Inhibition of Apoptosis Regulatory Targets by Punicalaginin Hepatocellular Carcinoma Cells

Sravanthi K[1*], Deepak Sharma[2]

Abstract:

Punicalagin, a naturally occurring compound found in pomegranate, has gained attention for its potential anticancer properties. This study investigated the impact of punicalagin on the expression levels of key apoptosis-related genes, specifically BAD, BCL2, BCL-xl, and Bax,in hepatocellular carcinoma (HCC) cells. The HepG2 cell line was subjected to varying concentrations of punicalagin (100 and 150 µg), and subsequent analysis focused on the expression levels of the target genes. The results revealed a significant decrease in mRNA expression levels of BAD, BCL2, and BCL-xl in punicalagin-treated HepG2 cells compared to untreated cells. Notably, the reduction in expression levels was more pronounced with the higher concentration of 150 µg. Interestingly, the treatment with punicalagin led to an upregulation of Bax in liver cancer cells.To explore the potential interaction between punicalagin and the apoptosis-regulating targets, molecular docking analysis was employed. The analysis substantiated a strong binding affinity between punicalagin and BAD, BCL2, BCL-xl, implying a direct interaction between the compound and these targets.Collectively,this study sheds light on the inhibitory effect of punicalagin on apoptosis-mediated targets in HCC cells. The findings propose that punicalagin might exert a regulatory influence on apoptosis in liver cancer cells. Further investigation is required to validate these findings and to elucidate the therapeutic potential of punicalagin in hepatocellular carcinoma tumorigenesis.

Keywords: Punicalagin, hepatocellular carcinoma, apoptosis, molecular docking, gene expression, therapeutic approach.

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Introduction

Cancer is a disease characterized by the dysregulation of cell proliferation and death in affected tissues. Tumor promotion occurs when initially damaged or mutated cells are stimulated to grow due to inflammation or injury. This leads to the formation of a clone of actively dividing premalignant/benign tumor cells, which may regress without further stimuli [1]. Clonal selection allows partially transformed cells to proliferate and acquire additional mutations necessary for malignant transformation. Malignant neoplasms possess distinct characteristics such as uncontrolled growth, invasiveness, and the ability to metastasize. These features are acquired progressively during tumor progression [2]. Genetic changes driving tumorprogression involve not only genes that regulate cell growth but also genes involved in angiogenesis, invasion, and metastasis [3].Hepatocellular carcinoma (HCC) is the most common form of liver cancer and represents a major cause of cancer-related deaths globally. It ranks sixth in terms of incidence and third in terms of mortality, contributing to approximately 7% of all cancer cases worldwide [4]. The highest rates of HCC are observed in regions such asEastern and Southern Asia, Middle and Western Africa, Melanesia, and Micronesia/Polynesia.Chronic liver disease and cirrhosis are the major risk factors for the development of HCC. Chronic viral hepatitis and excessive alcohol consumption are the leading risk factors globally. Chronic viral hepatitis, specifically

hepatitis B and C, is the primary cause of long-term liver inflammation, which can progress to cirrhosis and eventually lead to the development of HCC [5].

Despite advancements in prevention, diagnosis, and treatment, hepatocellular carcinoma (HCC) remains a formidable challenge with high mortality rates. Consequently, investigators are actively investigating more effective and less toxic approaches for the treatment of this cancer [6]. In this pursuit, the utilization of phytochemicals, which are compounds derived from plants, has garnered significant attention. Phytochemicals are known for their potent antioxidant and anti-inflammatory properties, making them promising candidates to improve HCC survival rates [7].The potent antioxidant activity of phytochemicals is crucial in combating oxidative stress, a hallmark feature of cancer development. Oxidative stress occurs when there is an imbalance between the production of reactive oxygen species (ROS) and the ability of the body's antioxidant defenses to neutralize them. ROS can damage cellular components, including DNA, proteins, and lipids, leading to tumor initiation and progression

[8]. Phytochemicals counteract this oxidative stress by scavenging free radicals and protecting cells from DNA damage and mutation, thus potentially inhibiting the development of HCC [9].

