In vitro and Pharmacoinformatics-based phytochemical screening for anticancer impacts of pistachio hull essential oil on AGS, PLC/PRF/5, and CACO2 cell lines

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Research Article

Keywords: Anti-cancer therapeutic agents, Essential oil, Pistachio hull, Molecular docking analysis, wnt pathway

Posted Date: August 29th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1775073/v2

Abstract

Background: The essential oil of *pistacia vera* (*cv.* Ohadi) hull (PHEO) was checked using gas chromatography mass spectrometry (GC/MS) analysis. It was studied the genes of the wnt pathway with a certain concentration of PHEO on Human gastric cancer (AGS), human hepatocellular carcinoma (PLC/PRF/5), and human colon cancer (CACO2) cell lines.

Methods and Results: After evaluating the survival rate of cancer cells by MTT test and determining IC50, pistachio hull essential oil (PHEO) was used for 24-hours to treat the cells. After RNA extraction, the expression of wnt pathway genes was evaluated by Real-Time PCR. Considering the crucial role of beta-catenin accumulation and its effect on the progression of gastrointestinal cancers, Western blot analysis was also used to determine the effect of PHEO in protein expression of β-catenin inhibition. Also, an *in silico* analysis was carried out to investigate the effect of PHEO extracted compounds on protein expression of β-catenin and FZD7 inhibition. According to the results, wnt pathway genes were changed in samples treated using PHEO. The results showed the up-regulation of GSK-3β and down-regulation of Wnt-1, LEF-1, TCF1, and CTNNB1 genes compared to the control.

Conclusion: We showed inhibition of β-catenin protein in cancer cell lines. Four compounds of PHEO were suggested to have an inhibition effect on β-catenin and FZD7. These compounds can be useful in the treatment of gastrointestinal cancers. Altogether, the inhibitory role of β-catenin protein can be very effective and can be considered one of the therapeutic goals in the treatment of gastrointestinal cancers.

1. Introduction

Gastrointestinal cancers are among the most commonly diagnosed cancers that cause mortality with high costs of treatment [1]. Gastric cancer occurs when cancer cells form on the lining of the stomach and spread from the stomach to other organs, especially the liver, lungs, bones, and lymph nodes. Liver cancer, which presents as a lump in the upper right abdomen with general symptoms such as jaundice and weakness, is more common in men than women [2]. Colorectal cancer or colon cancer will occur when cancer cells grow and multiply in the rectum or colon. Common treatments for gastrointestinal cancer usually include a combination of surgery, radiation therapy, and chemotherapy that have many side effects [3]. In invasive tumors of the gastrointestinal tract, especially liver and stomach cancer, most common treatments are ineffective [4]. Besides, the wnt pathway plays an important role in tissue development during the embryonic and postnatal periods as well as tissue homeostasis [5]. The signal pathway by the Wnt family is one of the basic mechanisms that lead the cell to cell proliferation, polarity, and cell fate during embryonic development and tissue homeostasis. As a result, mutations in the Wnt pathway are often associated with birth defects, cancer, and other human diseases. This signal pathway contains proteins that transmit signals and messages through cellular receptors to the cell [6]. The wnt signaling pathway has been shown to play several key cellular functions, including regulating gene expression, genomic reprogramming, gene expression activation, and other vital roles. Recently, many studies have shown that the wnt signaling pathway is not regulated in cancer tissues and cells; it is

suggested that these may be important contributors to tumorigenesis. Several studies have shown that the signaling pathway of wnt genes is frequently malfunctioned in several different cancers [7]. The first evidence to study the Wnt pathway and its role in the incidence of cancer was obtained in 1994 during a study on acute human myeloid leukemia. They were able to identify and isolate a population of AML cells with CD34 + / CD38 surface markers from a person with leukemia. Therefore, they should be considered for cancer treatment. Understanding and identifying this path will help us achieve this goal [8]. Due to the wide variety of roles of the wnt signaling pathway, it can be a starting point for functional studies to identify other genes and proteins that can be used to make new and specific drugs in gastrointestinal tumors [9]. On the other hand, today, herbal medicines play an essential role in the fields of biology and medicine and are one of the requirements of basic and applied research. So far, various studies on different types of cancer have been performed using herbal compounds [10]. Pistachio with the scientific name of *Pistacia vera* is a two-stemmed tree that grows geographically in large areas of the Mediterranean and the Middle East. Pistachios are highly nutritious and contain vitamins B1, B3, E and nutrients including protein, beneficial fatty acids including linoleic acid (omega 3, 6 and 9 fatty acids) and palmitic acid, stearic acid. Pistachios also contain nutrients such as iron, zinc, potassium and calcium. Pistachio hull is rich in phenolic and antioxidant compounds, including quercetin, epicatechin, luteolin and cyanidin-3-a-glucoside [11]. These compounds are referred to as natural antioxidants. Pistachio peel also has pharmacological activities including anti-diabetic, anti-tumor, anticholinesterase, antimicrobial and antifungal activity [12]. Numerous studies have been conducted on the therapeutic properties of pistachios and have shown that this plant has many therapeutic effects [13]. Also, this plant is a rich source of chemical drugs due to its ease of access and reduction of side effects compared to chemical drugs and its reasonable price [14]. In this study, due to the presence of beneficial compounds in different parts of pistachios and the fact that so far no research on the effect of these compounds in pistachio hull essential oil (PHEO) on the expression of Wnt / β-catenin signaling pathway genes in gastrointestinal cancer cells (stomach, liver and clone) Therefore, in this study, we decided to investigate the effect of PHEO on the expression of some of these genes.

