Synergistic Anticancer Effects of Essential Oil from *Pistacia atlantica* subsp *mutica* Peel and Doxorubicin on Gastrointestinal Cancer Cells

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Abstract

Background: Developing less toxic therapies for gastrointestinal cancers is crucial, as doxorubicin chemotherapy causes severe side effects and drug resistance. This research investigated combining doxorubicin with wild pistachio peel essential oil (WPPEO). Plant compounds like WPPEO possess antioxidant properties that may reduce doxorubicin-induced oxidative stress. Their anti-inflammatory effects could also sensitize cancer cells to treatment. The study focuses on this combination's potential to enhance therapeutic efficacy while mitigating the significant limitations of standard doxorubicin use.

Materials and Methods: MTT assays assessed the cytotoxicity of WPPEO and doxorubicin, individually and combined, in gastric (MKN), liver (HepG2), colorectal (SW480) cancer cells, and normal fibroblasts (SKM). Synergy was evaluated via isobologram analysis. RT-PCR quantified BAX and BCL-2 gene expression to measure apoptosis induction.

Results: Doxorubicin and WPPEO synergistically reduced HepG2 viability to 32.4% (CI<1). WPPEO selectively targeted cancer cells, exhibiting 2-2.5 times lower toxicity to normal cells (IC_50=251 µg/mL) compared to cancer cells (IC_{50}: 100-125 µg/mL). Isobologram analysis confirmed this synergy, which sensitized cancer cells to doxorubicin-induced cell death via the intrinsic apoptosis pathway, increasing the pro-apoptotic BAX/BCL-2 ratio by 30-fold.

Conclusions: Doxorubicin and WPPEO worked together to significantly reduce the viability of HepG2 cancer cells to 32.4% (with a confidence interval less than 1). WPPEO selectively targeted cancer cells, showing 2 to 2.5 times lower toxicity to normal cells (IC50 = 251 µg/mL) compared to cancer cells (IC50: 100-125 µg/mL). An isobologram analysis confirmed this synergistic effect, which made cancer cells more sensitive to cell death induced by doxorubicin through the intrinsic apoptosis pathway. This was evidenced by a 30-fold increase in the pro-apoptotic BAX/BCL-2 ratio.

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1. Introduction

Cancer is a major global health crisis and a leading cause of death, highlighting the need for the continuous development of new and improved therapies. Among the various types of cancer, gastrointestinal cancers, such as gastric, liver, and colorectal cancers, are particularly concerning due to their high prevalence and low survival rates [1]. Common treatments for these cancers include surgery, chemotherapy, and radiotherapy. However, these treatment options often come with significant side effects and may lead to drug resistance. Consequently, extensive research is being conducted to discover new compounds from natural sources that possess anticancer properties while minimizing toxicity to healthy cells [2, 3].

Traditional healing practices have historically relied on therapeutic botanicals and their bioactive constituents to manage a wide array of illnesses. *Pistacia atlantica subsp. mutica*, also known as wild pistachio, is a species from the Anacardiaceae family found in various regions, including Iran. Reflecting its history of use in folk medicine for specific ailments, the therapeutic properties of the plant's various parts have been the subject of scientific investigation [4, 5].

Essential oils are plant-derived volatiles with antimicrobial, antioxidant, and anti-inflammatory effects. In wild pistachio, unlike the cultivated type, the green peel is edible and likely serves as an evolutionary defense for the seed. Rich in phenolics, flavonoids, and alkaloids, it protects against pests and pathogens and has been traditionally used to treat nausea, diarrhea, and gum weakness [6]. Previous research has revealed the significant anticancer potential of various parts of plants from the *Pistacia* genus. Specifically, different parts of the *Pistacia atlantica* plant have shown notable effects; for instance, its oleoresin essential oil has been effective in inducing apoptosis in gastric cancer cells, while its fruit extract has demonstrated selective cytotoxicity against oral cancer cells. Furthermore, the plant's gum is capable of inhibiting the growth of gastrointestinal cancer cells through cell cycle arrest and, notably, has shown a synergistic effect with the drug doxorubicin in breast cancer studies. Other species in this genus, such as *Pistacia lentiscus* (mastic gum) and *Pistacia terebinthus*, have also been reported to be effective in inhibiting angiogenesis in colon cancer and exhibiting cytotoxicity in liver cancer, respectively, all of which underscores the therapeutic potential of this plant family [7]. Despite studies investigating the anticancer properties of the gum or fruit of various Pistacia species, the essential oil extracted from the green peel of *Pistacia atlantica subsp. mutica* remains largely unknown. In particular, its potential synergistic effect with chemotherapeutic drugs such as doxorubicin is an important research gap that this study aims to address. This study focuses on the essential oil extracted from the green peel of wild pistachio. This part of the plant, which is typically discarded during processing, represents a potential source for the extraction of compounds with medicinal value. Doxorubicin is a widely used chemotherapeutic agent from the anthracycline class that is extensively applied in treating a wide range of

