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Evaluation of the selective anticancer potential and the genetic mechanisms of the induction of apoptosis by walnut milk in human breast and prostate cancer cells.

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Abstract

In different cancer types, classical chemotherapy has several side effects due to the cytotoxic properties of the compounds and non-selective targeting of normal tissue. The aims of this study were to determine bioactive molecules and to investigate the genetic mechanisms of the anticancer properties of walnut special mixture, walnut milk (WM), as a potential anticancer treatment in DU145, MCF7 and TG/HA-VSMC cells. The bioactive molecules of WM were determined by LC-Q-TOF analysis. After treatment with the WM, cell viability was determined using the MTT assay and apoptosis induction was observed following cell membrane staining by annexin-V/propidium-iodide using a Tali-cytometer. The gene expression studies were carried out using a qRT-PCR assay. In the WM, we quantified five hormones, eight polyphenols, quercetin and juglone. Abscisic acid (63.07 ± 18.70 µg/l), gallic acid (3887.08 ± 155.06 µg/l), quercetin (245.26 ± 34.12 µg/l) and juglone (401.52 ± 16.60 µg/l) were major components of the quantified compounds. Our results indicated that WM dramatically reduces cell viability and selectively induces caspase-dependent apoptosis in DU145 and MCF7 cells without affecting TG/HA-VSMC non-cancerous cells by triggering intrinsic apoptotic signalling and increases in ROS production. Our results suggest that WM is a potential anticancer agent with selective apoptotic potential and special bioactive chemical constituents.

Keywords: *Juglans regia L*, Walnut milk, Anticancer activity, Apoptosis, Breast cancer, Prostate cancer.

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Introduction

Human health studies have revealed that there is a clear significant correlation between the regular consumption of natural products and a reduced incidence of several diseases, such as physiological disorders and lung, oral-pharynx, colon, pancreatic and endometrial cancers [1]. These anticarcinogen, radioprotective and chemopreventive characteristics have been attributed to the content of natural flavonoids (especially quercetin, mrycetin, naringenin, and apigenine), lectines, polyphenols, anthocyanin, and vitamins (ascorbic acid and tocopherols) [2-5]. Antioxidant and preventive roles of phenolic compounds was reported from *in vivo* and *in vitro* studies, and they are thought to be major bioactive molecules with human health benefits [6-8].

Walnuts, *Juglans regia L*, are commonly found in temperate areas of the Palearctic, Nearctic and Oriental zones and are commercially cultivated in several countries. In all cultivated areas, the shrubs, seeds, shells, bark, green husk and leaves of walnuts are used in complementary medicine and the pharmaceutical and cosmetic industries [1]. Several parts of walnuts are good sources of phenolic compounds [8-10]. Several phenolic molecules reduce the molecular damage associated with degenerative diseases by preventing cellular oxidative stress and inhibition of macromolecular oxidation [5,9,11,12]. Recent studies demonstrated the antioxidant, antiradical, antimicrobial and antiproliferative activity of phenolic products using different *in vitro* and *in vivo* estimation models [1,13-15].

Walnut milk (Ucan Adam Company, Turkey) is a novel walnut drink that is obtained by mixing fresh walnut sap with certain amounts of an aqueous extract of male flowers and internal membranes of fruit. This drink is patented by the Turkish Patent Office (Patent No.: TR 2010/06465- B) and is legal for human consumption as a dietary supplement. Some studies reported the potential antioxidant, antimicrobial, antiradical and antiproliferative effect of walnut extracts and fruits [9,15,16], leaves [1], and liqueurs produced from green fruits[17] and the green husk [1,9], but information about the male flower and internal membrane of fruits is almost non-existent. In addition, the combined anti-carcinogenic effect of extracts of several parts of walnuts has not been studied.

In this paper, we determined the polyphenol content and the amounts of quercetin and juglone and evaluated the cytotoxic and anticancer activity of walnut milk in DU145, MCF7 and TG/HA-VSMC cell lines. We also demonstrated for the first time to our knowledge the apoptotic affect and the role of the intrinsic apoptosis signalling pathway of walnut extracts on healthy and cancer cell lines via image-based cytometer and target gene expression profiles. The aim of this study was to investigate the genetic mechanisms of the anticarcinogenic properties of a special walnut mixture (walnut milk) as a potential anticancer treatment.

Materials and Methods

Chemicals

Three cell lines, human prostate carcinoma DU 145 (ATCC® HTB-81™), human metastatic breast adenocarcinoma MCF7 (ATCC® HTB-22™), and human normal aorta smooth muscle TG/HA-VSMC (ATCC® CRL-1999™) were purchased from the American Type Culture Collection (ATCC, Rockville, Maryland, USA). The cell culture materials HAMS F 12, Dulbecco's modified Eagle's medium (DMEM), L-glutamine, foetal bovine serum (FBS), and penicillin-streptomycin were supplied by MULTİCELL (Vısent Bioproducts, Canada). PBS-EDTA, dimethyl sulfoxide (DMSO), trypsin, yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and analytic standards for the phenolic compounds, quercetin and juglone were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). The PureLink® RNA Mini Kit, High Capacity cDNA Reverse Transcription Kit, SYBR® Select Master Mix and Tali® Apoptosis Kit - Annexin V Alexa Fluor® 488 and propidium iodide were supplied from Life Technologies (USA). Methanol, acetonitrile and ultrapure water (LiChrosolv® Reag) were purchased from Merck-Millipore (Darmstadt, Germany).

Walnut milk

The walnut milk and the extraction procedures were supplied by the manufacturer (Ucan Adam Inc. Turkey). This procedure is also found in the patent document (Patent No. TR 2010/06465- B, Turkish Patent Institute) and is given below. Walnut sap was collected from approximately 24 cm in the trunk of a walnut tree and stored in a stainless steel tank at <4°C until it was used. Walnut male flowers and the inner membrane of walnut fruits were collected in the Denizli province of Turkey. After collection, these materials was washed with distilled water and mechanically cut into very small parts and dried at room temperature in the dark. Shade dried plant materials were powdered with a tungsten carbide bit to approximately 60-mesh size and used for water extraction. Walnut milk was obtained from fresh walnut sap with certain amounts of an aqueous extract of male flower and the internal membrane of walnut fruits. This walnut extract was subsequently bottled and stored at <4°C.

