

Original Research

Changes in TP53 Gene Expression in PANC-1 Pancreatic Cancer Cells Treated with a Crude Aqueous Extract of *Syzygium aromaticum*

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Abstract

Pancreatic cancer is a highly aggressive and therapy-resistant malignancy. This study evaluated the cytotoxic effects of *Syzygium aromaticum* (clove) aqueous extract on PANC-1 pancreatic cancer cells and its effect on TP53 mRNA expression. PANC-1 cells were exposed to increasing concentrations of the extract (10-1000 µg/ml) for 48 h. We assessed cell viability using the MTT assay and calculated the half-maximal inhibitory concentration (IC₅₀) using four-parameter logistic regression. TP53 mRNA expression was quantified by quantitative real-time PCR (qRT-PCR). Statistical analyses were performed using one-way ANOVA followed by Dunnett's post hoc test, with $P < 0.05$ considered statistically significant. The extract significantly reduced PANC-1 cell viability in a concentration-dependent manner ($P < 0.0001$), with viability decreasing from 39.55% at 10 µg/ml to 9.01% at 1000 µg/ml. The calculated IC₅₀ was 2.743 µg/ml (95% CI: 0.5627-6.345 µg/ml). TP53 mRNA expression increased approximately 2.3-fold following treatment compared with untreated controls ($P = 0.0058$). *S. aromaticum* aqueous extract demonstrated potent antiproliferative activity against PANC-1 cells and increased TP53 mRNA expression. However, the functional significance of TP53



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upregulation and its contribution to apoptosis or other cellular mechanisms require further investigation.

Keywords

Syzygium aromaticum; pancreatic cancer; apoptosis; anticancer activity

1. Introduction

Pancreatic cancer is one of the most lethal malignancies of the gastrointestinal tract and remains a major global health challenge. It currently ranks among the leading causes of cancer-related mortality worldwide and is projected to become the second leading cause of cancer death by 2030 [1-3]. The mortality rate of pancreatic cancer closely parallels its incidence, reflecting its aggressive biology and the lack of effective early detection strategies [4, 5]. The poor prognosis of pancreatic cancer is largely due to its nonspecific early symptoms and frequent diagnosis at advanced stages. Therefore, developing sensitive biomarkers and advanced imaging strategies for earlier detection remains a critical clinical need [6, 7].

At the molecular level, pancreatic ductal adenocarcinoma is characterized by accumulating genetic and epigenetic alterations. Activating mutations in the proto-oncogene KRAS occur in over 90% of cases and activate downstream signaling pathways such as RAF/MEK/ERK and PI3K/AKT, leading to uncontrolled proliferation [8, 9]. Additional oncogenic alterations, such as MYC activation, contribute to tumor progression, invasion, and therapeutic resistance. Concurrently, inactivation of tumor suppressor genes, including CDKN2A (p16) and p53, facilitates cell cycle deregulation and genomic instability. Among tumor suppressors, p53 plays a central role in maintaining genomic integrity and is frequently mutated in 50-75% of pancreatic cancers [10-12].

Increased TP53 mRNA may reflect a cellular stress or regulatory response rather than restoration of functional p53 signaling [13, 14]. After DNA damage, p53 induces cell cycle arrest, DNA repair, senescence, or apoptosis by transcriptionally regulating downstream targets such as p21, Bax, and other cell-fate effectors [15, 16]. Mutant p53 may interact with oncogenic pathways such as Ras and NF- κ B, contributing to tumor progression and treatment resistance. Therefore, TP53 expression changes in PANC-1 cells should be interpreted in the context of their mutant TP53 background [17, 18].

In parallel with advances in molecular oncology, increasing attention has been directed toward natural bioactive compounds as potential anticancer agents. Among these, *Syzygium aromaticum* (clove), a member of the Myrtaceae family, is widely cultivated in tropical and subtropical regions and is rich in essential oils and polyphenolic compounds [19, 20]. Clove extracts contain bioactive constituents such as eugenol, eugenyl acetate, β -caryophyllene, and α -humulene, which exhibit antioxidant, anti-inflammatory, antimicrobial, and anticancer properties [21, 22]. The effects of crude aqueous clove extract cannot be attributed to a single compound, as its bioactivity may result from multiple constituents and their interactions [22, 23].