malignancies, including those investigated in the present research. However, Cardiotoxicity and acquired drug resistance in certain patients are major obstacles limiting doxorubicin's clinical utility. This highlights the urgent need for strategies that improve the drug's effectiveness while decreasing its toxic effects [8].

A promising strategy that has recently emerged involves employing natural compounds to act as adjuvant agents with chemotherapeutic drugs. These substances can enhance therapeutic outcomes via several mechanisms, including improving the chemosensitivity of malignant cells, inhibiting drug resistance mechanisms, and reducing drug toxicity to healthy cells [9].

Given the medicinal potential of wild pistachio and the necessity of discovering more effective therapeutic approaches for gastrointestinal cancers, this study aimed to investigate the anticancer effects of wild pistachio peel essential oil alone and in combination with the chemotherapeutic drug doxorubicin on human gastric cancer (MKN), liver cancer (HepG2), and colorectal cancer (SW480) cell lines. In this regard, the cytotoxic effects of these substances were analyzed using the MTT method, and isobologram analysis was performed to determine the type of interaction between the essential oil and doxorubicin. In addition, changes in the expression of apoptosis-related genes (BAX and BCL2) were also investigated. Our findings suggest the therapeutic potential of wild pistachio peel essential oil, either as a standalone anticancer agent or as an adjunct therapy for managing gastrointestinal malignancies.

2. Materials and Methods

2.1. Collection and Preparation of Wild Pistachio Peels

Green peels of wild pistachio were collected from Rabor County, Kerman Province, between early August and the end of October 2023. After collection, the peels were spread out in a shaded location to dry under ambient conditions. The dried peels were stored in a dark place until use. The plant material was examined and confirmed by botanists at the Rafsanjan Pistachio Research Center to belong to the *Pistacia atlantica* species, commonly known as wild pistachio (Beneh tree).

2.2. Essential Oil Extraction

Extraction of the essential oil from the pistachio peels was performed with a Clevenger-type apparatus. For this purpose, 150 grams of dried pistachio peel were mixed with 2000 milliliters of distilled water. The mixture was boiled for 4 hours to complete the extraction process. The obtained essential oil was dried using anhydrous sodium sulfate to remove residual moisture and stored in dark glass bottles at a temperature of -20°C [10].

2.3. Cell Culture

Human gastric cancer (MKN), liver cancer (HepG2), and colorectal cancer (SW480) cell lines, along with a normal human fibroblast cell line (SKM), were obtained from the Pasteur Institute of Iran. Cancer cell lines were cultured in RPMI 1640 medium, while fibroblasts were cultured in DMEM, both supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. All cells were maintained in a humidified incubator at 37°C with 5% CO_2 [11].

2.4 MTT Assay

Cytotoxic effects of wild pistachio peel essential oil and doxorubicin on cell lines were measured using the MTT method. Cells were cultured at a density of 10,000 cells per well in 96-well plates and incubated for 24 hours. Then, they were treated for 24 hours with different

concentrations of wild pistachio peel essential oil (5, 12, 25, 50, 100, and 200 micrograms per milliliter) and doxorubicin (0.1, 0.5, 1, 5, and 10 micromolar).

