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Research Article

In vitro REGULATION OF THE EXPRESSION OF THE SARS-CoV-2 RECEPTOR ANGIOTENSIN-CONVERTING ENZYME (*ACE2*) IN LUNG CANCER CELLS BY NATURAL PRODUCTS

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Abstract: The COVID-19 pandemic continues infecting people causing deaths globally. Although various medicines have been tried to combat with COVID-19, there is no medicine or treatment that has been validated yet. People have been using natural products for centuries against bacterial and viral illnesses. This study aimed to test the effects of the biomolecule oleuropein, whey collected from industrial waste and soaproot extracts obtained from *Gypsophila arrostii* Guss. var. *nebulosa* Boiss. & Heldr. and *Saponaria officinalis* L. on the expression of the human *ACE2* gene as SARS-CoV-2 receptor on the A549 adenocarcinoma cell-line by Real-Time Quantitative Polymerase Chain Reaction (qPCR). According to the cytotoxicity tests, *G. arrostii* var. *nebulosa* and *S. officinalis* extract treatments showed a dose dependent cytotoxic effect on the cells. The EC50 values of *G. arrostii* var. *nebulosa* and *S. officinalis* were found to be 54.3 µg/ml and 17.3 µg/ml, respectively. Oleuropein showed moderate cytotoxic effects with the EC50 value over 250 µg/ml. Whey (fermented and non-fermented) did not show any cytotoxic effect at the applied doses. The qPCR results showed that the *ACE2* mRNA level decreased by 89.8% and 35.2% due to the fermented and non-fermented whey extracts, respectively. Similarly, *G. arrostii* var. *nebulosa* and *S. officinalis* downregulated *ACE2* by 79.8% and 90.1%, respectively. In contrast, oleropein upregulated *ACE2* (102.8%). Our results showed that the natural supporting products produced from soaproot extracts and fermented whey can be used against COVID-19 by both cancer patients and people in potential risk groups.

Özet: COVID-19 pandemisi tüm dünyada küresel çapta insanları enfekte etmeye ve ölümlere neden olmaya devam etmektedir. COVID-19 ile mücadelede birçok ilaç denenmiş olmasına karşın henüz herhangi bir ilaç veya tedavi yöntemi onaylanmamıştır. İnsanlar yüzyıllardan bu yana hastalıklara karşı doğal ürünleri kullanmışlardır. Bu çalışmadaki amacımız bir biyomolekül olan oleuropein, endüstriyel atık olarak bertaraf edilen peynir altı suyu ve *Gypsophila arrostii* Guss. var. *nebulosa* Boiss. & Heldr. ve *Saponaria officinalis* L. bitkilerinden elde edilen ekstraktların A549 kanserli hücre hatlarında ACE2 reseptörünü kodlayan *ACE2* geninin anlatım seviyesi üzerine etkilerini Gerçek Zamanlı Kantitatif Polimeraz Zincir Reaksiyonu (qPCR) ile belirlemektedir. Yaptığımız sitotoksisite testlerine göre *G. arrostii* var. *nebulosa* ve *S. officinalis* ekstraktları sırası ile 54,3 µg/ml ve 17,3 µg/ml EC50 değerleri ile doza bağımlı sitotoksik etki gösterirken, oleuropein 250 µg/ml'nin üzerinde bir değerle orta dereceli sitotoksik etki göstermiştir. Öte yandan peynir altı suyu (ferment eve fermente edilmeyen), çalışmada kullanılan dozlarda sitotoksik etki göstermemiştir. qPCR sonuçlarına göre fermente edilmiş ve edilmemiş peynir altı suyunun *ACE2* genine ait mRNA seviyesini sırası ile %89,8 ve %35,2 oranlarında düşürdüğü belirlenmiştir. Benzer şekilde *G. arrostii* var. *nebulosa* ve *S. officinalis* ekstraktlarının *ACE2* geni mRNA seviyesini sırası ile %79,8 ve %90,1 oranında düşürdüğü belirlenmiştir. Bu sonuçların aksine oleuropein biyomolekülünün *ACE2* mRNA seviyesini %102,8 oranında arttırdığı belirlenmiştir. Çalışma sonuçlarına göre kullanılan bitki ekstraktlarının ve fermente edilmiş peynir altı suyunun COVID-19 ile mücadelede kanser hastalarında ve risk gruplarında kullanılabilecek doğal destek ürünlerinin üretilmesinde kullanılabileceğini göstermektedir.

