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Original Article

In silico and *in vitro* analyses to investigate the effects of vitamin C on VEGF protein

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الملخص

أهداف البحث: أجريت هذه الدراسة لتقييم تأثير فيتامين سي على جينات الاستماتة والتكاثر في خلايا "هيب جي 2" المصابة.

طريقة البحث: في تحليل السيليكو تم إجراء استخدام الالتحام الجزيئي للمركبات الكيميائية مع بروتين عامل النمو البطاني الوعائي. الأدوات الحسابية المختلفة المستخدمة هي "أوتودوك فينا" و"بايوفيا ديسكفري ستوديو" و "بايمول". تم تحليل تشابه الأدوية وسميتها بواسطة "سويس أدميت". تمت معالجة التقاء الخلايا 60-70 —% بتركيزات مختلفة من "اتش 2 أوه 2" (100-2000 مايكرومولر) وحمض الأسكوربيك (30، 60، 90 ميكروغرام / مل). تم إجراء اختبار موت الخلايا "ام تي تي" لمقارنة الإمكانات التكاثرية لخلايا "هيب جي 2" المعالجة بـ "اتش 2 أوه 2" وحمض الأسكوربيك باستخدام 96 لوحة جيدة.

النتائج: تمت ملاحظة أدنى طاقة ربط لـ بروتين عامل النمو البطاني الوعائي مع فيتامين سي -5,2 كيلو كالوري/مول وحمض الأسكوربيك-2 جليكوسيد -4.7 كيلو كالوري/مول في تحليل السيليكو. تم اختيار فيتامين سي لأنه يظهر تفاعلا عاليا مع بروتين عامل النمو البطاني الوعائي ويحقق قاعدة ليبينسكي، والقدرة على البقاء عن طريق الفم، والحركية الدوائية مقارنة بجليكوسيد حمض الأسكوربيك. أظهرت فحوصات صلاحية الخلية أن فيتامين سي كان له تأثيرات موت الخلايا المبرمج. بعد معالجة خلايا "هيب جي 2" بحمض الأسكوربيك، لوحظ انخفاض بروتين عامل النمو البطاني الوعائي (تكوين الأوعية) من خلال التعبير الجيني للموت المبرمج والتكاثري. أدى علاج حامض الأسكوربيك لخط خلايا "هيب جي 2" إلى تنظيم علامات التكاثر "بي سي ان ايه" و "كي آي 67" و تي أوه بي 2 ايه". وقد لوحظت زيادة في موت الخلايا المبرمج بعد العلاج بفيتامين سي بسبب تنظيم "بي53" و "أنيكسين في".

الاستنتاجات: بناء على النتائج، من الواضح أن فيتامين سي يثبط نمو الخلايا السرطانية وبالتالي يحمي خلايا "هيب جي 2" من الإجهاد التأكسدي. أظهر فيتامين سي نشاطا مضادا للتكاثر كما لوحظ في السيليكو، وفي المختبر، وكذلك النتائج المحتملة من تثبيط التعبير عن الجينات المشاركة في تخليق البروتين.

الكلمات المفتاحية: دورة الخلية؛ فيتامين سي؛ الجينات التكاثرية؛ موت الخلايا المبرمج؛ تنظيم الجينات

Abstract

Objectives: This study was conducted to evaluate the effects of vitamin C on apoptotic and proliferative genes in injured HepG2 cells.

Methods: *In silico* analysis was performed using molecular docking of chemical compounds with vascular endothelial growth factor (VEGF). The different computational tools used were AutoDock Vina, BIOVIA DISCOVERY studio, and PyMOL. Drug likeness and toxicity were analyzed by SWISS ADMET. Cells that were 60–70% confluent were treated with different concentrations of hydrogen peroxide (H_2O_2) (100–2000 μM) and ascorbic acid (30, 60, 90 μg/mL). The MTT cell proliferation assay was performed to compare the proliferative potential of HepG2 cells treated with H_2O_2 or ascorbic acid with untreated HepG2 cells using 96-well plates.

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Results: The lowest binding energy of VEGF with vitamin C −5.2 kcal/mol and L-ascorbic acid-2 glycoside −4.7 kcal/mol was observed by *in silico* analysis. Vitamin C was selected because it exhibited a high interaction with VEGF and fulfilled Lipinski's rule, and had better oral viability and pharmacokinetics compared to L-ascorbic acid-2 glycoside. Cell viability assays showed that vitamin C had significant apoptotic effects ($P < 0.0001$). After treating HepG2 cells with ascorbic acid, reduced VEGF (angiogenesis) was observed as determined by apoptotic and proliferative gene expression. Ascorbic acid treatment of HepG2 cells led to downregulation of the proliferation markers, proliferating cell nuclear antigen, Ki67, and DNA topoisomerase II alpha. Increased apoptosis after treatment with vitamin C was observed due to upregulation of p53 and annexin V.

Conclusion: The results of this study showed that vitamin C inhibited the growth of cancer cells, thus protecting HepG2 cells from oxidative stress. Vitamin C exhibited antiproliferative activity as observed *in silico* and *in vitro*, as well as by the inhibited expression of genes involved in protein synthesis.

Keywords: Apoptosis; Cell cycle; Gene upregulation; Proliferative genes; Vitamin C

Introduction

Cancer is predicted to become the leading cause of death in this century, as it is a disease that can affect any part of the body.[1,2] Cancer is characterized by the rapid growth of abnormal cells that exceed their normal limits and have the potential to invade nearby tissues and spread to other organs. This phenomenon is known as metastasis.[3,4]

Vitamin C and its derivatives, including L-ascorbic acid-2 glycoside (AA2G), have been investigated for their potential impact on cancer. Both compounds are forms of vitamin C and share antioxidant properties, which help neutralize free radicals and reduce oxidative stress that can contribute to cancer development. By reducing oxidative damage to cells, these compounds may theoretically aid in cancer prevention.[5] Vitamin C is essential for collagen synthesis, which is crucial for maintaining tissue integrity and preventing uncontrolled cell growth, a hallmark of cancer. Additionally, vitamin C supports the immune system, which plays a role in identifying and eliminating abnormal or cancerous cells. While AA2G is a stable derivative of vitamin C often used in skincare for its skin-brightening and antioxidant properties, its specific effects on cancer cells have not been as extensively studied as those of vitamin C. The effectiveness of vitamin C and its derivatives in preventing or treating cancer can vary depending on various factors including the type of cancer, stage of cancer, and individual patient characteristics.[6] Phytochemicals can help prevent the development of cancer by stimulating the production of detoxifying compounds that combat oxidative stress, which is facilitated by cell reinforcements. Antioxidants, which play a crucial role in eliminating reactive oxygen species (ROS) that are involved in various cellular processes, such as cell proliferation, differentiation, stress adaptation, and metabolic adjustment, are essential in this process.[7] Increased levels of ROS can prompt cell damage and increase chronic disease development. Cancer prevention agents regulate ROS levels and prevent cell damage through different mechanisms.[8] Experiments in animal models suggest that vitamin C may play a role in stopping the multiplication of malignant cells.[9]

The exact metabolic and redox actions of vitamin C on papillary thyroid cancer (PTC) cell lines have not been fully elucidated, but it is known that vitamin C slows down their proliferation.[10] Redox homeostasis is disrupted, leading to an increase in ROS and imbalances in antioxidants and electron carriers, which can result in both apoptosis and necrosis, two forms of cell death. This disruption in redox homeostasis is caused by oxidative stress, which also affects the metabolic processes in the tricarboxylic acid (TCA) cycle and glycolysis.[11] The pro-oxidant action of vitamin C in causing cytotoxicity in PTC cells indicates that the suppression of glycolysis and modification of the TCA cycle through NAD+ depletion may be key components of this mechanism.[12] Research has shown that high levels of vitamin C in PTC cells can inhibit tumor growth by disrupting the redox equilibrium, which impacts the NAD salvage pathway. This leads to impaired glycolysis and the TCA cycle, ultimately resulting in cell death. To further explore the potential use of vitamin C as an antitumor agent, additional studies, including *in vivo* experiments, are necessary to better understand the mechanisms underlying its cytotoxic effects on PTC-derived cells.[13]