2. Materials And Methods

Pistachio green shell variety of cultivar Ohadi was collected and taken between the beginning of August and the end of October 2021, then spread on a bed in the shade and dried at room temperature. The hull parts were stored in a dark place until used. The Clevenger system was used to extract the essential oil from the Pistachio hull. 150 g of Pistachio hull was weighed and immersed separately in 2,000 mL of distilled water for this purpose. When the contents of the Clevenger's balloons were boiled for 4 hours, the extraction time began. Sodium sulfate was used at the end of the extraction process to remove the moisture content of the samples, which were then stored at -20°C.

2.1 Gaschromatographymass spectrometry

An Agilent 7890A apparatus Co., USA (0.25 µm flm × 0.25 mm i.d. × 30 m length) interfaced with a quadruple mass detector and a computer equipped with the Wiley 7n.Llibrary was used for GC-MS

analyses. The following were the other analytical settings:

[50°C (5 minutes), 50°C-250°C (3°C/min), 250°C (10 minutes)]; injector temperature, 250°C; injection volume, 0.1 L; split ratio, 1:50; carrier gas, helium with 1.1ml/min flow rate; ionization potential, 70 eV; ionization current, 150 A; and mass range, 35–465.

Individual compounds were identified by comparing their mass spectra and retention indices (RI) to those of authentic samples and those found in the literature. The relative amount of the individual components was quantified using the area percentage without taking the calibration factor into account.

2.2 Cell culture

Human gastric cancer cell line (AGS), human hepatocellular carcinoma cell line (PLC/PRF/5), and human colon cancer cell line (CACO2) were purchased from Pasteur Institute of Iran in Roswell Park Memorial Institute (RPMI) 1640 Medium supplemented with (Fetal Bovine serum) FBS (10%). Human gingival fibroblast cell line (HGF1-PI 1) was obtained from Iran Pasteur Institute in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS and placed in an incubator in 5% CO2 at 37 ° C.

2.3 MTT assay

The MTT test is performed with a yellow compound called (1- 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide). This compound is reduced by reductase after entering the mitochondria of living cells and converted to purple material called formazan, so in cases where there are more living cells, due to the greater activity of mitochondrial reductase enzyme, MTT is converted to more formazan and as a result, more purple color is produced. In other words, more purple color indicates more living cells. 103–105 cells were seeded in each well of 96 well plate and different concentrations of the PHEO (12.5, 25, 50, 100, and 200 µg/ml) were used for 24 hours. After the incubation time, the culture medium was discarded and 150 µl fresh medium containing 5 mg/ml MTT was added for 4 hours. After that, 50 µl of DMSO was added to dissolve the crystals of formazan. Finally, the absorbance was read at 570 nm using micro-plate reader. All experiments were done in triplicate. The cytotoxicity (%) was measured at different concentrations and the IC50 values were distinguished [15–18].

2.4 Primer Design

The sequences of primers to amplify the region of the wnt pathway genes with the β-actin gene as an internal control gene were designed using Allele ID software (Table 1).

Table 1
Shows the sequences of primers to amplify the region of the wnt pathway genes with the β-actin gene as an internal control gene were designed using Allele ID software.

Genes	Forward Primer (5'→3')	Reverse Primer (5'→3')
Wnt-1	GCACAGAGCGGGCAAAGC	TGTAAGCAGGTTCGTGGAGGAG
GSK-3β	CTCCTACCCGCTCCTTCC	GCCAACAGACTCCACTTCC
LEF-1	TCGGCAAGGACGGTAACT	GAAGCAGAAACAAAGAGGGAACT
TCF1	CCACACTTCTCAGGACACA	TAGCCACCCAGGAAATGC
GAPDH	TCCCATCACCATCTTCCAGG	TGATGATCTTGAGGCTGTTGTCA
CTNNB1	ACCAAGAAAGCAAGCTCATCA	CTTCAGCACTCTGCTTGTGG
ACTB	CTTCGCGGGCGACGAT	CCACATAGGAATCCTTCTGACC

50 µg/ml was selected to treat the cells for 24 hours. After that, RNAs extracted, cDNAs synthesizes and RT-qPCR reaction done using takapouzist kit (Tehran, Iran) based on the instruction by the supplier. The expression of wnt pathway genes is examined in the presence of different treatments. β-actin and GAPDH genes were used as an internal control and data were analyzed using ΔΔCt method.