Eugenol, the principal phenolic compound, has been reported to inhibit tumor cell proliferation, induce apoptosis, and modulate reactive oxygen species (ROS) generation. Similarly, β -caryophyllene and α -humulene have demonstrated antiproliferative and pro-apoptotic effects [24,

25]. Considering the pivotal role of p53 in controlling cell-cycle progression, apoptosis, and genomic stability, the present study aimed to investigate changes in TP53 mRNA expression in PANC-1 pancreatic cancer cells after treatment with a crude aqueous extract of *Syzygium aromaticum*.

2. Materials and Methods

2.1 Cell Culture

PANC-1 cells were cultured in complete growth medium composed of DMEM supplemented with fetal bovine serum (FBS), penicillin-streptomycin, GlutaMAX, and non-essential amino acids (NEAA). Culture flasks were routinely examined under an inverted microscope to assess cell morphology and viability. Cells were maintained at 37°C in a humidified CO₂ incubator, and the culture medium was replaced as required.

Cells were passaged when they reached approximately 70-80% confluence. The culture medium was aspirated, and cells were gently rinsed with 1 mL sterile phosphate-buffered saline (PBS) to remove residual serum. Subsequently, 1 mL of 0.05% trypsin-EDTA was added, and the flask was incubated at 37°C for 7 minutes to facilitate detachment. Gently tap the flask to release adherent cells. Neutralize trypsin activity by adding 2 mL of complete medium. Transfer the resulting suspension to a 15 mL Falcon tube and centrifuge at 2000 rpm for 7 minutes. After discarding the supernatant, the cell pellet was resuspended in 7 mL of complete medium. Half of the suspension was transferred into a new culture flask, supplemented with fresh medium, and incubated for 72 hours under standard conditions.

2.2 Cryopreservation

For cryopreservation, cells were harvested using trypsinization as described above. After centrifugation at 2000 rpm for 7 minutes, the supernatant was removed, and the cell pellet was resuspended in a freezing mixture containing 1800 µL FBS and 200 µL DMSO. The suspension was transferred into cryovials, sealed with Parafilm, and wrapped in aluminum foil. To ensure gradual temperature reduction, cryovials were placed among ice pieces, stored at -20°C for 2 hours, and subsequently transferred to -80°C for long-term storage.

2.3 Clove Extraction

An aqueous clove extract was prepared by adding 25 g of clove powder to 500 mL of distilled water in a sterile beaker. The mixture was heated at 90°C using a heater-stirrer until the volume decreased to approximately 200 mL. After cooling, the solution was filtered through filter paper and reheated to evaporate the solvent completely. The concentrated extract was transferred to a sealed glass container and freeze-dried to obtain a powdered form. To prepare the stock solution, 0.1 g of the dried extract was dissolved in 9 mL DMEM and 1 mL PBS, yielding a final concentration of 10 µg/mL.

Because this study used a crude aqueous clove extract, we did not quantify the extract's total polyphenol content. Therefore, the concentrations reported throughout the study refer to the mass concentration of the crude extract rather than to a standardized concentration of individual or total polyphenolic compounds.

2.4 3-(4,5-Dimethylthiazol-2-yl) 2,5-Diphenyl Tetrazolium Bromide (MTT) Cell Viability Test

We evaluated cell viability using the MTT assay. The MTT reagent (Sigma-Aldrich, USA) was prepared by dissolving 500 mg of MTT powder in 15 mL PBS, then vortex mixing. The solution was protected from light and stored at 4°C. For the assay, cells were harvested, centrifuged at 2000 rpm for 5 minutes, and resuspended in complete medium to achieve approximately 3000 cells per 100 µL in each well of a 96-well plate and incubated for 48 hours at 37°C in a CO₂ incubator to allow cell attachment.