Ascorbate, at therapeutic concentrations, can help halt the synthesis of hydroxyl radicals in the Fenton reaction, making it an essential component in oxidative stress therapy for cancer cells. The generation of hydroxyl radicals and depletion of ATP caused by the activation of polymerase I are two key factors that contribute to ascorbate-induced cell death. Instead of disrupting bioenergetics, DNA damage is more likely to play a significant role in the anticancer effect of pharmaceutical ascorbate.[14]

Vitamin C, also known as ascorbic acid, is transported into cells using glucose transporters (GLUTs), including in cancer cells. Since ascorbic acid resembles glucose in terms of its structure, it can also be transported by GLUTs, which are responsible for absorbing both glucose and vitamin C. Compared to normal cells, cancer cells often exhibit altered metabolic pathways.[15,16] The Warburg effect is a hallmark of cancer metabolism, where cancer cells preferentially rely on glycolysis, a process that converts glucose into lactate, to generate energy, even when there is an adequate supply of oxygen. This metabolic shift is accompanied by a decrease in oxidative phosphorylation, which is the primary pathway for producing ATP using oxygen in the mitochondria. One theory suggests that the increased glycolytic activity in cancer cells may lead to a faster conversion of ascorbic acid to dehydroascorbic acid (DHA). DHA can be taken up by cells through glucose transporters and subsequently converted back to ascorbic acid. The elevated levels of ascorbic acid found within cancer cells may be due to this recycling process of ascorbic acid and DHA.[17]

Vitamin C has been shown to alter the metabolomics and epigenetic profiles of cancer cells, as well as kill cancer stem cells by activating ten eleven translocation (TET) proteins and upregulating pluripotency factors. It also induces the degradation of hypoxia inducible factor 1, which is necessary for tumor cells to survive in low oxygen environments. Additionally, vitamin C may stimulate the immune system by activating monocytes, natural killer cells, and T cells through dietary intake. However, further research is needed to fully understand the molecular and therapeutic implications of the ability of vitamin C to block the progression of cancer in multiple pathways.[18]

Because AA2G has advantageous effects on a variety of cell types and organs, it has been employed as a therapeutic. The ability to scavenge radicals is one of AA2G's most significant biological functions. One stable derivative of vitamin C is AA2G, which is a glycosylated form of ascorbic acid (AA).[19] Well-known for its exceptional antioxidant qualities and bioavailability is AA2G. By decreasing initial DNA damage and acting as a radical scavenger, AA2G can shield cells from radiation.[20]

In silico analysis allows researchers to investigate the connections between therapeutic drugs and various diseases by examining existing bioinformatics databases. These databases may provide information on gene expression, protein interactions, and pathways related to diseases.

Vitamin C and AA2G, well-known antioxidants, can neutralize oxidants that also help to protect cells from oxidative damage. In addition to contributing to abnormal cell proliferation, oxidative stress is linked to a number of diseases including cancer. Studies on the functions of these compounds in the context of anticancer activity are still limited. According to recent studies, natural substances such as vitamin C may have antiproliferative, antioxidant properties, and anti-inflammatory effects. The antioxidant compounds have demonstrated that these agents function biologically through inhibition of the antiproliferative pathway.

The aim of this study was to investigate the effects of vitamin C on apoptotic and proliferative genes in injured HepG2 cells.

Materials and Methods

Retrieved target protein

The Protein Data Bank (PDB) database was searched for information on vascular epidermal growth factor A (VEGF-A). The crystal structure of the receptor-binding domain of VEGF shows from the PDB database (PDB ID: 5T89) was downloaded from the Research Collaboratory for Structural Bioinformatics (RCSB) PDB database (https://www.rcsb.org/).[21]

Dataset of compounds

The following two compounds were selected for docking from PubChem (https://pubchem.ncbi.nlm.nih.gov): vitamin C (compound CID: 54670067) and AA2G (compound CID: 54693473).[22]

Ligand preparation

The three-dimensional (3D) structures of the compounds were generated using PubChem and then exported to PyMOL software. The improved ligands were saved in PDB format and subsequently exported to AutoDock Vina, where they underwent additional preparation such as hydrogen atoms, energy minimization, and removal of heteroatoms in ligands preparation and were saved in PDBQT file format.

Protein preparation and structure validation

The functional and stability prediction of the 3D crystal structure of VEGF (PDB ID: 5T89) was analyzed. The linked ligands, water molecules, and other heteroatoms were eliminated using PyMOL. The prepared protein was exported into AutoDock Vina, where the missing atoms were examined and mended, Kollman's charges were inserted, and the polar hydrogen was dissolved. It was then saved in PDBQT file format. The quality of the structure was validated using the Ramachandran plot[23] (Figure 2).

Molecular docking analysis

The following algorithms were used to analyze the functional and stability prediction of the 3D crystal structure of VEGF-A. AutoDock Vina to analyze the protein−ligand interactions was used to calculate the binding energy. The grid parameters in AutoDock Vina are important configurations that specify the 3D grid, which is used to assess the interactions between ligands and proteins. The primary grid parameters consist of grid center, grid dimension, and grid spacing. We used two compounds: vitamin C and AA2G. These compounds were used as ligands in PDB format, and VEGF-A was used as a receptor. AutoDock Vina was used to analyze the top-ranked docked model, and the most precise structure was selected using the docking score or binding energy. BIOVIA Discovery Studio and PyMOL was utilized to visualize the modeled complex (Figure 1).

Drug-likeness, pharmacokinetics, and toxicity profile of ligands

The SwissADME online server or URL (http://www.swissadme.ch) was used to examine the top-ranked absorption, distribution, metabolism, excretion, and toxicity (ADMET) characteristics and pharmacological properties of the two compounds. The selected compounds displayed significant biological features. By utilizing Lipinski's rule of five, the most distinct natural ligand underwent evaluation for this essential parameter. The ADMET web server, which is free to use, was employed for *in silico* ADMET screening and drug-likeness examination of multiple ligands.[24]

Chemicals and reagents

Human liver tissue containing high-grade differentiated hepatocellular carcinoma cells were used to develop the HepG2 cell line, which was purchased from the American Type Culture Collection (Rockville, MD, USA). Cells were grown in monolayer cultures in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin. The cells were incubated at 37 °C in a humidified environment with 5% CO_2. Trypsin was used to detach the

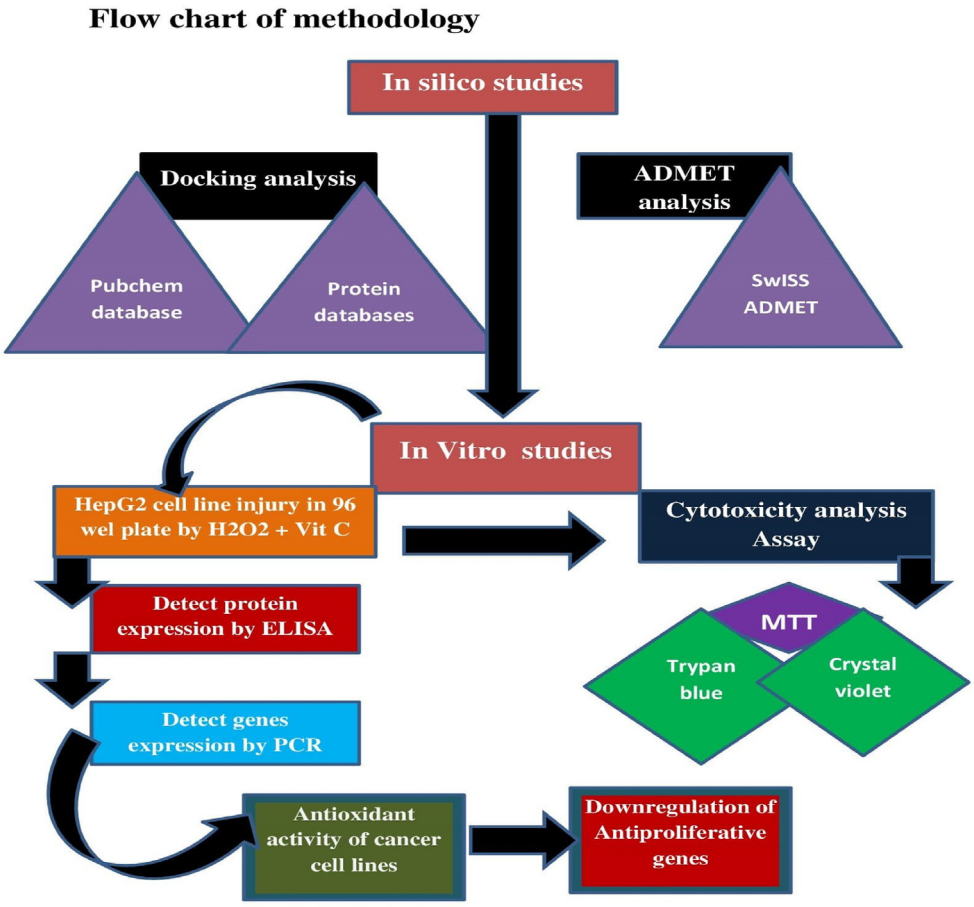

Figure 1: Flow chart of the Methodology.

