Cellular & Molecular Biology
Cell. Mol. Biol. 2015; 61 (7): 128-134
Published online November 30, 2015 (http://www.cellmolbiol.com)
Received on October 20, 2015, Accepted on November 26, 2015.
doi : 10.14715/cmb/2015.61.7.20

Induction of apoptosis by pistachio (*Pistacia vera* L.) hull extract and its molecular mechanisms of action in human hepatoma cell line HepG2

J. Fathalizadeh[1,2], V. Bagheri[1], H. Khorramdelazad[3], M. Kazemi Arababadi[1], A. Jafarzadeh[2,3], M. R. Mirzaei[3,4], A. Shamsizadeh[5] and M. R. Hajizadeh[3,6]✉

[1] Immunology of Infectious Diseases Research Center, Rafsanjan University of Medical Sciences, Rafsanjan, Iran
[2] Department of Microbiology and Immunology, Rafsanjan University of Medical Sciences, Rafsanjan, Iran
[3] Molecular Medicine Research Center, Rafsanjan University of Medical Sciences, Rafsanjan, Iran
[4] Department of Genetics, Rafsanjan University of Medical Sciences, Rafsanjan, Iran
[5] Department of Physiology and Pharmacology, Rafsanjan University of Medical Sciences, Rafsanjan, Iran
[6] Department of Clinical Biochemistry, Rafsanjan University of Medical Sciences, Rafsanjan, Iran

Corresponding author: M. R. Hajizadeh, Department of Clinical Biochemistry, Rafsanjan University of Medical Sciences, Rafsanjan, Iran. Email: hajizadehus@yahoo.com

Abstract

Several important *Pistacia* species such as *P. vera* have been traditionally used for treating a wide range of diseases (for instance, liver-related disorders). There is a relative lack of research into pharmacological aspects of pistachio hull. Hence, this study was aimed at investigating whether pistachio rosy hull (PRH) extract exerts apoptotic impacts on HepG2 liver cancer cell line. In order to evaluate cell viability and apoptosis in response to treatment with the extract, MTT assay and Annexin-V-fluorescein/propidium iodide (PI) double staining were performed, respectively. Moreover, molecular mechanism of apoptosis induced by the extract was determined using human apoptosis PCR array. Our findings showed that PRH extract treatment reduced cell viability (IC_{50} ~ 0.3 mg/ml) in a dose-dependent manner. Flow cytometric analysis revealed that the extract significantly induced apoptosis in HepG2 cells. In addition, quantitative PCR array results demonstrated the regulation of a considerable number of apoptosis-related genes belonging to the TNF, BCL2, IAP, TRAF, and caspase families. We observed altered expression of both pro-apoptotic and anti-apoptotic genes associated with the extrinsic and intrinsic apoptosis signaling pathways. These results suggest that the aqueous extract of PRH possesses apoptotic activity through cytotoxic and apoptosis-inducing effects on HepG2 cells.

Key words: Pistachio hull, *Pistacia vera* L., apoptosis, HepG2 cells, liver cancer.