At the end of the incubation period, the culture medium in each well was aspirated and 150 µL of a 5 mg/mL MTT solution in fresh medium was added. The plates were then maintained for another 4 hours to permit formazan crystal development. To solubilize these crystals, the MTT-containing medium was removed, and 50 µL of DMSO was dispensed into each well. Finally, the absorbance was recorded at 570 nm with a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Each assay was conducted in three independent replicates, and the proportion of viable cells was calculated for every concentration. For each cell line, the half-maximal inhibitory concentration (IC50), defined as the concentration causing a 50% reduction in cell survival, was subsequently determined for both doxorubicin and the essential oil using the ED50 Plus plugin in Microsoft Excel [12].

2.5. Isobologram Analysis

To determine the type of interaction between wild pistachio peel essential oil and doxorubicin in different cell lines, isobologram analysis was conducted on each cell line individually for 24 hours. For this purpose, different concentrations of doxorubicin and pistachio peel essential oil were prepared according to Table 1 and applied to 96-well plates, following the same procedure as the MTT assay [13].

Table 1. Concentrations of Doxorubicin and Pistachio Essential Oil used in Isobologram Assay on three different cell lines, MKN45, HepG2, and SW480.

Cell lines	Doxo (µM)	EO (µM)
MKN45	0.95	125
	1.90	125
	2.85	125
	3.81	31.25
	3.81	62.50
	3.81	93.75
HepG2	0.75	100
	1.51	100
	2.26	100
	3.02	25
	3.02	50
	3.02	75
	0.85	109
	1.7	109
	2.55	109

SW480	3.4	27.25
	3.4	54.5
	3.4	81.75

2.6. RNA Extraction and cDNA Synthesis

Total RNA isolation from treated cells was performed with an RNA extraction kit (Sinacolon, Iran), following the manufacturer's instructions. The concentration and purity of the resulting RNA were then determined using a NanoDrop spectrophotometer (Nano Mahan, Iran). High RNA purity was confirmed, with the A260/A280 ratio of absorbance for all preparations falling within the 1.8 to 2.0 range. Complementary DNA (cDNA) synthesis was carried out using a cDNA synthesis kit (Takapou Zist, Tehran, Iran) following the manufacturer's instructions. The synthesis steps included incubation at 25 °C for 5 minutes, 42 °C for 60 minutes, and 85 °C for 5 minutes.

2.7. Gene Expression Analysis Using RT-PCR

Quantitative real-time PCR (qRT-PCR) analysis was carried out using the Takapou Zist kit (Tehran, Iran) on a 96-well real-time PCR system (Applied Biosystems, USA). Cells from each line were exposed for 24 hours to different concentrations of wild pistachio essential oil (0, 12.5, 25, 50, 100, and 200 µg/mL) as well as doxorubicin (0, 0.1, 0.5, 1, 5, and 10 µM). All treatments were performed in triplicate. The negative control consisted solely of the culture medium without any treatment. The final volume of each qRT-PCR reaction was 20 µL, consisting of 10 µL of SYBR Green Master Mix, 1 µL of cDNA, 1 µL of primer mix (forward and reverse, with a final concentration of 0.5 µM for each primer), and 8 µL of nuclease-free water.

Primers specific to the target genes BAX (pro-apoptotic) and BCL-2 (anti-apoptotic) were employed, with β-actin serving as the internal reference gene for normalization. The primer sequences were generated using Allele ID software, verified with GeneRunner, and subsequently synthesized by Ampliqon (Denmark). The primer details are listed in Table 2. Gene expression data were analyzed using the ΔΔCt method [14].

Table 2. Information on primers used in the Real-time PCR reaction.

Genes	Gene Bank Number	TM (°C)	Sequences	Product (Basepare)
β-ACTIN	NM_001101	64	F: GGACATCCGCAAAGACCTGTA	189
			R: ACATCTGCTGGAAGGTGGACA	
BCL2	NM_138578	61	F: GTGGATGACTGAGTACCTGA	119
			R: AGCCAGGAGAAATCAAACAGA	
	NM_004324	61	F: TTTGCTTCAGGGTTTCATCC	154

BAX			R: CAGCTCCATGTTACTGTCCA	

Statistical Analysis

Comparisons among the experimental groups and the reference group were performed through one-way ANOVA, followed by Dunnett's multiple comparison test. This statistical approach is generally resilient to moderate non-normality. Results were regarded as statistically significant when the p-value was below 0.05.