Punicalagin is a bioactive compound found abundantly in pomegranate fruit and its related products. It has gained significant attention for its potential anticancer properties, including its effects on hepatic cellular carcinoma (HCC) [10]. Punicalagin exerts its biological activities through multiple mechanisms, making it an intriguing candidate for HCC treatment and prevention [11].One of the key mechanisms by which punicalagin exerts its effects in HCC is through the regulation of apoptosis, the programmed cell death process. Apoptosis plays a crucial role in maintaining tissue homeostasis by eliminating damaged or malignant cells. Dysregulation of apoptosis is a hallmark of cancer, including HCC. Punicalagin has been shown to induce apoptosis in HCC cells, leading to cell death [12]. It does so by modulating the expression of various apoptotic regulatory proteins, such as BAX, BCL-2, and BCL-xl, which are involved in the control of cell survival and death. Punicalagin has been found to reduce the expression levels of anti-apoptotic proteins (e.g., BCL-2, BCL-xl) and increase the expression of pro-apoptotic proteins (e.g., BAX) in HCC cells, promoting apoptosis and inhibiting tumor growth [13].Another important aspect of punicalagin's activity in HCC is its anti-inflammatory and antioxidant properties. Chronic inflammation and oxidative stress are closely linked to the development and progression of HCC [14].

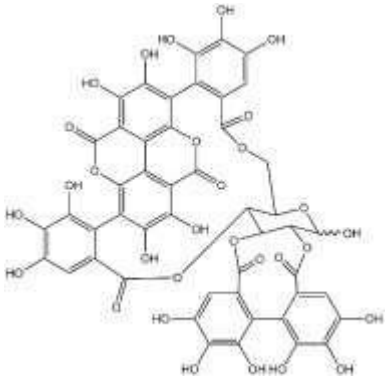


Fig 1. Structure of punicalagin

Punicalagin exhibits potent anti-inflammatory effects by suppressing the production of pro-inflammatory cytokines and inhibiting inflammatory signaling pathways [15]. Additionally, it acts as an antioxidant, scavenging free radicals and reducing oxidative stress, which can contribute to DNA damage and promote tumor growth. By reducing inflammation and oxidative stress, punicalagin may help inhibit the development and progression of HCC. This study investigates the effect of punicalagin shows inhibition of apoptotic targets in hepatocellular carcinoma [16].

Materials and methods

Reagents

The following laboratory supplies and reagents were obtained from HiMedia: Dulbecco's Modified Eagle's Medium (DMEM), Fetal Bovine Serum (FBS), penicillin/streptomycin antibiotics. The total RNA isolation kit was provided by Invitrogen, USA. Furthermore, the primers required for gene amplification, including BAD, BAX, BCL-2, and BCL-XL were supplied by Eurofins Genomics India Pvt. Ltd, Bangalore, India.

1.1 Cell culture

The Human hepatic carcinoma cell line, HepG2 was procured from the National Centre for Cell Science (NCCS). These cell lines were cultured and maintained in a CO_2 incubator at 37°C, utilizing DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. For the mRNA expression analysis, around $5x10^6$ of HepG2 cells were detached using 0.25% trypsin-EDTA and then seeded into a 6-well plate.

1.2 mRNA expression analysis

HepG2 cells were seeded at a density of 5×10^6 cells per well in a 6-well plate. The cells were cultured and treated with punicalagin in serum-free medium for 24 hours after an initial incubation period. The concentrations of punicalagin100 and 150µgwere determined. Following the treatment, the cells were collected, and RNA extraction was performed using theTRIR kit from Abgene, United Kingdom. The concentration of the extracted RNA was determined using spectrometric quantification, following the method described by Porichi et al., 2009, and expressed in micrograms (µg) [17].To synthesize complementary DNA (cDNA), 2 µg of total RNA was used in conjunction with a reverse transcriptase kit from Eurogentec (Seraing, Belgium) according to the manufacturer's instructions. For Real-Time PCR analysis,a reaction mixture was prepared using Takara SyBr green master mix, and specific forward andreverse primers were designed for BAD, BAX, BCL-2, and BCL-XL. The primer sequences and reaction conditions are listed in Table 1. The Real-Time PCR cycles consisted of pre- denaturation at 94 ºC for 2 minutes, followed by 35 cycles of denaturation at 94 ºC for 1minute, annealing at 57 ºC for 30 seconds, extension at 72 ºC for 30 seconds, and a final extension at 72 ºC for 10 minutes. Melting analysis was performed, with β-actin serving as an intrinsic positive control. The CT values of each sample were normalized to β-actin, and the
relative expression ratio of each gene was determined using the geometric mean of three samples in each group. The CFX96 Touch Real-Time PCR detection system from the USA wasutilized for this process. The obtained results were plotted on a graph generated by the PCR equipment, providing insights into the expression levels of the target genes.

