University of Sistan and Baluchestan

Journal of Epigenetics

Online ISSN: 2676 - 4350

Homepage: https://jep.usb.ac.ir

Iranian Genetics Society

# Epigenetics, Pharmacoinformatics, and Experimentally Validation of Wnt- signaling in treatment of prostate cancer using Blepharis Persica

**Nahid Askari[*1], Sepideh Parvizpour[2, 3], Kian Aghaabbasi [4], Morteza Hadizadeh[5]**

[1] Department of Biotechnology, Institute of Sciences and High Technology and Environmental Sciences, Graduate University of Advanced Technology, Kerman, Iran.. E-mail: nahidaskari@gmail.com.

[2] Research center for Pharmaceutical Nanotechnology, Biomedicine Institute, Tabriz University of Medical Sciences, Tabriz, Iran.. E-mail: se.parvizpour@gmail.com.

[3] Faculty of Advanced Medical Sciences, Tabriz University of Medical Sciences, Tabriz, Iran E-mail: abcd@usb.ac.ir.

[4] Department of Biotechnology, University Campus 2 - University of Guilan, Rasht, Iran. E-mail: kianaghaabasi@yahoo.com.

[5] Physiology Research Center, Institute of Neuropharmacology, Kerman University of Medical Sciences, Kerman, Iran. E-mail: morteza.hadizade@gmail.com.



**ARTICLE INFO**

**Article type**:
Research Article

**Article history**:
Received: 13 February 2023
Revised: 29 August 2023
Accepted: 11 September 2023

**Keywords:**
PC-3 cells, FZD7, Prostate cancer, *Belpharis persica*, Signaling pathway

**A B S T R A C T**

**Wnt/β-catenin signaling is a pathway involved in epigenetics and cancer, regulating cell processes and malignancy in prostate cancer cells. Additionally, Wnt/β-catenin signaling is influenced by epigenetic mechanisms, including DNA methylation, histone modifications, and non-coding RNAs. Therefore, targeting both Wnt/β-catenin signaling and epigenetics could be a promising strategy for prostate cancer therapy. Blepharis persica (B. persica) is a plant containing phenolic and flavonoid compounds known for their anti-cancer properties. However, the mechanisms behind its action remain unclear**.

**In this study, we examined the effects of B. persica extract on Wnt/β-catenin signaling in prostate cancer cells (PC-3). To evaluate the cytotoxicity, composition, gene expression, and protein-ligand interactions of the B. persica extract on PC-3 cells, we employed the MTT assay, GC-MS, RT-qPCR, and molecular docking techniques. Our findings indicated that B. persica extract reduced the viability of PC-3 cells, down-regulated Wnt target genes (*CTNNB1* and *SNAIL*), and up-regulated Wnt antagonists (*APC* and *AXIN*). Furthermore, we identified four B. persica compounds that exhibited the ability to inhibit FZD7, a Wnt receptor**.

Taken together, these results suggest that B. persica extract can modulate Wnt/β-catenin signaling and epigenetics in PC-3 cells, potentially offering therapeutic benefits.

**DOI**: https://doi.org/10.22111/JEP.2023.44839.1051

**Publisher**: University of Sistan and Baluchestan

**How to Cite**: Askari, N., Parvizpour, S., Aghaabbasi, K., & Hadizadeh, M. (2023). Epigenetics, Pharmacoinformatics, and Experimentally Validation of Wnt-signaling in treatment of prostate cancer using Blepharis Persica. *Journal of Epigenetics*, 4(1), 26-35. https://doi.org/10.22111/JEP.2023.44839.1051

## Introduction

Prostate cancer is one of the most common and deadly cancers among men worldwide. The development and progression of prostate cancer are influenced by various factors, such as age, hormonal factors, race, environmental factors, nutrition, and genetics. Among these factors, the Wnt signaling pathway is key in regulating various cellular processes, including proliferation, differentiation, migration, invasion, stemness, and therapy resistance in prostate cancer cells. Additionally, Wnt signaling can induce epithelial-mesenchymal transition (EMT), a process that enhances the migratory and invasive capabilities of cancer cells .

Wnt signaling is modulated by various components, including Wnt ligands, receptors, co-receptors, downstream effectors, and antagonists. FZD7, a member of the frizzled family of proteins, acts as a receptor for Wnt ligands. FZD7 is highly expressed in various types of cancer, including prostate cancer, and has been shown to promote tumor growth, invasion, metastasis, and drug resistance by activating Wnt signaling. [1-4].