2.5 Western blot

50 µg/ml was selected to treat the cells for 24 hours and the protein content of the cells was extracted. After washing the cells using PBS, they were lysed in a specific buffer (150 mM NaCl, 1 percent NP-40, 0.1 percent SDS, 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride [PMSF]).. The lysates were centrifuged at 12,000 g for 20 minutes at 4 ° C, and the protein content was measured using the Bradford assay. Proteins (50 g) were separated by SDS-PAGE (12%) and transferred to nitrocellulose membrane. Cellular lysates were incubated with the β-catenin antibodies overnight at 4 ° C, followed by 2 hours at 4°C with secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). β-actin was used as a control.

2.6 Statistical analysis

Data was analyzed using SPSS software version 18. T-test was used to compare the mean of the experimental groups with the control group and a p-value less than 0.05 was statistically significant in all tests.

2.7 Molecular docking based virtual screening

The protein data bank was used to obtain the 3D structure of FZD7 (PDB ID: 5T44) and *β*-Catenin protein (PDB ID: 1JDH). The preparation process was carried out to convert the macromolecular structures into appropriate forms to be used in computational experiments. These include removing water molecules, adding and optimizing hydrogen bonds, and removing atomic clashes, which are required prior to the

molecular docking of a protein structure. PubChem was used to retrieve the structure of compounds in SDF format. Then, their structure was subjected to energy minimization and bond angle optimization.

Molecular docking was used to estimate the binding pose of all the retrieved ligands to the target proteins. The molecular docking study is now widely used in computer-aided drug design (CADD) to determine the best drug binding mode to a specific macromolecule. The docking was carried out using the AutoDock Vina tool from PyRx virtual screening software. PyRx is a tool used in CADD for the virtual screening of libraries of compounds against a specific drug target. It provides a more reliable and user-friendly docking tool and uses the Lamarckian genetic algorithm (LGA) as a scoring function in both AutoDock 4 and AutoDock Vina. The tool is used to search through a library of substrates for a specific drug target. Default configuration parameters of the PyRx program were used for docking. The complexes with the lowest binding energy (kcal/mol) were chosen for further investigation. The binding interaction between the pair of ligand and protein was also observed using the BIOVIA Discovery Studio Visualizer software.

2.8 Pharmacokinetics study

The drug candidates were assessed for their potential for absorption, distribution, metabolism, and excretion (ADME) characteristics. The assessment was carried out based on the pharmacokinetics study to look into the behavior of candidates in these four characteristics before their practical test in clinical trials. The ADME profile optimization is critically important to obtain success in the clinical and commercial test of a drug. The ADME profile influences physicochemical properties including hydrophobicity, lipophilicity, gastrointestinal environment, and blood-brain barrier before the drug is excreted from the body. The Swiss-ADME online server was used to evaluate the ADME properties of the compounds.

2.9 Toxicity analysis

Another assessment was conducted to investigate the toxicity of the drug compounds. The toxicity test determines the safety profile of the compound in drug development. The aim of the preclinical toxicity test is to look for certain hazards in the profile of the compounds. These include a quantitative and qualitative test of mutagenicity, carcinogenicity, and immunotoxicity. The toxicity test of the compounds was carried out using the ADMETlab 2.0 web server. Different toxicological pathways are provided by the server such as stress response pathways and nuclear receptor signaling pathways.

3. Results

3.1 GC/MS Analysis

Chemical characterizations of the PHEO analyzed by GC–MS are shown in Table 2. This pistachio hull oil had been patented (Patent number: US9732305B2). In this study we evaluated the chemical composition and some biological activities of the PHEO from Pistacia Ohadi. The yield of PHEO was 0.5% (v/w) on a dry weight basis. Fifty six constituents were detected in the PHEO accounting for 99.8% of the total PHEO

composition. The major components were DL-Limonene (61.08%), Alpha Phellandrene and Alpha Thujene (11.65%), Alpha Pinene (8.11%), and Alpha Terpinolene (7.8%) respectively.