Table 1: Real-Time PCR Primers

Gene(s)	Primer 5'-3'	References
BAD	F –CAGTGATCTGCTCCACATTC R- TCCAGCTAGGATGATAGGAC	Chen S et al., 2015
Bax	F-TTCTGACGGCAACTTCAACTG R-TGAGGAGTCTCACCCAACCA	Chen S et al., 2015
Bcl-2	F-GACGCTTTGCCACGGTGGTG R-GGGGCAGGCATGTTGACTTCAC	Chen S et al., 2015

BCL-XL	F- AGGATACAGCTGGAGTCAG R - TCTCCTTGTCTACGCTTTCC	Chen S et al., 2015
β-actin	F-AACAAGATGAGATTGGCA R-AGTGGGGTGGCTTTTAGGAT	Chen S et al., 2015

F- Forward; R- Reverse

Molecular docking analysis

The binding interactions between punicalagin and apoptosis-regulating targets, including BAD, BAX, BCL-2, and BCL-XL, were studied using the molecular docking program AutoDock 4.2. Crystal structures of these proteins, namely BAD (PDB ID: 7Q16), BAX (PDB ID: 1F16), BCL-2 (PDB ID: 2XA0), and BCL-XL (PDB ID: 1BX1), were obtained from the Protein Data Bank (https://www.pdb.org/pdb).During the docking process, a grid box with dimensions of 90Å × 90 Å × 90 Å and a grid spacing of 0.45 Å was used. The docking calculations employedthe Lamarckian genetic algorithm (LGA), and 100 genetic algorithm runs were performed. The resulting docking outputs were analyzed to identify high-pose interactions between punicalagin and the apoptosis-regulating targets. The binding affinities of the ligand-receptor interactions were also evaluated to characterize the binding mode. The three-dimensional structure of the complex obtained from the docking results was visualized using BIOVIA Discovery Studio.

1.3 Statistical analysis

The data obtained from the experiments were presented as the mean ± standard deviation (S.D.). Statistical analysis was conducted using GraphPad Prism 8 software. A t-test was performed to assess the significance of the data. p-values *< 0.05, **<0.01, and ***<0.001 were considered statistically significant.

Results

Regulation of apoptotic targets by punicalagin

Our investigation aimed to uncover the underlying molecular mechanisms of treating punicalagin against apoptotic signaling targets. It is well established that the Bax family of proteins and activation of BAD are crucial in the process of cell apoptosis. To examine these mechanisms, HepG2 cells were treated with 100µg and 150µg concentrations of punicalaginfor 48 hours, and the mRNA expression levels of BAD, BAX, BCL-2, and BCL-XL were measured using real-time PCR.Comparing the results to the untreated group, we observed a significant increase in the mRNA expression levels of the pro-apoptotic genes Baxin HepG2 cells treated with punicalagin. In contrast, the expression of the anti-apoptotic genes BAD, Bcl-2 and BCL-XL was reduced upon treatment with punicalagin in hepatic cell line, as depicted inFigure 2.These findings suggest that punicalagin triggers an upregulation of pro-apoptotic genes and a downregulation of anti-apoptotic genes in HepG2 cells. The increased expressionof Bax, indicates their involvement in the apoptotic pathway, promoting cell death. Conversely,the decreased expression of BAD, Bcl-2 and BCL-XL, which are known to inhibit apoptosis, further supports the pro-apoptotic effects of punicalagin in HCC cells.

Table 2: Molecular docking results

S. no	Drug	Protein	Binding energy (kcal/mol)	No. of H bonds involved	Amino acid residues
1.	Punicalagin (CID: 44584733)	BAD (7Q16)	-10.3	3	GLN120, THR60, GLY48
2.		BAX (1F16)	-10.1	2	PHE105, TRP107
3.		BCL2 (2XA0)	-10.8	4	GLU160, HIS120,ASP140, HIS184
4.		BCL-XL (1BX1)	-10.00	2	ASP11, GLU44

