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Echinacoside decreases cell proliferation and inhibits cell invasion in PC3 androgen-independent prostate cancer cells

Ekinakozit PC3 androjen bağımsız prostat kanseri hücrelerinde hücre proliferasyonunu azaltır ve hücre invazyonunu inhibe eder

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Abstract

Purpose: The aim of this study was to determine the effects of echinacoside on cell proliferation, invasion and mRNA expression changes of invasion-related genes in PC3 androgen-independent prostate cancer cells.

Material and methods: The effect of echinacoside on cell proliferation in PC3 cells was determined by XTT method. Anti-invasive efficacy was achieved using the transwell chamber. Total RNA isolation was performed by Trizol and cDNA was subsequently synthesized. mRNA expression changes in *MMP2,MMP9, TIMP1, TIMP2* and *TIMP3* were also performed in RT-PCR with SYBER Green.

Results: In this study, the IC50 dose of echinacoside in PC3 cells was determined as 55.21 μM at 48h. It was determined that echinacoside inhibited cell invasion in PC3 cells and reduced the invasion by 66% in the dose group. In addition, it was found statistically significant that echinacoside increased *TIMP1* mRNA expression 1.96 times, *TIMP2* mRNA expression 2.60 times, while decreasing *MMP2* expression 3.82 times and *MMP9* mRNA expression 1.54 times in IC50 dose group according to control.

Conclusion: In conclusion, it was revealed that echinacoside has an anti-proliferative effect on PC3 prostate cancer cells. It has also been shown that invasion-related genes can suppress invasion by regulating expression changes. With this study, preliminary data were presented in terms of detailed molecular biological studies to be carried out on echinacoside and its effect on prostate cancer.

Key words: Echinacoside, PC3 cells, androgen-independent prostate cancer.

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Öz

Amaç: Bu çalışmanın amacı ekinakozitin PC3 androjen bağımsız prostat kanseri hücrelerinde hücre proliferasyonuna, invazyonuna ve invazyon ilişkili genlerin mRNA ekspresyon değişimleri üzerine etkilerini belirlemektir.

Gereç ve yöntem: Ekinakozitin PC3 hücrelerinde hücre proliferasyonuna olan etkisi XTT yöntemiyle belirlenmiştir. Anti-invaziv etkinliği transwel chamber kullanılarak gerçekleştirilmiştir. Total RNA izolasyonu Trizol aracılığıyla gerçekleştirilmiş ve takiben cDNA sentezlenmiştir. *MMP2, MMP9, TIMP1, TIMP2* ve *TIMP3*'ün mRNA ekspresyon değişimleri SYBER Green ile RT-PCR da gerçekleştirilmiştir.

Bulgular: Bu çalışmada Ekinakozitin PC3 hücrelerinde IC50 dozu 48. saate 55,21 μM olarak tespit edilmiştir. Ekinakozitin PC3 hücrelerinde hücre invazyonunu inhibe ettiği doz grubunda %66 oranında invazyonu azalttığı belirlenmiştir. Ayrıca ekinakozitin kontrole göre IC50 doz grubunda, *TIMP1* mRNA ekspresyonunu 1,96 kat, *TIMP2* mRNA ekspresyonunu 2,60 kat arttırken *MMP2* ekspresyonunu 3,82 kat, *MMP9* mRNA ekspresyonunu 1,54 kat azaltması istatistiksel olarak anlamlı bulunmuştur.

Sonuç: Sonuç olarak, ekinakozitin PC3 prostat kanseri hücreleri üzerinde antiproliferatif etki gösterdiği ortaya koyulmuştur. Ayrıca invazyon ilişkili genlerin ekspresyon değişimlerini regüle ederek invazyonu da baskılayabileceği gösterilmiştir. Bu çalışma ile ekinakozit ve prostat kanseri üzerindeki etkisi ile ilgili olarak bundan sonraki yapılacak olan detaylı moleküler biyolojik çalışmalar açısından ön veriler ortaya koyulmuştur.

Anahtar kelimeler: Ekinakozit, PC3 hücreleri, androjen bağımsız prostat kanseri.