Then, cells were treated with clove extract at concentrations of 10, 25, 50, 100, 250, 500, 750, and 1000 µg/ml. Blank wells (without cells and extract) and negative control wells (cells without extract) were included. Each concentration was tested in six replicates, and the plates were incubated with the extract for 48 hours before assessment of cell viability. Following treatment, the medium was discarded, and 25 µL of MTT solution was added to each well and incubated for 3-4 hours. The MTT solution was then removed, and 100 µL of isopropanol was added to dissolve the formazan crystals. After gentle shaking for 15 minutes, absorbance was measured at 570 nm using an ELISA reader (BioTek, USA). The IC₅₀ value was defined as the concentration of clove extract that reduced cell viability to 50% relative to the untreated control.

2.5 RNA Extraction

Total RNA extraction was performed using the SinaClon RNA extraction kit (SinaClon BioScience Co., Iran) according to the manufacturer's instructions. The kit components included 50 mini spin columns, 50 collection tubes, 20 mL lysis solution, 15 mL precipitation solution, 20 mL Wash I solution, 40 mL Wash II solution, and RNase-free water (2 × 1250 µL). Purified RNA was eluted using RNase-free water and stored at -80°C until further analysis. RNA concentration and purity were evaluated spectrophotometrically.

2.6 Complementary DNA (cDNA) Synthesis

cDNA was synthesized using the SinaClon cDNA synthesis kit (SinaClon BioScience Co., Iran). Extracted RNA was first mixed with DEPC-treated water and oligo(dT) primer and incubated at 70°C for 6-7 minutes. In a separate tube, 5× buffer solution, RNase inhibitor, and reverse transcriptase enzyme were prepared. The two mixtures were combined and subjected to the following thermal program: 25°C for 5 minutes, 50°C for 40 minutes, and 70°C for 15 minutes.

2.7 Quantitative Real-Time PCR (qRT-PCR)

Primer sequences for the target gene *p53* and the reference gene *β-actin* (*ACTB*) were obtained from the [26] study and validated for specificity using BLAST analysis via the National Center for Biotechnology Information (NCBI) database. Primer stocks and working solutions were prepared according to the manufacturer's instructions. For qRT-PCR analysis, PANC-1 cells were treated with clove extract at the experimentally determined IC₅₀ concentration of 2.743 µg/ml. The treatment duration was 48 hours, consistent with the MTT assay. The primer sequences and corresponding information are presented in Table 1.

Table 1 Primer sequences used for quantitative real-time PCR analysis.

Genes	Primers	Sequences	Ref.
<i>p53</i>	Reverse	5'-ATGAGCCAGATCAGGGACTG-3'	[26]
	Forward	5'-CCCCTCCATCCTTTCTTCTC-3'	
<i>β-actin</i>	Reverse	5'-GGGCACGAAGGCTCATCATT-3'	[26]
	Forward	5'-AGCGAGCATCCCCCAAAGTT-3'	

TP53, tumor protein p53; *ACTB*, β-actin.

We performed q-RT-PCR using the SinaClon kit (SinaClon BioScience Co., Iran). The amplification program consisted of an initial denaturation step at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 54°C for 40 seconds, and extension at 72°C for 40 seconds, with a final extension at 72°C for 5 minutes using a MIC qPCR thermocycler (BioMolecular Systems, Queensland, Australia). All qRT-PCR reactions were performed in technical triplicate for each of three independent biological replicates per group. Cycle threshold (Ct) values for TP53 and ACTB were recorded, and relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method. Statistical analysis was performed using the mean expression value obtained from the technical replicates for each independent biological replicate. Data analysis and normalization were performed using GenEx software.

2.8 Statistical Analysis

All statistical data were analyzed using Prism®9.4.1 software (GraphPad Software Inc., LaJolla, CA) by considering a significant level less than 0.05. Normality tests, including D'Agostino-Pearson, Anderson-Darling, Shapiro-Wilk, and Kolmogorov-Smirnov, were performed. Independent t-test and one-way ANOVA were applied to compare the results among the study groups.

3. Results

Assessing the cytotoxic activity of *Syzygium aromaticum* extract against PANC-1 cells using the MTT assay following 48 h of exposure to concentrations ranging from 10 to 1000 µg/ml demonstrated a marked, concentration-dependent decrease in cell viability (Figure 1). Relative cell viability declined from 39.55% at 10 µg/ml to 9.01% at 1000 µg/ml, demonstrating a robust inhibitory effect even at low concentrations.