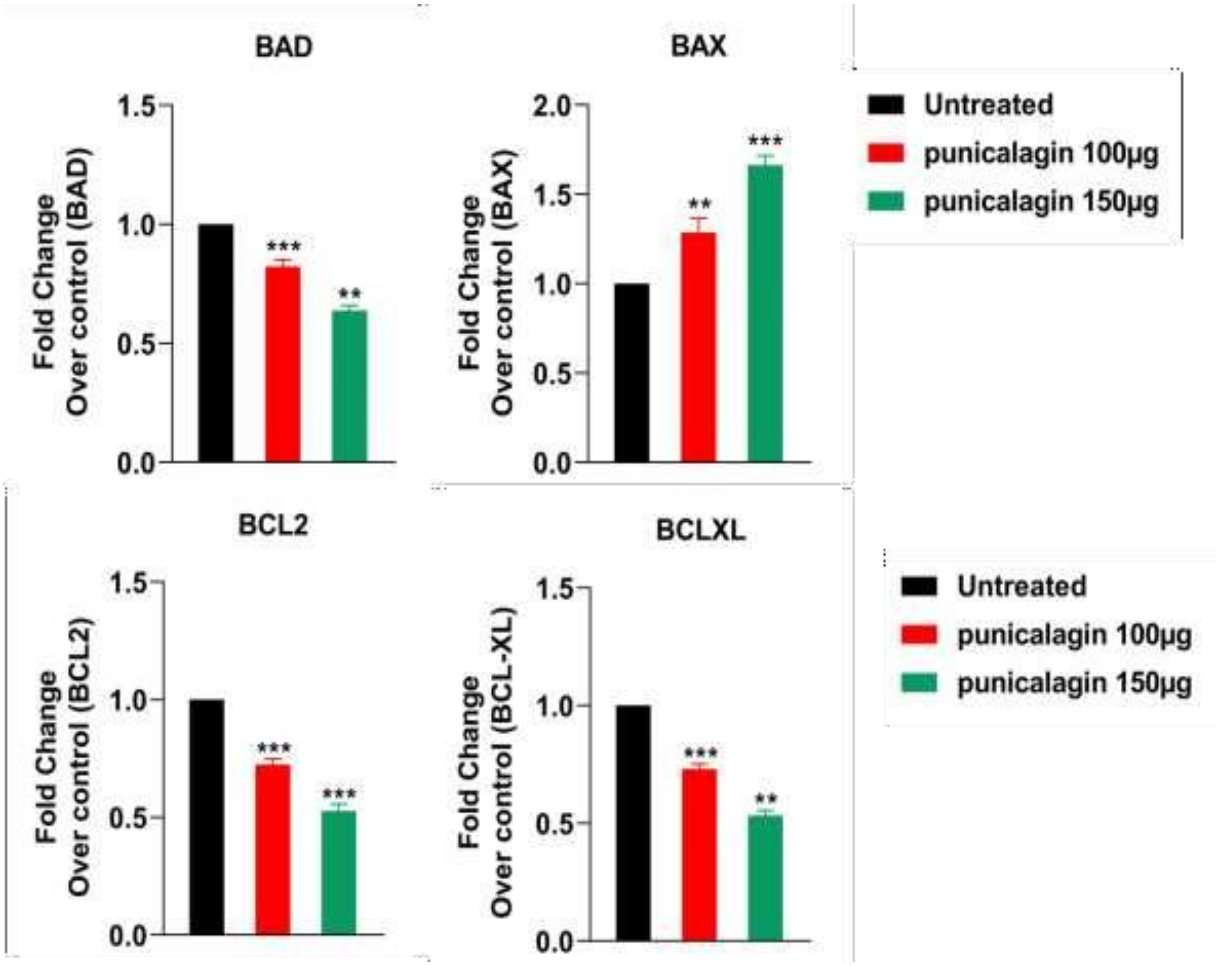
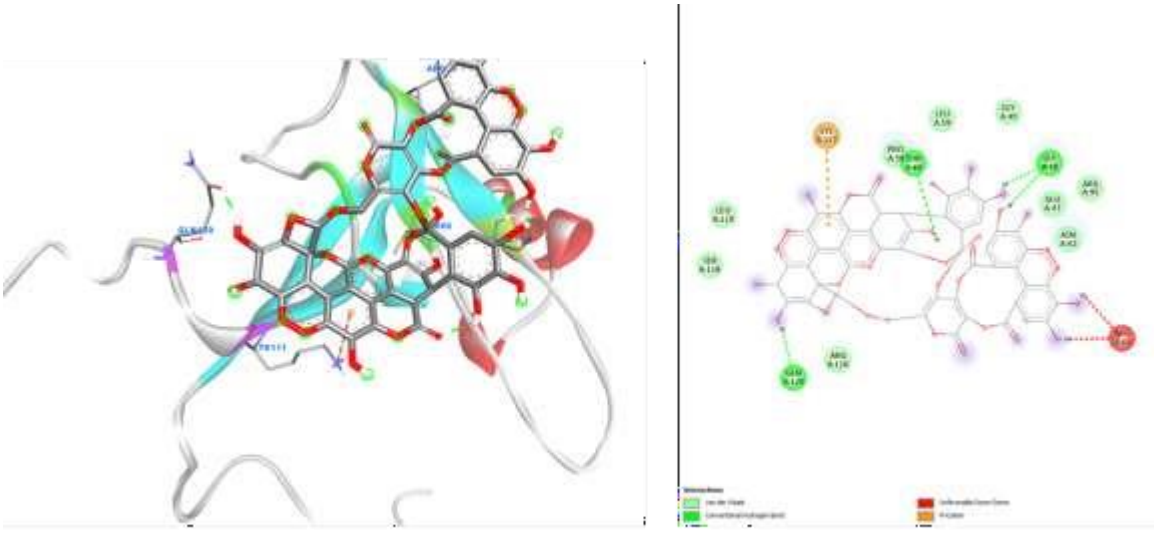
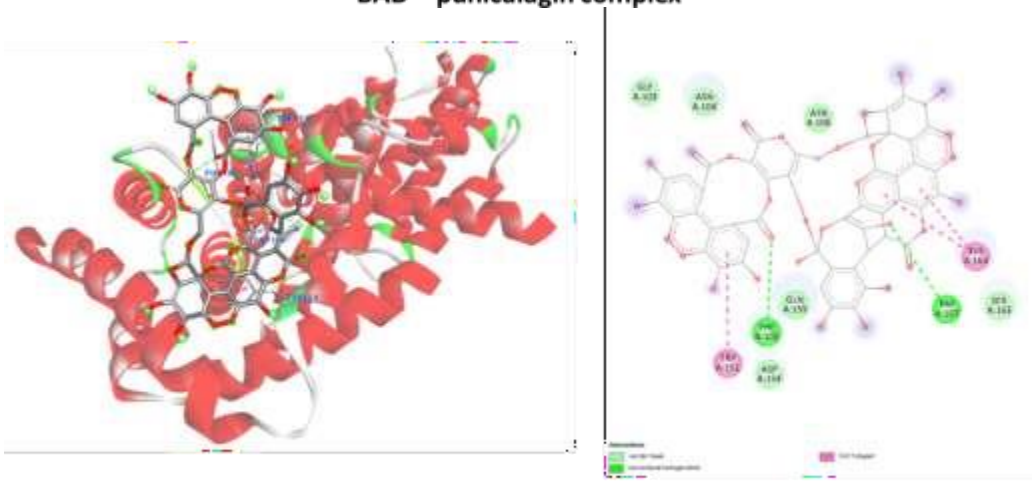