Introduction

Since December 2019, when the severe, acute respiratory syndrome coronavirus 2 (SARS-CoV-2 or COVID-19) was detected in Wuhan, China, the disease infected more than 173 million people and caused 3.7 million deaths globally. The *ACE2* gene, which encodes receptor of the angiotensin-converting enzyme-2 was proven to be the main gateway for both the SARS-coronavirus (SARS-CoV) and the human coronavirus (HCoV NL63) (Zhou *et al.* 2020). In vitro tests showed that there is a positive correlation between *ACE2* gene expression and COVID-19 infection (Hofmann *et al.* 2004, Li *et al.* 2007). Phylogenetic studies showed that COVID-19 and the SARS-CoV have many similar sequences, and their spike proteins have 76.5% sequence similarity (Xu *et al.* 2020). Therefore, the spike protein of COVID-19 is predicted to have a binding ability to *ACE2*. Studies indicated ACE2 receptor as the potential target to develop therapeutics for COVID-19 (Zhang *et al.* 2020). Despite the great efforts of researchers, there exists no validated therapeutics available for the disease. The COVID-19 pandemic not only affected healthy people, but also people who have major lung diseases. The patients with lung cancer cohort with COVID-19 are at greater risk due to both diseases damaging their lungs (Wang *et al.* 2019, Chen *et al.* 2020, Liang *et al.* 2020, Wang & Zhang 2020). Besides, Feng *et al.* (2011) showed that the overexpression of *ACE2* inhibits angiogenesis on tumor cells both in vitro and *in vivo*.

Plants have been used as medicines for thousands of years because of their healing effects. Plant secondary metabolites have important pharmaceutical effects on many diseases. For instance, the phenolic compounds of olive (*Olea europea* L.), particularly the oleuropein, show high anti-inflammatory and anticancer activities by inhibiting the tumor growth (Carrera-González *et al.* 2013). Oleuropein has also been reported to have anti-viral, anti-cancer, and anti-inflammatory effects (Haris Omar 2010).

Milk and colostrum are health-enhancing natural products due to their protein and peptide contents. Whey is a by-product of the dairy industry during the manufacturing of milk products. Studies showed that the whey proteins lactoferrin and alpha-lactalbumin have antiviral and antitumor activities, and casein has antitumor activity (Almehdar *et al.* 2015, Kanwar *et al.* 2009, Zimecki & Kruzel 2007).

Ribosome-inactivating-proteins (RIPs) are immunotoxins and antiviral reagents and saporins are the basic types of type-1 RIPs. Soaproot is woody roots of some perennial plants. Seven plant species, *Ankyropetalum gypsophiloides* Fenzl., *Gypsophila arrostii* Guss. var. *nebulosa* (Boiss. & Heldr.) Bark., *G. bicolor* (Freyn & Sint.) Grossh., *G. eriocalyx* Boiss., G. *graminifolia* Bark., *G. perfoliata* L. and *G. venusta* Fenzl. are used to obtain soaproot in Turkey (Koyuncu *et al.* 2008). *Saponaria officinalis* L., which is also used to obtain soaproot in Europe, but not in Turkey, contains RIPs on its seeds and leaves (Carzaniga *et al.* 1994). It is also known that soaproot has an antiviral effect (Serkedjieva *et al.* 1990).

In this study, we aimed to test the effects of oleuropein, whey (fermented and non-fermented), and two types of soaproot extracts (*G. arrtosii* var. *nabulosa* and *S. officinalis*) on the expression of *ACE2* gene on the A549 human adenocarcinoma cell-line by qPCR. This is the first study that shows how biomolecules and natural products affect *ACE2* gene expression on the adenocarcinoma cell-line.

Materials and Methods

Obtaining the plant extracts and whey

Oleuropein obtained from BLD Pharmatech Pvt Ltd (India) (Cat. No: BD1777) was used. It was dissolved in dimethyl sulfoxide (DMSO) (final concentration in medium did not exceed 0.5%) before used.