Introduction

Pistachio (*Pistacia vera* L.), a member of the Anacardiaceae family, is mainly found in Greece, Iran, Italy, Syria, Turkey, and USA (1, 2). Various parts of plant, including fruit, leave, resin, and seed have pharmacological properties such as anti-inflammatory, antioxidant, and antimicrobial activities (3-8). Fruits of *Pistacia* species have been widely used in the traditional Iranian medicine (TIM) for the treatment of liver, kidney, heart, and respiratory system diseases. Phytochemical studies have shown that different parts of *P. vera*, including kernel, seed, and skin contain phenolic compounds (9). Hull, which is a part of the fruit, is one of pistachio by-products produced every year at harvest time and can lead to environmental pollution. In comparison to other parts of pistachio plant, hull has higher levels of phenolics and antioxidant activity (10). Among several solvents examined, water is able to extract a higher content of hull phenolic compounds (11). Furthermore, antioxidant, antimicrobial and antimutagenic properties of pistachio green hull aqueous extract have been previously reported (12). Globally, liver cancer is the sixth most common type of cancer and also the third cause of cancer mortality (13). International trends show a rise in liver cancer incidence rates in developed countries (14). Currently, there is no effective therapies for liver cancer, and therefore, the development of novel strategies to treat this cancer is of critical importance. Polyphenols are phytochemicals with antioxidant and anti-inflammatory properties which have been suggested as preventive and therapeutic agents against hepatocarcinogenesis (15). Apoptosis, programmed cell death, is a biological process with specific morphological and biochemical features playing a role in development, aging, and maintaining homeostasis (16). We now know that anticancer drugs use apoptosis induction to exert their cytotoxic actions. Thus, several direct or indirect apoptosis-inducing strategies can be developed to target cancer cells (17). Many natural products have the potential to trigger apoptosis in numerous human cancer cell types. Hence, it is necessary to search for new plant-derived products as inducers of apoptosis (18). Additionally, a number of plant-based agents or/and their semisynthetic derivatives are clinically used in cancer treatment, including vinblastine, vincristine, camptothecin, podophyllotoxin, and paclitaxel (Taxol) (19, 20).

In the present study, we investigated cytotoxic and apoptosis-triggering effects of PRH extract on HepG2 cells. The mechanism of action of extract on apoptosis was also evaluated.

Materials and methods

Plant collection and extract preparation

Pistachio fruits (*Pistacia vera* L.) were collected from Rafsanjan, Kerman province (September 2013) and authenticated by Physiology-Pharmacolo-

gy Research Center, Rafsanjan University of Medical Sciences. Then, rosy hulls were separated, air-dried at room temperature for one week, and ground into powder. The powdered sample (50 g) was macerated with water (200 mL) for 48 h at room temperature under continual shaking. The extract was filtered by Whatman filter paper under vacuum and then evaporated at 40 °C using rotary evaporator. Finally, the dried extract was stored at -20 °C until further investigation.

Cell culture

The human hepatocellular carcinoma HepG2 cells were kindly provided by Dr. Asadi (Kerman University of Medical Sciences, Kerman, Iran). HepG2 cells were cultured in RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum (Gibco), penicillin (100 U/ml), streptomycin (100 µg/ml) and incubated at 37 °C in a 5% CO2 incubator.

MTT assay

The cytotoxicity of extract was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in triplicate. Briefly, HepG2 cells were plated in 96-well plates at 1×10^4 cells per well in 100 µL RPMI 1640 medium and incubated overnight at 37 °C. After incubation, cells were treated with 100 µL of fresh medium containing different concentrations of PRH extract (0.02-0.5 mg/ml) for 24 h. MTT (Sigma-Aldrich) stock solution was made in PBS (5 mg/ml) and stored at 4 °C. After treatment, 20 µL of MTT stock solution was added to each well and the plates were incubated. After 4 h incubation, the culture media was removed and 100 µL of DMSO was added to each well to dissolve formazan crystals. Subsequently, the plates were agitated for 15 min and absorbance (A) of each well was read at 570 nm on an ELISA plate reader. Cell viability was determined using $(A_{sample}/A_{control}) \times 100$.

Apoptosis assay

In order to evaluate induction of apoptosis by PRH extract, Annexin-V-FLUOS staining kit (Roche Diagnostics) was employed, according to manufacturer's instructions. This double staining method differentiates between apoptotic and necrotic cells. Briefly, cells were treated with two different concentrations of extract (0.1 and 0.3 mg/ml) at 37 °C for 24 h. After treatment, 1×10^6 cells were washed with PBS and centrifuged at 200 × *g* for 5 min. The cell plet was resuspended in 100 µL of Annexin-V-FLUOS labeling solution containing Annexin-V-FLUOS labeling reagent and propidium iodide solution. The cell suspensions were then incubated for 15 min at 25 °C. Triplicate samples were analyzed on a BD FACSCalibur flow cytometer.