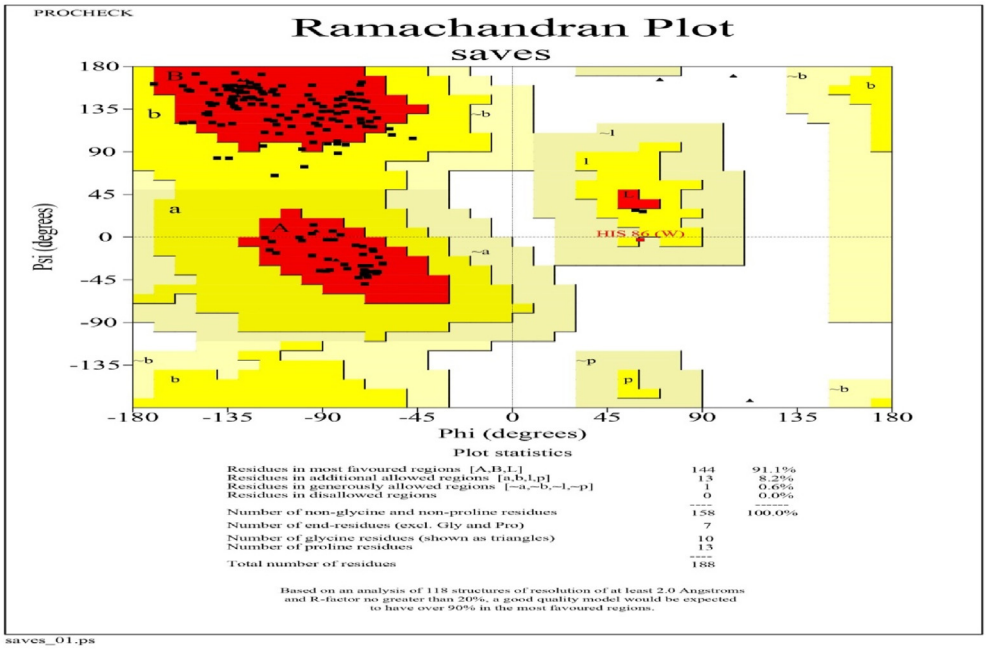

Figure 2: Protein structure validation.

confluent cells, and then the cells were subcultured at a split ratio of 1:4. All cell culture supplies were purchased from Sigma–Aldrich (Milan, Italy).

Cell line

The HepG2 cell line was cultivated in a cell culture laboratory at The University of Lahore (Lahore, Pakistan). All experiments were conducted in this laboratory using analytical grade reagents. To establish liver cells, HepG2 cell lines were employed and maintained in a monolayer culture in DMEM supplemented with 10% FBS in an incubator with 5% CO_2 at 37 °C. Once the cells reached confluence, they were prepared for treatment (Figure 1).

Treatment of cells

The cells were expanded in 96-well microtiter plates at a starting density of 2×10^4 cells per well and 5% FBS along with growth media. The cells were incubated overnight in a CO_2 incubator and then fixed to the wells. After cells reached 60–70%, they were treated with different concentrations of H_2O_2 (100, 200, 500, 1000 μM) and ascorbic acid (30, 60, and 90 μg/mL).

Evaluation of cytotoxicity

Trypan blue, crystal violet, tryptophan, and enzyme-linked immunosorbent assay (ELISA) were used to measure cytotoxicity. Optical measurements were performed using a reader plate and optical measurements were taken at 540 nm. Total cell viability was determined by using the average absorbance values of the controls[25] (Figure 1).

MTT assay

In this assay, we measured the cell viability of the untreated and treated groups using trypan blue and the crystal violet assay (Figure 1).

Cell viability assay

The MTT assay was performed to compare the proliferative potential of HepG2 cells treated with ascorbic acid and normal HepG2 cells cultured in 96-well plates. One group was controlled and the remaining three groups were treated and placed in a CO_2 incubator at 37 °C for 24 h. Viable cells converted MTT assay into a purple formazan product with absorbance taken at 570 nm[26] (Figure 1).

ELISA

ELISA was performed on HepG2 cell lines with VEGF, p53, and Annxien V. This assay was used to determine the peptide ligands in large molecules in cells[27] (Figure 1).

Evaluation of antioxidative enzymes

The medium was saved and used to measure antioxidant enzymes including catalase (CAT), glutathione, and 1,1-diphenyl-2-picrylhydrazyl (DPPH). The assay was done in 96-well plates and after addition of the appropriate reagents, the absorbance was taken at different wavelengths[28] (Figure 1).

Gene expression

RNA isolation

RNA extraction from the cells was performed 24 h after the medium was discarded. The quantity of total RNA was determined using a spectrophotometer, and a mixture of trizole, chloroform, 70% ethanol, and distilled water was added and centrifuged at 12,000 rpm for 10 min to isolate the RNA[29] (Figure 1).

cDNA (complementary DNA) synthesis

cDNA was synthesized using 1 μL primer (10 mM dNTPaSE), 2 μL oligo dT, and 10 μL RNA, and the mixture was incubated at 65 °C for 5 min. Then 2 μL buffer 10 XM-MuLV, 1 μL (reverse transcriptase) and 4 μL RNA were added to the mixture, followed by incubation in a PCR machine for 1 h at 42 °C. The total reaction mixture volume was 20 μL. Next, a mastermix was made, 1 μL cDNA was added, and tubes were incubated in a PCR machine at 42 °C for 60 min, then at 70 °C for 15 min and 4 °C for 5 min[30,31] (Figure 1).

Data analysis

All data were analyzed by GraphPad Prism.[32]

Abbreviations

ROS	Reactive oxygen species
H_2O_2	Hydrogen peroxide
VEGF	Vascular epidermal growth factor
CAT	Catalase
PCR	Polymerase chain reaction
GR	Glutathione reductase
PDB	Protein Data Bank
ELISA	Enzymelinked immunosorbent assay
TET	Ten eleven translocation
TCA	Tricarboxylic acid
AA2G	Ascorbic acid 2-glucoside

Results

To evaluate the potential of natural compounds employed as inhibitors, this research was planned to digitally screen the

Table 1: Molecular docking of ligand binding affinities against VEGF with their interacting residues.

Parameter	VEGF/Vitamin C	VEGF/AA2G
Pubchem ID	54670067	54693473
Total Binding energy	−5.2 kcal/mol	−4.7 kcal/mol
Hydrogen bond	2	4
Direct interacting residue	LYS-107, CYS-61	SER-60, ASP-63, CYS-61, CYS-68
Distance	2.96 Å, −3.21 Å, 2.96 Å	3.09 Å, 3.16 Å, 3.15 Å, 3.12 Å.
Surrounding interacting residue of VEGF	GLU-64, LEU-66, CYS-63, ASP-63, GLU-67, ASN-62, CYS-63, GLY-59	ASP-34, ASN-62, PHE-36, GLY-69, GLU-64, LEU-66, GLU-67