3. Results

3.1. MTT Assay

Outcomes of the MTT assay demonstrated that both the wild pistachio essential oil and doxorubicin exhibited concentration-dependent cytotoxic effects on the studied cell lines. This cytotoxicity was generally more pronounced in the cancer cell lines (MKN45, HepG2, and SW480) compared to the normal fibroblast cell line (SKM). Moreover, doxorubicin showed significantly stronger cytotoxicity than the essential oil (Figure 1). The IC50 values for each treatment in the different cell lines are summarized in Table 3.

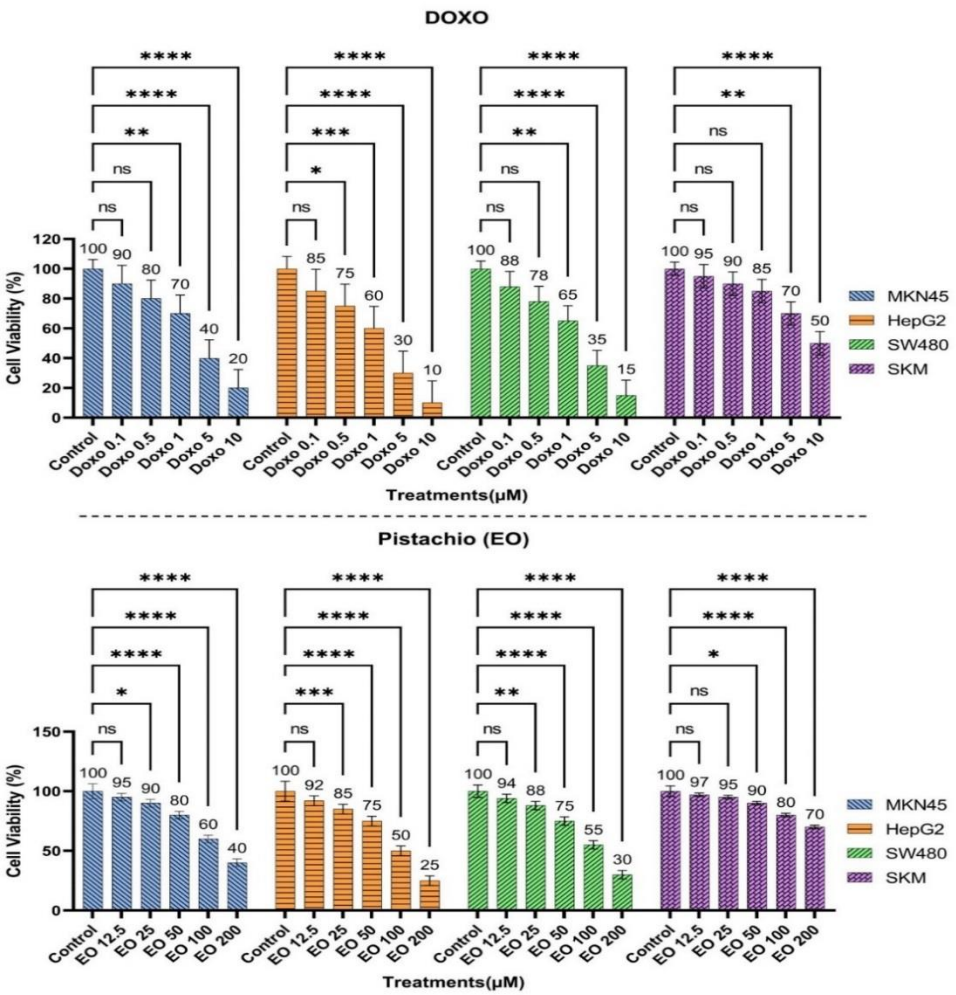

Fig 1. Bar graphs showing the percentage viability of four cell lines (MKN45, HepG2, SW480, and SKM) after 24-hour treatment with varying concentrations of doxorubicin (Doxo) and wild pistachio peel essential oil (EO).

Standard deviations from the mean are depicted as error bars, and asterisks denote statistically significant differences between groups (* indicates p-value <0.05).