Table 2
Chemical composition of *pistacia vera* hull essential oil (PHEO)

No.	Compound	%	Boiling point (⁰C)
1	DL-Limonene	61.08	176
2	Alpha Phellandrene	11. 65	171–172
3	Alpha Thujene		
4	Alpha Pinene	8.11	155
5	Alpha Terpinolene	7.8	185
6	Delta 3-Carene	1.82	167–169
7	Beta Myrcene	1.79	166–168
8	Acetic acid	1.79	118–119
9	Bicyclo[2.2.1]heptan-2-ol,		
10	Camphene	1.62	159
11	Beta Pinene	1.47	165–167
12	gamma Terpinene	0.80	183
13	3-Cyclohexen-1-ol	0.66	
14	Alpha Terpinene	0.54	173.5-174.8
15	Sabinene	0.35	163–164
16	1,3,6-Octatriene,	0.35	
17	(+)-4-Carene (+)-2-CARENE	0.16	
18	2-Pentadecanone	0.0	

3.2 MTT assay analysis

The effect of PHEO on cell life at different concentrations (12.5, 25, 50, 100, and 200 µg/ml) showed that the percentage of cell viability depends on the concentration of PHEO. The IC50 values for PLC/PRF/5, Caco2, and AGS cells were 180, 172, and 195 µg/ml in the 24-hour test, respectively.

3.3 Real time RT-qPCR

After statistical analysis by t-test (SPSS software), the significance of the results was confirmed and a concentration of 50 µg/ml was selected as the appropriate concentration for the final treatment in 24

hours. The expression level of Wnt-1, GSK-3β, LEF-1, TCF1, and CTNNB1 genes in the Wnt signal pathway were changed in PLC/PRF/5, Caco2, AGS cells treated with PHEO at a concentration of 50 µg/ml for 24 hours. Multiplication and quantitative comparison of the effect of PHEO on gene expression showed that the expression of GSK-3β gene transcripts increased at a concentration of 50 µg/ml compared to the control, while the expression of Wnt-1, LEF-1, CTNNB1 and TCF1, and genes increased. All experiments were performed three times (Fig. 2).

3.4 Western Blot

The increase in β-catenin after treatment using PHEO was also confirmed by Western blot method. β-catenin protein which was over expressed before treatment was detected on nitrocellulose membrane containing total protein extracted from the cells 24 hours after treatment (Fig. 3).

3.5 Molecular docking analysis

The molecular docking study was conducted to identify the best intermolecular framework formed between the FZD7 (*PDB* ID: 5T44) and *β-Catenin* (*PDB* ID: 1JDH) proteins and the phytochemical compounds. The study was carried out using AutoDock Vina wizard from the PyRx software. The study specifies that the phytocompounds have binding affinities within a range between – 3.0 and – 7.0 kcal/mol. Among phytochemicals, four compounds on the top of the list were selected based on a lower binding affinity. Table 3 shows the binding affinity of the extracted phytochemicals of PHEO with *β-Catenin and FZD7 proteins.* The four highest binding affinities that show lower energy in these tables are bolded. The visualized details of the interactions between *β-Catenin* and Delta 3-Carene also FZD7 and Alpha Terpinolene are represented in Fig. 4.

Table 3
The binding affinity of extracted phytochemicals of *pistacia vera* hull essential oil (PHEO) with *β-Catenin and FZD7* proteins.

Compounded name	Pubchem CID	*β-Catenin*	*FZD7*
DL-Limonene	440917	-4.6	-5.7
Alpha Phellandrene	7460	-4.4	**-6.0**
Alpha Thujene	6451618	-4.6	-5.1
Alpha Pinene	6654	-4.8	-4.8
Alpha Terpinolene	11463	**-4.9**	**-6.8**
Delta 3-Carene	442461	**-5.1**	-5.2
Beta Myrcene	31253	-4.4	-5.1
Acetic acid	176	-3.0	-2.8
Bicyclo [2.2.1] heptan-2-ol,	79028	-4.0	-4.0
Camphene	6616	-4.7	-4.7
Beta Pinene	14896	**-5.0**	-4.8
gamma Terpinene	7461	-4.7	**-6.3**
3-Cyclohexen-1-ol	556685	-3.7	-3.9
Alpha Terpinene	7462	-4.7	-5.4
Sabinene	18818	-4.4	-4.8
1,3,6-Octatriene,	5367382	-3.8	-5.0
(+)-4-Carene (+)-2-CARENE	78249	**-4.8**	**-5.8**
2-Pentadecanone	61303	-3.8	-4.3