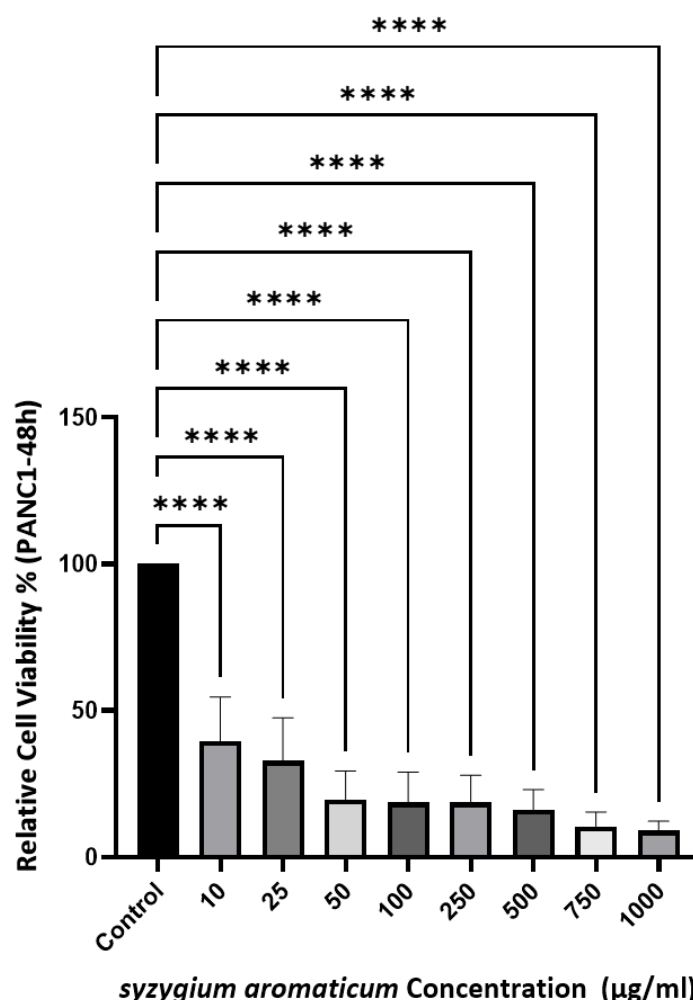


Figure 1 Effect of *Syzygium aromaticum* extract on PANC-1 cell viability after 48 h of treatment, assessed by the MTT assay. Cells were exposed to increasing concentrations of the extract (10-1000 µg/ml). Bars represent the mean $\pm$ SD of five replicates per group. All concentrations significantly reduced cell viability compared with the untreated control, as determined by Dunnett's multiple comparisons test (**** $P < 0.0001$). Metabolic activity declined dose-dependently, with the highest concentrations producing near-complete inhibition of cell viability.

One-way ANOVA revealed a highly significant overall effect of treatment on cell viability [$F(8, 36) = 44.02$, $P < 0.0001$, $R^2 = 0.9073$]. Dunnett's post-hoc test indicated that all extract concentrations significantly reduced viability compared with the untreated control (all adjusted $P < 0.0001$), with mean differences ranging from 60.45% at 10 µg/ml to 90.99% at 1000 µg/ml. The Brown-Forsythe test showed no evidence of heteroscedasticity ($P = 0.0919$), indicating homogeneity of variances among treatment groups.

Residual evaluation confirmed that the data met normality assumptions. D'Agostino-Pearson, Anderson-Darling, Shapiro-Wilk, and Kolmogorov-Smirnov tests all reported non-significant P values ($P > 0.05$ for all), supporting normal distribution of residuals. This was consistent with the QQ plot, in which residuals aligned closely with the reference line (Figure 2).