Whey is discarded as an industrial waste in Iğdır province of Turkey. We obtained whey from Has Mandıra Dairy Products Company (Iğdır, Turkey) in Iğdır Organised Industrial Site with its fat and pellet. After discarding the fat, the whey was titrated by 0.1 N NaOH and the pH was adjusted to 6.0. We boiled the mixture and filtered the precipitation. Then, we added yeast extract (0.75%), $MnSO_4$ (20 mg/l) and $CaCO_3$ (1.5%), and sterilised the mixture by autoclaving at 121°C and 1.5 ATM. The sterilised whey was fermented by *Lactobacillus casei* at 37°C for 48 h. Non-fermented and fermented forms of whey were used in the study.

Gypsophila arrostii var. *nebulosa* was collected from Isparta (Turkey – approx. N37.7, E030.5) in May 2020, and the collected specimens were identified by taxonomist Ahmet Zafer Tel from Iğdır University, Department of Agricultural Biotechnology by using the identification key in the Flora of Turkey and the East Aegean Islands (Davis 1970). After cleaning and grinding the roots, we obtained the soaproot extract. We also commercially ordered a second soaproot extract powder, which was made from the roots of *S. officinalis*, from İstanbul Agricultural Products and Food Industry Trade Ltd Company (İstanbul, Turkey) (Cat. No: SAPO-4434) to be tested in the study. It was dissolved in DMSO before the treatment.

Experimental design, cytotoxicity tests and the treatment of the cells with the biomolecules and the natural products

Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), trypsin, penicillin/streptomycin mixture were purchased from Sigma-Aldrich Chemical Company (St Louis, Missouri, USA). The MTT Cell Proliferation Assay Kit was purchased from BioVision, Inc. (USA). All the other chemicals and solvents were obtained from commercial sources at the highest grade of purity available.

A549 cells (European Collection of Cell Cultures. ECACC, UK) were cultured in DMEM containing 10% fetal bovine serum, 100 U ml^{-1} penicillin and 100 μg/ml streptomycin mixture in a humidified atmosphere with 5% CO_2 under the normal oxygen conditions at 37°C and were passaged every 2-3 days. The cytotoxic effects of isolated molecules and extracts were determined by using the effects of the MTT (3-(4,5-dimethyltiazole-2-yl) -2,5-diphenyltetrazolium bromide) assay as triplicates. For this purpose, we seeded A549 cells in 96-well plates at a density of 5000 cells/well and incubated for 24 h for attachment. The cells were then exposed to different concentrations of isolated molecules and extracts for another 24 h. We incubated the treated and control cells for 24 h at 37°C in humidified 5% CO_2 atmosphere. After 24 h, the medium was removed, and fresh medium was added to each well. After that, 10 µl of the MTT reagent was added to each well and incubated for 4 h in the incubator. After 4 h, the medium was removed carefully and 50 µl of DMSO added to each well. The amount of formazan formed was determined by measuring the absorbance at 590 nm using a microplate reader (Epoch, BioTek). We used three replicated wells for each experimental condition. Viability was expressed as a percentage of the control.