Table 3. IC50 values of pistachio essential oil and doxorubicin in different cell lines, MKN45, HepG2, SW480, and SKM.

Cell lines	Pistachio Essential Oil IC50 (µM)	±SD	Doxorubicin IC50 (µM)	±SD
MKN45	125	8.5	3.81	0.12
HepG2	100	6.8	3.02	0.10
SW480	109	10.2	3.40	0.15
SKM	251	7.8	10	0.58

3.2. Isobologram Analysis Results

In all three cancer cell lines, increasing the variable concentration of the essential oil while keeping the doxorubicin concentration constant led to enhanced cytotoxic effects. Analyses were performed using 2-way ANOVA (Dunnett's method) in Prism software and showed CI < 1. Similarly, increasing variable concentrations of doxorubicin in the presence of a fixed concentration of the essential oil resulted in further reductions in cell viability. In this condition, the overall cell viability was lower than when each agent was used alone (Figure 2).

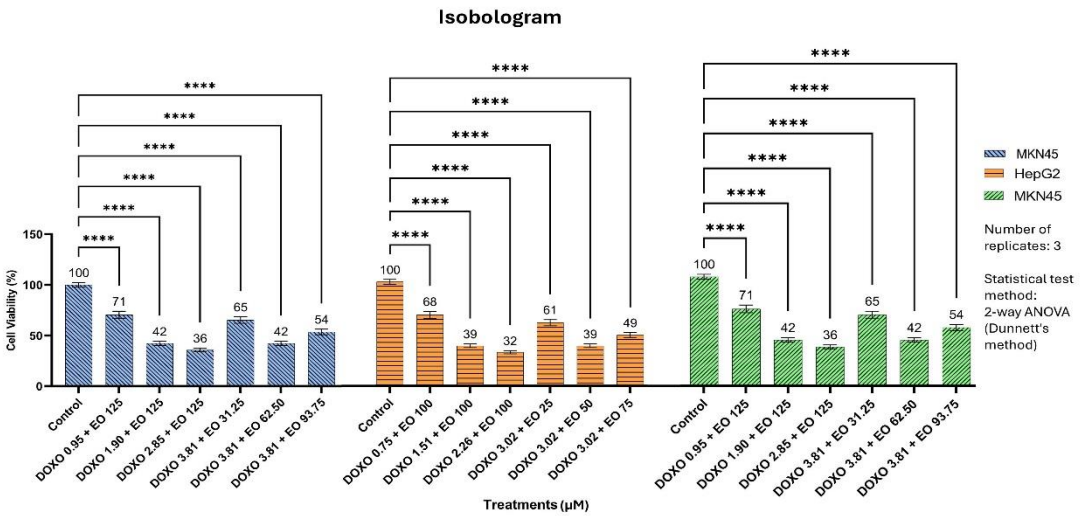

Fig 2. Results of the isobologram assay showing the viability percentage of gastrointestinal cancer cell lines following treatment with various combinations of Doxo (doxorubicin) and EO (pistachio essential oil). The x-axis represents different drug treatment combinations at varying concentrations, and the y-axis indicates cell

viability percentage. Standard deviations from the mean are depicted as error bars, and asterisks mark statistically significant variations relative to the control.

3.3. Results of Real-Time PCR Analysis

Gene expression results demonstrated that both pistachio essential oil and the chemotherapeutic agent doxorubicin significantly affected (p-value <0.05) the expression of key apoptotic genes in cancer cells but not in SKM cells. However, these changes were generally more pronounced in the doxorubicin-treated groups. Separate administration of doxorubicin and essential oil produced a notable, dose-dependent increase in *BAX* transcript levels throughout all treatments. In addition, *BCL*-2 gene expression was significantly and dose-dependently downregulated in all treatment groups. The detailed expression levels are presented in Figure 3.