3.6 Pharmacokinetics study

The pharmacokinetic characteristics of the compounds in interaction with the target macromolecules were investigated based on the evaluation of the ADME properties of the selected compounds. The favorable and unfavorable pharmacological features of a drug candidate are determined through the study. These include absorption, distribution, metabolism, and excretion. A drug candidate may be rejected before further continuing the drug design process based on the information related to its unfavorable features. The SwissADME webserver was used to evaluate the ADME properties of selected compounds. The ADME properties of four low-energy selected compounds generated by the SwissADME server are represented in Table 4 including pharmacokinetics and physicochemical properties, lipophilicity, water-solubility, drug-likeness, and medicinal chemistry. A key factor of a drug molecule

across the biological barrier is its permeability which is affected by the molecular weight. The permeability of a drug is decreased with a higher molecular weight. Lipophilicity is another property that influences the absorption of the drug molecule within the human body. It is calculated by the logarithm of the inorganic and aqueous phase partition coefficient of the target molecule (LogP). There is a correlation between the higher value of this parameter and the lower absorption of the drug candidate. The water solubility is another property of a drug candidate that is calculated by the LogS parameter. The lower value of solubility indicates the higher solubility of the molecule. The number of donors and acceptors of hydrogen bonds identifies the capacity of a drug molecule to cross the membrane bilayer. From the results in Table 4, it is confirmed that all compounds can successfully use for further investigation as drug candidates.

Table 4
Pharmacokinetics properties of the selected four compounds of *pistacia vera* hull essential oil (PHEO).

	Properties	**PubChem CID: 7460**	**PubChem CID: 11463**	**PubChem CID: 442461**	**PubChem CID: 78249**	**PubChem CID: 14896**
Physicochemical properties	Molecular Weight (g/mol)	136.23	136.23	136.23	136.23	136.23
	Heavy atoms	10	10	10	10	10
	Arom. heavy atoms	0	0	0	0	0
	Rotatable bonds	1	0	0	0	0
	H-bond acceptors	0	0	0	0	0
	H-bond donors	0	0	0	0	0
Lipophilicity	Log Po/w	2.97	3.40	3.42	3.12	3.42
Water solubility	Log S (ESOL)	-2.64	-3.50	-3.44	-2.48	-3.31
Pharmacokinetics	GI absorption	LOW	LOW	LOW	LOW	LOW
Drug-likeness	Lipinski	Yes	Yes	Yes	Yes	Yes
Medi.Chemistry	Synth. Accessibility	4.15	2.98	3.48	3.84	3.73

3.7 Toxicity test

A toxicity test is another key step in drug design through the in-silico approach, which examines the safety of a drug candidate. The toxicity of the compounds was examined by submitting them to the admetlab 2.0 online server. The server assesses several properties related to toxicity such as AMES, hERG, carcinogenicity, P-glycoprotein inhibitor (Pgp), and Rat (LD50) value. The results are shown in Table 5 for four selected compounds. From the results in Table 5, it is confirmed that all compounds except (PubChem CID: 11463) have no toxicity effect.

Table 5
Toxicity test results of four selected compounds of *pistacia vera* hull essential oil (PHEO) produced by admetSAR online server.

Compound ID	PubChem CID: 7460	PubChem CID: 11463	Pubchem CID: 442461	PubChem CID:78249	Pubchem CID: 14896
HERG toxicity	N	N	N	N	N
AMES toxicity	N	N	N	N	N
Carcinogenicity	M	Y	N	N	N
Lipinski Rule	Accepted	Accepted	Accepted	Accepted	Accepted
Rat (LD50)	N	N	N	N	N
Pgp-inhibitor	N	N	N	N	N

4. Discussion

Natural drugs have been shown to be effective against gastrointestinal cancers through a variety of mechanisms of action, including modulation of inflammatory mediators, anti-oxidative stress defense, antioxidant performance, enzymatic activity, and cytoprotective properties [19].

Several studies have confirmed the antioxidant effects of Pistacia essential oil, which could be considered one of its anti-oxidant mechanisms.

GC/MS method was used to measure the phytochemicals of PHEO. The chromatogram showed 18 peaks that are related to various compounds. The essential oil of this extracted oil was found to have anti-cancer activity. The administration of various doses of essential oil demonstrated a significant restriction effect against gastrointestinal cancer cell invasion in a dose dependent manner. Cell viability was significantly reduced after high dose administration of PHEO (200 µg/ml) was used compared to control.

Besides, Delazar et al 2004, identified eleven compounds in the essential oil extracted from the oleoresin of *Pistacia atlantica var.* mutica, the main constituents of which were -pinene (70 percent), citral (5.72 percent), myrtenol (5.31 percent), carveol (2.18 percent), epoxypinene (2.15 percent), and -pinene (1.94 percent) [20].

As a result, the concentration of Alpha pinene in the current essential oil was significantly higher than that reported by Delazar and his colleagues. Sharifi et al 2011, reported that seven compounds were identified in essential oil from *Pistacia atlantica var.* kurdica oleoresin, the most abundant of which was pinene (97.18 percent) and -pinene (1.26 percent) [21].

