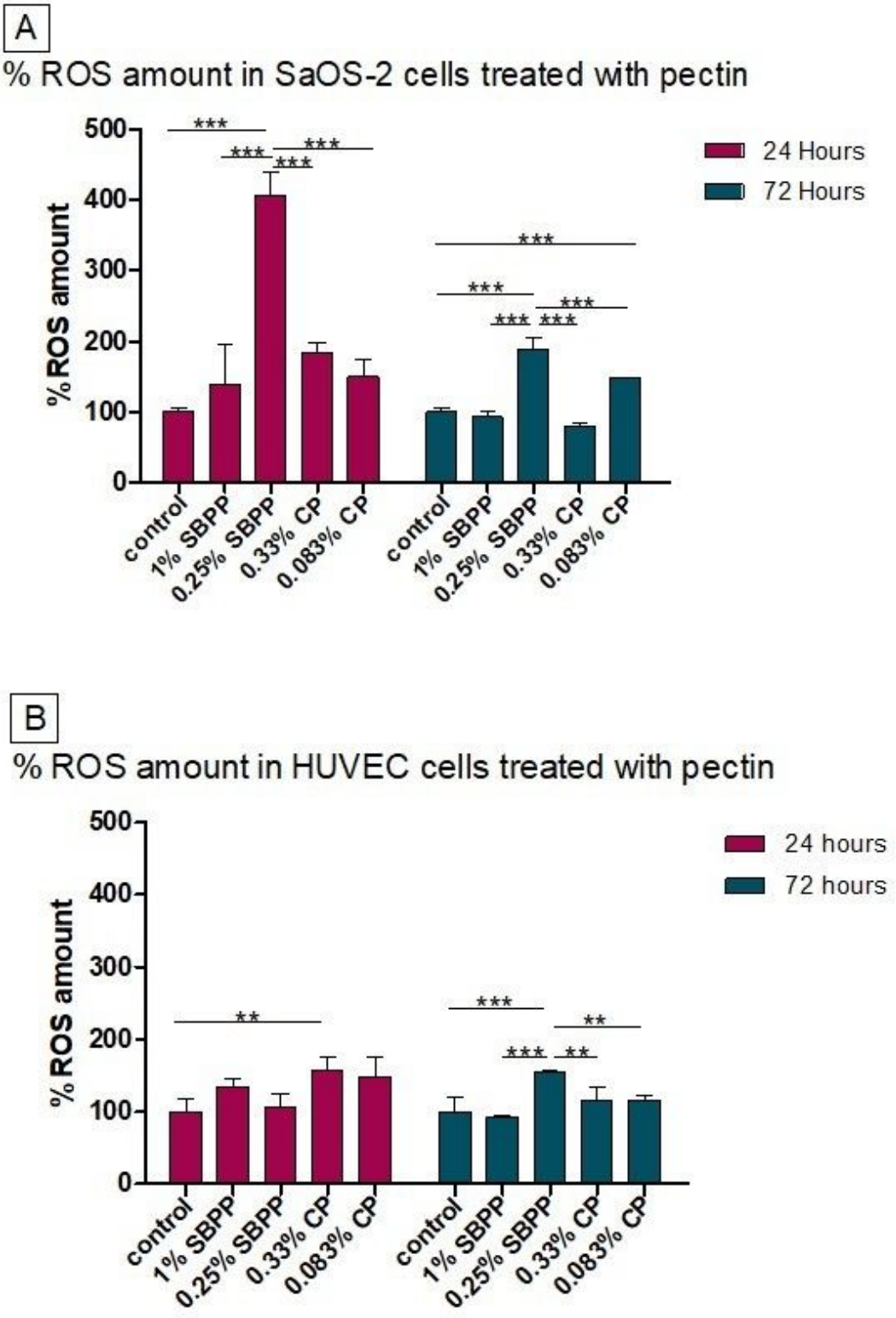

Figure 4

Change in the cellular ROS level in cells treated with different concentrations of SBPP and CP for 24 and 72 hours. **A)** SaOS-2 cells **B)** HUVEC cells. Error bars represent standard deviation of replicas of each group. Pink colored bars represent ROS level after 24 hours, blue colored bars represent after 72 hours of pectin treatment in each cell line. Data in ROS measurement experiments were analyzed with one-way ANOVA (**$p < 0.01$ ***$p \leq 0.0001$)