Primer design, RNA extraction, cDNA synthesis and qPCR

We designed the primers targeting the human *ACE2* gene referencing *Homo sapiens* ACE-related carboxypeptidase *ACE2* mRNA, complete CDS (GenBank accession: AF291820.1) as ten alternatives using the National Center for Biotechnology Information (NCBI) Primer-BLAST tool (optimal annealing temperature is 60°C and product size range is 80-110bp). We selected the human glyceraldehyde3phosphate dehydrogenase (*GAPDH*) gene as the reference gene (Goulter *et al*. 2004) (5′-CGGAGTCAACGGATTTGGTC-3′ and 5′-TGAGGTCAATGAAGGGGTCA-3′) for normalisation of the qPCR results. The A549 cells (1×10^7 cells) were seeded to plates and exposed to maximum non-toxic doses dosed of test materials and harvested after a 24 h treatment. Total RNA was extracted by using InnuPREP RNA Mini Kit (Analytic Jena, Germany). Extracted RNA was quantified spectrophotometrically at 260/280 nm, and the integrity was checked using 1% agarose gel electrophoresis. We converted 2.5 µg of RNA to cDNA by EasyScript™ cDNA Synthesis Kit according to the manual provided by the supplier (ABM, Canada) and the cDNA was stored at -80°C for further use. We confirmed the cDNA synthesis by performing end-point PCR using the reference gene *GAPDH*. The qPCR analyses were performed by using SYBR® Green fluorescent dye-containing master mix (KiloGreen 2X qPCR Master Mix, ABM, Canada). qPCR reactions were performed with 10 µl KiloGreen 2X qPCR Master Mix, (ABM, Canada) 0.6 µl (200 nM) of each primer, and 10 ng cDNA template. qPCR was performed on the Applied Biosystems StepOnePlus Real-Time PCR systems (Applied Biosystems, USA). All amplifications were as 95°C for 10 m initial denaturation followed by 45 cycles at 95°C 15 s for denaturation, 60°C 60 s for annealing, and 72°C 60 s for elongation. Melting curve analysis with a ramp rate of 0.5°C/step was added after amplification to confirm specificity of the primers. All the experiments were performed as three biological and three technical replicates. We calculated PCR efficiencies for each primer pairs according to (Ruijter *et al.* 2009), and used the primer only with efficiency value between 90-105% (Forward 5′-TGAAGGCCCTCTGCACAAAT-3′ and 5′-ATGCTAGGGTCCAGGGTTCT-3′). We calculated the gene expression differences according to the comparative Delta Delta Ct method ($2^{-\Delta\Delta C_t}$) (Livak & Schmittgen 2001).

Statistical Analysis

All data presented are mean values of each qPCR treatments. Data were analysed using the statistical program JASP (0.14.1). The analysis of variance (ANOVA) was followed by Fisher's protected LSD test to identify homogenous groups within the means. Significant differences among treatments were considered at the $P\leq0.05$ level.

Results

The cytotoxicity of the pure compounds and extracts on A549 cells was measured by MTT test. *Gypsophila arrostii* var. *nebulosa* and *S. officinalis* extract treatments showed a dose-dependent cytotoxic effect on A549 cells (Fig. 1). The EC50 values of the *G. arrostii* var. *nebulosa* and *S. officinalis* were found to be 54.3 µg ml^{-1} and 17.3 µg ml^{-1}, respectively. Oleuropein showed moderate cytotoxic effects (EC50 value was over 250 µg ml^{-1}), while whey (fermented and non-fermented) did not show any cytotoxic effect at applied doses.

We obtained high quality and sufficient amounts of RNA for the cDNA synthesis. The successful amplification of the *GAPDH* gene by conventional PCR confirmed the cDNA synthesis success.

Preliminary tests were carried out to determine the changes in *ACE2* mRNA levels with respect to extracts and pure compounds. For this purpose, maximum non-toxic doses of the test materials (250 µg ml^{-1} for fermented and non-fermented whey extract, 10 µg ml^{-1} for *S. officinalis* extract, 12.5 µg ml^{-1} for *G. arrostii* var. *nebulosa* extract, and 100 µg ml^{-1} for oleuropein) were selected and applied to the cells for 24 hours. Statistical analysis revealed that there were significant changes in expression levels of *ACE2*. The qPCR results showed that the *ACE2* expression level decreased to 89.8% and 35.2% as a result of the fermented and non-fermented whey extract, respectively (Fig. 2). Similarly, *G. arrtosii* var. *nebulosa* and *S. officinalis* decreased the *ACE2* expression to 79.8% and 90.1%, respectively. On the contrary, oleropein increased the *ACE2* expression level to 102.8%.