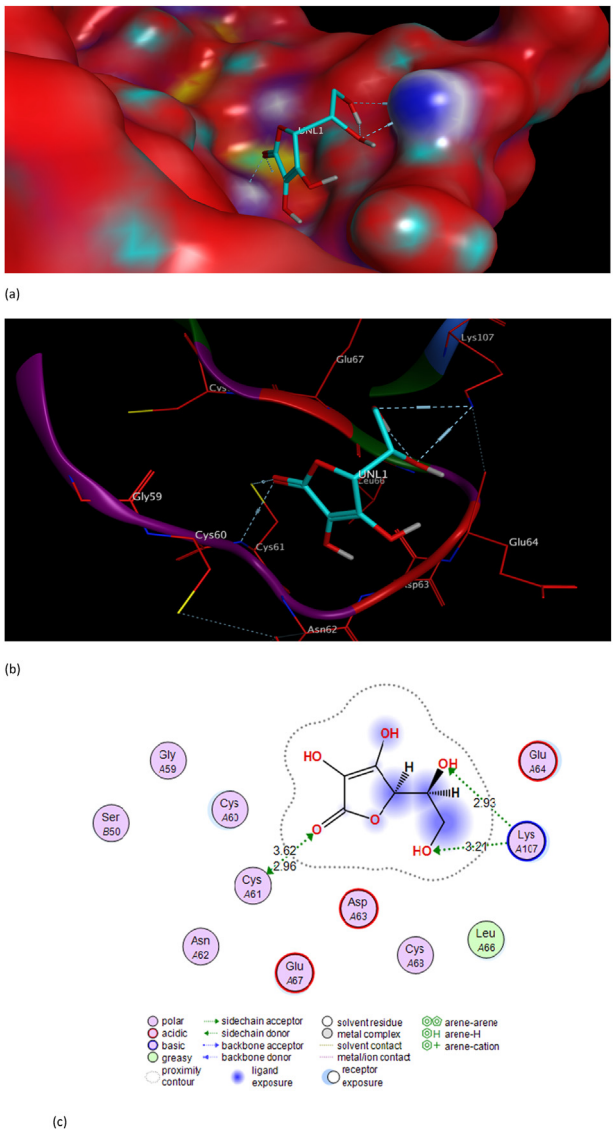

Figure 3: *In silico* modeled structure representation of complex VEGF/vitamin C. (a): 3D Spherical cartoon structure of VEGF represent the interaction with ligand and binding pocket cavity interacting of residue of VEGF with vitamin C. (b): Surrounding interacting residue of VEGF included GLU-64, LEU-66, CYS-63, ASP-63, GLU-67, ASN-62, CYS-63, and GLY-59. (c): 2D structure of VEGF with vitamin C. Dotted line shows the polar interaction.

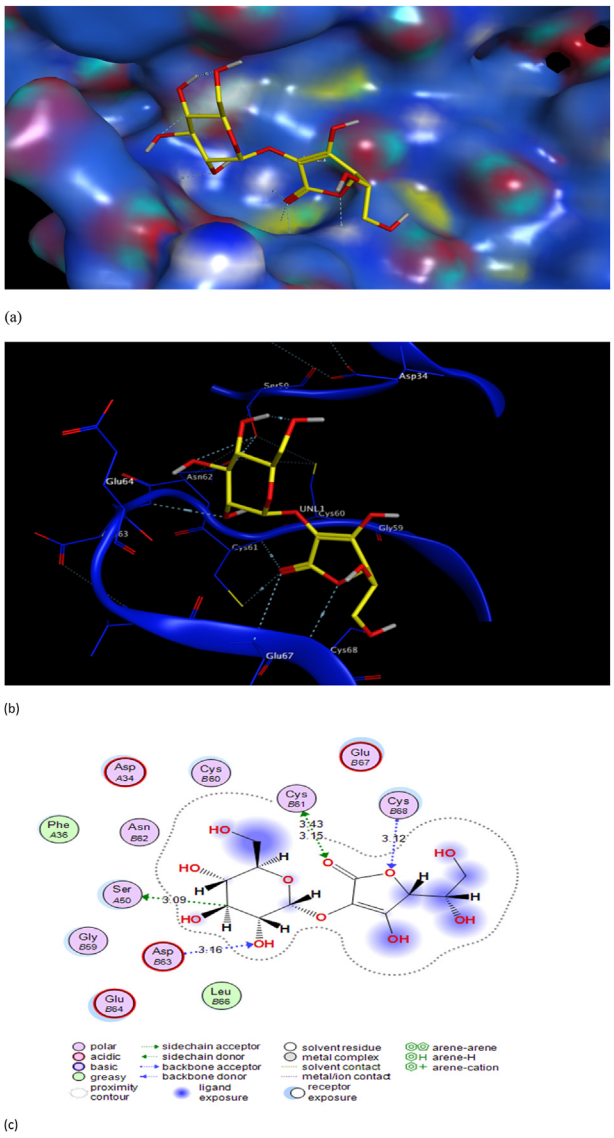

Figure 4: *In silico* modeled structure representation of complex VEGF/AA2G. (a): 3D spherical structure of VEGF represents the interaction with ligand and binding pocket cavity interacting residue of VEGF with vitamin C. (b): Interacting residue of VEGF included ASP-34, ASN-62, PHE-36, GLY-69, GLU-64, LEU-66, and GLU-67. **(c):** 2D structure of VEGF with LAA2G. Dotted line shows polar interaction.

VEGF protein in proliferative markers by performing *in silico* and *in vitro* analyses.

Molecular docking

In the *in silico* study, the structure of the VEGF was chosen as the interaction receptor. It has two chains A and B which contain residue 131, and the natural chemical structure was chosen as the ligand. The 3D crystallographic models were visualized by using PyMOL tools. The traits utilized to evaluate structural interactions include the energy binding score, residues, and atoms that are created by the contact of the receptor and ligand. Natural chemicals were collected using molecular docking techniques. The two compounds that scored the highest overall from a combination of the available compounds were revealed to be novel compounds.

Table 2: Drug-likeness profile of multiple ligands.

Ligand Name	Vitamin C	AA2G
Lipinski's Rule	Yes	No
Hydrogen bond acceptor	6	11
Hydrogen bond donor	4	7
Molecular weight (g/mol)	176.12 g/mol	338.26 g/mol
cLog	−1.42	2.85

cLog = log of the partition coefficient.

Table 3: ADMET profile of ligands.

Ligand Name	Toxicity		PK		Oral bioavailability score
Vitamin C	Tumorigenic	No	P-gp substrate	No	0.56
	Reproductive effect	No	CYP2D6 inhibitor	No	
	Irritant	No	BBB permeant	No	
	Mutagenic	No	GI absorption	High	
AA2G	Tumorigenic	No	P-gp substrate	Yes	0.11
	Reproductive effect	No	CYP2D6 inhibitor	No	
	Irritant	No	BBB permeant	No	
	Mutagenic	No	GI absorption	Low	

P-gp = P-glycoprotein, CYP2D6 = cytochrome 2D6, BBB = blood brain barrier, GI = gastrointestinal.

AutoDock Vina tool was used to evaluate the binding posture of each docked ligand. The binding energy, binding affinity, and docking pose confirmation inside the binding pocket were used to score docking poses. The top docking results for docked ligands are displayed in Table 1.

The range of binding energy of vitamin C and AA2G compound was −5.2 and −4.7 kcal/mol by using the AutoDock Vina tool. The protein was prepared and a receptor grid with box diameter X = 29.01, Y = −5.20 and Z = −25.08 was generated depending on the previously ob-

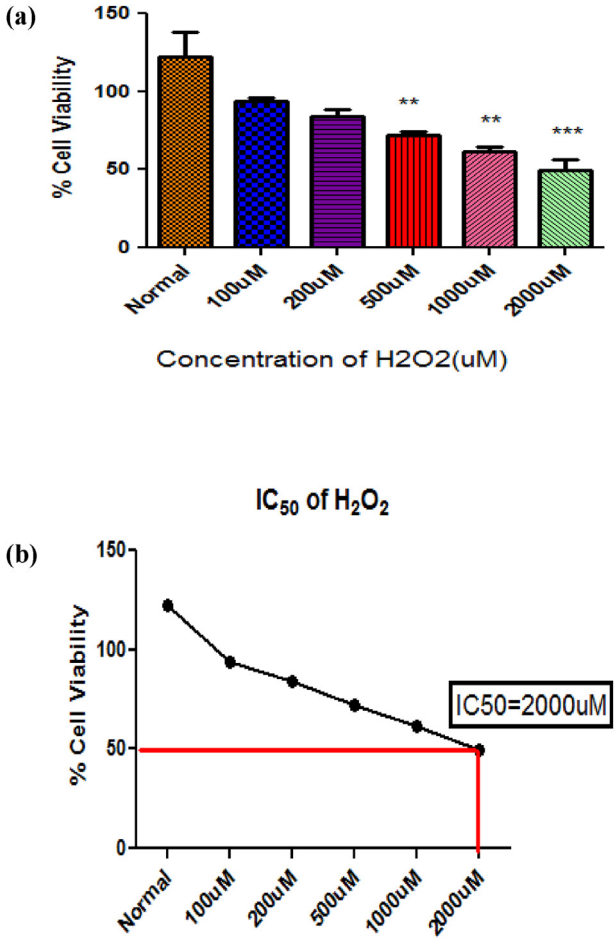

Figure 5: Analysis of cell viability. (a) MTT assay (b) IC_{50}. H_2O_2 to produce oxidative stress that targets malignant cells. The values are presented as the mean ± standard error of the mean (SEM) where $P < 0.0003$ and * indicate significant differences between the different concentrations of H_2O_2-treated and untreated cells.