For the first time, we confirmed the PHEO anti-cancer effect on a gastrointestinal cancer cell lines. Furthermore, a western blot confirmed that the PHEO had protective effects. Alpha-Pinene, as one of the main components of the essential oil, may be responsible for the PHEO gastrointestinal-protective functions. Besides, Wnt signaling dysregulation contributes to the progression of several major human cancers, including colorectal, liver, and prostate cancer [22].

We demonstrated activation of some components of the Wnt pathway in gastrointestinal cancer cells by using plant essential oil. In recent years, the role of changes in the expression of wnt pathway genes in the metastasis and recurrence of cancer has been investigated. This pathway has also developed resistance to treatment, which can be also involved in the metastasis of cancer cells [23]. This pathway also plays an important role in the development of tissues during the embryonic period and after birth. Studies have shown that the Wnt focal pathway, which is dependent on the beta-catatin protein, plays an important role in the control of cancer cells [24]. Beta-catenin interacting protein (CTNNBIP1) is known as an inhibitory protein of interaction between beta-catenin protein and members of the TCF / LEF family, which acts as a negative regulator of the Wnt / β-catenin signaling pathway. In cancer cells, the expression of this protein decreases while the expression of beta-catatin protein inside the cancer cell is higher than usual [25]. Our data confirmed that the expression level of β-catenin decreased after treatment at mRNA and protein level using real time and western blot tests. On the other hand, due to the fact that gastrointestinal cancers are increasing in developed countries [26] and these cancers have shown drug resistance in most cases [27]. Therefore, the effort to find new treatments against this type of cancers has become extremely crucial and also the pistachio tree is very widespread all around the world. Although such a study has not been done before, this study aimed to determine the difference in cytotoxicity of the PHEO on gastrointestinal cancer cells. PHEO based on GC analysis mainly contains beta-pinene, gamma-terpinene, paracimen and limonene. Due to the importance of studying the mechanism and factors involved in the development and progression of gastrointestinal cancers, we study the results of treatment of PLC/PRF/5, Caco2, AGS cell lines gene expression and also protein analysis.

It was found that the β-catenine protein level was reduced after treating cells using PHEO. In the absence of Wnt protein, the cytoplasmic protein of beta-catinine is continuously degraded by the Axin complex. The Axin complex consists of the Axin protein scaffold, a product of the tumor suppressor gene for adenomatous polyposis (APC), casein kinase 1 alpha (CK1α) and glycogen synthase kinase 3 (GSK3). CK1α and GSK3 phosphorylate the amino terminal region of beta-catenin, respectively. As a result, beta-catenin is identified by b-Trcp and the E3 unit of ubiquitinin ligase, after which beta-catenin is ubiquitinated and degraded by the proteasome. This continuous deletion of beta-catenin prevents beta-catenin from reaching the nucleus, and as a result, Wnt target genes are suppressed by DNA-binding

proteins of the DNA cell / lymphoid-boosting factor (TCF / LEF). The Wnt / b-catenin pathway is activated when the Wnt ligand binds to receptor through the Frizzled (Fz, Fzd) and its helper is a low-density lipoprotein receptor associated with protein 6 (LRP6), or close to LRP5. The formation of the Wnt-Fz-LRP6 complex, together with the arrival of the dishevelled scaffold protein (Dvl), leads to the phosphorylation of LRP6 and the activation and entry of the Axin complex into the receptor. These events inhibit the phosphorylation of β-catenin by Axin, thereby stabilizing β-catenin, which results in its accumulation and send to the nucleus to form a complex with TCF / LEF and activation of Wnt target genes, target gene expression. Beta-catenin is a multifunctional protein that, in addition to participating in cellular connections, is a component of the Wnt signaling pathway [28]. The cytoplasmic concentration of this protein in normal cells is completely controlled. Increased beta-catenin activity has been reported in many human tumors, including hepatocellular carcinoma (liver), breast cancer, colon cancer, lung cancer, ovarian cancer, and uterine mucosal cancer [29]. Beta-catenin interacting protein (CTNNBIP1) is a beta-catenin-interacting protein that encodes its own gene and negatively controls the Wnt / β-catenin signaling pathway. This protein works by inhibiting the interaction between the β-catenin protein and members of the TCF family. In fact, this protein acts as an inhibitor of beta-catatin [30]. Our data also confirm that the expression level of CTNNBI was increased while the β-catatin protein was decreased after treatment with the PHEO. Furthermore, compounds of PHEO were investigated for their inhibition effect on β-catenin. The results illustrated that compounds extracted from PHEO can be useful in the treatment of gastrointestinal cancers.

Although, when we examined the molecules involved, we discovered that the molecular patterns differed greatly between cancer cell lines and control. It is preferable to conduct bioinformatic and biological research to investigate the underlying exact mechanisms.