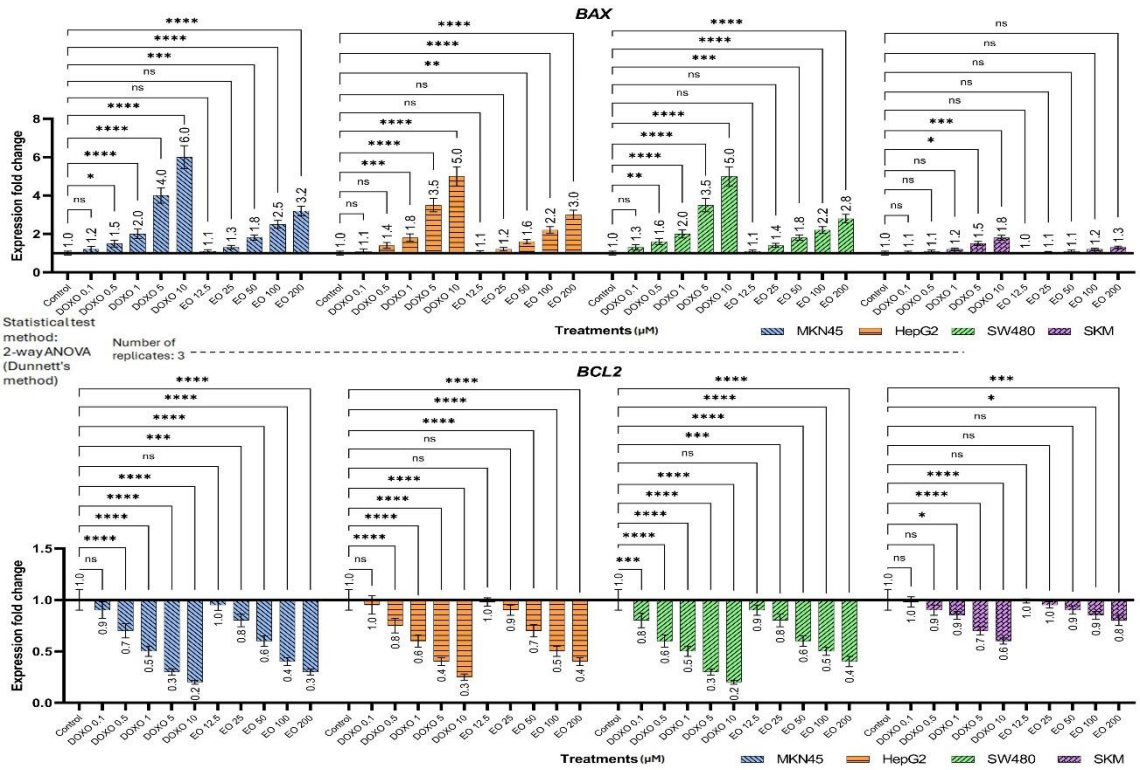

Fig 3. Expression changes of apoptotic genes Bax and Bcl-2 in MKN45, HepG2, SW480, and SKM cell lines after treatment with various concentrations of doxorubicin (Doxo) and pistachio essential oil (EO). Gene expression was measured using Real-Time PCR and is presented as fold change relative to the control group. Standard deviations from the mean are depicted as error bars, and asterisks mark statistically significant variations relative to the control.

4. Discussion

Doxorubicin, a widely used chemotherapeutic agent from the anthracycline class, exerts its antitumor effects through multiple mechanisms. The processes include insertion into DNA and suppression of the enzyme topoisomerase II, which subsequently causes DNA damage and activates apoptotic pathways. Doxorubicin also induces cancer cell

apoptosis by elevating ROS levels, thereby exacerbating oxidative stress and mitochondrial damage [8].

Nevertheless, the clinical application of doxorubicin is limited because of its systemic toxicity, particularly cardiotoxicity, as well as the emergence of treatment resistance [15]. The mechanisms of drug resistance vary across different cancer cell lines, highlighting the need to develop combination strategies to enhance efficacy and overcome these limitations. For instance, studies have shown that combining compounds such as curcumin with doxorubicin can produce synergistic effects in colorectal cancer cells like the HCT-116 cell line, increasing cytotoxicity even at lower doses [16, 17].

Recently, plant-derived natural compounds have attracted considerable attention in research because of their ability to combat cancer while exhibiting lower toxicity than conventional chemotherapeutic agents. Due to their complex composition of bioactive volatile compounds, essential oils have drawn considerable interest for exhibiting a wide range of biological activities, encompassing antimicrobial, antioxidative, and anti-inflammatory actions [18]. *Pistacia atlantica* is one such natural source that has traditionally been used in folk medicine. Previous studies have reported various biological activities for different parts of this plant. Essential oils extracted from different *Pistacia* species have demonstrated notable antioxidant and anti-inflammatory properties, which may contribute to their anticancer effects [19].