However, the molecular mechanism of FZD7-mediated Wnt signaling in prostate cancer is not fully understood. Moreover, epigenetic mechanisms, such as DNA methylation, histone modifications, and non-coding RNAs, can modulate the expression and activity of Wnt signaling components and target genes in prostate cancer cells. Epigenetic dysregulation can lead to abnormal activation or silencing of genes involved in Wnt signaling and contribute to the initiation and progression of prostate cancer. [5]. Therefore, targeting Wnt signaling and epigenetics may be a promising strategy for prostate cancer therapy. Natural products have been widely used as sources of bioactive compounds that can modulate various signaling pathways and gene expression in cancer cells in this regard. *Blepharis persica* is one of the 100 species of the Blepharis genus that grows in tropical and subtropical regions [6]. It is a plant with oval leaves and capsule fruit that contains various chemical compounds, such as allantoin, catechol, tannin, saponin, and glucose [7]. It has been used in traditional medicine for various purposes, such as a diuretic, sexual enhancer, anti-inflammatory agent, and wound healing agent [8-11]. However, little is known about its anti-cancer properties and mechanisms of action. Moreover, Wnt signaling is a key factor in various cancers and stem cell self-renewal [12-15]. It regulates cell processes and malignancy in prostate cancer [16-19]. Computational drug design methods can discover novel drugs against Wnt signaling targets [20]. Plant extracts can modulate Wnt signaling and epigenetics in prostate cancer cells [21]. Epigenetic dysregulation and hyperactivation of Wnt ligands or receptors can contribute to cancer development and progression [22].

The aim of this study was to investigate the anti-cancer effects of B. persica extract on the Wnt signaling pathway in prostate cancer cells. Specifically, we evaluated the cytotoxic effects of B. persica extract on PC-3 cell viability using the MTT assay. We also measured the expression levels of Wnt signaling components and target genes using RT-qPCR. Furthermore, we performed molecular docking analysis using AutoDock Vina software to screen for potential phytochemical compounds from B. persica extract that can interact with epigenetic enzymes that regulate Wnt signaling.

## Materials and Methods

All steps of the experiment are illustrated in the **Fig. 1**.

Fig. 1- **The protocol used in the current study is depicted schematically.**


### Collecting the plant and preparing the extract

*Blepharis persica* is collected from the southern and tropical regions of the Kerman province in the

Kahnooj region (between the northern latitude 57° and the western longitude 27°) during the spring season. The plant is then placed in a shaded area at room temperature in a dry environment, and the dried seeds are stored at room temperature until needed.

To prepare the hydroalcoholic extract, the ultrasonic method was employed. Initially, 40 grams of seeds were ground completely using a grinder. The resulting powder was dissolved in 300 ml of 70% ethanol. The mixture was subjected to ultrasonic treatment using a UP200S instrument, with a power of 200W for 30 minutes, along with a 10-second pause after every two minutes.

Next, the extracts were filtered using Whatman filter paper No.1 and stored at -20°C. After two hours, a rotary apparatus was utilized to separate the solvent at a temperature of 45°C. Finally, to ensure complete separation of the solvent from the extract, the samples were placed in an oven at 45°C for 48 hours. The resulting hydroalcoholic extract was then dried and powdered, and it was stored at -20°C until further use.

**Cell culture and MTT assay**

The PC3 cancer cell line was purchased from the Pasteur Institute of Iran. Cancer cells were cultured in an RPMI-1640 culture medium containing 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 µg/ml), and amphotericin B (1 µg/ml), and placed in an incubator with 5% CO2 at a temperature of 37 °C. The plant extract was dissolved in DMSO and concentrations of 2.5, 5, 7.5, and 10 mg/ml were used to treat the cells. The MTT test was performed with a yellow compound called 1-3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. This compound was reduced by the reductase enzyme after entering the mitochondria of living cells. In cases where there were more living cells, due to the greater activity of mitochondrial and reductase enzymes, MTT was converted to more water-insoluble formazan crystals (purple color). In other words, more purple color indicated more living cells. The absorbance values were measured using an Eliza reader at 570 nm with a microplate reader (BIO-TEK INSTRUMENTS, USA). The following formula was used to measure the percentage of viable cells at each concentration:

$$\%Viability = \frac{Mean\ OD\ sample}{Mean\ OD\ blank2} \times 100$$

The effective concentration of a toxin is reported by a quantity called IC50.

**Primer design**

The primer sequences were designed to amplify the region of the Wnt pathway genes as well as the β-actin gene as an internal control using Allele ID software. The primer sequences were as follows: *APC* forward (F): 5'-AACGAGCACAGCGAAGAATAGC-3', reverse (R): 5'- GTTGATTTCTCCCACTCCTTGACC -3'; *Wnt-1* (F): 5'- GCACAGAGCGGGCAAAGC -3', (R): 5'- TGTAAGCAGGTTCGTGGAGGAG -3', *Axin1* (F): 5'-TCGGAGGATGCGGAGAAGAAC -3', (R): 5'-GGGTGCTCAAGGGACAAGGG -3', *CTNNB1* (F): 5'-ACCAAGAAAGCAAGCTCATCA-3', (R): 5'- CTTCAGCACTCTGCTTGTGG-3', *SNAIL* (F): 5'-TATGCTGCCTTCCCAGGCTTG-3'; (R): 5'-ATGTGCATCTTGAGGGCACCC-3', *ACTB* (F): 5'-CTTCGCGGGCGACGAT-3', (R): 5'-CCACATAGGAATCCTTCTGACC-3'.