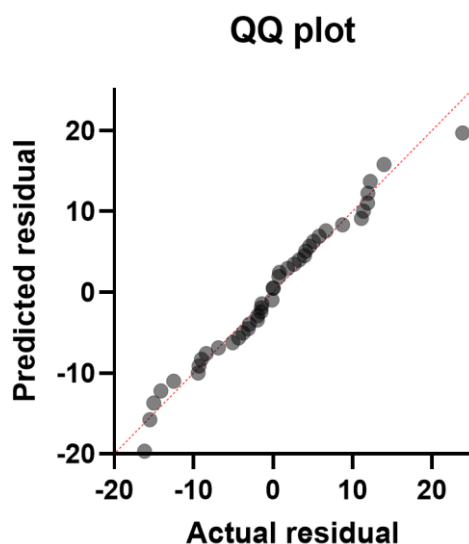


Figure 2 QQ plot of standardized residuals from the one-way ANOVA evaluating the effects of *Syzygium aromaticum* extract on PANC-1 cell viability. The plotted points represent observed residuals compared with those expected under a normal distribution (red dashed reference line). The close alignment of points with the reference line indicates that the residuals meet normality assumptions, consistent with multiple statistical normality tests.

A variable-slope four-parameter logistic model was fitted to determine the IC_{50} value of the extract. The model showed excellent goodness of fit ($R^2 = 0.9138$), with an estimated IC_{50} of 2.743 $\mu\text{g/ml}$ (95% CI: 0.5627-6.345 $\mu\text{g/ml}$). The Hill slope was -0.3723 (95% CI: -0.5088 to -0.2525), indicating a gradual inhibitory transition across the concentration range. *Syzygium aromaticum* extract exerts strong cytotoxic effects on PANC-1 cells, with significant reductions in viability across all tested concentrations and a low IC_{50} value in the sub-10- $\mu\text{g/ml}$ range.

To investigate the effect of *Syzygium aromaticum* extract on p53 mRNA expression in PANC-1 cells, relative p53 transcript levels were quantified by qRT-PCR following 48 h of treatment with the extract at its IC_{50} concentration (2.743 $\mu\text{g/ml}$). As shown in Figure 3, treatment with the extract significantly increased p53 mRNA expression compared with the untreated control group. The mean fold-change increased from 1.00 in the control to 2.289 in the treated cells. An unpaired t-test with Welch's correction confirmed that this increase was significant ($t = 13.12$, $df = 2.000$, $P = 0.0058$). The difference between group means was 1.289 ± 0.0983 (95% CI: 0.8663-1.712), a marked difference in relative p53 mRNA expression between the groups ($P < 0.0001$).

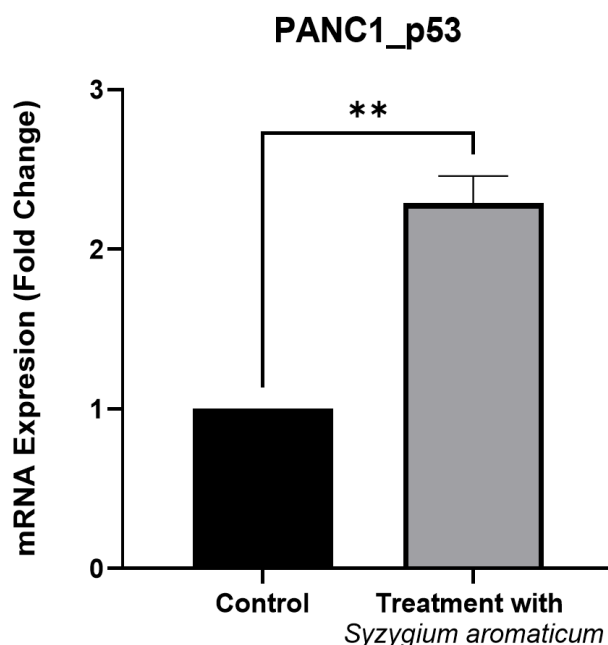


Figure 3 Relative p53 mRNA expression in PANC-1 cells following treatment with *Syzygium aromaticum* extract. Relative TP53 mRNA expression in PANC-1 cells following treatment with *Syzygium aromaticum* extract. Relative TP53 mRNA expression was determined by qRT-PCR after 48 h of treatment with clove extract at its IC₅₀ concentration (2.743 µg/ml) and normalized to ACTB. Each biological sample was analyzed in technical triplicate. Data are presented as mean ± standard deviation (SD) from three independent biological replicates per group. The comparison was performed using an unpaired t-test with Welch's correction; P = 0.0058 versus the untreated control.