Determination of polyphenols, plant hormones, quercetin and juglone

During chemical analysis, standards for the phenolic compounds and the plant hormones quercetin and juglone were individually loaded onto the micro liquid chromatography (Exigent Micro LC 200, Abi-Sciex, USA)-Electron Spray Ionisation-Triple Quadropol-Time of Flight Spectrometer (Micro LC-ESI- Q-TOF, Abi-Sciex, USA) system to determine fragments and analysis conditions. Afterwards, mixtures of the 15 analytic standards were prepared with concentrations ranging from 0.100 to 200 µg/kg to set up eight points for calibration. All data were opened with Peak View (ABI-Sciex, USA) software, and mass and fragment results were checked with Master View (ABI-Sciex, USA) options. Mass and specific fragments were exported to MultiQuant software (ABI-Sciex, USA) to generate calibration curves for each type of compound. Samples (2 µl) were analysed by Micro LC-ESI-Q-TOF using an Eksigent MicroLC 200 Plus system coupled with an Applied Biosystems 4600 Triple Quadropol-TOF. Chromatographic separation was carried out on a Eksigent 2.7 µ*3 cm C18 halo column at 30°C. The solvent gradient was as follows: 100 % A (99.8% UPW: 0.2% formic acid) to 100% B (99.8% acetonitrile: 0.2% formic acid) over 10 min. The gradient profile for chemicals was applied as follows: (t (min)/A%): (0/90), (1/90), (2/10), (6/10), (7/50), (9/90), (10/90). Phenolic compounds, hormones, quercetin and juglone analyses were performed using a DuoSpray source and Electrospray (ESI) probe. An IDA method was used containing a TOF-MS survey of 70 ms and up to 20 dependent TOF-MS/MS scans of 25 ms accumulation time. The mass range was set to 100-960 da for MS and the product ion mass range was set to 30-960 da for MSn. The curtain gas was set at 20 a.u.; the source temperature was 400°C; and the ion source gases 1 and 2 were both 30 a.u. The declustering potential was set at -100 V. The source voltage was -4500 V. A Collision Energy (CE) of -20 V and Collision Energy Spread (CES) of -15 V were used. Other data are given in (Table 1).

Table 1: *Micro LC-Q-TOF condition for biological active molecule analyses and chemical composition of walnut milk (mean±SE).*

Analyte	Transition	Scan Mode	Linear Regression Equation	Correlation Coefficient	Amount (µg/l)
Gallic acid	169 [125]	-	y=60.3682 x+388.2263	0.9994	3887.08 ± 155.06
2-5 dihydroxybenzoic acid	153 [109]	-	y=91.9236 x+1176.0827	0.9996	195.42 ± 14.21
Syringic acid	197 [182]	-	y=9.8103 x+8.3439	0.9995	10.04 ± 1.83

Caffeic acid	179 [135]	-	y=327.6805 x+1408.9109	0.9997	161.55 ± 9.20
Protocatechuic acid	153 [109]	-	y=97.4971 x+688.3555	0.9984	165.27 ± 9.24
Ferulic acid	193 [134]	-	y=34.8124 x+44.1034	0.9995	26.79 ± 2.68
P -coumaric acid	163 [119]	-	y=308.8506 x+815.8047	0.9996	71.86 ± 28.26
Sinapic acid	223 [208]	-	y=25.7638 x+72.2770	0.9993	5.18 ± 1.03
Gibberellic acid	345 [143]	-	y=9.9546 x+4.7564	0.9996	4.57 ± 1.41
Indole-3-acetic acid	174 [130]	-	y=6.0935 x+74.9215	0.9993	3.62 ± 0.97
Abscisic acid	263 [153]	-	y=70.5488 x+256.0263	0.9995	63.07 ± 18.70
Salicylic acid	137 [93]	-	y=228.2290 x+137.8879	0.9994	0.39 ± 0.15
Jasmonic acid	209 [59]	-	y=53.6581 x+13.5492	0.9998	0.69 ± 0.20
Jugnole	174 [118]	-	y=24.7915 x+1450.0543	0.9982	401.52 ± 16.60
Quercetin	301 [151]	-	y=430.9054 x+15896.4599	0.9978	245.26 ± 34.12

Cell culture and treatment conditions

Cells were cultured in a 1:1 mixture of Dulbecco's Modified Eagle Medium (DMEM) and HAM's Nutrient Mixture F12 with 5% heat-inactivated foetal bovine serum (FBS), 1% L-glutamine and 100 IU/ml penicillin- streptomycin. Cells were maintained at 37°C in a CO_2 incubator with a humidified atmosphere of 5% CO_2 in 75 ml sterile cell culture flasks. The experiments works began at the 5^{th} passage of all cells.

MTT assay

The MTT assay was performed to determine cell viability. In this method, DU145, MCF7, and TG/HA-VSMC cells were sown in a 96-well sterile microplate at a density of approximately 7500 cells/well in 200 µl of medium 24 h before treatment. The plates were incubated in a 5% CO_2 incubator at 37°C for 24 h to permit the cells to attach to the well. After incubation, the cells were treated with 200 µl of walnut milk and mixed medium which was filtered twice through 45 micron and 0.20 micron disposable PTFE filters (Chromex, USA) prepared at different concentration levels of 1.25, 2.50, 5.00, 7.50, 10.0 (10 fold), 15.0, 20.0 (5-fold) and 25 % (Walnut Milk/ Medium, V/V). After incubation for 24 and 48 h, MTT solutions (20 µl/200 µl per well of a 5 mg/ml solution) were added and the plates were incubated for 4 h at 37°C. The blue formazan crystals were dissolved in DMSO (200 µl/well) and the absorbance was measured at 490 nm with a Thermo Multiscan Go Microplate Reader Spectrophotometer (Thermo Scientific, USA). The calculation of absorbance for each concentration of walnut extract mix was compared with the water treated control.