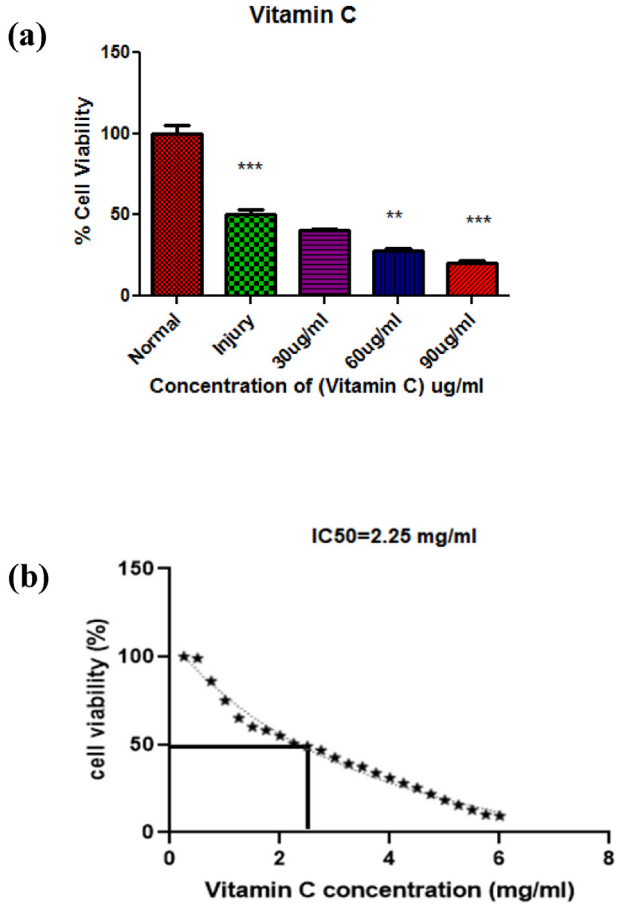

Figure 6: Analysis of cell viability: (a). H_2O_2-pretreated HepG2 cells (injured) after ascorbic acid treatment. (b). The IC_{50} value of vitamin C was calculated as 2.25 mg/mL in hepatocellular carcinoma. The values are presented as the mean ± SEM where $P < 0.0001$ and * indicate significant differences between different concentrations of vitamin C and untreated cells.

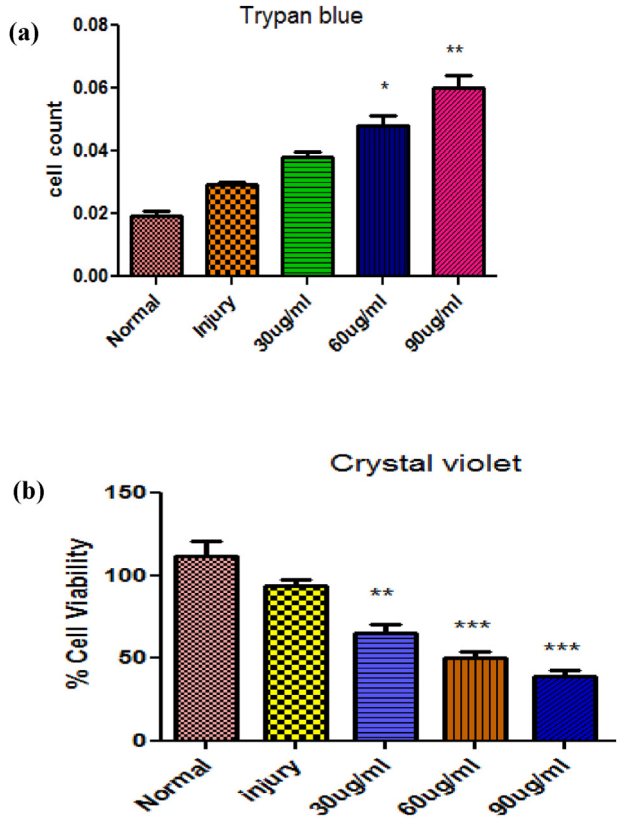

Figure 7: Cell viability assay. (a) Trypan blue (b) Crystal violet. (a). The bar graph shows a lower number of dead cells treated with ascorbic acid. (b)The bar graph shows an increase in the viability of cells treated with ascorbic acid. The values are shown as the mean ± SEM. $P < 0.0008$ and $P < 0.0001$ and * indicate significant differences between different concentrations of vitamin C and untreated cells.

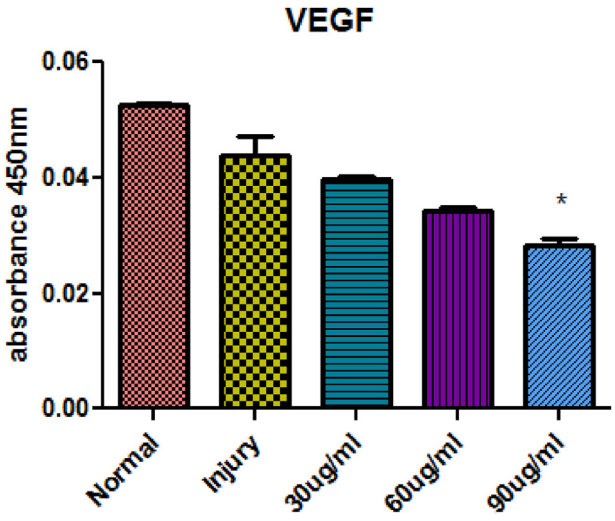

Figure 8: Expression of VEGF. The effect of ascorbic acid at 90 µg/mL in HepG2 cells showed significantly reduced VEGF gene expression in the presence of H_2O_2, where $P < 0.0001$ and * indicate significantly different treatments from untreated cells.

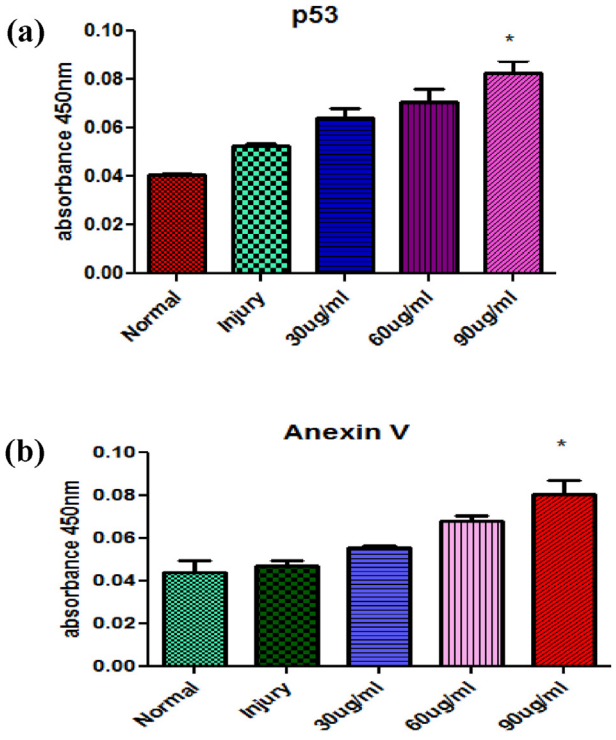

Figure 9: Analysis of apoptotic proteins via ELISA. (a) p53 (b) Annexin V. Compared with the control group, the bar graph shows higher apoptotic gene expression after ascorbic acid treatment at 90 µg/mL. $P < 0.0001$ and * indicate significantly different treatments from untreated cells.

tained binding site to the chain A. Two drugs were selected, vitamin C and AA2G, against the VEGF structure. The interactions between vitamin C and the VEGF residues were analyzed and a binding energy of −5.2 kcal/mol was found. Critical residues, including ASN-125, HIS-117-, CYS-127, ARG-111, were involved in hydrogen bonding with vitamin C. The VEGF residues within the binding pocket formed four hydrogen bonds with vitamin C at distances of −1.7 Å, −1.4 Å, −2.2 Å, and 1.5 Å, as shown in Figure 3. We also analyzed the binding energy between the VEGF structure and AA2G, which was −4.9 kcal/mol. Critical residues for this interaction included CYS-121, ARG120-, and ASN-115, which were situated within the binding pocket and formed three hydrogen bonds with AA2G at distances of −2.7 Å, −2.9 Å, and 2.3 Å, respectively, as shown in Figure 4. The binding pocket of the VEGF structure is displayed by a 2D diagram, which includes amino acids residue such as ASN-55, Val-170, CYS-42, VAL-115, TYR-113, ALA-121, TYR-128, and GLU-123. The VEGF protein exhibited a binding pocket where the ligands could fit best, indicating a strong potential for inhibitory interactions that could inhibit its activity.