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Introduction

Cancer is a complex, genetic and pathological condition in which cells grow unevenly and multiply uncontrollably [1]. Cancer continues to be one of the leading causes of death worldwide. The data reported by the International Agency for Research on Cancer (IARC) show that a total of 19.3 million new cancer cases were seen in 2020 and the number of cancer-related deaths was 10.0 million. In addition, this report predicts that the global burden will be 28.4 million cases in 2040 [2]. Prostate cancer (PSa) is the most common malignancy in men and ranks second in cancer-related deaths in men after lung cancer [3]. In approximately 90% of prostate cancer cases, the cancer is still organ-confined or has only progressed locally at the time of diagnosis. This allows effective treatment to be provided, such as local radiotherapy or prostatectomy. However, cancer progression is usually detected in 30-40% of patients [4, 5]. In this process, where tumor growth is dependent on androgens, the most effective treatment option is androgen-deprivation therapy, in which hormone secretion or activity is blocked [5, 6]. The progression of prostate cancer is initially androgen dependent, allowing androgen ablation to remain the mainstay of therapy for patients with advanced cancer [7]. Although this hormone deprivation is palliative in more than 50% of prostate cancer patients, its effects are temporary. However, in most of these patients, it is observed that the cancer metastatically and transition to the incurable androgen-independent stage occurs in the course of time [8]. The exact molecular mechanisms that contribute to androgen independence are not fully known. However, recent scientific developments highlight the role of the tumor microenvironment along with changes in androgen receptor-related functions [8, 9].

Conditions such as high toxicity, side effects and development of resistance in cancer treatment have triggered the development of non-toxic treatment strategies for normal cells or the increase of studies on the discovery of new natural effective herbal components [10]. Phenylethanoid glycosides (PhGs) are water-soluble compounds that can be found in many horticultural and medicinal plants. Especially in recent years, there has been an increasing interest in the use of PhGs as potential agents in the treatment of various diseases [11, 12]. Echinacoside is a natural small phenylethanoid glycoside molecule first isolated from Echinacea root. Echinacoside can also be extracted from Cistanche, which is widely used in Europe and is known to be good for colds and infections [13, 14]. Echinacoside has been shown to have a wide variety of biological effects in various studies. Neuroprotective, hepatoprotective, immune modulator, antidiabetic and anticancer activities of echinacoside have been demonstrated in previous studies [15-19]. It has been reported that echinacoside exhibits anticancer activity through cellular mechanisms such as triggering apoptosis, cell cycle arrest, and inhibition of cell proliferation in various cancers such as breast [14] colon [15], pancreas [20], ovary [21] and liver [22]. There is no comprehensive molecular mechanistic study on echinacoside and prostate cancer in the literature. In this study, it was aimed to investigate the effects of echinacoside on cell proliferation and invasion in PC3 androgen-independent prostate cancer cells. In addition, the action mechanism on anti-invasive properties via matrix metalloproteinases (MMPs) expression regulation has been tried to be clarified.

Materials and methods

Cell culture

In this study, PC3 human androgen-independent prostate cancer cell line was used and grown in RPMI Media 1640 (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco), 20 units/ml penicillin and 20 µg/mL streptomycin (PAN Biotech), 0,1 mM amino acid solution (PAN Biotech) and 1mM sodium pyruvate (Gibco) and cultured at 37°C in 5% CO_2. Echinacoside was obtained from Chem Faces (Cat. No: CFN98105). Ecchinocoside was treated to the PC3 cells via different doses including 25 µM, 50 µM, 75 µM, 125 µM, 250 µM, 500 µM to investigate the anti-proliferative activity at 24h and 48h.

XTT Assay

Anti-proliferative effects of echinacoside on PC3 cells were detected by XTT (2,3-bis(2-methoxy-4 nitro-5- sulfophenyl)-2H-tetrazolium-5-carboxanilide) assay at a concentration of 3×10^4 cells per well in 96-well plates according to the kit protocol (Cell Proliferation Kit ; Biotium,

USA). Following the adhesion of the cells, echinacoside at different concentrations in the dose range of 25 µM and 500 µM were treated to the cells for 24 and 48 hours. After the dosing periods were over, the XTT mixture was applied in accordance with the dose and time specified by the manufacturer. Formazan formation was measured spectrophotometrically by a microplate reader (Biotek) at 450 wavelengths (reference wavelength 630 nm) and colorimetrically. Cell viability (%) was calculated using the specified formula via absorbance values with small modifications according to Secme et al. [23]. Since echinacoside is a soluble molecule in the medium, no additional solvent was used.