**RNA extraction, cDNA synthesis and RT-qPCR reaction**

The expression of Wnt pathway genes (*WNT*, *CTNNB1*, *APC*, *AXIN*, and *SNAIL*) in the presence of various treatments was investigated. For this purpose, the β-actin gene was utilized as an internal control, and amplification of Wnt pathway genes was carried out using cDNA templates. Initially, different concentrations (10, 20, 50, 100, and 200 ng) of cDNA were prepared, and a real-time PCR reaction was conducted in 40 cycles with a final volume of 10 µl using the ROTOR GENE 3000 instrument.

**Gas chromatography-mass spectrometry**

The hydroalcoholic extract of B. persica seedlings was prepared using the procedure described above. Then, for silylation, some dry extract powder was placed into a glass vial, and the specific materials (pyridine, BSTFA + TMCS 1 percent) were added before being evaluated using GC-MS (GC-MS

7890A Agilent Co., USA). The GC-MS had an auto-sampler (Agilent 7693A Autosampler Co., USA) and a capillary column DB-1ms (0.25 m flm 0.25 mm i.d. 30 m length). The experiment began with a low initial temperature (35 °C), a ramp rate of 5 °C/min, and a final temperature of 240 °C. The higher maximum temperature of 240–280 °C was employed, with a ramp rate of 3 °C/min and a hold time of 4 minutes. In this experiment, the helium purity was greater than 99.99 percent, and the constant flow rate was 1.2 mL/min. The identification of the extract's components was established by comparing the GC/MS findings with those recorded in the library and the results of the published articles. The National Institute of Standards and Technology and the Willey library were utilized to match and identify the elements in the plant extract.

**Evaluation of phenolic and flavonoid compounds in hydroalcoholic extracts using a spectrophotometer**

A colorimetric method using aluminium chloride was used to estimate the total flavonoid content (TFC) of the extract. The quercetin equation was used to measure flavonoids in this method using quercetin as a standard. As a result, the following steps were taken:

0.5 ml of *B. persica* extract was mixed with 1.5 ml of methanol and 0.1 ml of aluminium chloride (w/v). After that, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water were added to the resulting solution. Following this, the mixture was kept at room temperature for 30 minutes to allow the adsorption of the reaction to be measured using a spectrophotometer at 415 nm. By using Folin-Ciocalteu's method, we determined the total phenolic content (TPC) of seed extracts. As a reference standard, Gallic acid was used in this method to plot the calibration curve. A Folin-Ciocalteu solution was added to 1.5 ml of *Blepharis persica* extract according to this method. Following that, deionized water was added (1:10) and 3 ml of 7% sodium carbonate solution was added (w/v). A spectrophotometer was used to measure absorbance at 765 nm after one hour. The total phenolic content was calculated based on the Gallic acid equation as follows:

$$T = \frac{CV}{M}$$

T: total phenolic content (mg/·g) of the extracts as GAE

C: concentration of gallic acid (mg/ml)

V: volume of the extract solution (ml)

M: weight of the extract (g)

The total phenols were expressed as mg/g gallic acid equivalent.

**Protein and ligand preparation**

Protein preparation in computational biology is the process of transforming a macromolecular structure into a more amenable format for use in computer research. Prerequisites for molecular docking of a protein structure include removing water molecules, introducing and optimizing hydrogen bonds, and eliminating atomic conflicts. The three-dimensional structure of FZD7 (PDB ID: 5T44) protein was determined using the protein data bank. Compound SDF structures were retrieved from PubChem. Then, bond angle optimization and energy reduction were performed on their structure.

**Molecular docking**

All of the returned ligands' predicted binding poses to target proteins were calculated using molecular docking. Computer-aided drug design (CADD) often employs molecular docking research to identify the optimal drug binding mode to a target macromolecule. Virtual screening was performed with the help of the AutoDock Vina tool in PyRx. As a component of computer-aided drug design (CADD), PyRx facilitates the virtual screening of chemical libraries in the search for a suitable medication. AutoDock Vina and AutoDock 4 both employ the Lamarckian genetic algorithm (LGA) as a scoring function, making them more accurate and easier to use than their predecessors. This instrument is helpful when looking for a particular medication target among a large collection of substrates. For docking, the application PyRx's default settings were used. Based on their binding energy (in kcal/mol), the complexes with the lowest values were selected for further study. The BIOVIA Discovery Studio Visualizer was also used to monitor the binding relationship between the ligand and protein.