RT² Profiler PCR array

RT² Profiler PCR Array (SABiosciences) was used to analyze expression of a panel of genes involved in apoptosis pathway (Human Apoptosis PCR Array, Catalog No. PAHS-012). The PCR array contains 84 apoptosis-related genes, five housekeeping genes, and three RNA and PCR quality controls. Cells were treated with either medium containing 0.3 mg/ml of extract or control medium at 37 °C. After 24 h of treatment, cells were then washed with PBS and harvested for RNA isolation. RNA was extracted using RNeasy Mini kit (Qiagen) according to manufacturer's instructions. Spectrophotometry and agarose gel electrophoresis were used to assess RNA purity and integrity. The first strand cDNA was synthesized using RT² First Strand kit (SABiosciences) from 1.0 µg of total RNA for 96-well plate. A CFX96 Real-Time PCR Detection System (Bio-Rad, USA) was used for real-time PCR analysis. For each PCR array plate, experimental cocktail was made containing 1275 µL of RT² qPCR master mix, 102 µL of first strand cDNA synthesis reaction, and 1173 µL of ddH_2O. A 25 µl reaction volume of the experimental cocktail was aliquoted into each well of the PCR Array. The real-time PCR thermal cycling program was as follows: one cycle at 95 °C for 10 min; 40 cycles at 95 °C for 15 s, 55 °C for 40 s, and 72 °C for 30 s. Finally, resulting threshold cycle (C_T) values were exported and analyzed using web-based software RT² Profiler PCR Array Data Analysis version 3.5 (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php). Fold changes were calculated by using the ΔΔCT method. PCR array was done in triplicate for each sample (extract-treated sample and control sample).

Statistical analysis

We performed all tests in triplicate. The results were expressed as mean ± standard error of the mean (SEM). Data were analyzed by one-way analysis of variance (ANOVA) and $P < 0.05$ was considered to be a statistically significant difference.

Results

Effect of PRH extract on cell viability

We used MTT viability assay to evaluate the potential cytotoxicity of PRH extract against HepG2 cell line. In this study, different concentrations of extract (0.02, 0.05, 0.07, 0.1, 0.2, 0.3, 0.4, and 0.5 mg/ml) were applied. In a dose-dependent manner, extract exhibited a decrease in cell viability rate compared to control after 24 h treatment (Fig 1). For instance, the results showed that viability decreased significantly ($P < 0.05$) from 0.1 mg/ml to 0.5 mg/ml with an IC_{50} value of approxima-

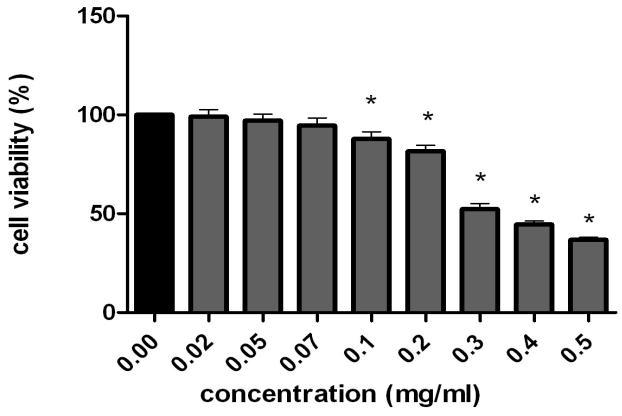

Figure 1. Cytotoxic effects of PRH extract on HepG2 cell line. Cell viability was measured after 24 h incubation with different concentrations (0.02-0.5 mg/ml) of the extract using MTT assay. Values represent mean ± SEM of three different experiments. *Statistically significant difference compared with control ($P < 0.05$)