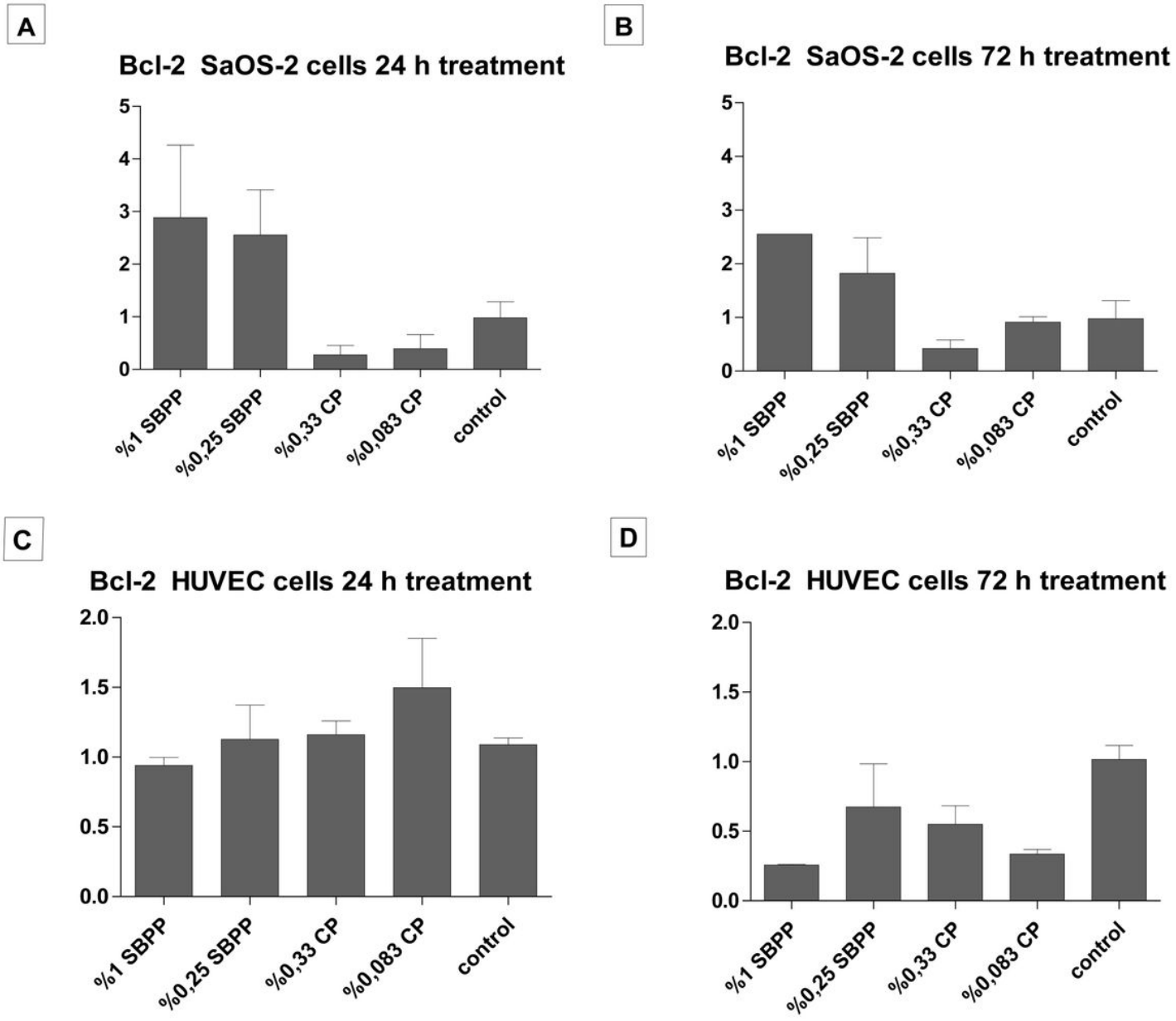

Figure 5

Bcl-2 expression level in cells after 24 and 72 hours of treatment with SBPP and CP. **A)** Bcl-2 gene expression level in SaOS-2 cells after 24 hours and **B)** 72 hours of pectin treatment. **C)** Bcl-2 gene expression level in HUVEC cells after 24 hours and **D)** 72 hours of pectin treatment. Error bars represent standard error of the mean (SEM) of replicas in each group.

Supplementary Files

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- Table1.jpg

- SupportingInformationGulcihanGulseren.docx