Tali cytometer and gene expression

Cells were sown in 6-well sterile plates including 2 ml of medium and routinely grown for 24 h in a CO_2 incubator. After this incubation, 5-fold- and 10-fold-diluted walnut extract mixes (filtered through 0.45- and 0.20-micron sterile syringe filters) diluted with medium were added to each well for 24 and 48 h. Each plate contained approximately 6 well X 5-10 10^5 cells. Three were used in the Tali Cytometer assay and the others were used in gene expressions studies.

Tali image based cytometer assays

Cell apoptosis, viability and dead cells were determined by Annexin V and PI (Tali® Apoptosis Kit; Life Technologies) according to the manufacturer's instructions. After trypsinization, the cell suspension (5-10. 10^5 cells) was centrifuged at 800 g at 4°C for 2.5 min, and the supernatant was discarded. The cell pellet was suspended in 100 µl 1X annexin binding buffer, and 5 µl of Annexin V Alexa Fluor® 488 was added. The mixture was incubated at room temperature in the dark for 20 min. At the end of the incubation period, the mixture was centrifuged and the supernatant was discarded. After suspension in 100 µl of 1X Annexin binding buffer, an aliquot of the suspension was mixed with 1 µl of Tali® Propidium Iodide and incubated for 5 min at room temperature. For the Tali Analysis, 25 µl of the stained cells was loaded into a Tali® Cellular Analysis Slide and cell viability, dead cells and apoptotic cells were determined by Tali® Image-Based Cytometer software.

Gene expression assays

Isolation of total RNA and cDNA synthesis: Total RNA was isolated from three cell lines cultured in six-well plates using the PureLink® RNA Mini Kit (Life Technologies, USA) according to the manufacturer's instructions. The RNA concentrations were measured using the Qubit® Fluorometer (Life Technologies, USA). The concentration of total RNA was adjusted to 100 ng/µl for the synthesis of the first strand of cDNA using a High Capacity cDNA Reverse Transcription Kit (Life Technologies, USA). cDNA synthesis was performed using the thermal cycler Applied Biosystems® Veriti® (Step 1: 25°C, 10 min; Step 2: 37°C, 120 min; Step 3: 85°C, 5 min). The cDNA was stored at -20°C for subsequent steps of the analysis procedure.

Quantitative real-time PCR (qRT-PCR) analysis: Expression levels of antioxidant enzymes [CuZn-superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), Mn-SOD], apoptosis inhibitor groups [survivin, livin, X-linked inhibitor of apoptosis (XIAP), inhibitor of apoptosis protein (c-IAP1, c-IAP2), B-cell lymphoma 2 (BCL-2), B-cell lymphoma-extra-large (BCL-XL)], apoptosis group [tumour necrosis factor alpha (TNF-A), tumour suppressor (P53), apoptosis regulator (BAX), apoptotic protease activating factor 1 (APAF-1), cytochrome C (Cyt-C), caspase 3] and cell cycle proteins [cyclin-dependent kinase inhibitor (P21Cip1), cyclin-dependent kinase inhibitor 1B (P27Kip1), cyclin-D1 (CCND1)] genes in response to walnut extract exposure were analysed by qRT-PCR using the SYBR® select master mix (Life Technologies, USA) on an ABI 7500 Real-Time PCR system (1 cycle of 2 min at 50°C and 10 min at 95°C followed by 40 cycles of denaturation at 95°C for 15 s, annealing and extension at 60°C for 1 min) with the primer pairs shown in Table 2. Gene expression was determined as the relative fold change compared to the control and normalized to GADPH: F (5'-TTGGTATCGTGGAAGGACTCA-3') and R (5'-TGTCATCATATTTGGCAGGTTT-3') mRNA expression. The comparative cycle threshold (Ct) method (User Bulletin 2, Applied Biosystems, CA) was used to analyse the expression levels of the mRNAs. In addition, differences in the degree of the relative fold change resulting from gene expression due to walnut extract applications were compared using analysis of variance (ANOVA) with Duncan's separation of means test using SPSS 18 software at a significance level of $p \leq 0.05$.

Table 2: Genes, related mechanisms, and primer sequences used in the reverse transcription-polymerase change reaction (qRT-PCR).