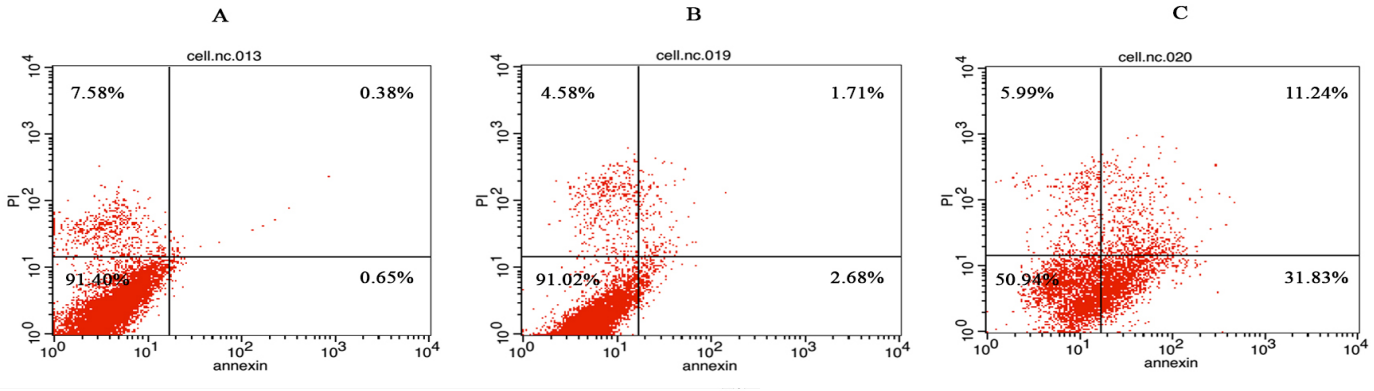

Figure 2. Flow cytometric analysis of HepG2 cells after 24 h treatment with medium (A), 0.1 mg/ml (B), and 0.3 mg/ml PRH extract (C). Scatter plots consist of four quadrants: upper left (Annexin-V$^-$/PI$^+$, necrotic cells), upper right (Annexin-V$^+$/PI$^+$, late apoptotic cells), lower left (Annexin-V$^-$/PI$^-$, viable cells), lower right (Annexin-V$^+$/PI$^-$, early apoptotic cells).

Table 1. Induction of apoptosis evaluated by Annexin-V-FLUOS/PI staining after 24 h treatment with PRH extract.

Treatment	Necrotic cells (%)	Late apoptotic cells (%)	Viable cells (%)	Early apoptotic cells (%)
Control	6.51 ± 0.58	0.45 ± 0.06	92.36 ± 0.49	0.67 ± 0.09
PRH (0.1 mg/ml)	5.41 ± 0.49	0.51 ± 0.11	92.45 ± 0.32	1.61 ± 0.53
PRH (0.3 mg/ml)	6.42 ± 0.34	7.76 ± 0.76	58.22 ± 2.17	27.58 ± 2.21

tely 0.3 mg/ml. In contrast, no significant difference was observed between concentrations below 0.1 mg/ml and control group.

Effect of PRH extract on apoptosis

In order to investigate induction of apoptosis by PRH extract in HepG2 cells, Annexin-V-fluorescein/propidium iodide (PI) double staining was performed (Fig 2). After exposure to 0.1 and 0.3 mg/ml of extract for 24 h, the number of both early and late apoptotic cells increased in comparison with untreated group. According to Table 1, however, a sharp increase in the rate of early apoptosis from 0.67 ± 0.09 to 27.58 ± 2.21 was observed at concentration of 0.3 mg/ml. These results clearly showed that a decline in the number of viable cells is directly associated with an increase in apoptosis.