IC_{50} dose of echinacoside on PC3 cells was determined by AAT bioquest online tool (https://www.aatbio.com/tools/ic50-calculator). After the IC_{50} dose was determined, this dose was selected and applied as the dose group for the invasion transwell chamber and RT-PCR experimental steps in this study.

Matrigel-Invasion assay

Transwell invasion chambers (Thermo Fisher Scientific, Denmark, 140629, CC INSERT MD24) were used for determination of anti-invasive effects of echinacoside in PC3 cells. 2 x 10^4 cells were seeded into matrigel chambers for control and dose groups in serum free medium. On the lower floor of the chamber, a medium containing serum was used to act as a chemoattractant. A cotton-tipped swab was used for cleaning non-invasive cells, methanol was used for fixation, and crystal violet was used for staining invasive cells in accordance with the manufacturer's instructions at the end of the incubation period. Percentage of invasive cell was calculated accroding to Dodurga et al. [24] study.

Real-Time PCR Assay

Total RNA isolation from cells was performed using Trizol (Ambion) according to the manufacturer's protocol. cDNA synthesis was performed using cDNA Reverse Transcription Kit (OneScript® Plus cDNA Synthesis Kit, Cat no: G236). mRNA expression changes of *MMP2, MMP9, TIMP1, TIMP2,TIMP3* were evaluated by RT-PCR (Applied Biosystem, StepOne Plus). *Beta-actin* was used as housekeeping gene. The primer sequences were used in this study were given in Table 1. The primer sequences were used from studies performed by Eroglu et al. 2018 and 2021 [25, 26]. Real-time PCR tests were performed according to the SYBR Green qPCR Master Mix (ABT 2x qPCR SYBR-Green Master Mİx,Turkey Cat No: Q03-02-05) protocol.

Statistical analysis

RT-PCR data were analzed according to ΔΔCt method. The comparison of the groups has been analyzed via "RT^2 Profiles PCR Array Data Analysis", which is assessed statistically using the "student *t*-test." $P<0.05$ was considered to indicate statistically significant.

Results

XTT Assay

Upon treatment with echinacoside, cell viability of PC3 androgen-independent prostate cancer cells was determined by the colorimetric

Table 1. Primer sequences of the genes used in this study [25-26]

Gene	Primer sequence	PCR product size (bp)
MMP2	F:5-TGGCAGTGCAATACCTGAA-3 R:5-GCATGGTCTCGATGGTATTCT-3	147
MMP9	F:5-GCAGACATCGTCATCCAGTT-3 R:5-ACAACTCGTCATCGTCGAAAT-3	139
TIMP1	F:5-GCGTTATGAGATCAAGATGACCA-3 R:5-AACTCCTCGCTGCGGTT-3	141
TIMP2	F:5-GCTGCGAGTGCAAGATCA-3 R:5-CTCTTGATGCAGGCGAAGAA-3	136
TIMP3	F:5-GCAAGATCAAGTCCTGCTACTAC-3 R:5-GGATGCAGGCGTAGTGTTT-3	123
ACTB	F:5-AGCACGGCATCGTCACCAACT-3 R:5-TGGCTGGGGTGTTGAAGGTCT-3	179

based XTT assay. Reduce in viability of PC3 cells was investigated in a time- and dose-dependent manner. The changes in cell viability were observed by treatment with various concentration of echinacoside at the 24 and 48 hour. In this study, IC_{50} doses (inhibitory concentration where 50% of the cells die) of echinacoside was detected as 55.21 µM at the 48th hour. The change in cell viability in cells treated with echinacoside depending on the dose and time is showed in Figure 1.

Transwell Invasion Assay

As observed by the transwell chamber experiment figure 2, the cell invasion was significantly inhibited in the PC3 echinacoside treated group, while this inhibition was not evident in the PC3 control group (Figure 2a). Echinacoside treatment was found to reduce invasion in cells in the dose group by 66% compared to the control cells (Figure 2b).

