

Investigation of the Apoptotic Effects of *Pistacia vera* Nut Skin Methanol Extract on Human Gastric Cancer Cell Line HGC-27 †

Abdullah Tuncay Demiryürek ^{1,*}, Ahmet Saracaloglu ¹, Ebru Temiz ², Seniz Demiryürek ³ and Bilge Sener ⁴

¹ Department of Medical Pharmacology, Faculty of Medicine, University of Gaziantep, Gaziantep 27310, Turkey; ahmetsaracal@gantep.edu.tr

² Department of Medical Biochemistry, Faculty of Medicine, Harran University, Şanlıurfa 63300, Turkey; ebrutemiz90@gmail.com

³ Department of Physiology, Faculty of Medicine, University of Gaziantep, Gaziantep 27310, Turkey; sdemiryurek@gantep.edu.tr

⁴ Department of Pharmacognosy, Faculty of Pharmacy, Gazi University, Ankara 06330, Turkey; bilgesener11@gmail.com

* Correspondence: demiryurek@gantep.edu.tr

† Presented at the 2nd International Cell Death Research Congress, Izmir, Turkey, 1–4 November 2018.

Published: 5 December 2018

Abstract: Gastric cancer (GC) is one of the most prevalent malignant types in the world and an aggressive disease with a poor 5-year survival. GC is the third greatest global cause of cancer-related deaths. It is well recognized that tumor heterogeneity, a fundamental feature of malignancy, plays an essential role in the cancer development and chemoresistance. In this study, we investigated the apoptotic effects of *Pistacia vera* nut skin methanol extract on human gastric cancer cell line HGC-27. The results of this study demonstrate for the first time the protective effect of *Pistacia vera* nut skin methanol extract on gastric carcinoma.

Keywords: apoptotic activity; gastric cancer; *Pistacia vera* extract

1. Introduction

Gastric cancer (GC) is the third leading cause of cancer-related deaths worldwide. GC represents one of the most aggressive cancer tumors, in which chemotherapy and molecularly targeted agents have had limited efficacy. Despite the improvement in conventional therapies for advanced GC, including surgery, chemotherapy and radiotherapy, the length or quality of life of patients with advanced GC is still poor [1,2]. Therefore, there is a need to develop novel therapy for GC. Bioactive natural products are a good source for development of novel cancer preventive and therapeutic drugs. In this study, we investigated the apoptotic activity of methanol extract of the pistachio (*Pistacia vera* L.) nut skin on the human GC cell line HGC-27.

2. Materials and Methods

HGC-27 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum. When cells reached 70% of confluence, they were treated with water extract or saline during 24 h. Cells were treated with methanol extracts (25, 50, 100, or 150 µg/mL) and supernatant nitric oxide (NO) levels were measured using NO/ozone chemiluminescence method.

NO measurements: Nitrate and nitrite are the primary oxidation products of NO and therefore the nitrate/nitrite level in plasma and tissue homogenates can be used as an indicator of NO formation. The plasma samples were deproteinized with absolute ethanol at 0 °C in a 1:2 *v/v* mix, incubated 30 min at 0 °C followed by centrifugation at 14,000 rpm for 5 min. The pellets were discarded and the supernatant was used to measure NO levels. For measurement of NO, we employed the NO/ozone chemiluminescence technique (Model 280i NOA, Sievers Instruments, Boulder, CO, USA). Briefly, a saturated solution of the reducing agent (vanadium-III chloride dissolved in 1 M HCl) was prepared and filtered before use. Five milliliters of this agent was added to purge the vessel with nitrogen for 5–10 min before use. The purge vessel was equipped with a cold-water condenser and a water jacket to permit heating of the reducing agent to 95 °C using a circulating water bath. The HCl vapors were removed by a gas bubbler containing 15 mL of 1 M NaOH. Samples and standards were injected into the purge vessel to react with the reducing agent, which converted nitrate, nitrite and S-nitroso compounds to NO. A continuous stream of pure nitrogen purged the resultant NO from the reaction vessel to the chemiluminescence chamber. A standard curve was established with a set of serial dilutions (0.1–300 µM) of sodium nitrate. The concentrations of NO metabolites in the samples were determined by comparison with the standard curve and expressed as µM. Data collection and analysis was performed using the NOAnalysis™ software (version 3.21, Sievers, Boulder, CO, USA) [3].

Apoptotic cells were quantified by Annexin-V/7AAD-positive staining, using an Annexin-V-FITC/7AAD kit from Beckman Coulter. The kit was used according to the manufacturer's instructions. In brief, treated and untreated (background control) cells (10⁵ cells/sample) were washed with DMEM cell culture medium supplemented with 2% FCS and centrifuged for 5 min at 400 g. Each sample was resuspended in 500 µL of DMEM with 2% FCS. Then 7-AAD was added to obtain concentrations of 1, 5, 10, and 20 µg/mL. Samples were incubated for 20 min on ice in darkness. After incubation, all samples were centrifuged (400 g, 5 min, 4 °C), washed once with 1 mL of PBS with 2% FCS (4 °C), centrifuged again (400 g, 5 min, 4 °C), and resuspended in 500 µL of PBS with 2% FCS (4 °C). Samples were stored on ice and analyzed by flow cytometry within 1 h on a flow cytometer (NAVIOS Beckman Coulter, Miami, FL, USA). Fluorescence intensity values between late apoptotic/dead and viable cells were considered to represent apoptotic cells.