Drug-likeness, pharmacokinetics, and toxicity profile of ligands

Drug-likeliness, or oral bioavailability, is a result of the physicochemical composition of a drug. The results of these computations for different ligands are shown in Table 2. The

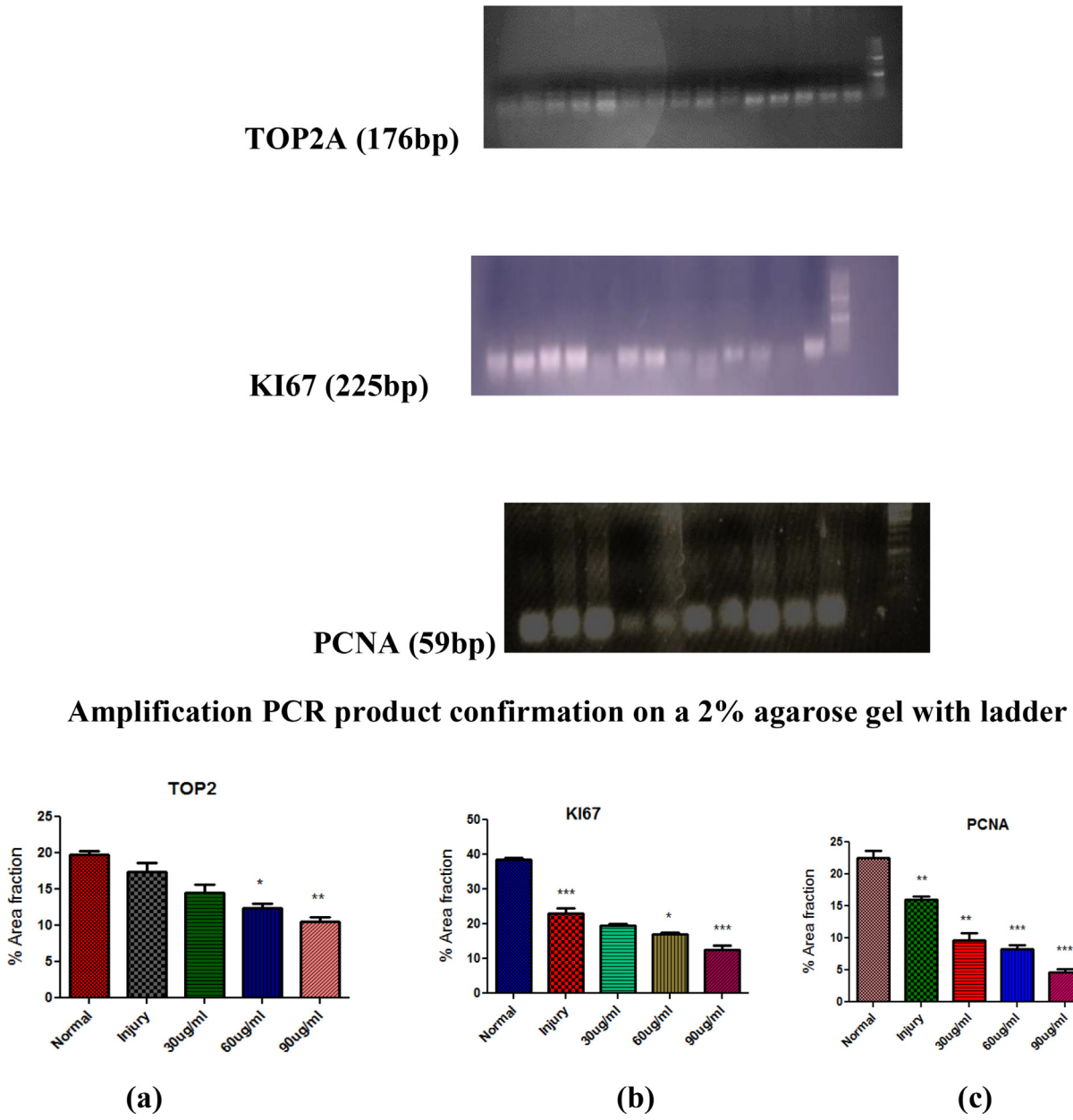

Figure 10: Gene expression analysis. (a) TOP2A (b) Ki-67 (c) PCNA. The bar graph represents the decrease in proliferative gene expression compared to that in the untreated group where $P < 0.0001$ and * shows significant differences between treated and untreated cells.

pharmacokinetic (PK) and toxicity profiles of different ligands are shown in Table 3. Vitamin C was selected because it exhibits high interaction with VEGF and fulfills Lipinski's rule, oral viability, and PK compared to AA2G.

Evaluation of cytotoxicity

Effect of H_2O_2 on cell viability

The MTT assay was conducted to assess the proliferative capacity of HepG2 cells treated with ascorbic acid compared with normal HepG2 cells. It was observed that higher concentrations of H_2O_2 inhibited cell proliferation. H_2O_2 generates oxidative stress, which selectively targets the malignant characteristics of cells. The determined IC_{50} value was found to be less than 2000 μM H_2O_2, as illustrated in Figure 5.

Effects of different concentrations of ascorbic acid on cell viability

To further increase the knowledge on the potential defensive impact of cancer prevention agents, cancer cells were treated with various concentrations of vitamin C, and