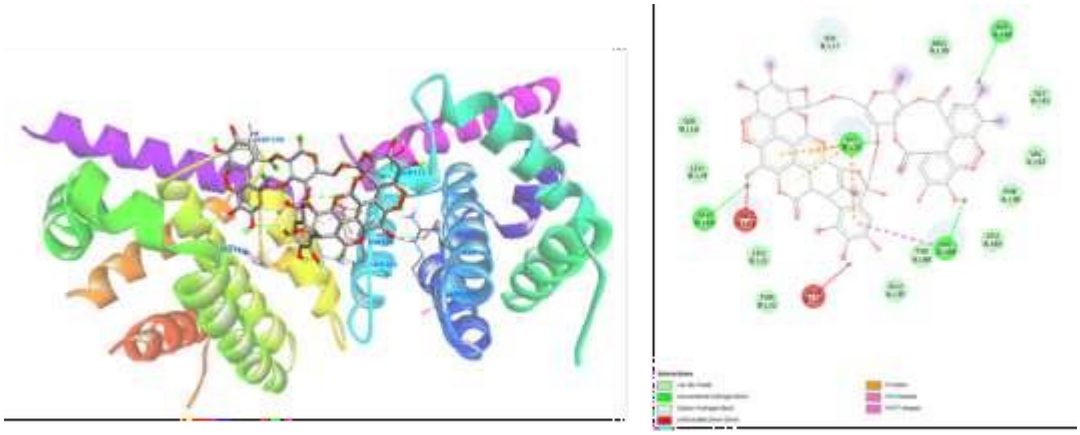
Fig 1.mRNA expression of BAX, BAD, Bcl-2, and BCL-XL in HepG2 cells