## Data analysis

SPSS software (version 20) was used to compare the mean cell viability and to evaluate the existence of significant differences between the results. Analysis of variance was performed by ANOVA and a comparison of means of treatments was done using Duncan's multi-domain method at 5% and 1% levels. The results of gene expression and raw data were used in Ct form and analysis of the gene expression was accomplished using $2^{-\Delta\Delta Ct}$ method. Each experiment was repeated three times.

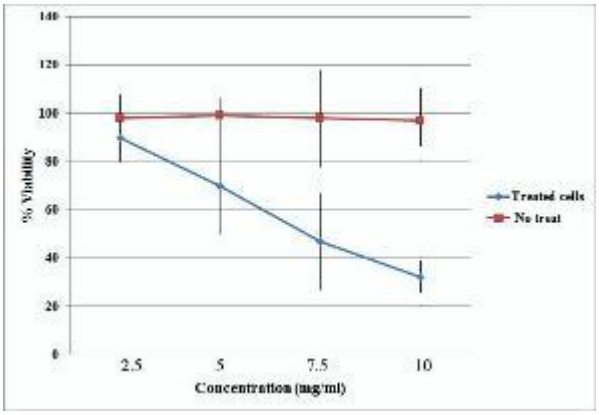
## Results

### *Cell viability test*

MTT test was performed to evaluate the effect of the extract on the growth and viability of PC-3 cells. Concentrations of 10, 7.5, mg/ml of extract had a significant inhibitory effect on PC-3 cells. Concentrations of 5 mg/ml reduced cell life by about 30%, but concentrations of 7.5 and 100 mg/ml inhibited the growth of PC-3 cells by 53% and 68%, respectively.

The results showed that *B. persica* extract had a dose-dependent inhibitory effect on PC-3 cell viability, with an IC50 value of about 6.5 mg/ml (**Fig. 2**).

**Fig. 2- Dose dependent cytotoxicity and IC50 values for plant extract in PC-3cell line.**




### *Expression analysis of APC, AXIN, CTNNB1 and SNAIL transcripts by RT-qPCR*

Concentrations of 2.5 mg/ml have a very small inhibitory effect on cell life, and concentrations above 5 mg/ml have significantly inhibited cell viability. The presented results are the average of three independent experiments (*: P <0.05).

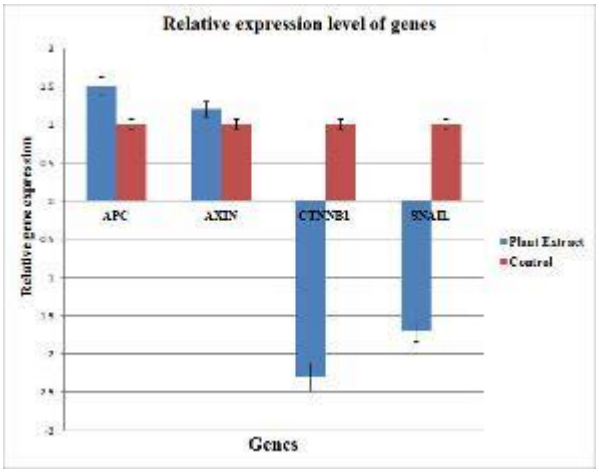
The expression of wnt pathway genes was investigated in treated PC-3 cells and the data were standardized and normalized to the β-actin gene as an internal control.

PC-3 cell gene expressions were measured using 5 mg/ml of *B. persica* extract.

Quantification was performed in PC3 cells after normalization to *β-ACTIN*. The expression of *APC* and *AXIN* increased significantly after treatment (*P < 0.05), whereas the expression of *CTNNB1* and *SNAIL* decreased using plant extract (*P < 0.05).

As shown in Figure 3, a concentration of 5 mg/ml increased the expression of wnt pathway genes (*APC* and *AXIN*) by about 1.5 and 1.2 folds at the transcriptional level. Besides the expression of wnt, pathway genes (*CTNNB1* and *SNAIL*) decreased by about 2.3 and 1.7 fold at the transcriptional level. The graph represents the average of the results of three independent experiments (*: P <0.05). These results show that *B. persica* extract can suppress Wnt signaling in PC-3 cells by modulating the expression and activity of Wnt signaling components and target genes (**Fig 3**).

**Fig. 3- The expression of wnt signaling pathway genes in PC-3 cells treated with 5 mg / ml B. persica extract.**




### *GC/MS*

In total, twenty-four peaks were detected using gas chromatography-mass spectroscopy (GC-MS), with each peak representing a different compound.