Gene		Primer		Gene		Primer	
Antioxidant Enzymes Genes				**Cell Cycle Control Genes**			
SOD	F 5'	GTTCGGTGACAACACCAATG	3'	P21Cip1	F 5'	GGCGTTTGGAGTGGTAGAAA	3'
	R 5'	GGAGTCGGTGATGTTGACCT	3'		R 5'	GACTCTCAGGGTCGAAAACG	3'
CAT	F 5'	TACGAGCAGGCCAAGAAGTT	3'	P27Kip1	F 5'	CCGGCTAACTCTGAGGACAC	3'
	R 5'	ACCTTGTACGGGCAGTTCAC	3'		R 5'	TGGATCCAAGGCTCTAGGTG	3'
GPX	F 5'	TGGGACCAGCAAGTAAAACC	3'	CCND1	F 5'	AACAGAAGTGCGAGGAGGAG	3'
	R 5'	TCGCGAATG TAGAACTCGTG	3'		F 5'	TGAGGCGGTAGTAGGACAGG	3'
SOD2	F 5'	TCTGAAGAAGGCCATCGAGT	3'	**Apoptosis Inhibitors Genes**			
	R 5'	GCAGATAGTAGGCGTGCTCC	3'	SURVIVIN	F 5'	GACGACCCCATAGAGGAACA	3'
Pro-Apoptotic-Apoptotic Genes					R 5'	GACAGAAAGGAAAGCGCAAC	3'
TNF-α	F 5' R	AGGCGCTCCCCAAGAAGACA	3'	LIVIN	F 5'	TGGCCTCCTTCTATGACTGG	3'
	R 5'	TCCTTGGCAAAACTGCACCT	3'		R 5'	ACCTCACCTTGTCCTGATGG	3'
P53	F 5'	CACGAGCGCTGCTCAGATAGC	3'	XIAP	F 5'	GGGGTTCAGTTTCAAGGAC	3'
	R 5'	ACAGGCACAAACACGCACAAA	3'		R 5'	TGCAACCAGAACCTCAAGTG	3'
BAX	F 5'	TTCATCCAGGATCGAGCAGA	3'	c-IAP-1	F 5'	GCATTTTCCCAACTGTCCAT	3'
	R 5'	GCAAAGTAGAAGGCAACG	3'		R 5'	ATTCGAGCTGCATGTGTCTG	3'
Cyt-C	F 5'	AGTGGCTAGAGTGGTCATTCATTTAC/	3'	c-IAP-2	F 5'	GCATTTTCCCAACTGTCCAT	3'
	R 5'	TCATGATCTGAATTCTGGTGTATGAG/	3'		R 5'	ATTTTCCACCACAGGCAAAG	3'
APAF-1	F 5'	GATATGGAATGTCTCAGATGGCC	3'	BCL-XL	F 5'	GTAAACTGGGGTCGCATTGT	3'
	R 5'	GGTCTGTGAGGACTCCCCA	3'		R 5'	TGGATCCAAGGCTCTAGGTG	3'
Caspase 3	F 5'	GGTATTGAGACAGACAGTGG	3'	BCL-2	F 5'	ATGTGTGTGGAGAGCGTCAA	3'
	R 5'	CATGGGATCTGTTTCTTTGC	3'		R 5'	ACAGTTCCACAAAGGCATCC	3'

Results

Phenolic compounds, plant hormones, quercetin and juglone

The micro LC-ESI-Q-TOF analysis of a mixture of aqueous extracts of walnut sap, male flowers and the inner membrane of walnut fruit revealed the presence of several phenolic acids, plant hormones, and flavonoid derivatives. Five plant hormones, eight phenolic acids, and quercetin (flavonoid) were quantified. Although juglone (5 hydroxy-1,4- naphthoquinone) has low solubility and volatile characteristics, it was present in walnut milk extract. While ABA was the major plant hormone (63.07 ± 18.70 µg/L), JA and SA were present at trace levels. Walnut milk exhibited a rich phenolic acid profile, in which gallic acid was the major compound (3887.08 ± 155.06 µg/L), followed by 2-5 dihydroxybenzoic acid (195.42 ± 14.21 µg/L). Syringic acid was a minor compound, present at 10.04 ± 1.83 µg/L (Table 1). In this study, quercetin and juglone were the other major components among the quantified plant compounds, present at 245.26 ± 34.12, 401.52 ± 16.60 µg/L, respectively.

Determining the effective dose and time of walnut milk

The first step in determining the influence of a walnut extract treatment was to calculate the effect of walnut milk (WM) on

the viability and growth inhibition of cancer and normal cells. Following treatment with increasing concentrations of walnut milk at time points, the cells were assayed with MTT to determine the growth inhibition and lethal potential of WM. We observed the ability of aqueous WM to lower the viability of cancer cells, including human breast (MCF 7) and prostate (DU 145) adenocarcinoma cell lines. The cancer cell lines MCF7 and DU 145 were on average inhibited to a higher degree than the normal cell line TG/HA-VSMC. While a significant dose- and time-dependent inhibition were observed in both cancer cells, there was no significant correlation between cell viability and doses of walnut extract in normal cells at both incubation times. The cell viability in walnut milk-treated MCF 7 and DU 145 cells showed a significant decrease of 7.5-25% and 2.5-25% walnut milk concentrations after 24 and 48 hours of exposure compared with their respective control group (Figure 1). However, at 48 hours, the inhibitory effect of walnut milk was 4.25- and 2.69-fold higher than at 24 hours in MCF 7 and DU 145 cells, respectively. At 48 hours treatment, the IC50 and IC80 concentrations of walnut milk were calculated as 11.34-20.39 % ($y=-3.3168x + 87.63$; $R^2=0.932$) and 10.54-20.34% ($y=-3.1587x+83.316$; $R^2=0.908$) in MCF7 and DU 145 cells, respectively. The inhibitory effects for the 10-fold (10%) and 5-fold (20%) diluted concentrations of the walnut milk were 21.6-31.4% and 30.3-72.6 inhibition compared to controls for the MCF 7 cells; 12.7-47.4% and 46.4-64.2% inhibition for the DU 145 cells; and 16.1-16.5% and 12.9-15.5% inhibition for the normal TG/HA-VSMC cells (Figure 1).

To further estimate the anticancer properties of WM, we wanted to investigate its role in cell death mechanisms and its selectivity to cancer cells. Our results revealed that WM specifically induces cell death in both MCF 7 and DU 145 cancer cells in a dose- and time-dependent manner, as shown by the increase in red fluorescence signal sources from propidium iodide-positive cancer cells exposed to WM (Figure 2). Additionally, this effect was selective, as healthy smooth muscle cells remained unaffected by WM treatment at the same concentrations and time-points (Figures 1 and 2). These outcomes were confirmed using image-based cytometry to assess the percentage of viable, dead and apoptotic cells. In the Tali analysis, the cells were stained with the annexin V–Alexa Fluor® 488 conjugate for determining the apoptotic cell population. Annexin V and propidium iodide (PI) stained cells that were dead radiated red or yellow signals (annexin V-/PI+or annexin V+/PI+, respectively), and irradiated green signals when apoptotic (annexin V+/PI-). In this study, while there were no significant changes in cell viability observed in smooth muscle cells at all-time points after WM treatment, compared with the controls, the live cell population of both cancer cells was significantly reduced with increased WM concentration under the same experimental conditions. We determined a 15-26% and 1-27% increase in PI and annexin V-PI positive dead cells following 48 hours of WM exposure, and a 24-47% and 47-60% annexin-V positive increase in the same DU 145 and MCF 7 cells, confirming the induction of the apoptosis pathway, respectively (Figure 2).