For the cell cycle assay, BD Cycletest™ Plus DNA Reagent kit (BD Biosciences, San Jose, CA, USA) was used. Cell cycle analysis was performed by using flow cytometer (NAVIOS Beckman Coulter, Miami, FL, USA).

For gene expression study, mRNA was isolated from cells by using miRNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). Then cDNA was produced with the Ipsogen RT Kit (Qiagen GmbH). qRT-PCR was performed by Rotor Gene 6000 (Qiagen GmbH, Hilden, Germany). Protein expressions were analyzed with western blot method. Bicinchoninic acid protein assay kit (Thermo Fisher Scientific, IL, USA) was used to determine protein concentration. All samples were electrophoresed in 10% SDS polyacrylamide gels. The proteins were then transferred to polyvinylidene difluoride (PVDF) membranes. PVDF membranes were incubated overnight with primary antibodies and β-actin antibody was used as a loading control. Immunoreactivities of the bands were visualized with chemiluminescence substrate Super Signal West Pico (cat. no.34080, Thermo Fisher Scientific, IL, USA). The density of each band was measured using an automatic electrophoresis gel image analysis system (Image Lab, Bio-Rad, USA), then normalized to beta-actin levels.

Statistical analyses: All values were shown as the mean ± SD, SEM or percentage. The unpaired Student's t test was used for comparisons of the differences between mean values of two groups. Statistical analysis was carried out using GraphPad InStat version 3.05 (GraphPad Software Inc., San Diego, CA, USA). The *p* values of 0.05 or less were considered to be statistical significant.

3. Results

The supernatant of the cells treated with 150 mg/mL methanol extract produced 11.0 ± 3.5% inhibition in NO levels. Apoptosis of the HGC-27 cells was not stimulated with methanol extract

(0.6%) when compared to DMSO (0.5%). Proliferation assessed by cell cycle assay showed that the methanol extract decreased the G0/G1 phase of cell cycle (methanol extract: G0/G1 60.5%, G2 0.0%, and S 39.6%; DMSO: G0/G1 60.0%, G2 0.3%, and S 39.7%, $p > 0.05$). NFkB (2.5 fold), p16 (5.2 fold), p27 (2.4 fold), and p53 (2.2 fold) gene expressions were significantly augmented with methanol extract treatment. Similarly, NFkB (1.6 fold), p16 (4.4 fold), p27 (2.9 fold), and p53 (1.3 fold) protein expressions were significantly modified with methanol extract treatment.

4. Discussion

Our results showed that methanol extract of the *Pistacia vera* nut skin exhibited its anti-tumor activity against HGC-27 cells through elevation of apoptosis-related proteins. In a recent study, Rahman [4] has studied anticancer activities of mastic gum resin from *Pistacia atlantica* and found that the mastic gum resin suppressed proliferation of gastric adenocarcinoma (CRL-1739) cells. It was demonstrated in this study that the apoptotic effect to mastic gum resin was via the mitochondrial as shown by the up-regulation of Bax, down-regulation of Bcl-2 genes, and activation of caspase-9 and -3 activities [4]. Our findings are the first to demonstrate that marked increases in p16, p27, and p53 gene and protein expressions may mediate the anticancer activity of the *Pistacia vera* nut skin methanol extract.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Wu, K.; Nie, Y.; Guo, C.; Chen, Y.; Ding, J.; Fan, D. Molecular basis of therapeutic approaches to gastric cancer. *J. Gastroenterol. Hepatol.* **2009**, *24*, 37–41, doi:10.1111/j.1440-1746.2008.05753.x.
2. Venerito, M.; Vasapolli, R.; Rokkas, T.; Malfertheiner, P. Gastric cancer: Epidemiology, prevention, and therapy. *Helicobacter* **2018**, *23*(Suppl. 1), e12518, doi:10.1007/s10120-018-0876-0.
3. Alasehirli, B.; Demiryürek, S.; Arica, E.; Gürsoy, S.; Demiryürek, A.T. No evidence for an association between the Glu298Asp polymorphism of the endothelial nitric oxide synthase gene and fibromyalgia syndrome. *Rheumatol. Int.* **2007**, *27*, 275–280, doi:10.1007/s00296-006-0199-z.
4. Rahman, H.S. Phytochemical analysis and antioxidant and anticancer activities of mastic gum resin from *Pistacia atlantica* subspecies *kurdica*. *OncoTargets Ther.* **2018**, *11*, 4559–4572, doi:10.2147/OTT.S170827.



© 2018 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>).