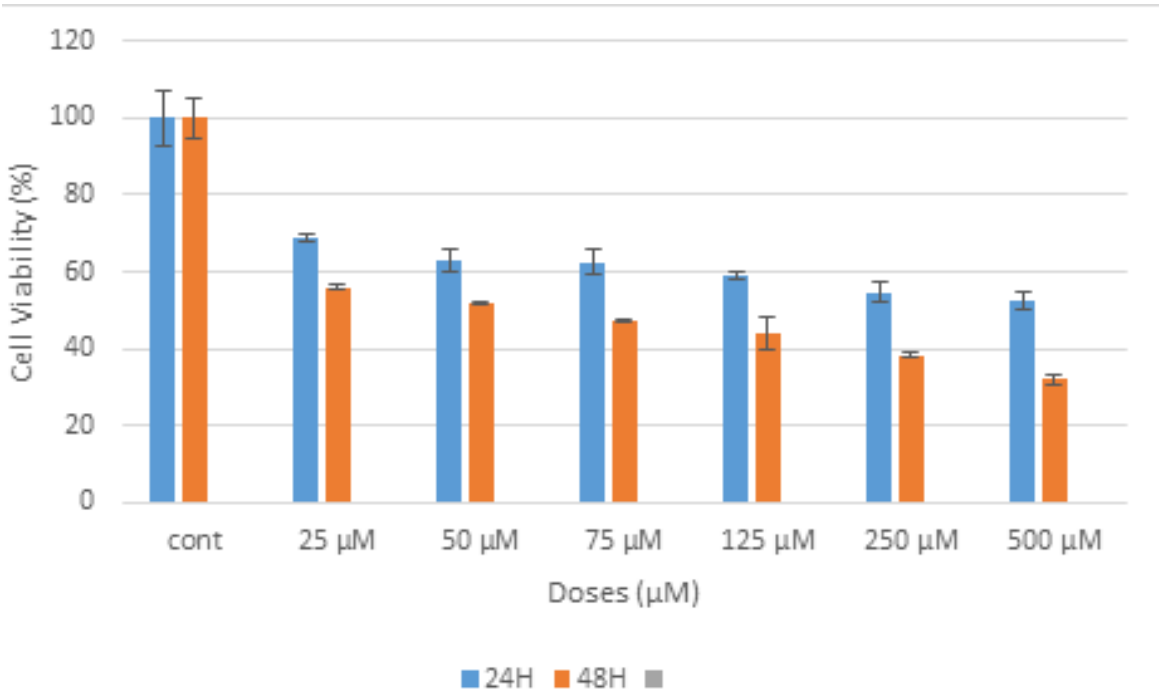

Figure 1. Effect of Echinacoside on the viability of PC3 cells. Following treatment with Echinacoside at different concentrations and time intervals, cellular proliferation was assessed by XTT assay. Data shows the average results of three independent experiments. IC_{50} dose of Echinacoside in PC3 prostate cancer cells was detected 55.21 µM at 48h.

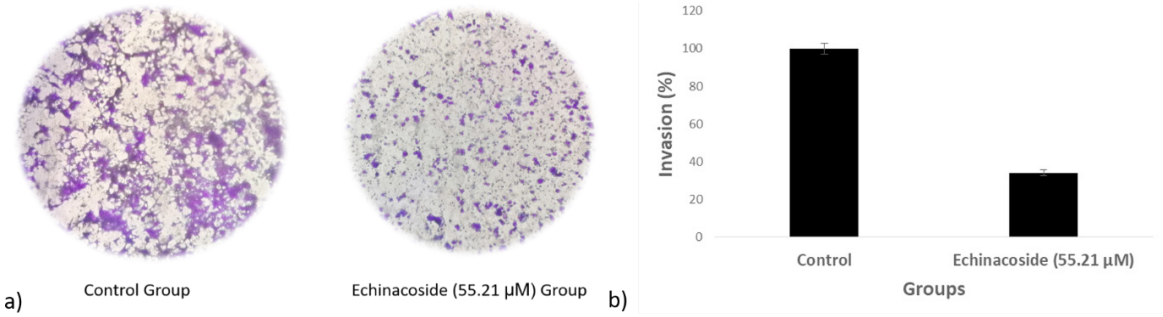

Figure 2. a) Echinacoside exhibits an anti-invasive potential to PC3 prostate cancer cells. Descriptive micrograph images of PC3 cell invasion b) Invasion (%) graph of the groups

RT-PCR Assay

The effects of echinacoside on mRNA expression changes of genes related to invasion in PC3 prostate cancer cells were evaluated by RT-PCR. Fold regulation and *p* value of the gene expression changes were given in Table 2. The expression analysis of *MMP2, MMP9, TIMP1, TIMP2 and TIMP3* were investigated by Real-Time PCR.