Gene expression profiles in apoptosis signalling induced by WM

Several therapies used to treat cancer, such as chemotherapy, γ-irradiation, immunotherapy and alternative medicine, have an antitumor effect principally by activating apoptosis in cancer cells. Several anticancer agents, especially phenolic compound-inducible molecules, have been linked to the activation of apoptosis signal transduction pathways in cancer cells. For this reason, we investigated the apoptotic effect of WM on cancer and healthy cells using gene expression profiles of four principle components of the intrinsic pathway: antioxidant, apoptosis inhibitor, pro-apoptotic and cell cycle.

Oxidative stress is a key factor in the stimulation of several cell death processes, especially apoptosis. The antioxidant genes are rapid and sensitive biomarkers that monitor oxidative stress in tissue and cells. Significant increases in SOD, CAT, GPX and SOD2 gene expression were observed in DU 145 cells at both treatment times after WM treatment (Figure 3). These increases were observed in both reactive oxygen species scavenger genes (cytosolic CuZn-SOD, CAT and mitochondrial Mn SOD) in a range of approximately 1.6- to 3.7-fold, and the lipid peroxidation preventive gene (GPX) in the range of 1.5- to 5.6-fold.

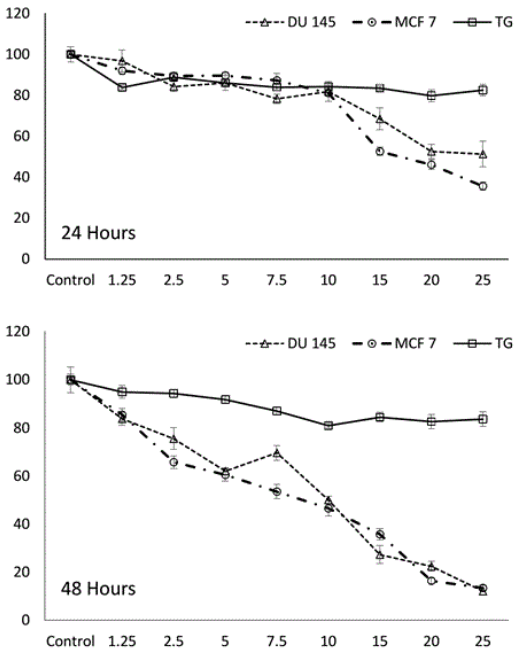

Figure 1: *The cell viability (%) of human prostate (DU145), breast (MCF7) cancer cells and smooth muscle cells (TG/HA-VSMC) treated with 1.25-25% (Walnut Milk/ Medium, V/V) for 24 and 48 hours. Values are presented as the means (n=8) ± S.E.*

Our results indicate that the overexpression of antioxidant genes could not prevent the reduction in viability (Figures 1-2), signifying that the apoptotic effect of WM in DU 145 cells is correlated with oxidative damage. In MCF 7 cells because no differences were observed among control and WM treated

groups in the qRT-PCR data for SOD, SOD2 and CAT genes, the significant increases (15.5- to 23.3-fold) were determined only in the *GPX* gene for the 5- and 10-fold diluted WM treatment at 48 hours compared to the control. This indicates that WM induced lipid peroxidation and produced hydrogen peroxide radicals in MCF 7 cells. WM treatment did not cause oxidative stress in normal cell lines, in contrast to cancer cell lines. Moreover, lower expression levels of antioxidant genes were detected in all genes, with two exceptions (Figure 3).

To determine the apoptotic mechanisms under the effect of WM in IC50 and IC80 doses, selected genes belonging to the apoptosis inhibitor, apoptosis activator and cell cycle control groups were assessed by qRT-PCR analysis. Our results indicate that the apoptotic effect of WM was particularly strong after 48 hours of treatment. In DU 145 cells, while apoptosis inhibitor genes exhibited slight expression levels, except cIAP2, apoptosis regulator genes of the intrinsic apoptosis pathway and BAX were overexpressed (26.5-fold at 48 hours) and triggered the release of Cyt-C (10.8-fold) from mitochondrial membranes. This gene linked the APAF 1 (2.6-fold) and activated caspase 3 (5.1-fold). Additionally, the G1/S transition checkpoint gene *p21cip1* (overexpressed 19.5-fold) and the cell cycle arrest *p27Kip1* gene stopped the cycle in the G1 phase. In DU 145 cells, the combined effect of these genes completed the apoptosis cycle, especially after 48 hours of WM treatment (Figures 4 and 5).

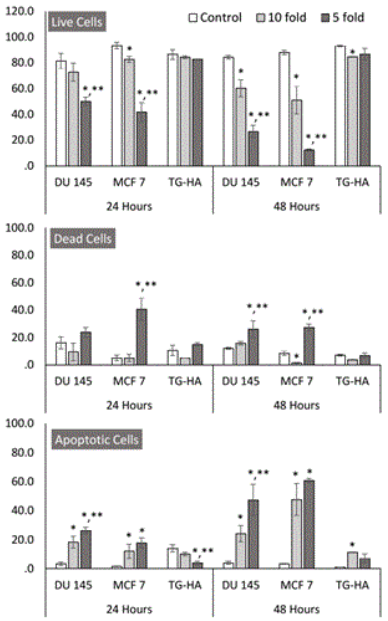

Figure 2: *Percentage result of live, dead and apoptotic cells stained with annexin V/ propidium iodide as determined using an Tali image based cytometer. All data are given as the mean values of percentages for each group ± SE. n=3* indicates significantly different values compared to their respective controls and ** indicates significantly different values compared to other treatment groups, analysed by one-way ANOVA, Duncan test (p ≤ 0.05).*