Effect of PRH extract on apoptosis-related genes

To profile the expression of key genes involved in programmed cell death in response to 24 h treatment of PRH extract (0.3 mg/ml), we carried out real-time PCR using the Human Apoptosis RT² Profiler PCR Array. Multiple members of families, including TNF/TNFR domain proteins, BCL2 and BAG domain proteins, BIR domain proteins, TRAF domain proteins, and caspases were analyzed. Genes with fold-change values greater than 2 (fold-regulation > 2) and less than 0.5 (fold-regulation < -2) were considered to be differentially expressed. Table 2 shows the fold regulation of genes examined on the PCR array. Among selected genes, 8 genes were up-regulated, whereas 14 genes were down-regulated (Fig 3). Accordingly, genes with altered expression were TNF/TNFR family members CD27, FASLG, LTA, TNF, TNFRSF10A, TNFRSF21; BCL2 and BAG family members BAG4, BAX, BCL2A1, BCL2L11; BIR family members BIRC3, XIAP; TRAF family members TRAF2; caspase family members CASP10, CASP14, CASP3, CASP4, CASP6, CASP7, CFLAR, CRADD, PYCARD. However, the most strongly upregulated genes included lymphotoxin alpha (LTA), PYD and CARD domain containing (PYCARD), CD27 molecule (CD27), and caspase 6, apoptosis-related cysteine peptidase (CASP6). In addition, the most strongly downregulated genes included CASP8 and FADD-like apoptosis regulator (CFLAR), TNF receptor-associated factor 2 (TRAF2), baculoviral IAP repeat containing 3 (BIRC3), BCL2-related protein A1 (BCL2A1), caspase 7, apoptosis-related cysteine peptidase (CASP7), BCL2-like 11 (BCL2L11), and CASP2 and RIPK1 domain containing adaptor with death domain (CRADD).

Discussion

The incidence of the most common cancers are decreasing, while liver cancer incidence rate is on the increase (21). Despite all efforts to find new drugs, sorafenib has been demonstrated to be the only effective treatment option for patients with advanced liver cancer (22). Hence, development of new anticancer agents against this type of cancer is of crucial importance. *Pis-*

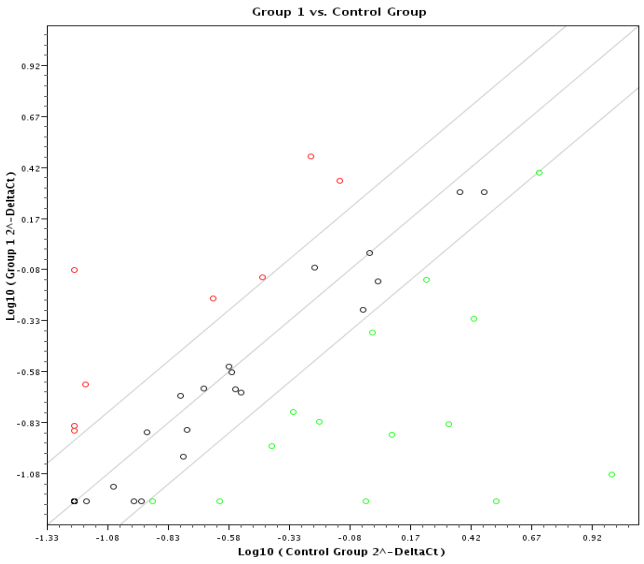

Figure 3. The scatter plot created in the PCR array data analysis webportal compares the normalized expression of each gene on PCR array between 2 groups. The upper left section (red dots) indicates upregulated genes, while the lower right section (green dots) indicates downregulated genes.

Table 2. Expression of apoptosis-related genes evaluated by RT² Profiler PCR Array in HepG2 cells treated with PRH extract for 24 h.