BAD – punicalagin complex



BAX – punicalagin complex



BCL2 – punicalagin complex

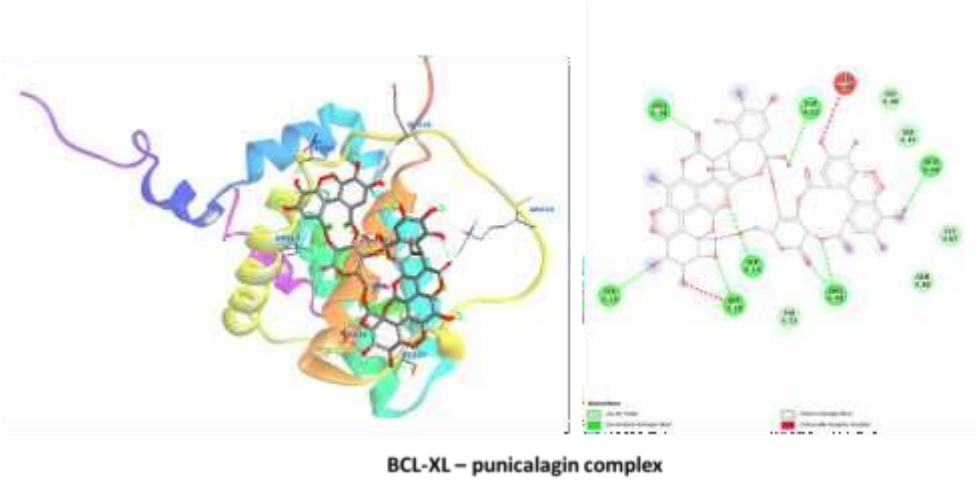


Fig 2: Molecular docking analysis of BAX, BAD, Bcl-2, and BCL-XL Molecular docking

To explore the potential regulation of apoptotic-regulating targets by Punicalagin, molecular docking studies were conducted. The objective was to evaluate the interaction between punicalaginand crucial proteins involved in apoptosis, including BAD, BAX, BCL-2, andBCL-XL. The results of the molecular docking revealed the binding energies between Punicalagin, as shown in Figure 3. Notably, punicalaginexhibited higher binding energies with BAD (-10.3 kcal/mol), BAX (-10.1 kcal/mol), BCL-2 (-10.8 kcal/mol), and BCL-XL (-10.00 kcal/mol) indicating a strong potential for interaction as shown in table 2. This suggests that punicalaginmay directly interact with these proteins and potentially modulate their activity.Furthermore, the molecular docking studies revealed the formation of hydrogen bonds between Punicalaginand the active sites of GLN120, THR60, and GLY48 for BAD, PHE105, TRP107 for BAX, GLU160, HIS120, ASP140, and HIS184 for BCL-2, and ASP11, GLU44 for BCL-XL. These hydrogen bonds are crucial for the stability of the protein-ligand complex, indicating that punicalaginmay disrupt the protein structure, potentially leading to the inhibition of their activity.The results obtained from the molecular docking studies align with the observed anticancer effects of punicalaginin HepG2 cancer cells. These findings suggest that punicalaginmay interfere with the apoptosis signaling pathway, ultimately leading to cell death. However, further investigations are necessary to fully comprehend the underlying molecular mechanisms of the interaction between punicalaginand these apoptotic-regulating targets.