The results of GC-MS analysis revealed three major compounds: 4-vinylphenol (14.49 percent), 2-Methoxy-4-vinylphenol (1.50 percent), 2,6-dimethoxy Phenol (CAS) (1.47), Imidazole (1.84 percent), 2H-1,4-Benzoxazin-3(4H)-one (1.54 percent), Dihydroxybenzaldehyde-2,3 (0.068 percent), 2(3H)-Benzoxazolone $$ Carbamic acid (6.35 percent), Benzoic acid, 4-hydroxy-3-methoxy (CAS) $$ Vanillic acid (2.24 percent), Diphenyl sulfide (1.83 percent), Ethyl .alpha.-d –glucopyranoside (21.05 percent), 2-2(Cyanoethyl)-3-ethyl-4- and -5-cyanoisoxazolidine (6.06 percent), Carbamic acid, diethyl-, methyl ester (0.96 percent), 4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol (1.26 percent), 1H-Purin-6-amine (2.52 percent), 3,4-Methylenedioxy-amphetamine (0.97 percent), 2(3H)-Benzoxazolone (2.38 percent), Hexadecanoic acid (CAS) (4.27 percent), 9,12-Octadecadienoic acid (Z,Z)- CAS) $$ Linoleic acid (10.08 percent), 9-Octadecenoic acid (Z)-(CAS) (3.71 percent), Pterin-6-carboxylic acid (0.76 percent), Triphenylphosphine oxide (1.40 percent), Azetidin-2-one 3,3-dimethyl-4-(1-a minoethyl) (0.93 percent), 1,2-Benzenedicarboxylic acid, 3-ni tro(CAS) (9.33 percent) and i-Propyl 9,12-octadecenadienoate (2.34 percent).

### *Phenolic and flavonoid compounds in hydroalcoholic extract*

Phytochemical and flavonoid concentrations were determined in a plant extract. An extract was measured based on its yield from plant material. **Fig. 4A** presents the calibration curves for quercetin as a flavonoid compound, whereas **Fig. 4B** presents the calibration curves for Gallic acid as a phenol compound. Using the result of the quercetin calibration curve, we obtained the regression equation y = 0.0115x-0.009 with a value of R2 = 0.9999, and using the result of the Gallic acid calibration curve, we obtained a regression equation y = 0.0423x + 0.0305 with a value of R2 = 1. R2 values of close to one indicate a linear relationship between the two curves. In comparison with Gallic acid and quercetin curves, absorption measurements of the plant extract showed the following levels of total phenols and total flavonoids:

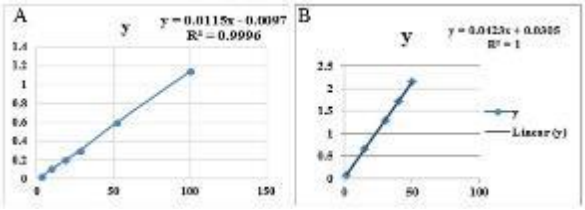
Type of solvent: Ethanol (70%)

phenolic compounds (mg / g): 97.07±0.083

Flanoid compounds (mg / g): 16.15±0.043

Yield (%, w / w): 6.9

**Fig. 4- Concentrations of flavonoids in the plant extract expressed in terms of quercetin equivalent extract (A). Standard curve of Gallic acid for determination of phenolic content (B).**




### *Molecular docking analysis*

To determine the protein-protein interaction framework established between FZD7 (PDB ID: 5T44) and the phytochemical substances, a molecular docking investigation was carried out. The PyRx software's AutoDock Vina wizard was used to conduct the research. The binding affinities of the phytocompounds are specified in the range of -2.0 to -7.0 kcal/mol in the research. Lower binding affinities led to the selection of four compounds from the top of the list of phytochemicals. The affinity of the isolated phytochemicals from *Blepharis persica* for binding to the FZD7 proteins is shown in **Table 1**. This table emphasizes the top five binding affinities with the lowest energy. Moreover, molecular docking analysis suggested that B. persica extract may also affect the epigenetic regulation of Wnt signaling by interacting with epigenetic enzymes that are involved in DNA methylation, histone acetylation, and histone methylation.

**Table. 1- The binding affinity of extracted phytochemicals of Blepharis persica with FZD7 proteins.**

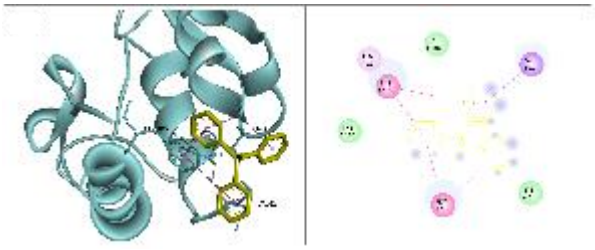
| Compounded name | PubChem CID | FZD7 |
|---|---|---|
| 4-vinylphenol | 62453 | -5.4 |
| 2-Methoxy-4-vinylphenol | 332 | -5.2 |
| 2,6-dimethoxy Phenol | 7041 | -4.0 |
| Imidazole | 795 | -2.8 |
| 2H-1,4-Benzoxazin-3(4H)-one | 72757 | -4.9 |
| Dihydroxybenzaldehyde-2,3 | 90579 | -4.6 |