GeneBank	Symbol	Gene Name	Description	Fold Regulation
NM_001242	CD27	S152/S152. LPFS2/T14/TNFRSF7/Tp55	CD27 molecule	3.40
NM_001250	CD40	Bp50/CDW40/TNFRSF5/p50	CD40 molecule, TNF receptor superfamily member 5	1.05
NM_000043	FAS	ALPS1A/APO-1/APT1/CD95/FAS1/FASTM/TNFRSF6	Fas (TNF receptor superfamily, member 6)	-1.74
NM_000639	FASLG	ALPS1B/APT1LG1/APTL/CD178/CD95-L/CD95L/FASL/ TNFSF6	Fas ligand (TNF superfamily, member 6)	2.22
NM_000595	LTA	LT/TNFB/TNFSF1	Lymphotoxin alpha (TNF superfamily, member 1)	13.73
NM_002342	LTBR	CD18/D12S370/LT-BETA-R/TNF-R-III/TNFCR/TNFR-RP/ TNFR2-RP/TNFR3/TNFRSF3	Lymphotoxin beta receptor (TNFR superfamily, member 3)	-1.68
NM_000594	TNF	DIF/TNF-alpha/TNFA/TNFSF2	Tumor necrosis factor	2.35
NM_003810	TNFSF10	APO2L/Apo-2L/CD253/TL2/TRAIL	Tumor necrosis factor (ligand) superfamily, member 10	1.05
NM_003844	TNFRSF10A	APO2/CD261/DR4/TRAILR-1/TRAILR1	Tumor necrosis factor receptor superfamily, member 10a	-2.08
NM_003842	TNFRSF10B	CD262/DR5/KILLER/KILLER/DR5/TRAIL-R2/TRAILR2/ TRICK2/TRICK2A/TRICK2B/TRICKB/ZTNFR9	Tumor necrosis factor receptor superfamily, member 10b	-1.21
NM_002546	TNFRSF11B	OCIF/OPG/TR1	Tumor necrosis factor receptor superfamily, member 11b	1.00
NM_014452	TNFRSF21	BM-018/CD358/DR6	Tumor necrosis factor receptor superfamily, member 21	-3.96
NM_003790	TNFRSF25	APO-3/DDR3/DR3/LARD/TNFRSF12/TR3/TRAMP/WSL-1/ WSL-LR	Tumor necrosis factor receptor superfamily, member 25	1.00
NM_001561	TNFRSF9	4-1BB/CD137/CDw137/ILA	Tumor necrosis factor receptor superfamily, member 9	1.00
NM_004323	BAG1	BAG-1/HAP/RAP46	BCL2-associated athanogene	-1.78
NM_004281	BAG3	BAG-3/BIS/CAIR-1/MFM6	BCL2-associated athanogene 3	-1.18
NM_004874	BAG4	BAG-4/SODD	BCL2-associated athanogene 4	2.66
NM_004324	BAX	BCL2L4	BCL2-associated X protein	-2.02
NM_000633	BCL2	Bcl-2/PPP1R50	B-cell CLL/lymphoma 2	-1.50
NM_004049	BCL2A1	ACC-1/ACC-2/BCL2L5/BFL1/GRS/HBPA1	BCL2-related protein A1	-14.68
NM_138578	BCL2L1	BCL-XL/S/BCL2L/BCLX/BCLXL/BCLXS/Bcl-X/PPP1R52/ bcl-xL/bcl-xS	BCL2-like 1	-1.29
NM_020396	BCL2L10	BCL-B/Boo/Diva	BCL2-like 10 (apoptosis facilitator)	1.00
NM_006538	BCL2L11	BAM/BIM/BOD	BCL2-like 11 (apoptosis facilitator)	-5.69
NM_004050	BCL2L2	BCL-W/BCL2-L-2/BCLW/PPP1R51	BCL2-like 2	-1.30