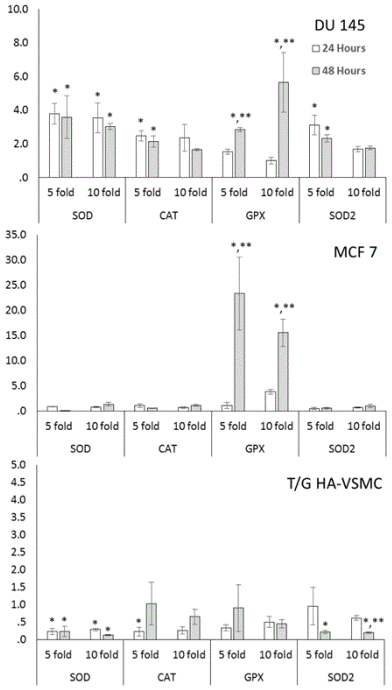

Figure 3: *Relative fold change determined by quantitative real time PCR (qRT-PCR) analysis of antioxidant systems, as follows: Cu-Zn-superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and Mn- superoxide dismutase (SOD2) genes in MCF7, DU 145 and TG HA-VSMC cells treated with 5-fold- and 10-fold-diluted WM. All data are normalized to GADPH expression and given as relative to control. (Control=1 not shown in figure) n=3* indicates significantly different values compared to their respective controls and **indicates significantly different values compared to other treatment groups, analysed by one-way ANOVA, Duncan test (p ≤ 0.05)*

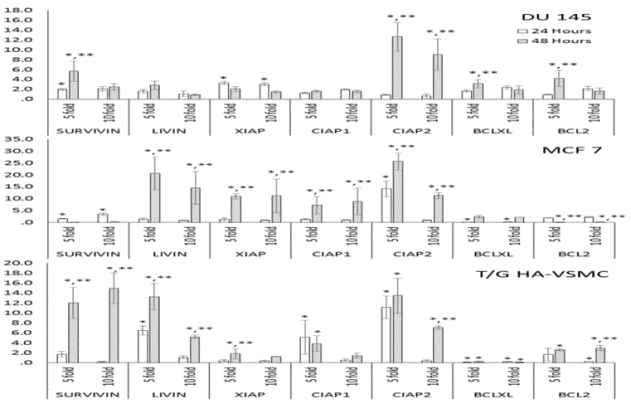

Figure 4: *Relative fold change determined by quantitative real time PCR (qRT-PCR) analysis of apoptosis inhibitor family proteins (IAPs) survivin, livin, XIAP, cIAP1, cIAP2 and BCL-XL and BCL2 genes in MCF7, DU 145 and TG HA-VSMC cells treated with 5-fold- and 10-fold-diluted WM. All data are normalized to GADPH expression and given as relative to control. (Control=1 not shown in figure) n=3* indicates significantly different values compared to their respective controls and **indicates significantly different values compared to other treatment groups, analysed by one-way ANOVA, Duncan test (p ≤ 0.05)*

Treatment of MCF-7 with the 5-fold-diluted WM effectively increased the expression of apoptosis inhibitor mRNA levels, including the IAP groups and livin genes (Figure 4). Conversely, in the same conditions, BCL-2 gene expression was suppressed, and this reduction was triggered by the increase in pro-apoptotic genes (BAX, APAF1, Cyc-C and Caspase 3) belonging to the intrinsic apoptosis pathway approximately in the range of 6.9- to 27.9-fold (Figure 5). While a significant increase was also observed for tumour suppressor P53 and tumour necrosis factor TNFα genes, the cell cycle control genes did not exhibit dose- and time-dependent expression. In this study, normal cells were also affected by WM treatment, and Cyc-C and APAF 1 were induced by WM treatment. However, the BAX and Caspase 3 genes were unaffected by this increase, in addition to all apoptosis inhibitor genes except for BCl-XL and XIAP, which were overexpressed especially after 48 hours of WM treatment. There were no significant changes in the expression of other genes responsible for the cell cycle (Figure 6). For this reason, the apoptotic effect of WM on healthy cells remained at a low level.

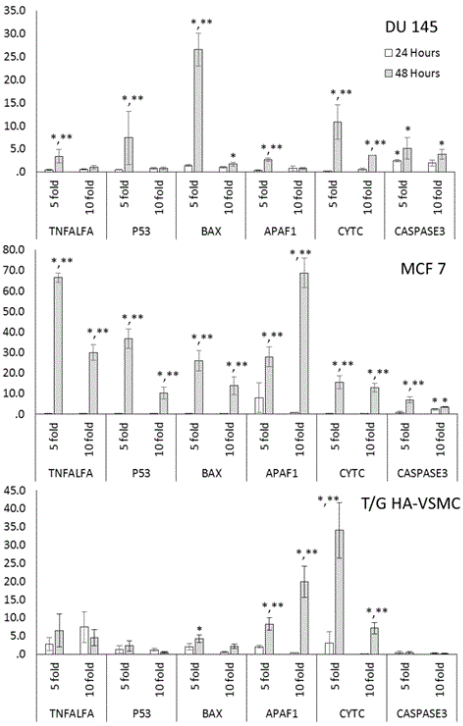

Figure 5: *Relative fold change, determined by quantitative real time PCR (qRT-PCR) analysis of tumour necrosis factor alfa (TNF-α), tumour suppressor (P53), apoptosis regulator (BAX) and apoptotic protease activating factor 1 (APAF 1), cytochrome-c and caspase 3 genes in MCF7, DU 145 and TG HA-VSMC cells treated with 5-fold- and 10-fold-diluted WM. All data normalized with GADPH expression and given as relative to control. (Control=1 not shown in figure) n=3* indicate significantly differences values compared to their respective controls and **indicate significantly differences values compared to other treatment groups, analysed by one-way ANOVA, Duncan test (p ≤ 0.05).*