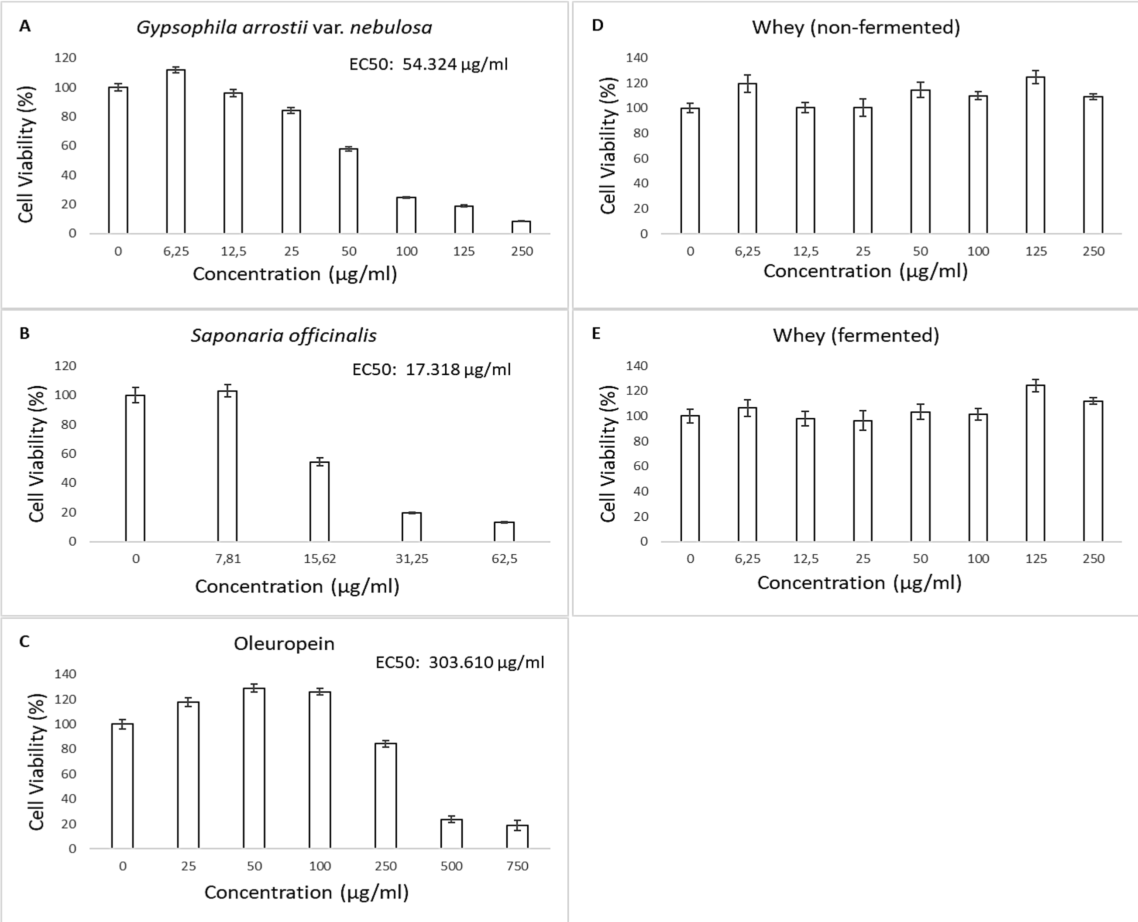

Fig. 1. The cytotoxicity levels of the pure compounds and the extracts measured by MTT test. The EC50 values given on each graph with cytotoxicity effect. *Gypsophila arrostii* var. *nebulosa* and *S. officinalis* (A and B) showed dose-dependent cytotoxicity, oleuropein (C) showed moderate cytotoxicity, and whey (D and E) showed no cytotoxicity. The error bars represent standard deviation values.