4. Discussion

The present study demonstrated that the extract of *Syzygium aromaticum* exhibited a very low IC₅₀ value (2.743 µg/ml), indicating substantial antiproliferative potency even at relatively low concentrations. In parallel, treatment significantly increased TP53 mRNA, suggesting that modulation of p53-related signaling may be associated with the observed antiproliferative effect.

The strong cytotoxic effect observed in the current study is broadly consistent with findings reported by Kim et al. using purified eugenol in PANC-1 cells [27]. Their study also showed that eugenol sensitized pancreatic cancer cells to TRAIL-mediated apoptosis by upregulating death receptor-5 (DR5), activating caspase-3 and caspase-8, and cleaving PARP. Importantly, they reported that eugenol increased p53 expression and induced ROS-mediated endoplasmic reticulum (ER) stress, ultimately promoting apoptotic cell death [27]. However, these findings were obtained using purified eugenol rather than crude aqueous *S. aromaticum* extract and therefore cannot be directly extrapolated to the present preparation.

The observed upregulation of p53 is mechanistically important because p53 is one of the most critical tumor suppressor proteins involved in cellular stress responses, DNA repair, apoptosis, and cell cycle arrest. However, interpreting TP53 modulation in PANC-1 cells requires consideration of their hotspot TP53 p.R273H mutation and loss of normal wild-type p53 function. Thus, increased TP53 mRNA in this model should not be interpreted as restoration of functional wild-type p53

signaling and may instead reflect a cellular stress or regulatory response. Activation of p53 can induce transcription of pro-apoptotic genes such as *BAX*, *PUMA*, and *NOXA*, while simultaneously suppressing anti-apoptotic mediators including *BCL-2* [28, 29]. Increased p53 activity also promotes mitochondrial outer membrane permeabilization, cytochrome c release, and downstream activation of caspase-dependent apoptosis [30, 31]. In pancreatic cancer cells, restoring or enhancing p53 signaling may partially overcome intrinsic resistance to apoptosis and improve susceptibility to cytotoxic agents [32]. Therefore, the observed increase in TP53 mRNA may be associated with the cellular response to *S. aromaticum* treatment; however, its contribution to apoptosis or mitochondrial signaling cannot be established from the present data [33, 34].

ROS-mediated mechanisms may also explain the observed cytotoxicity of *Syzygium aromaticum*, involving ROS generation. Although eugenol is often described as an antioxidant under physiological conditions, increasing evidence suggests that in cancer cells it may exert pro-oxidant effects, resulting in excessive ROS accumulation and oxidative stress-mediated apoptosis [33, 35, 36]. Cancer cells are particularly vulnerable to oxidative imbalance because they already maintain elevated basal ROS levels to sustain rapid proliferation. In cancer cells, excessive ROS can potentially contribute to mitochondrial dysfunction, DNA damage, ER stress, and apoptotic signaling; however, these mechanisms were not directly assessed in the present study [37, 38]. Although previous studies of purified eugenol in PANC-1 cells have implicated ROS generation and ER-stress signaling through the PERK/eIF2 α /ATF4/CHOP axis [27, 39], the present study did not directly measure ROS production, mitochondrial membrane potential, or ER-stress markers. Therefore, these mechanisms should be considered hypothetical rather than demonstrated effects of the crude aqueous extract.

Previous investigations also support these findings by evaluating other natural phytochemicals against pancreatic cancer cells. Capsaicin has been shown to suppress PANC-1 viability through inhibition of the PI3K/Akt pathway and activation of caspase-3-dependent apoptosis [40-42]. Similarly, zerumbone induced apoptosis in PANC-1 cells through ROS generation and upregulation of p53 and p21 proteins. Ginger-derived compounds such as [6]-shogaol and [6]-gingerol also demonstrated significant antiproliferative activity against pancreatic cancer through ROS-mediated autophagic and apoptotic cell death [43, 44].