NM_021960	MCL1	BCL2L3/EAT/MCL1-ES/MCL1L/MCL1S/Mcl-1/TM/bcl2-L-3/ mcl1/EAT	Myeloid cell leukemia sequence 1 (BCL2-related)	-1.87
NM_001166	BIRC2	API1/HIAP2/Hiap-2/MIHB/RNF48/c-IAP1/cIAP1	Baculoviral IAP repeat containing 2	-1.11
NM_001165	BIRC3	AIP1/API2/CIAP2/HAIP1/HIAP1/MALT2/MIHC/RNF49/c-IAP2	Baculoviral IAP repeat containing 3	-15.92
NM_016252	BIRC6	APOLLON/BRUCE	Baculoviral IAP repeat containing 6	-1.42
NM_033341	BIRC8	ILP-2/ILP2/hILP2	Baculoviral IAP repeat containing 8	1.09
NM_004536	NAIP	BIRC1/NLRB1/psiNAIP	NLR family, apoptosis inhibitory protein	1.00
NM_001167	XIAP	API3/BIRC4/IAP-3/ILP1/MIHA/XLP2/hIAP-3/hIAP3	X-linked inhibitor of apoptosis	-2.31
NM_021138	TRAF2	MGC:45012/TRAP/TRAP3	TNF receptor-associated factor 2	-54.69
NM_003300	TRAF3	CAP-1/CAP1/CD40bp/CRAF1/IIAE5/LAP1	TNF receptor-associated factor 3	1.00
NM_004295	TRAF4	CART1/MLN62/RNF83	TNF receptor-associated factor 4	1.00
NM_033292	CASP1	ICE/IL1BC/P45	Caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)	-1.04
NM_001230	CASP10	ALPS2/FLICE2/MCH4	Caspase 10, apoptosis-related cysteine peptidase	-2.52
NM_012114	CASP14	-	Caspase 14, apoptosis-related cysteine peptidase	2.10
NM_032982	CASP2	CASP-2/ICH1/NEDD-2/NEDD2/PPP1R57	Caspase 2, apoptosis-related cysteine peptidase	1.20
NM_004346	CASP3	CPP32/CPP32B/SCA-1	Caspase 3, apoptosis-related cysteine peptidase	-3.45
NM_001225	CASP4	ICE(rel)II/ICEREL-II/ICH-2/Mih1/TX/TX	Caspase 4, apoptosis-related cysteine peptidase	-2.91
NM_004347	CASP5	ICE(rel)III/ICEREL-III/ICH-3	Caspase 5, apoptosis-related cysteine peptidase	1.43
NM_032992	CASP6	MCH2	Caspase 6, apoptosis-related cysteine peptidase	3.01
NM_001227	CASP7	CASP-7/CMH-1/ICE-LAP3/LICE2/MCH3	Caspase 7, apoptosis-related cysteine peptidase	-9.66
NM_001228	CASP8	ALPS2B/CAP4/Casp-8/FLICE/MACH/MCH5	Caspase 8, apoptosis-related cysteine peptidase	1.00
NM_001229	CASP9	APAF-3/APAF3/ICE-LAP6/MCH6/PPP1R56	Caspase 9, apoptosis-related cysteine peptidase	-1.49
NM_003879	CFLAR	CASH/CASP8AP1/CLARP/Casper/FLAME/FLAME-1/ FLAME1/FLIP/I-FLICE/MRIT/c-FLIP/c-FLIPL/c-FLIPR/c-FLIPS	CASP8 and FADD-like apoptosis regulator	-121.65
NM_003805	CRADD	MRT34/RAIDD	CASP2 and RIPK1 domain containing adaptor with death domain	-4.16
NM_013258	PYCARD	ASC/CARD5/TMS/TMS-1/TMS1	PYD and CARD domain containing	5.16