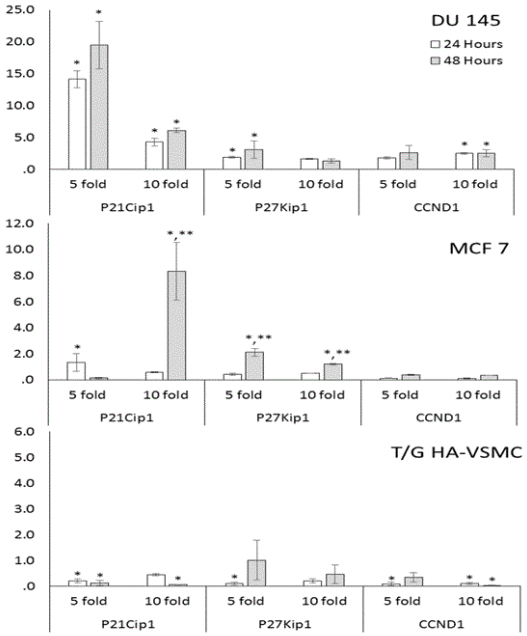

Figure 6: *Relative fold change, determined by quantitative real time PCR (qRT-PCR) analysis of cyclin-dependent kinase inhibitor (P21Cip1), cyclin-dependent kinase inhibitor 1B (P27Kip1), cyclin-D1 and CCND1 genes in MCF7, DU 145 and TG HA-VSMC cells treated with 5-fold- and 10-fold-diluted WM. All data are normalized to GADPH expression and given as relative to control. (Control=1 not shown in figure) n=3* indicates significantly different values compared to their respective controls and **indicates significantly different values compared to other treatment groups, analysed by one-way ANOVA, Duncan test (p ≤ 0.05).*

Discussion

Although single and complex extracts obtained from walnut (*Juglans regia L.*) seeds, green husks and leaves exert an anticarcinogenic effect, including antiproliferative and growth inhibition of some cancer cells [1,18-20], the underlying mechanisms and molecular targets remain unclear. In this report we determined for the first time the selective anticancer capability and effect on cell signalling belonging to the intrinsic apoptosis pathway of WM, a special walnut drink, in human breast (MCF7) and prostate (DU145) cancer cell lines and a non-cancerous (TG-HA-VSMC) cell line.

The LC-Q-TOF analysis of WM revealed a rich phenolic content compared to several plant parts (leaf, bloom, green husk and seed) of walnut trees. Additionally, we detected four plant hormones, especially ABA, in WM. This finding is in agreement with several studies of the phenolic composition of different parts of walnut trees, with the exception of the plant hormones, in which the phenolic compounds were reported to be catechin, epicatechin, caffeic, chlorogenic, ellagic, ferulic, gallic, sinapic, protocatechuic, syringic and vanillic acids; myricetin, and juglone from the walnut green husk [17], juglone, p-coumaric, caffeic, gallic, ellagic, syringic, ferulic and sinapic acids for walnut seeds [4,14] and nine phenolic complexes, three hydroxycinnamic acid derivatives and six

flavonols, especially heterosides of quercetin, for walnut leaves [21]. Several cell culture and animal model studies revealed that biologically active secondary metabolites, such as phenolic acids and quercetin, are the main phytochemicals with antioxidant and antiproliferative characteristics in walnut plants [1,22,23]. Phenolic acids move easily through the cell membrane and participate in several biochemical reactions, such as the Fenton reaction, with their redox properties, and play an important role in scavenging free radicals, quenching singlet oxygen, and chelating heavy metals in antioxidant defence mechanisms of cells [24]. It is well documented that the polyphenols, flavonoids and plant hormones such as gallic acid [25], ellagic acid [26], chlorogenic acid, epicatechin [7], quercetin [27,28] and ABA [29,30] could cause apoptosis or trigger several anticancer mechanisms in different cell lines and animal models.

Our results indicated that WM treatment effectively reduced the viability of cancer cells and induced apoptosis in a dose- and time-dependent manner, while there were no significant changes in the percentages of live, apoptotic and dead non-cancerous cells, according to the MTT and Tali Cytometer Assays (Figures 1 and 2). Our results confirmed previous research [1] conducted in A-498, 769-P and Caco-2 cells, indicating that the walnut extract caused significant cell growth inhibition in a concentration-dependent manner and has no potent cytotoxic ability or toxic effects on healthy cells. In this study, the two and four fold increase in the Annexin V stained cells compared to Annexin V/PI positive and PI positive in cancerous cell populations indicated that the marked decreases in cell viability as determined by the MTT assay arose from caspase-dependent apoptosis. The phosphatidylserine located on the cytoplasmic surface of the cell membrane in living cells is translocated from the inner to the outer membrane in apoptotic cells via caspase activation. Annexin V stains phosphatidylserine only in the outer membrane, and green irradiated cells are apoptotic [31,32]. Apoptosis is a programmed cell death, and almost all targeted and selective anticancer therapy has been connected with stimulation of apoptosis signalling pathways in cancer cells, such as the intrinsic and/or extrinsic pathway [33]. Caspases are one of the major players of apoptosis, which act as common apoptotic molecules in a number of different substrates in the cytoplasm in various forms of cell death [34].