5. Conclusion

The current study confirmed the use of Pistachio oil in traditional medicine as a dietary supplement and complementary medicine for the treatment of GI cancers. The results showed that PHEO, which mainly contains DL-Limonene, Alpha Phellandrene, Alpha Thujene, and Alpha Pinene, can reduce the proliferation of gastrointestinal cancer cells. Treatment with the PHEO also decreases the expression of β-catatin gene. β -catenin is one of the major proteins in the Wnt signal pathway and the decreased expression of β-catenin gene in a sample treated with the PHEO indicates the inhibitory role of this material at a specific concentration on the signal pathway. Therefore, the relationship between b-catenin levels and GI cancers has the potential to improve cell death in the future.

Declarations

Acknowledgments

The authors would like to express their sincere gratitude to the Pistachio Safety Research Center, Rafsanjan University of Medical Sciences (RUMS), Rafsanjan, Iran for their valuable assistance. This

study was funded by Rafsanjan University of Medical Sciences, Rafsanjan, Iran under ethics code IR.RUMS.REC.1400.061 at RUMS.

Funding

This study was financially supported by Rafsanjan University of Medical Sciences, Rafsanjan, Iran, under the ethical code of IR.RUMS.REC.1400.061 at RUMS.

Conflicts of interest/Competing interests

All authors declare that they have no conflict of interest.

Availability of data and material

Not applicable

Authors' contributions

N.A. and S.K.F.; contributed to design and implementation of the research and also contributed substantially to the drafting, writing and revising of the manuscript. S.M.B.M. and Z.P.; participated in the collection of data. F.B. contributed to the analysis and interpretation of data. All authors have approved the final version of the manuscript.

Code availability

Not applicable

Ethics approval

This project was approved by the Ethics Committee of Rafsanjan University of Medical Sciences, Rafsanjan, Iran under the ethical approval number “IR.RUMS.REC.1400.061” at RUMS.

Consent to participate

Not applicable

Consent for publication

Not applicable

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Figures

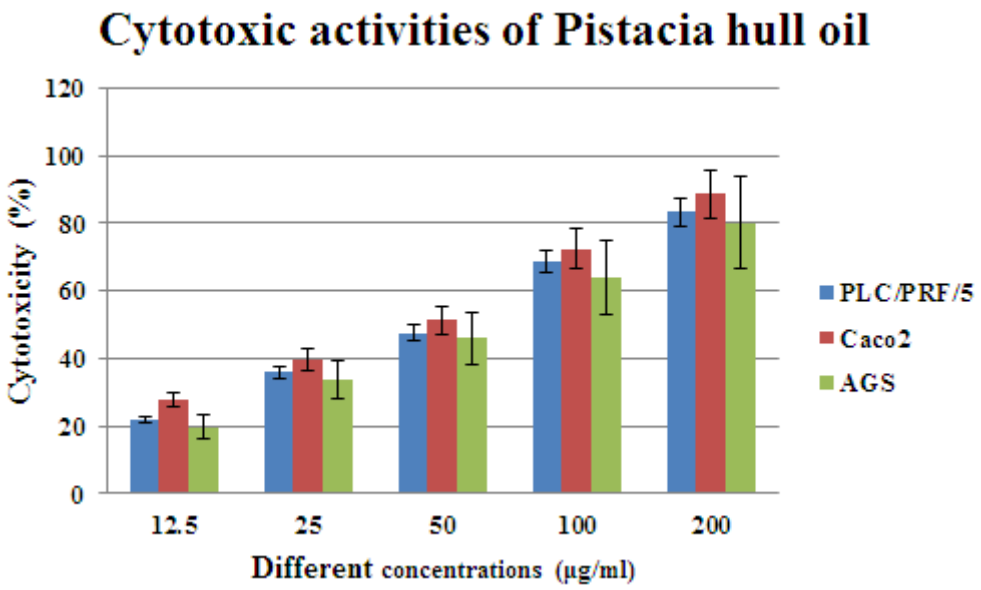

Figure 1

Shows the cytotoxicity effect of different concentrations of *pistacia vera* hull essential oil (PHEO) on PLC/PRF/5, Caco2, and AGS determined by MTT assay ($p<0.05$)