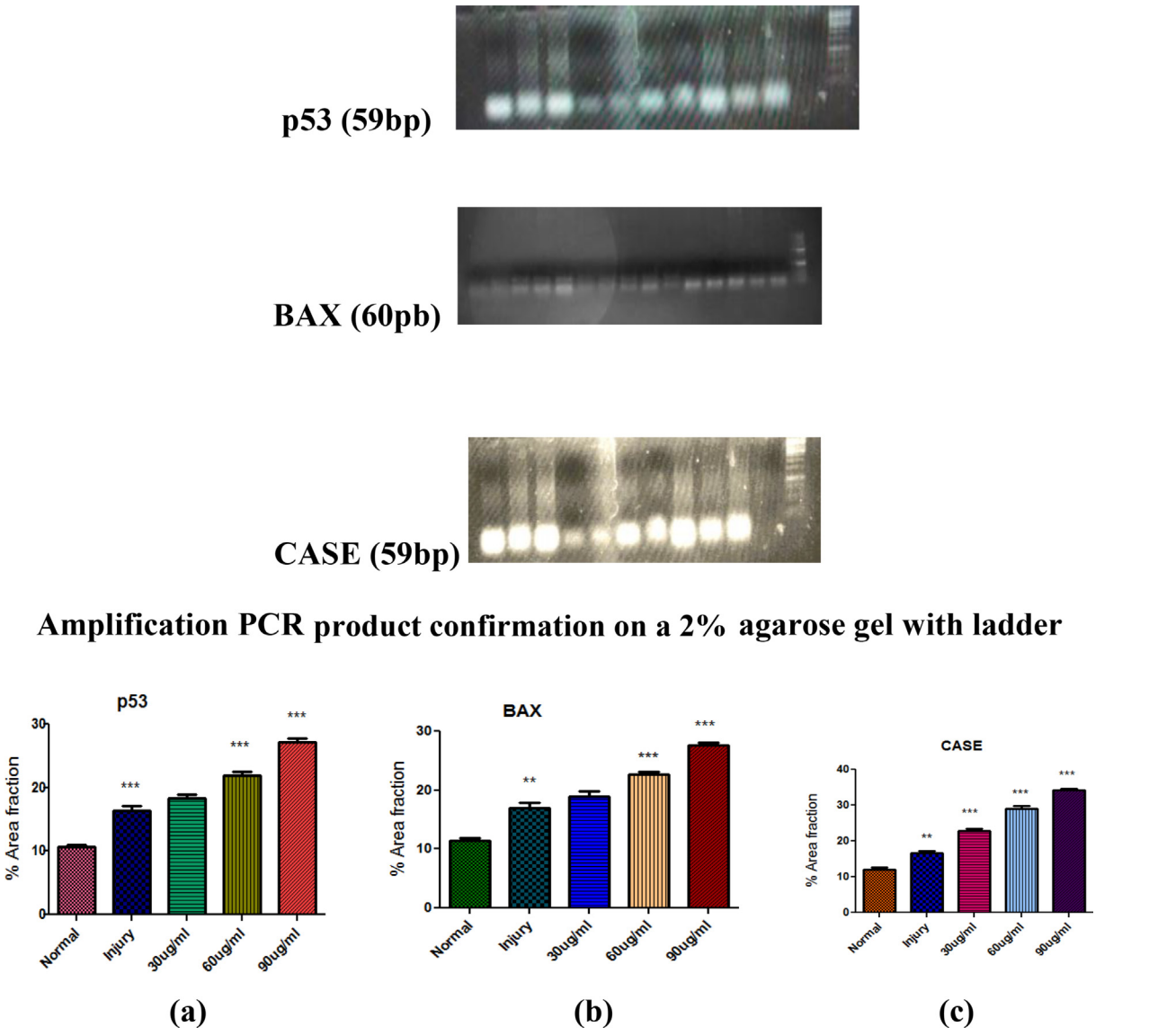

Figure 11: Analysis of gene expression. (a) p53 (b) B-cell lymphoma 2-associated X protein (c) Caspase-3. More apoptotic gene expression was shown in the bar graph than in the untreated group, where P = 0.0001 and * indicate that the treated and untreated cells significantly differed from one another.

cell viability was assessed. Generally, elevated levels of vitamin C (30, 60, 90 µg/mL) were seen as antioxidant agents, except less than 30 µg/mL vitamin C (Figure 6).

Determination of cell viability using trypan blue and crystal violet assays

When cells die, the adhering cells detach from the cell culture plates. When cells were stimulated with substances that cause cell death, these properties can be used to indirectly quantify cell death and identify variations in proliferate. To assess the effect of ascorbic acid in the HepG2 cell line, cell viability was assessed using trypan blue and crystal violet to distinguish between dead and live cells. Cell viability was increased after treatment with ascorbic acid as a lower number of dead cells were observed by trypan blue and crystal violet ($P < 0.0008$ and $P < 0.0001$, respectively) (Figure 5).

Downregulation of VEGF expression

HepG2 cells treated with ascorbic acid showed diverse mean ± SEM values, and reduced VEGF (angiogenesis) was observed by ELISA at an absorbance of 450 nm (Figure 6).

Upregulation of p53 and annexin V by ascorbic acid

HepG2 cells treated with ascorbic acid showed diverse mean ± SEM values and induced apoptosis in different types

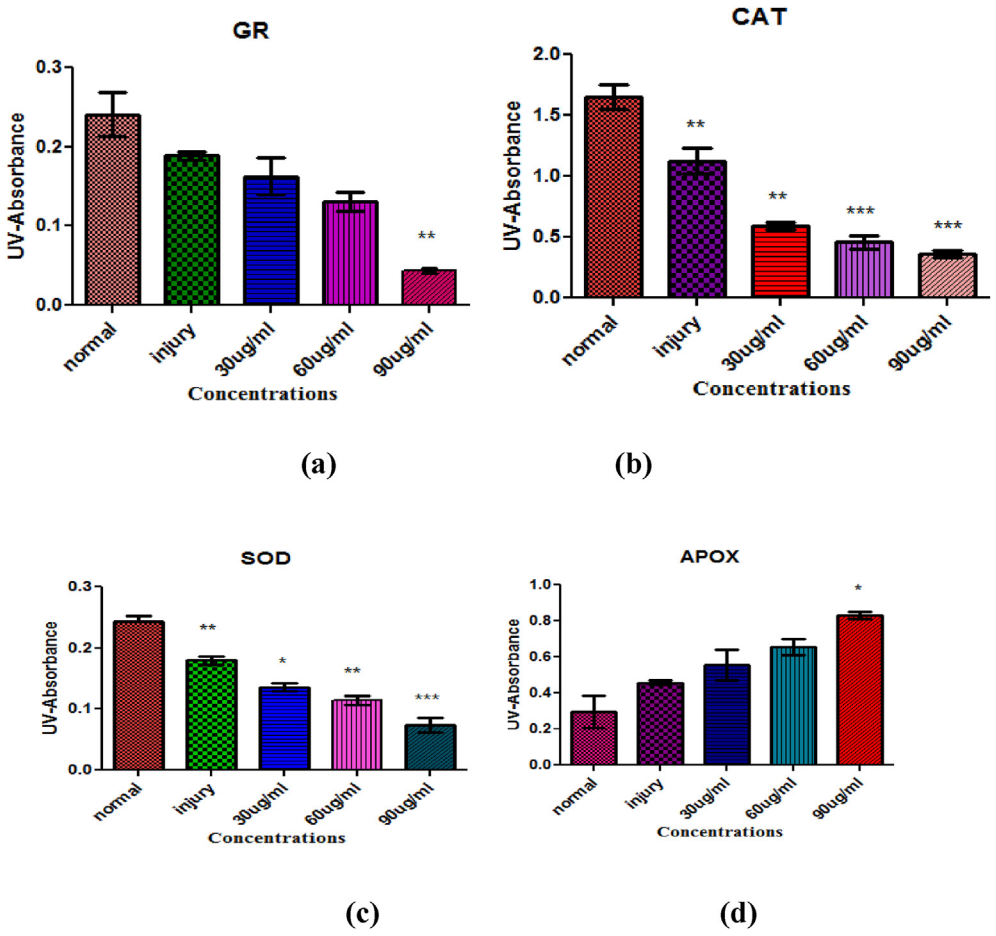

Figure 12: Analysis of antioxidants. (a) Glutathione reductase (b) catalase (c) superoxide dismutase (d) Ascarbate peroxidase. Compared with the untreated group, the ascorbic acid-treated group showed both a decline and an increase in antioxidant levels in the bar graph. $P < 0.0001$ and * indicate a significant difference between treated and untreated cells.

of tumor cells. As indicated by the results, treatment of cells with ascorbic acid in the presence of H_2O_2 upregulated apoptosis in malignant cells compared to normal cells. Figure 9 shows p53-induced apoptosis and annexin V.

Downregulation of proliferating cell nuclear antigen, topoisomerase II alpha, and Ki-67

After H_2O_2 treatment, cell lines damaged by oxidative stress were challenged. Topoisomerase II alpha (TOP2A), Ki-67, proliferating cell nuclear antigen (PCNA), are proliferation markers, which were downregulated after HepG2 cells were treated with ascorbic acid.

Evaluation of antioxidant activity

Different vitamin C concentrations were used to treat both cancerous and healthy cells to determine their antioxidant levels.

Discussion

VEGF is a signaling protein that plays a critical role in the angiogenesis process, which is essential for the growth and spread of tumors. By promoting the division of preexisting blood vessels into new ones, VEGF encourages angiogenesis.[33] Tumors with higher VEGF expression are often more aggressive and have a worse prognosis. VEGF not only affects angiogenesis but also directly impacts cancer cell behavior, enhancing their ability to grow, survive, and resist programmed cell death.[34]

In our *in silico* analysis, we found that predicting binding areas on the surface of VEGF through docking and subsequently verifying them through ADMET can be a promising approach for locating probable blockage. We chose vitamin C because it demonstrated high interaction with the VEGF protein, fulfilled Lipinski's Rule, had good oral viability, and showed favorable PK compared to AA2G. Ascorbic acid is a key component in anticancer activity, as it exhibits toxic effects on cancer cells treated with H_2O_2. Its strong antioxidant properties reduce the levels of ROS and may interact with preventive mechanisms against oxidative stress caused by H_2O_2.[35,36] According to our research, a high dose of ascorbic acid reduced the level of angiogenesis and other proliferative genes.