TIMP1 (p=0.000379) and TIMP2 (p=0.046970) gene expression increased, and *MMP2 (p=0.001293), MMP9 (p=0.0012305),* expressions were reduced significantly in the PC3 cell line when compared with the control group cells (Table 1). A 1.96-fold increase in *TIMP1* mRNA expression and a 2.60-fold increase in *TIMP2* expression were detected in the dose group cells treated to echinacoside. A 3.82-fold decrease in *MMP2* expression and 1.54-fold decrease in *MMP9* expression were determined in echinacoside -treated dose group cells. Although there was a 4.48-fold increase in expression in *TIMP3*, it was not found to be statistically significant (Table 2).

Table 2: The mRNA expression changes of invasion related genes in PC3 prostate cancer cells after treatment of echinacoside

Genes	*Fold Regulation*	*p-value*
MMP2	*-3.82*	*0.001293*
MMP9	*-1.54*	*0.0012305*
TIMP1	*1.96*	*0.000379*
TIMP2	*2.60*	*0.046970*
TIMP3	*4.48*	*0.370686*

Discussion

Echinacoside is a small phenylethanoid glycoside molecule that is an active ingredient of natural herbal plants such as Cistanche and Echinacea [15, 27]. Various molecular biological studies on the anti-cancer activity of echinacoside have been demonstrated both *in vitro* and *in vivo* in some studies. In these studies, echinacoside has been reported to reduce cell proliferation, cell invasion and migration-inhibited colony formation of various cancer cell lines [14, 15, 20-22, 27]. It was recently reported that echinacoside inhibits breast cancer cell growth under *in vitro* conditions via Wnt/β-catenin signaling pathway. It was also reported that echinacoside could effectively inhibit cell proliferation and invasion in MDA-MB-231 and MDA-MB-468 triple negative breast cancer cells. 50 and 100 μM doses of echinacoside have shown anti-proliferative, anti-migratory and anti-invasive effects in breast cancer cells via suppressing the expression of genes, including cyclin D1, LEF1 and CD44, which are well-known Wnt/b-catenin target genes. Similarly, in the in vivo MDA-MB-231 xenograft model, it was demonstrated that echinacoside inhibited the Wnt/b-catenin signaling pathway, and that it showed this function by regulating the expressions of the proteins involved in this pathway [27]. In another similar study, it was found that *Echinacea angustifolia* DC extract, which also contains the echinacoside active substance, induced cell cycle arrest and apoptosis in MDA-MB231 and MCF-7 breast cancer cells, and also showed a synergistic effect when combined with Paclitaxel. In the study, in which the strong antioxidant capacity of this *Echinacea angustifolia* plant was revealed, the extract presented cytotoxicity in the breast cancer cells without affecting the normal epithelial MCF-10 cells [28].

In another recent mechanistic study, in which the effect of echinacoside on breast cancer was observed, echinacoside inhibited cell proliferation in MCF-7 breast cancer cells at a dose range of 5-40 μg/ml, and also suppressed the cancer cell progression by downregulating miR-4306 and miR-4508 expressions [14]. In a study designed by Dong et al. [15], it has been shown that 50 μM dose treatment of echinacoside stimulated apoptosis, induced oxidative DNA damage and also decreased colony formation capacity in SW480 colorectal cancer cells. According to their results, the researchers suggested that echinacoside may be a new specific anticancer agent in colon

cancer. In a study published by Ye et al. [29] in 2018, the anticancer activities of echinacoside in liver cancer were revealed both in vitro and in vivo. The effect of echinacoside on the PI3K/AKT pathway, which is one of the important mediators of cell proliferation, was investigated through the total and phosphorylated forms of AKT and IGF-1. According to the results obtained, it has been shown that echinacoside significantly reduces IGF1-induced cell proliferation and p-AKT level in HepG2 cells. Furthermore, it was demonstrated that echinacoside alleviates DEN-induced HCC in mice under *in vivo* conditions [29]. In another study in liver cancer, it was reported that echinacoside reduced cell proliferation, invasion, migration and induced apoptosis in Huh-7 and Hepg2 hepatocellular cancer cells. It has also been shown that it achieves this effect by modulating the miR-503-3p/TGF-β1/Smad signaling pathway in liver cancer cells [22].