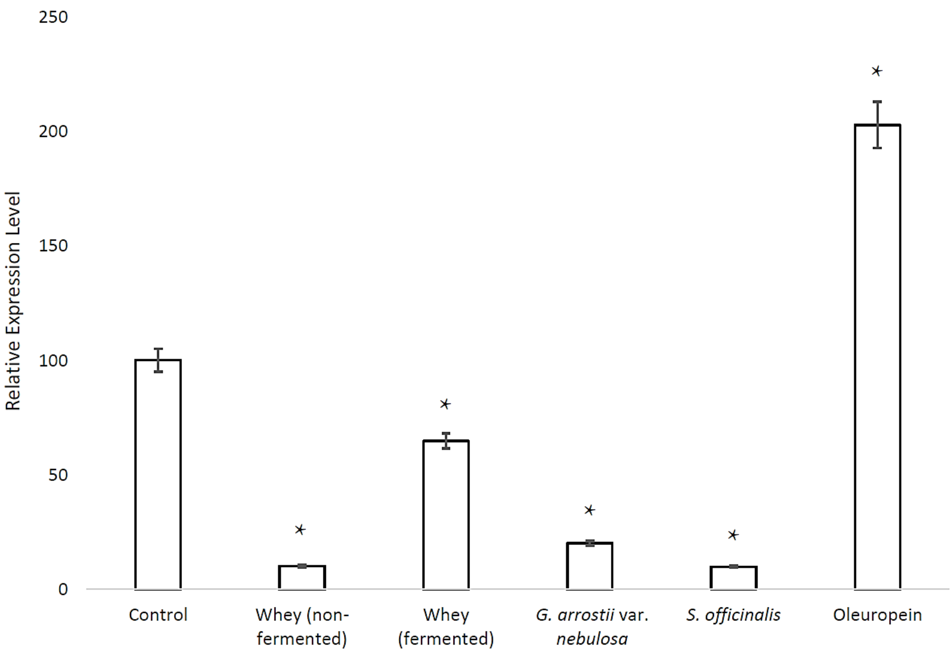

Fig. 2. The qPCR results of the biomolecules and natural products on the expression of the Human *ACE2* gene expression calculated using the Comparative Delta Delta Ct ($2^{-\Delta\Delta C_t}$) method. The values on the Y-axis represent the percentages. The error bars represent standard errors. Statistically significant ($P \leq 0.05$) values were indicated with asterisks.

Discussion

In this study, we investigated the effects of the secondary metabolite oleuropein, industrial waste whey (non-fermented and fermented) and soaproot extracts of the medicinal plants *G. arrostii* var. *nebulosa* and *S. officinalis* on the expression of the Human *ACE2* gene, which has a gateway role for COVID-19, on the A549 adenocarcinoma cell-line. Our cytotoxicity analyses, which helped us to determine the appropriate doses, showed that oleuropein has moderate cytotoxic effects (EC50 value was over 250 µg ml^{-1}), both non-fermented and fermented whey have no cytotoxic effect, and the soaproot extracts have a dose-dependent cytotoxic effect (*G. arrostii* var. *nebulosa* is 54.3 µg ml^{-1}, and *S. officinalis* is 17.3 µg ml^{-1}). The qPCR results showed that oleuropein upregulated the *ACE2* gene by 102.8%, while whey (fermented 89.8% and non-fermented 35.2%) and the two soaproot extracts (*G. arrostii* var. *nebulosa* 79.8% and *S. officinalis* 90.1%) downregulated. These findings make this study the first that shows biomolecules and natural products can regulate the Human *ACE2* expression on adenocarcinoma cells.

Patients with cancer background (both history and active patients) were concluded to be more likely to develop COVID-19 in China (Wang & Zhang 2020, Xia *et al.* 2020). Therefore, COVID-19 patients with cancer cohort are at greater risk. The pathology reports of cancer patients, particularly those with lung cancer, which have adenocarcinoma, cohort with COVID-19 developed oedema, proteinaceous exudate and inflammatory cellular infiltration in their lungs besides the tumours (Tian *et al.* 2020). Researchers concluded that sensitivity to COVID-19 in these patients was related to the excessive expression levels of *ACE2* gene (Jia *et al.* 2020). Therefore, focusing on developing medicines and supporting products for cancer patients is important.

Developing and testing vaccines needs more time than supporting products. Researchers are trying to find therapeutical effects of various biomolecules, active compounds, natural products and easy-to-find plant-derived products on COVID-19. Although most plant-derived products, which have phenolics, secondary metabolites etc., have antiviral effects, no study has been performed so far on Human *ACE2* gene with cancer.

Oleuropein, the only biomolecule that upregulated *ACE2* expression in our study, is the main phenolic component of olive. This is the first study that shows the effects of oleuropein on *ACE2* expression. Previous studies stressed its high anti-inflammatory, anticancer and antiviral effects (Carrera-González *et al.* 2013, Haris Omar 2010). Its inhibitory effect on *ACE1* expression was also reported (Msomi & Simelane 2017). Despite these properties, oleuropein caused a two fold increase of *ACE2* expression on cancer cells we used. The upregulation on *ACE2* might be because of the complex cell differentiation of the cancer cells. *ACE2* expression was reported to depend on the state of cell differentiation (Jia *et al.* 2006). In that study, researchers showed the correlation between cell differentiation and *ACE2* expression on A549 cells. Due to high differentiation rate of cancer cells, oleuropein might induce *ACE2* expression. Researchers indicated the correlation of *ACE2* expression and COVID19 infection (Hofmann *et al.* 2004, Li *et al.* 2007). Therefore, upregulation of *ACE2* by exposing to oleuropein will cause an increment on the ACE2 receptor on the membrane of the adenocarcinoma cells. This case proves that COVID-19 the gateway for the entrance into the cell.