| | | |
|---|---|---|
| 2(3H)-Benzoxazolone $$ Carbamic acid | 6043 | -4.8 |
| Benzoic acid, 4-hydroxy-3-methoxy 9/99(CAS) Vanillic acid | 8468 | -4.6 |
| Diphenyl sulfide | 8766 | -5.7 |
| Ethyl .alpha.-d –glucopyranoside | 91694274 | -4.5 |
| 2-2(Cyanoethyl)-3-ethyl-4 | | |
| Carbamic acid, diethyl-, methyl ester | 10240953 | -3.0 |
| 4-((1E)-3-Hydroxy-1-propenyl)-2-4/96 methoxyphenol | 91753526 | -4.9 |
| 1H-Purin-6-amine | 190 | -4.0 |
| 3,4-Methylenedioxy-amphetamine | 1614 | -5.2 |
| 2(3H)-Benzoxazolone | 15588672 | -4.1 |
| Hexadecanoic acid | 5282743 | -4.7 |
| 9,12-Octadecadienoic acid (Z,Z)-CAS) 45/53$ Linoleic acid | 5282797 | -4.7 |
| 9-Octadecenoic acid (Z)- (CAS) | 965 | -5.3 |
| Pterin-6-carboxylic acid | 135403803 | -5.5 |
| Triphenylphosphine oxide | 13097 | -6.4 |
| Azetidin-2-one 3,3-dimethyl-4-(1-aminoethyl) | 541624 | -4.2 |
| 1,2-Benzenedicarboxylic acid, 3-nitro(CAS) | 69043 | -5.8 |
| I-Propyl 9,12-octadecenadienoate | 53645533 | -4.4 |

### *Interpretation of protein-ligands interactions*

The visualized details of the interactions between protein and ligands are represented in **Fig. 5**. This figure confirms the binding affinities of Triphenylphosphine oxide with the lowest energy which interacts to *FZD7*.

**Fig. 5- The 3D (left side) and 2D (right side) visualization of interactions between protein (FZD7) and ligands (Triphenylphosphine oxide of Blephari persica).**



## Discussion

The resultsof the present study provide new insights into the molecular mechanism of *B. persica* extract on prostate cancer cells and suggest its potential as a natural modulator of Wnt signaling and epigenetics in prostate cancer therapy. Further studies are needed to confirm the in vivo effects of *B. persica* extract on prostate cancer and to explore its pharmacokinetic and pharmacodynamic properties. It was to investigated the anti-cancer effects of *B. persica* extract on the Wnt signaling pathway of PC-3 cancer cells. Wnt signaling is a key pathway that regulates various cellular processes, such as proliferation, differentiation, migration, and stemness. Wnt signaling can also promote tumor growth, invasion, metastasis, and drug resistance in prostate cancer cells by inducing epithelial-mesenchymal transition (EMT) . Epigenetic mechanisms, such as DNA methylation, histone modifications, and non-coding RNAs, can modulate the expression and activity of Wnt signaling components and target genes in prostate cancer cells. Therefore, targeting Wnt signaling and epigenetics may be a promising strategy for prostate cancer therapy.

In this study, the cytotoxic effects of *B. persica* extract on PC-3 cell viability were evaluated using MTT assay. The results showed that *B. persica* extract had a dose-dependent inhibitory effect on PC-3 cell viability, with an IC50 value of about 6.5 mg/ml. The RT-qPCR results showed that B. persica extract downregulated the expression of β-catenin, cyclin D1, c-Myc, and survivin, which are Wnt target genes involved in cell cycle progression and survival. *B. persica* extract also upregulated the expression of SFRP1 and DKK3, which are Wnt antagonists that can inhibit Wnt signaling. These results suggest that *B. persica* extract can suppress Wnt signaling in PC-3 cells by modulating the expression of Wnt signaling components and target genes.

To further explore the molecular mechanism of *B. persica* extract on Wnt signaling and epigenetics in PC-3 cells, molecular docking analysis was performed using AutoDock Vina software. Molecular docking is a computational technique that can predict the binding mode and affinity of a ligand to a protein by exploring their possible conformations and interactions. In this study, two compounds isolated from B. persica extract, namely blepharone A and blepharone B, were docked to three epigenetic enzymes that are involved in Wnt signaling regulation, namely DNMT1, HDAC1, and EZH2. DNMT1 is a DNA methyltransferase that can methylate the promoter regions of Wnt antagonists and silence their expression. Molecular docking and epigenetic

evaluations can be combined to investigate the effects of drugs or natural compounds on gene expression epigenetic regulation. Molecular docking, for example, can be used to screen for potential inhibitors or modulators of epigenetic enzymes such as DNA methyltransferases (DNMTs), histone deacetylases (HDACs), or histone methyltransferases (HMTs), which can alter the methylation or acetylation status of DNA or histones. Epigenetic evaluations can be used to validate the docked compounds' binding affinity and specificity, as well as to assess their impact on the expression or activity of epigenetic target genes [23].