Both the intrinsic and extrinsic apoptosis pathways are closely related to different types of caspase activations, and these mechanisms regulate gene expression, including antioxidant, apoptosis inhibitor, apoptotic and cell cycle arrest genes. Reactive oxygen species (ROS)-mediated oxidative stress is a generalized phenomenon in most cancer therapy-induced cell damage. The induction role of oxidative stress in different apoptosis mechanisms has been well documented [35]. Earlier studies have recommended that cancer cells, with different chemical environments in the cytoplasm, are more dependent on cellular response processes against oxidative damage and have used this property to selectively target cancer cells [36]. While we observed a significant increase in all antioxidant gene expression at both treatment time and dose in DU 145 cells, only GPX was overexpressed in MCF 7 cell lines under the same treatment. Antioxidant enzymes in non-cancerous cell lines did not significantly affect the WM application. In DU 145 cells, the increases in SOD, CAT, GPX and SOD2 gene expression levels at both concentrations can be explained by the ROS scavenging roles of these antioxidant enzymes. However, oxidative stress in MCF 7 cells was more obvious than DU 145 due to decreased SOD and CAT levels, causing insufficient scavenging of H_2O_2 radicals. Our hypothesis that overexpression of the *GPX* gene is the precursor of an excess amount of hydroxyl radicals, which is closely related to lipid peroxidation in MCF 7 cells. Increased ROS and hydroxyl radical levels in the cells are a potent activator of lipid peroxidation and DNA damage and high concentrations are known to affect cellular homeostasis, which ultimately leads to apoptotic/ necrotic cell death [37]. Additionally, ROS and hydroxyl radicals mediate metabolic instability in the cells, possibly triggering apoptosis by the activation of several related genes in apoptosis pathway signalling, which is linked to cellular damage and glutathiolation levels [38,39].

WM was associated with different apoptosis signalling patterns in two cancer cell lines. In DU 145, only the survivin and *cIAP 2* genes were significantly expressed by WM treatment at a high dose, especially at 48 hours. Other apoptosis inhibitors were slightly increased, but the apoptosis regulator *BAX* gene was overexpressed and this signal triggers the release of Cyt-C from mitochondrial membranes. Finally, a 3-fold increase in APAF1 protein and Cyt-C complex activated Caspase 3 completed the intrinsic apoptosis circle. On the other hand, we observed a significant increase in *P21Cip1* and *p27Kip1* gene expression in DU145 cells. These cell cycle arrest genes are closely related to *P53* and *BAX* [40]. Biotic and abiotic cellular stresses initiate induction of p21 expression by both p53-dependent and -independent mechanisms [40]. The effected mechanisms of overexpression of p21 include C6 –ceramide-induced apoptosis in the human hepatoma (Hep3B) cell line. In that study, overexpression of p21 triggered the proapoptotic protein Bax, thus modulating the molecular ratio of Bcl-2/ Bax in Hep3B cells [41]. Similarly, p21 and Bax were overexpressed and resulted in effective apoptosis in Hep3B cells exposed to retinoic acid [42]. In this study, we thought that WM induced apoptosis as observed by the Tali cytometer could be caused by crosstalk to cell cycle arrest and intrinsic apoptosis pathway signalling in human prostate cancer (DU 145) cells. Cell cycle arrest signals play a major role in the apoptosis pathway and the probable function of p21 that triggers molecules responsible for regulating BAX and other apoptotic molecules in the inhibition of cyclin-dependent protein kinase and causes growth arrest in the G1/S sub-phase of the cell cycle [43].

Human breast cancer (MCF 7) cells expressed high levels of the inhibitor of apoptosis protein family (IAP) and pro-apoptotic genes in an extended exposure (48 h) to WM treatment. The major role of IAPs is caspase inhibition [44,45] but they also affect several cell survival processes such as cell division, cell cycle progression, and signal transduction pathways [46]. They are also a good biological indicator to

monitor cell damage such as lipid peroxidation, DNA damage, and disrupting survival proteins due to significant expression, especially against stimuli of death signalling [47]. In this study, although the IAPs group genes were significantly overexpressed at both high doses and long-term application, we observed a number of death cells and apoptotic bodies by MTT and Tali assay in a population of MCF 7 cells. For this reason, the increases in IAPs expression levels can be explained by the protective roles due to enhanced efficiency of the cell survival systems of cells. However, it could be said that the marked increases in apoptotic cells can be explained by suppression of the *BCL 2* genes by long exposure to high concentrations of WM. In the MCF 7 cell line, our results indicated that *P53* gene expression was upregulated by WM treatment and anti-apoptotic Bcl-2 was downregulated, whereas the expression of pro-apoptotic protein BAX, Cyt-C, APAF 1 and Caspase 3 were upregulated in cells exposed to WM, which are considered as excellent signals to determine the intrinsic apoptosis pathway [48,49]. We observed that WM treatment did not cause oxidative stress in TG/HA-VSMC cell lines at both treatment dose and time. While significant increases were determined, the expression levels of IAPs group genes and anti-apoptotic BCL2 as well as pro-apoptotic APAF1 and Cyt-C, the main player of apoptotic genes, such as Caspase 3 and P21, were significantly down regulated. We thought that in TG/HA-VSMC cells, overexpressed IAPs genes allow enough time to repair the survival molecules, in the meantime increasing the level of BCL2 and suppressing caspase 3 activation signals that occur as a response to WM application, thus preventing apoptosis and non-cancerous cells remaining viable.

A number of studies conducted on both cell lines and animal tissues emphasised that the natural plant extract, with multiple bioactive molecules, has several advantages compared to single therapeutics [1,50]. Natural aqueous extracts are suitable for human consumption and most of them may be administered orally. Moreover, rich bioactive molecules within natural extracts not only effect many survival mechanisms in cells via a synergistic enhancement of anticancer activities but also may possibly decrease the resistance to chemotherapy [51]. In conclusion, our results demonstrate that 5-fold-diluted WM, especially at 48 hours, is selective in inducing apoptotic cell death in both human breast (MCF7) and prostate (DU 145) cancer cells by targeting intrinsic apoptosis signalling pathways. The same experimental exposure is well tolerated by non-cancerous TG/HA-VSMC cells. Compared to previous findings from studies on the anticancer effect of phenolic compounds on cancer cells, the mechanisms of action for WM seem more likely related to its phenolic ingredients. According to our findings, we suggest that WM is a potential anticancer agent with a selective apoptotic potential and special bioactive chemical constituents against at least human prostate and breast cancer.

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