The natural product whey, which is discarded as a waste of manufacturing from various milk products, was tested in this study for the first time on *ACE2* gene and downregulated its expression. We tested two types of whey as non-fermented and fermented. The non-fermented whey drew out the fermented type by almost silencing the *ACE2* with 89.8% downregulation ratio. The fermented whey also had a downregulation effect on *ACE2* by 35.2%. Milk and particularly colostrum are important sources of proteins that have many bioactivities. Whey also has various proteins including a group of milk protein lactoferrin (Teo *et al.* 2016). Lactoferrin was reported as an antiviral, antifungal, antibacterial, antitumor and immune enhancer whey protein (Ng *et al.* 2015). It can bind Heparan Sulfate Proteoglycans (HSPGs) and *ACE2*. Therefore, researchers reported that lactoferrin might have a preventive and therapeutic value for COVID-19 (Kell *et al.* 2020). Alphalactalbumin and lactoglobulin, other proteins included in whey, have an inhibition effect on HIV reverse transcriptase (Ng *et al.* 2015). Due to COVID-19 being a RNA virus, these proteins might affect COVID-19 reverse transcriptase, as well. In another study, fresh buttermilk cultured using paneer whey was reported as ACE enzyme inhibitor (Parekh *et al.* 2017). Supporting our results, having both *ACE2* receptor binding ability and decreasing the *ACE2* gene expression, whey might be a conspicuous natural product against COVID-19 in cancer patients. We think that re-fermentation of whey inhibited its bio-functional properties.

Gypsophila arrostii var. *nebulosa* is being used to obtain soaproot mostly in Anatolia (Koyuncu *et al.* 2008). Since there is no available study about the effects of *Gypsophila* sp. on *ACE2* expression, our result of 79.8% downregulation will open a new avenue for researchers in the field of pharmaceutics. The phytochemical studies on *G. arrostii* var. *nebulosa* showed that triterpene saponins are present in its roots (Arslan *et al.* 2013). Due to their modifying effect on cell membranes, saponins have a potential pharmaceutical value (Mostad & Doehl 1987). Although saponins are commonly found in higher plants, triterpene saponins are very rare in nature (Arslan & Cenzano 2020). A recent review by Arslan & Cenzano (2020) concluded that triterpene saponins have been used in cancer therapies since 1976 (Ebbesen *et al.* 1976). Recent studies showed significant anticancer activities for saponins (Cheng *et al.* 2016). Although it is known that *Panax notoginseng* (Burkill) F.H.Chen saponins have inhibitor effects on *ACE2* expression (Guo *et al.* 2010), there is no study on the triterpene saponins in the literature. We think that *ACE2* inhibition effect might be related with triterpene saponins in the roots of *G. arrostii* var. *nebulosa*.

According to our results, soaproot extract obtained from *S. officinalis* had the most inhibition effect on *ACE2* expression by 90.1% which may be regarded as silencing of the gene. The main bioactive compound of *S. officinalis* has triterpene saponins, as in *Gypsophila* species. The immune-stimulant effects of triterpene saponins were reported before (Press *et al.* 2000). Koike *et. al.* (1999) discovered new types of saponins in *S. officinalis* such as saponariosides and Saponarioside C. Although there are no studies on the immunological effects of these molecules, they may have immune-stimulant effects similar to triterpene saponins.

Due to the high infection rate, COVID-19 spread throughout the world. The people in the high-risk group, particularly those suffering from cancer, need more attention. In this study, we showed that the natural products whey and soaproot extracts can downregulate the *ACE2* gene, which is the main gateway for COVID-19. Both whey and soaproot extracts have anti-cancer and antitumor effects. Therefore, we conclude that the food-supporting products or medicines made from these natural products would be a good protector against COVID-19 in cancer patients. The results of the study will open an avenue for more clinical studies of natural products.

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