Previous research has evaluated the cytotoxic effects of *Pistacia atlantica* essential oil on human gastric cancer cell lines (MKN-45 and AGS), showing its influence on the expression of apoptosis-related genes *BAX* and *BCL*-2 and the induction of apoptosis [7]. Additionally, the cytotoxic effects of the ethanolic extract from *Pistacia atlantica* subsp. *kurdica* fruit has been investigated on oral cancer cell lines (KB) and human gingival fibroblasts (HGF), with findings indicating cytotoxicity against cancer cells and induction of apoptosis, while showing lower toxicity towards normal HGF cells. These findings suggest that other parts of *Pistacia atlantica* also possess selective cytotoxic and pro-apoptotic effects on cancer cell lines [20].

Studies have shown that the gum of *Pistacia atlantica*, particularly the *kurdica* subspecies, exhibits significant anticancer effects. The growth of gastrointestinal tumors, including cholangiocarcinoma, pancreatic, gastric, and colorectal types, is restrained by these compounds in a concentration- and time-related manner. This effect is mediated through apoptosis induction (characterized by Bax upregulation, BCL-2 downregulation, and the activation of caspases-9 and -3) along with G2/M phase cell cycle blockade, without harming non-malignant cells. [21]. Furthermore, in breast cancer (MCF-7), the gum has shown synergistic effects with doxorubicin by enhancing apoptotic markers (caspase-3 and p53) and decreasing cyclin D1 levels [22]. A nanostructured formulation containing the essential oil of this plant has also been shown to increase cytotoxicity against SKBR3 cells [23]. In addition to its anticancer properties, this plant exhibits antioxidant activity (attributed to its high polyphenol content) [24]. Anti-inflammatory effects (e.g., reducing IL-6 and CRP levels) [25], antimicrobial activity (against *S. aureus* and *E. coli*) [26], and metabolic regulatory effects (e.g., anti-diabetic properties) [27]. Traditionally, it has been used to treat wounds and gastrointestinal disorders [28]. Its main bioactive components include terpenoids

(such as α-pinene) and phenolics (such as gallic acid) [29].

The present study, employing a novel approach, investigates the therapeutic potential of wild pistachio (*Pistacia atlantica*) green hull essential oil as an adjuvant agent against gastrointestinal malignancies. This research simultaneously evaluates the essential oil's ability to increase the sensitivity of cancer cells to doxorubicin and to reduce potential drug resistance, alongside assessing its cytotoxicity on cancer cell lines and a normal fibroblast cell line. The use of pistachio hull, an agricultural by-product, not only offers potential therapeutic value but also presents economic and environmental benefits. In this study, the cytotoxic effects and molecular mechanisms of wild pistachio hull essential oil and doxorubicin, both individually and in combination, were investigated in three human gastrointestinal cancer cell lines (MKN45, HepG2, and SW480) and one normal cell line (SKM fibroblasts). Results from the MTT assay showed that both the essential oil and doxorubicin reduced the viability of cancer cells in a dose-dependent manner across all three cancer cell lines. This finding aligns with the well-established efficacy of doxorubicin as a potent chemotherapeutic agent. Additionally, results from the SKM normal fibroblast cell line showed that, at concentrations effective against cancer cells, the essential oil exhibited lower cytotoxicity toward normal cells. On the other hand, the results of the isobologram analysis indicated a synergistic interaction between wild pistachio hull essential oil and doxorubicin in inducing cytotoxicity in the studied cancer cell lines. The combined use of varying concentrations of both agents led to greater cell death compared to treatment with each agent alone. This synergism suggests that the effective dose of doxorubicin required to achieve desirable anticancer effects can be reduced when combined with the essential oil. Lowering the clinical dose of doxorubicin could significantly reduce adverse effects such as cardiotoxicity while maintaining or even enhancing therapeutic efficacy.