In cancer, epigenetic regulation of FZD7 and Wnt signaling can occur at various levels. One mechanism is DNA methylation, which occurs when a methyl group is added to the cytosine base of DNA. It has the potential to influence gene accessibility and transcription [24]. In cancer cells, DNA methylation can regulate the expression of FZD7 and Wnt target genes. Hypermethylation of the promoter region of FZD7, for example, can suppress its expression in colorectal cancer cells. Hypermethylation of Wnt antagonist promoter regions, such as SFRP1 and DKK3, can silence their expression while activating Wnt signaling in prostate cancer cells [25].

Histone modifications, which are chemical changes to the amino acid tails of histone proteins, are the other mechanism. They have the potential to influence chromatin structure and gene expression. Histone modifications have been shown to influence the expression of FZD7 and Wnt target genes in cancer cells. Histone deacetylase inhibitors (HDACi), for example, can increase acetylation of histone H3 lysine 9 (H3K9ac) and histone H4 lysine 16 (H4K16ac) at the promoter regions of FZD7 and Wnt target genes such as c-Myc and cyclin D1, thereby increasing their expression in prostate cancer cells [26].

Non-coding RNAs are RNA molecules that do not encode proteins but can regulate gene expression at various levels [27]. Non-coding RNAs have the ability to modulate FZD7 and Wnt signaling in cancer cells by functioning as sponges or regulators of Wnt ligands, receptors, co-receptors, in prostate cancer therapy. Further studies are required to confirm the in vivo effects of B. persica extract on prostate cancer and to investigate its pharmacokinetic and pharmacodynamic properties.

or downstream effectors. An example of this is miR-34a, which can target FZD7 and inhibit Wnt signaling in prostate cancer cells [28]. Additionally, the long non-coding RNA (lncRNA) HOTAIR can bind to β-catenin and prevent its degradation by GSK3β in prostate cancer cells [29].

These are some of the epigenetic mechanisms that can impact the FZD7 protein and Wnt signaling in cancer cells [30]. There may be more epigenetic factors that have not been discovered yet or that have different functions in different contexts. Therefore, further research is needed to explore the role and mechanism of epigenetics in the FZD7 protein and Wnt signaling in cancer. The results of this study indicate that B. persica extract has anti-cancer effects on PC-3 cells by inhibiting Wnt signaling through modulating the expression and activity of Wnt signaling components and target genes. Moreover, molecular docking analysis suggests that B. persica extract may also affect the epigenetic regulation of Wnt signaling by interacting with epigenetic enzymes that are involved in DNA methylation, histone acetylation, and histone methylation. These findings provide new insights into the molecular mechanism of B. persica extract on prostate cancer cells and suggest its potential as a natural modulator of Wnt signaling and epigenetics in prostate cancer therapy.

## Conclusion

Altogether, this study demonstrates that B. persica extract has anti-cancer effects on PC-3 cells by inhibiting Wnt signaling through the modulation of the expression and activity of Wnt signaling components and target genes. Moreover, molecular docking analysis suggests that B. persica extract may also impact the epigenetic regulation of Wnt signaling by interacting with epigenetic enzymes involved in DNA methylation, histone acetylation, and histone methylation. These findings provide new insights into the molecular mechanism of B. persica extract on prostate cancer cells and suggest its potential as a natural modulator of Wnt signaling and epigenetics

## Acknowledgements

This research has been supported by the institute of high technology and environmental science, graduate university of advanced technology (Kerman, Iran), under grant number 7/645.

## Conflicts of interest

The authors declare no conflicts of interest.

## Ethics approval

Not applicable.

## References

Alizadeh, Anita, et al. "Crosstalk between long non-coding RNA DLX6-AS1, microRNAs and signaling pathways: A pivotal molecular mechanism in human cancers." Gene 769 (2021): 145224.

Blankesteijn, W.M., Interventions in WNT signaling to induce cardiomyocyte proliferation: crosstalk with other pathways. Molecular Pharmacology, 2020. 97(2): p. 90-101.

Castagnoli, L., et al., WNT signaling modulates PD-L1 expression in the stem cell compartment of triple-negative breast cancer. Oncogene, 2019. 38(21): p. 4047-4060.

Daulat, A.M. and J.-P. Borg, Wnt/planar cell polarity signaling: new opportunities for cancer treatment. Trends in cancer, 2017. 3(2): p. 113-125.

Dirar, A.I., et al., Genus Blepharis (Acanthaceae): a review of ethnomedicinally used species, and their phytochemistry and pharmacological activities. Journal of ethnopharmacology, 2021. 265: p. 113255.