The low IC₅₀ observed in the present study may reflect the combined activity of multiple constituents present in the crude aqueous extract. However, because the individual compounds were not quantified or tested separately, synergistic effects cannot be confirmed. *Syzygium aromaticum* contains multiple phenolic and flavonoid compounds including gallic acid, ellagic acid, β -caryophyllene, and tannins, many of which possess independent anticancer properties. These constituents may contribute collectively to the biological activity of the extract; however, the specific contribution or interaction of individual compounds remains undetermined [45, 46]. Previous studies of eugenol-related anticancer mechanisms have emphasized its capacity to regulate multiple cellular targets simultaneously, including cyclin-dependent kinases, inflammatory mediators, angiogenic pathways, and apoptotic regulators [47, 48]. Such multitargeted actions may be particularly advantageous in pancreatic cancer, which is characterized by extensive molecular heterogeneity and adaptive resistance mechanisms.

Another important consideration is the potential relationship between an increase in TP53 mRNA expression and metabolic regulation in pancreatic cancer cells. Emerging evidence suggests that p53 not only induces apoptosis but also suppresses the Warburg effect by shifting cancer metabolism away from aerobic glycolysis toward oxidative phosphorylation [49, 50]. Since pancreatic cancer

cells rely heavily on glycolytic metabolism for survival and proliferation, restoration of p53 activity may impair metabolic adaptation and sensitize tumor cells to oxidative stress [51-53]. Thus, TP53 modulation may influence metabolic and apoptotic responses; however, the present study did not assess metabolic activity or functional p53 signaling, and these potential relationships require experimental validation.

Several limitations should be considered when interpreting these findings. First, the study was limited to a single pancreatic cancer cell line, and normal pancreatic epithelial cells were not evaluated; therefore, tumor selectivity and potential cytotoxicity toward nonmalignant pancreatic cells could not be determined. Second, although increased TP53 mRNA was observed, p53 protein abundance and functional activity were not assessed. Furthermore, downstream apoptotic markers, including Bax, Bcl-2, caspases, and PARP, as well as ROS production, mitochondrial membrane potential, and cell-cycle progression, were not directly evaluated. Therefore, the involvement of p53-mediated apoptosis, oxidative stress, mitochondrial dysfunction, or cell-cycle regulation remains speculative. Third, the qRT-PCR analysis was based on only three independent biological replicates per group, resulting in limited statistical power and precision; thus, the observed increase in TP53 mRNA should be considered preliminary and confirmed in adequately powered experiments with more independent replicates. Fourth, the study was performed entirely *in vitro*, and no *in vivo* efficacy, pharmacokinetic, toxicological, or clinical investigations were conducted. Consequently, the bioavailability, systemic safety, therapeutic efficacy, and clinical relevance of the extract remain unestablished. Future studies should include additional pancreatic cancer models, normal pancreatic cells, protein-level and functional assays of p53 and apoptosis, ROS and mitochondrial assessments, larger qRT-PCR sample sizes, and appropriate *in vivo* pharmacokinetic, toxicity, and efficacy studies.

5. Conclusion

The present study demonstrated that a crude aqueous extract of *Syzygium aromaticum* exerted substantial cytotoxic effects against PANC-1 pancreatic cancer cells in a concentration-dependent manner. Treatment with the extract was also associated with a significant increase in TP53 mRNA expression. However, because PANC-1 cells harbor a mutant TP53 background and the present study evaluated only TP53 transcript abundance, these findings should not be interpreted as evidence of restoration or activation of functional wild-type p53 signaling.

Author Contributions

Concept development (provided idea for the research): H.H and N.F. Supervision (provided oversight, responsible for organization and implementation): H.H and N.F. Methodology, data collection, and data analysis: H.H.M, H.H, and N.F. Literature search (performed the literature search and writing of the manuscript): H.H.M, H.H, and N.F. Data resource: H.H. Drafting the manuscript (responsible for writing a substantive part of the manuscript): H.H.M, H.H, and N.F.

Competing Interests

The authors declared no conflict of interest.

Data Availability Statement

Data are available upon a reasonable request to corresponding author [Dr. Hadi Habibollahi].

AI-Assisted Technologies Statement

Artificial intelligence (AI) tools were used solely for basic grammar correction and language refinement in the preparation of this manuscript. Specifically, ChatGPT was used to improve the language and grammar of the English text. All scientific content, data interpretation, and conclusions were developed independently by the authors. The authors have thoroughly reviewed and edited the AI-assisted text to ensure its accuracy and accept full responsibility for the content of the manuscript.

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