The synergistic interaction between wild pistachio hull essential oil and doxorubicin, quantitatively confirmed by CI<1, translated into a profound cytotoxic effect. This was exemplified in the HepG2 cell line, where the combination treatment reduced cancer cell viability to just 32.4%. Data from Real-Time PCR further elucidated the molecular mechanism underlying this synergy. According to the results, the treatments modulated the intrinsic apoptotic signaling by enhancing the levels of *BAX*, a pro-apoptotic marker, while suppressing *BCL-2*, an anti-apoptotic marker. This created a critical shift in the *BAX/BCL-2* ratio; for instance, doxorubicin alone was capable of increasing this ratio by up to 30-fold. The powerful synergy observed in combination therapy strongly suggests a potentiation of this molecular switch, confirming that the essential oil effectively sensitizes cancer cells to doxorubicin-induced apoptosis. This shift in the *BAX/BCL*-2 ratio toward apoptosis strongly correlated with the reduction in cell viability observed in the MTT assay. An elevated *BAX/BCL-2* ratio is a pivotal event in the intrinsic apoptotic pathway, as it triggers mitochondrial outer membrane permeabilization (MOMP). This process results in the translocation of essential pro-apoptotic factors, particularly cytochrome c, from the mitochondria into the cytosol. Once released, cytochrome c promotes apoptosome assembly, thereby triggering the caspase activation cascade, starting with caspase-9 and leading to the activation of effector caspases such as

caspase-3, which ultimately execute cell death. Therefore, while not directly measured, the pronounced shift in the *BAX/BCL-2* ratio strongly implies that the observed cytotoxicity is executed through this canonical mitochondrial-caspase pathway [30].

This study represents a significant step toward the development of combination therapies with lower toxicity and higher efficacy for gastrointestinal cancers by leveraging the potential of a native natural resource. However, focusing on only two key genes in the apoptotic pathway despite apoptosis being regulated by a complex network of factors constitutes a limitation. Moreover, the in vitro nature of the study limits its ability to fully replicate the complex tumor microenvironment and systemic physiological responses. The pharmacokinetics and bioavailability of WPPEO's active components are unknown, and understanding how they are absorbed, distributed, metabolized, and excreted is essential for clinical applicability. Another significant hurdle for natural products is the inherent variability in their chemical composition due to factors like harvest time and geographical origin. Therefore, standardization and rigorous quality control would be crucial prerequisites for developing a reliable therapeutic agent from this essential oil. To build upon these findings, a clear roadmap for future research is essential. The next critical step is to validate these in vitro results in preclinical animal models, such as xenografts of human gastrointestinal tumors, to evaluate the combination's efficacy on tumor growth and overall survival. Within these in vivo studies, comprehensive pharmacokinetic profiling should be conducted to determine the bioavailability, tissue distribution, and clearance rates of the essential oil's key components. To directly address the clinical challenge of chemoresistance, the synergistic effects should be tested specifically in doxorubicin-resistant cancer models to confirm WPPEO's potential as a chemosensitizing agent. Furthermore, to overcome issues of low bioavailability and improve tumor targeting, developing advanced delivery systems, such as nanocarrier formulations (e.g., liposomes or polymeric nanoparticles), should be explored. These future studies will be crucial for translating the therapeutic potential of this natural resource into a viable clinical strategy.

5. Conclusion

The results of this study indicate that the essential oil of Pistacia atlantica subsp. mutica has significant anticancer properties against gastrointestinal cancers. This effect appears to be achieved through the induction of apoptosis while maintaining very low toxicity toward healthy gastrointestinal cells, making it a natural and potentially safe option, pending in vivo validation. Importantly, this edible essential oil demonstrates a remarkable synergistic effect when combined with the chemotherapeutic agent doxorubicin. Our findings suggest that, due to its oral bioavailability and high safety profile, this essential oil could serve as an effective natural adjuvant in combination therapy protocols for gastrointestinal cancers. This approach may even allow for a reduction in the required dosage of doxorubicin, thereby alleviating its adverse gastrointestinal effects. Additionally, the long-standing role of this plant in traditional medicine further emphasizes its potential as a safer and more efficient option for developing oral therapies against gastrointestinal malignancies.

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Conflict of Interest

The authors declare no conflicts of interest

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