Discussion

The present study aimed to investigate the molecular mechanisms underlying the treatment of punicalagin against apoptotic signaling targets in HepG2 cells. The Bax family of proteins and the activation of BAD are well-known players in the process of cell apoptosis. Real-time PCR was employed to assess the mRNA expression levels of BAD, BAX, BCL-2, and BCL-XL in HepG2 cells following treatment with 100µg and 150µg concentrations of punicalagin for 48 hours.Comparing the results to the untreated group, significant upregulation of the pro- apoptotic gene Bax was observed in HepG2 cells treated with punicalagin. This finding suggests that punicalagin treatment stimulates the expression of Bax, which is known topromote apoptosis and contribute to cell death. On the other hand, the expression levels of the anti-apoptotic genes BAD, Bcl-2, and BCL-XL were reduced upon treatment with punicalagin. These genes typically act as inhibitors of apoptosis, and their downregulation further supports the pro-apoptotic effects of punicalagin in HepG2 cells.The observed changes in gene expression indicate that punicalagin treatment induces a shift in the balance between pro- apoptotic and anti-apoptotic factors, favoring apoptosis. The upregulation of Bax suggests its involvement in the apoptotic pathway,

where it can promote cell death. The downregulation of BAD, Bcl-2, and BCL-XL further reinforces the pro-apoptotic effects of punicalagin, as these genes typically counteract apoptosis and promote cell survival.

In order to shed light on the potential regulation of apoptotic-regulating targets by Punicalagin, molecular docking studies were conducted. The objective of these studies was to investigate the interaction between Punicalagin and key proteins involved in apoptosis, including BAD, BAX, BCL-2, and BCL-XL.The results of the molecular docking revealed the binding energies between Punicalagin and the target proteins, as shown in Figure 3. It is noteworthy that Punicalagin exhibited higher binding energies with BAD, BAX, BCL-2, and BCL-XL, indicating a strong potential for interaction. These results suggest that Punicalagin may directlyinteract with these proteins and potentially modulate their activity.Furthermore, the molecular docking studies unveiled the formation of hydrogen bonds between Punicalagin and specific active sites on the target proteins. For example, Punicalagin formed hydrogen bonds with GLN120, THR60, and GLY48 on BAD, PHE105 and TRP107 on BAX, GLU160, HIS120,

ASP140, and HIS184 on BCL-2, and ASP11 and GLU44 on BCL-XL. These hydrogen bonds play a critical role in the stability of the protein-ligand complex, indicating that Punicalagin may disrupt the protein structure and potentially inhibit their activity.The findings obtained from the molecular docking studies align with the observed anticancer effects of Punicalagin inHepG2 cancer cells. By directly interacting with apoptotic-regulating targets, Punicalagin may interfere with the apoptosis signaling pathway, ultimately leading to cell death. The ability of Punicalagin to modulate the activity of these proteins suggests its potential as a therapeutic agent in the treatment of hepatocellular carcinoma.

Overall, the findings from this study provide evidence that punicalagin treatment leads to an upregulation of pro-apoptotic genes (Bax) and a downregulation of anti-apoptotic genes (BAD, Bcl-2, BCL-XL) in HepG2 cells. This modulation of apoptotic signaling pathways may contribute to the induction of apoptosis and the suppression of cell survival in HCC cells. These findings support the potential of punicalagin as a therapeutic agent in hepatocellular carcinoma by targeting apoptotic regulatory pathways. Further investigations are needed to elucidate the underlying mechanisms of punicalagin's effects and to explore its potential in preclinical and clinical settings for the treatment of HCC.it is important to note that molecular docking studies provide a theoretical framework and further investigations are necessary to

validate these findings in experimental models. Additional studies, such as in vitro and in vivo experiments, are required to fully understand the underlying molecular mechanisms of the interaction between Punicalagin and apoptotic-regulating targets. These studies will provide deeper insights into the potential of Punicalagin as a therapeutic agent for the treatment of hepatocellular carcinoma and may pave the way for its future clinical application.

Conclusion

The present study provides evidence that punicalagin treatment leads to the modulation of apoptotic signaling targets in HepG2 cells. The upregulation of the pro-apoptotic gene Bax and downregulation of the anti-apoptotic genes BAD, Bcl-2, and BCL-XL suggest that punicalagin promotes apoptosis and suppresses cell survival in HCC cells. Molecular docking studies further support the potential interaction between punicalagin and apoptotic-regulating targets, indicating its ability to directly modulate their activity. These findings highlight the therapeutic potential of punicalagin in hepatocellular carcinoma by targeting apoptotic regulatory pathways. However, further experimental investigations are warranted to validate these findings and elucidate the precise mechanisms of punicalagin's effects. Such studies may pave the way for the future development of punicalagin as a promising therapeutic agent for the treatment of HCC.

Conflict of Interest

No conflict

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