In this 3.8 Å structure of VEGF protein, the number of residues in ramachandran favored region was good (90%) (Figure 2). To enhance the interaction between VEGF protein and vitamin C, it is possible to activate antioxidant

and apoptotic genes.[37] Following molecular docking, the selected ligand was examined for its binding energy with VEGF. The lowest binding energies observed for VEGF with vitamin C and AA2G were −5.2 and −4.7 kcal/mol, respectively. The amino acid residues in chain A of VEGF that interacted with vitamin C and exhibited the lowest binding energy (−5.2 kcal/mol) were PRO-135, Val-120, CYS-142, ARG-141, TYR-128, ALA-121, GLU-123, and HIS-186. Four hydrogen bonds were formed between these residues and vitamin C, with bond distances ranging from 1.5 to 2.5 Å. In addition, the amino acid residues CYS-142, ARG-141, and ASN-155 in chain A of VEGF interacted with AA2G and exhibited the lowest binding energy (−4.7 kcal/mol). Three hydrogen bonds were formed between these residues and AA2G, with bond distances ranging from 1.7 Å to 2.0 Å. These findings suggest that vitamin C could be used to inhibit VEGF in HepG2 cells, as shown in Table 1 and Figures 3 and 4. The bioavailability screening results for the ligands are presented in Table 2. According to Lipinski's rule, ligands with a molecular weight below 500 kDa and a hydrogen bond acceptor value below 10 may not have excellent oral absorption, as they violate two of the indices.[38] However, compound vitamin C has passed the test and can be considered orally bioavailable. It is important to note that while vitamin C has excellent antioxidant and anticancer properties, its enhanced oral bioavailability may be beneficial for its use.[39,40]

The PK study was conducted to evaluate the role of therapeutic compounds within biological systems. In Table 3, the ADMET profiles of ligands are presented. Poor ADMET profiles are a major contributor to the high failure rates of drug candidates during the clinical stage of development. Therefore, profiling drug candidates before advancing to the clinical stage is a cost-effective and rational drug discovery strategy.[41] Vitamin C has a better gastrointestinal absorption potential and interaction with P-glycoproteins (P-gps) compared to AA2G. The ligands were discovered to be non-substrates of P-gps, which means they may not be affected by P-gp efflux activity, leading to a significant increase in serum concentration and successful therapeutic effect. Vitamin C showed the best oral bioavailability, with a score of 0.56. The data in Table 3 also suggest that none of the ligands were able to cross the blood−brain barrier, which may be beneficial as it indicates that they would not have negative effects on the central nervous system when used as medications. The results displayed in Table 3 revealed that none of the ligands functioned as absolute inhibitors of cytochrome p450 enzymes. This suggests that they possess a significant likelihood of undergoing metabolism, biotransformation, and elimination from the biological system. Moreover, when the toxicity of the ligands was assessed using mutagenicity, tumorigenic effects, reproductive impact, and irritating effects as endpoints, it was observed that none of the numerous ligands exhibited any of these tendencies. As a result, they may not pose a substantial risk of toxicity.[42]

In the HepG2 cell line model, we found that the effects of ascorbic acid on injured and untreated HeG2 cell lines for 3 h to improve cell viability, apoptosis, and reduced oxidative stress against H_2O_2.[43,44] To investigate the role of H_2O_2 in HepG2 cells, we comprehensively examined changes in cell biological functions, including viability, proliferation, cell cycle, and apoptosis. Specifically, H_2O_2 could essentially decay cell appropriateness and obstruct extension at around 2000 mM, which was resolved at the IC_{50}.[45,46] In this study, H_2O_2-treated HepG2 cells showed oxidative stress which caused the death of cancer cells (Figure 5).

Hepatocellular carcinoma (HCC) is linked to an increased risk of Wilson's disease and hemochromatosis.[46] This association is supported by intricate and multifaceted mechanisms. Chronic inflammation, oxidative stress, and free radical-induced cellular component damage may all play a role in the onset and development of HCC. Furthermore, various signaling pathways involved in cell proliferation, survival, and apoptosis may be impacted by the deregulated regulation of copper and iron homeostasis under these circumstances, potentially promoting carcinogenesis.[47]

In this study, injured HepG2 cells treated with ascorbic acid showed increased oxidative stress, reduced cell viability, and increased apoptosis. The MTT assay, which measures the ability of viable cells to reduce tetrazolium salt to blue formazan crystals, was used to analyze cell viability as shown in Figure 6. Trypan blue and crystal violet were also used to analyze cell viability through absorbance[48,49] (Figure 7). The use of vitamin C as an anticancer agent was investigated in injured and treated HepG2 cell lines. It was administered at pharmacological doses (30, 60, and 90 μg/mL) over the course of 3 h as a dietary supplement or at pharmacological doses. Recent research has provided several hypotheses regarding its mechanism of action. Vitamin C is transported into cells through glucose transporter (GLUT) pathways, with GLUT1 and GLUT3 being the two main isoforms. The uptake of vitamin C through these pathways may indirectly affect VEGF expression and angiogenesis by altering intracellular availability.[50] In the present study, ascorbic acid-treated injured HepG2 cell lines was shown to reduce cell viability and increase apoptosis (Figure 8).

The process of angiogenesis serves as a proliferative marker and plays a crucial role in preventing the development and growth of cancer. We evaluated gene and protein expression levels using PCR and ELISA, respectively. Our findings revealed that ascorbic acid supplementation, administered either preventively or therapeutically, resulted in the reduced expression of the VEGF protein.[51] In the present study, we found that the ascorbic acid-treated injured HepG2 cell lines did not have increased expression of proliferative genes. Apoptosis is a crucial process in the development and growth of cancer. We found that the gene and protein expression of p53 and annexin V was upregulated, as detected by PCR and ELISA, respectively. Apoptosis is a natural process that inhibits tumor formation (Figure 9). In our study, we observed the anticancer effects of ascorbic acid on HepG2 cells by determining whether ascorbic acid induces morphological features associated with apoptosis. We found that protein expression of the checkpoint gene p53 and apoptotic genes B-cell lymphoma 2-associated X protein, p53, and Caspase-3 was increased, while expression of the antiproliferative genes Ki-67, PCNA, and TOP2A was decreased.[52,53] We found that ascorbic acid-treated injured HepG2 cells showed decreased expression of proliferative genes (Figures 10 and 11).

The effect of vitamin C on cancer cells in decreasing GSH, CAT, and DPPH resulted in an increase in ROS levels. It was

observed that ROS levels increased in cancer cells treated with high doses of ascorbic acid. Vitamin C prevents the formation of nitrosamines and blocks the metabolic activity of carcinogens, and its cancer preventive effects may be related to its ability to protect against oxidative stress.[54,55] In the present research, we found that ascorbic acid-treated injured HepG2 cells showed increased level of antioxidants (Figure 12).

Limitation of the study

The limitation of this study is that a significant amount of computer power may be needed for complex simulations, and not all research groups or institutions may have access to high-performance computing. Therefore, to confirm and expand these findings, more research, including *in vivo* investigations and clinical trials, are required.

Conclusion

The objective of the study was to evaluate the effects of vitamin C on apoptotic and proliferative genes in injured HepG2 cells. *In silico* analysis was performed using molecular docking of the chemical compounds with VEGF. Different computational tools were used AutoDock Vina, BIOVIA DISCOVERY studio, and PyMOL. The MTT assay was performed to compare the proliferative potential of HepG2 cells treated with H_2O_2, ascorbic acid, and normal HepG2 using 96-well plates. The results of research exhibited that vitamin C inhibited the growth of cancer cells hence protected HepG2 cells from oxidative stress. It has been observed that vitamin C showed an antiproliferative activity. Vitamin C inhibited the expression of genes involved in protein synthesis. Therefore, it is suggested that to confirm and expand on these findings, more research including molecular dynamics analysis and *in vivo* investigations may be performed.

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Conflict of interest

The authors declare that they have no financial or non-financial competing interests.

Ethical approval

No ethical approval was required as this study did not involve human participants or laboratory animals.

Authors contributions

Conceptualization: SJA. Methodology: AS. Validation: MUK. Formal analysis: TM, Investigation: SS, Resources: HMZ, Data curation: HM. Writing of Original Draft: AS. Writing, review, and editing: SUK. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

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