In a recent study on OVCAR3 and SKOV3 ovarian cancer cells, echinacoside was shown to inhibit cell viability with increasing dose in the dose range of 6.25 to 400 μM, and IC_{50} values were determined as 58.86 μM in OVCAR3 cells and 41.35 μM in SKOV3 cells, respectively. Anti-invasive activity of echinacoside has been observed in ovarian cancer cells, and it has been demonstrated by the transwell chamber method that echinacoside inhibits cell invasion in parallel with the increasing dose at 25, 50 and 100 μM echinacoside concentrations [21]. In a study on SW1990 pancreatic adenocarcinoma cells, echinacoside was shown to suppress cell proliferation, trigger apoptosis by regulating mitochondia membrane potential and the mitogen-activated protein kinase pathway [20]. In a study by Dong et al. [30], it was shown that echinacoside suppressed cell proliferation and reduced colony formation in Human MG-63 osteosarcoma, SK-HEP-1 hepatocarcinoma, MCF7 breast cancer, SW480 colorectal cancer cells. They also reported that echinacoside triggered apoptosis in MG-63 cells via inhibition of the nucleotide pool sanitizing enzyme MTH1.

In the literature, there is no study on the anticancer activities of echinacoside directly on PC3 cells. According to our results, echinacoside exhibits anti-proliferative activity in PC3 cells. It decreases cell viability with increasing dose. The IC_{50} dose of echinacoside was determined as 55.21 μM in PC3 cells. It was determined that the cell viability was reduced in PC3 cells at dose rates similar to those in studies with other cancer cell lines. While the cell viability did not fall below 50 percent in the doses treated in the first 24 hours, it was observed that it suppressed the cell viability more effectively for 48 hours. This shows us that echinacoside decreases cell proliferation in PC3 prostate cancer cells depending on dose and time.

Matrix metalloproteinases are enzyme groups that play roles in harmony with many extracellular matrix proteins responsible for degradation during organogenesis, growth and tissue transformation. A significant increase is observed in various tissue pathologies such as inflammatory diseases, tumor development and metastases, which cause undesired tissue destruction [31]. The invasion process is a multifactorial biological process and the availability of proteases that degrade the extracellular matrix, such as matrix metalloproteinases and their inhibitors, is also important in this process [32].

As a result of the experiments performed with the help of the transwell chamber in our study, it was observed that the number of invading cells in the dose group decreased when compared to the control group. *In vitro* studies with other cancer cells have demonstrated the anti-invasive activity of echinacea. In this study, echinacoside showed similar activity in parallel with the studies performed in other cancer lines. In addition, the significant decrease in the expressions of *MMP2* and *MMP9*, which are important invasion biomarkers, in the cells treated with echinacoside, and the significant increase in the expressions of MMP inhibitors *TIMP1*, *TIMP2* in the cells treated with echinacoside, suggest that the anti-invasive effect of echinacoside may be achieved by regulating the expressions of these invasion-related genes.

In conclusion, in our study, the anti-proliferative activity of echinacoside in PC3 androgen independent prostate cancer cells was shown as dose and time dependent manner. In addition, the anti-invasive effect of echinacoside has been demonstrated and it has been suggested that this mechanism of action may be related to downregulation of *MMP2* and *MMP9* mRNA expression and upregulation of *TIMP1* and *TIMP2* mRNA expression. In line with

the data obtained, it is thought that echinacoside will provide preliminary information for the determination of anticancer activity on androgen independent prostate cancer cells and for more comprehensive *in vitro* and *in vivo* experiments.

Conflict of interest: No conflict of interest was declared by the authors.

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Contributions of the authors to the article

M.S. and Y.D. was involved in data collection. M.S. and Y.D. performed the statistical analysis. M.S. interpreted data and prepared the manuscript draft. M.S. and Y.D. critically reviewed the final version of the manuscript. All authors approved the final version of the manuscript.