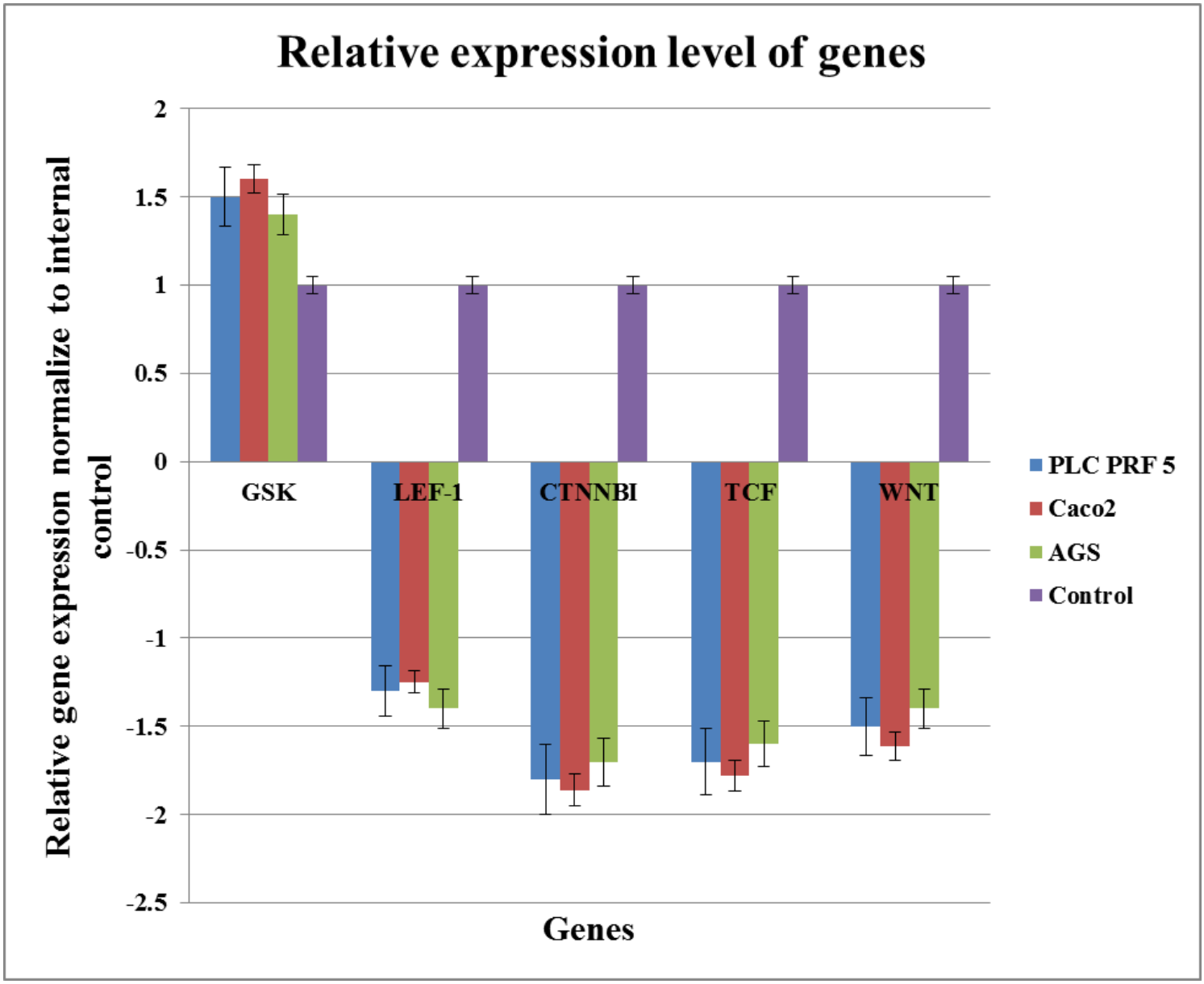

Figure 2

Shows the Bar chart of the effect of pistachio hull essential oil (PHEO) on the level of gene expression (Pvalue <0.05 *, significance of gene expression of the treated sample versus the control sample by T-test).

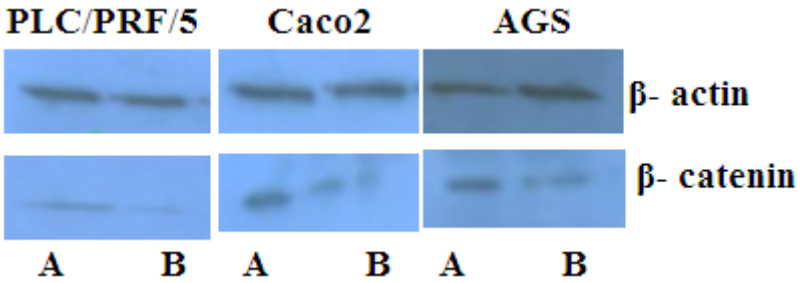

Figure 3

Shows the western blot analysis of B-catenin protein demonstrates its expression in the cells after treatment using pistachio hull essential oil (PHEO), A: before treatment, B: after treatment.

Figure 4

Shows the 3D (left side) and 2D (right side) visualization of interactions between protein and ligand (A) *β-Catenin and* Pistachio hull essential oil (PHEO), and (D) *FZD7 and* PHEO.