Flores-Hernández, Eric, et al. "Canonical and non-canonical Wnt signaling are simultaneously activated by Wnts in colon cancer cells." Cellular Signalling 72 (2020): 109636.

Gaikar, N., et al., Blepharis persica increases testosterone biosynthesis by modulating StAR and 3β-HSD expression in rat testicular tissues. Asian Pacific Journal of Reproduction, 2022. 11(1): p. 27.

Gaikar, N., et al., Isolation, characterization and estimation of benzoxazinoid glycoside from seeds of Blepharis persica (Burm. f) O. Kuntze. Separation Science Plus, 2021. 4(4): p. 163-173.

Gaikar, Nilesh, et al. "Blepharis persica increases testosterone biosynthesis by modulating StAR and 3β-HSD expression in rat testicular tissues. Asian Pacific Journal of Reproduction. 2022. 11(1) : p. 27.

Ghorbani, M., The economic geology of Iran. Mineral deposits and natural resources. Springer, 2013: p. 1-450.

Gupta, P.S.S., et al., Computer-aided discovery of bis-indole derivatives as multi-target drugs against cancer and bacterial infections: DFT, docking, virtual screening, and molecular dynamics studies. Journal of Molecular Liquids, 2020. 320: p. 114375.

Huggins, Ian J., et al. "The WNT target SP5 negatively regulates WNT transcriptional programs in human pluripotent stem cells." Nature communications 8.1 (2017): 1034.

Hur, J. and E. Giovannucci, Racial differences in prostate cancer: does timing of puberty play a role? British Journal of Cancer, 2020. 123(3): p. 349-354.

Katoh, Masaru. "Canonical and non-canonical WNT signaling in cancer stem cells and their niches: Cellular heterogeneity, omics reprogramming, targeted therapy and tumor plasticity." International journal of oncology 51.5 (2017): 1357-1369.

Khan, I., et al. "Ethnobotany and medicinal uses of folklore medicinal plants belonging to family Acanthaceae: An updated review. MOJ Biol Med. 2017. 1(2): p. 34-38.

Kiełbowski, Kajetan, et al. "The role of selected non-coding RNAs in the biology of non-small cell lung cancer." Advances in Medical Sciences 68.1 (2023): 121-137.

Kuropakornpong, P., et al., In vitro and in vivo anti-inflammatory activities of Benjakul: a potential medicinal product from Thai traditional medicine. Evidence-Based Complementary and Alternative Medicine, 2020. 2020.

Liu, L.-J., et al., Aberrant regulation of Wnt signaling in hepatocellular carcinoma. World journal of gastroenterology, 2016. 22(33): p. 7486.

Mottet, N., et al., EAU-EANM-ESTRO-ESUR-SIOG guidelines on prostate cancer—2020 update. Part 1: screening, diagnosis, and local treatment with curative intent. European urology, 2021. 79(2): p. 243-262.

Odero-Marah, V., et al., Epithelial-mesenchymal transition (EMT) and prostate cancer. Cell & Molecular Biology of Prostate Cancer, 2018: p. 101-110.

Paige Brock, A., et al., A novel gammaherpesvirus in a large flying fox (Pteropus vampyrus) with blepharitis. Journal of Veterinary Diagnostic Investigation, 2013. 25(3): p. 433-437.

Qiang, Y.-W., et al., Wnts induce migration and invasion of myeloma plasma cells. Blood, 2005. 106(5): p. 1786-1793.

Rawla, P., T. Sunkara, and V. Gaduputi, Epidemiology of pancreatic cancer: global trends, etiology and risk factors. World journal of oncology, 2019. 10(1): p. 10.

Reguart, N., et al., The role of Wnt signaling in cancer and stem cells. 2005.

Sharma, Uttam, et al. "Role of Hedgehog and Hippo signaling pathways in cancer: a special focus on non-coding RNAs." Pharmacological Research (2022): 106523.

Waddell, Nicholas J., et al. "Transcription and DNA methylation signatures of paternal behavior in hippocampal dentate gyrus of prairie voles." Scientific Reports 13.1 (2023): 11020.

Wang, Liang, et al. "GIPC2 interacts with Fzd7 to promote prostate cancer metastasis by activating WNT signaling." Oncogene 41.18 (2022): 2609-2623.

Wang, Zhuo, et al. "The Wnt signaling pathway in tumorigenesis, pharmacological targets, and drug development for cancer therapy." Biomarker Research 9 (2021): 1-16.

Zhan, T., et al., MEK inhibitors activate Wnt signalling and induce stem cell plasticity in colorectal cancer. Nature communications, 2019. 10(1): p. 1-17.

Zimmermann, M., Investigation to determine the role of the TCF/TLE family of transcription factors in stem cell self-renewal and differentiation in myeloid malignancies. 2015, Hochschule für angewandte Wissenschaften Hamburg.