tacia vera L. (Pistachio) is a nut tree species distributed in the Middle East, United States and Mediterranean countries (23). In traditional medicine, *Pistacia* species have had many different uses for diseases such as toothache, periodontal disease, blood clotting, gastralgia, dyspesia, peptic ulcer, asthma, jaundice, diarrhea, throat infections, and renal stones (24). In this study, we examined apoptotic potential of PRH aqueous extract against human hepatoma HepG2 Cells. Moreover, to determine the molecular mechanisms of apoptosis induced by extract, we employed PCR array technology. The results revealed that extract reduced cell viability and also induced apoptosis. It was also found that extract was able to alter expression of genes related to apoptosis. Aqueous extract was selected due to the highest amount of phenolic compounds that has been noted by previous studies. In vitro cytotoxic effect of extract on HepG2 cells was measured by MTT assay, which has been widely used for evaluating the cytotoxicity and cell viability. This test is based on MTT conversion into water-insoluble formazan crystals through dehydrogenases in the mitochondria of viable cells (25). We observed that the extract remarkably inhibited proliferation of HepG2 cells (IC_{50} ~ 0.3 mg/ml). Phenolics comprise a high proportion of plants secondary metabolites that play a role in plant defense against UV radiations, pathogens, and parasites (26). Numerous studies have shown that plant phenolics have in vitro cytotoxic impacts on different cell lines. For example, cytotoxicity of several plant-derived phenolic compounds has been reported against MCF-7, HepG2, B16, and A375 cell lines (27-29). It is notable that anticancer activities of some species of *Pistacia* (*P. lentiscus* and *P. integerrima*) have also been revealed (30-32). Dysregulation of the natural process of apoptosis (inactivation of pro-apoptotic proteins and upregulation of anti-apoptotic proteins) can cause a large number of cancers. In addition to cancer development, defects in the regulation of apoptotic pathways can result in resistance to cancer chemotherapy. One of strategies to combat cancer is to design new therapeutic agents, which act through upregulation of pro-apoptotic molecules or downregulation of anti-apoptotic molecules (33, 34). In the next step, cells were double-stained with Annexin-V and PI for flow cytometric detection of apoptotic cells. Annexin-V is a phospholipid-binding protein that binds to phosphatidylserine on the outer leaflet of the plasma membrane, detecting apoptotic cells in cell populations. On the other hand, application of propidium iodide which stains DNA of leaky necrotic cells allows the discrimination of necrotic cells from Annexin-V-stained cells (35). The results indicated that the apoptosis rate increased significantly after treatment with PRH extract. It has been previously demonstrated that natural phenolics trigger apoptosis in cancer cells. For instance, phenolic compounds from blueberries, and red wine extract possess the ability to induce apoptosis in HepG2 and Jurkat cells, respectively (36, 37). After apoptosis, we assessed expression of apoptosis-related genes by quantitative real-time PCR array technology to gain a better understanding of molecular mechanism. This PCR array includes several gene families involved in apoptosis such as TNF ligands and their receptors, members of the bcl-2, caspase, IAP, and TRAF. Our data showed altered expression of many genes in the extrinsic and intrinsic apoptosis signaling pathways. This was particularly important in the case of anti-apoptotic genes, supporting the hypothesis that PRH extract exerts its apoptotic effects mainly by downregulation of these genes. Among the 17 pro-apoptotic genes, 8 genes were upregulated (CD27, FASLG, LTA, TNF, BAG4, CASP14, CASP6, and PYCARD), whereas the remaining 9 genes (TNFRSF10A, TNFRSF21, BAX, BCL2L11, CASP10, CASP3, CASP4, CASP7, and CRADD) were downregulated. Nevertheless, anti-apoptotic genes (BCL2A1, BIRC3, XIAP, TRAF2, and CFLAR) were downregulated. CFLAR (CASP8 and FADD-like apoptosis regulator), also known as cellular FLICE-like inhibitory protein (c-FLIP), negatively regulates apoptotic pathway and has been reported to be overexpressed in several different cancer cell lines and tissues. Due to its important role in regulation of apoptosis, CFLAR downregulation has been considered to be an interesting target for future cancer therapies (38, 39). It has been demonstrated that p43-FLIP, N-terminal fragment of c-FLIP long isoform ($c\text{-}FLIP_L$), interacts with TRAF2 and activates NF-κB (40). Consequently, NF-κB activation generally upregulates anti-apoptotic genes in cancer cells, leading to cell survival (41). Hence, it is interesting to note that significant downregulation of c-FLIP and TRAF2 in the present study suggests a potential mechanism by which decreased activation of NF-κB may contribute to the triggering of apoptosis.

Collectively, the results of our study suggest for the first time that PRH aqueous extract shows apoptotic potential. The extract not only decreases cell viability but also induces apoptosis in HepG2 cells. It also affects the expression levels of some genes involved in apoptosis pathways. However, further experiments are required to identify bioactive compounds in hull extract and evaluate their pharmacological properties separately. Based on the presented findings, pistachio hull could be regarded as a source of pharmacologically valuable compounds against human cancer cells.

Acknowledgements

This project was funded through the Rafsanjan University of Medical Sciences (grant number 5676).

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