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ORIGINAL ARTICLE

In silico and *in vitro* Evaluation of the Cytotoxic Potential of Hinokitiol against Osteosarcoma by Targeting Glycogen Synthase Kinase 3β

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ABSTRACT

Objectives: The present study aimed to assess the antiproliferative and pro-apoptotic effects of hinokitiol in osteosarcoma cells *via in vitro* and in silico targeting of glycogen synthase kinase 3*β* (GSK3*β*).

Materials and Methods: The (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to evaluate the cytotoxic potential of hinokitiol in osteosarcoma cells. Various concentrations of hinokitiol (5, 10, 20, 40, 60, and 80 µg/mL) were tested, and the half-maximal inhibitory concentration (IC_{50}) was calculated. Cell morphology, migration (scratch assay), and gene expression analysis using real-time polymerase chain reaction for pro-apoptotic studies were conducted, with the IC_{50} dose of hinokitiol utilized in all these experiments. Additionally the anti-proliferative effect of hinokitiol on GSK3*β* was also examined using *in silico* and gene expression methods.

Results: Hinokitiol significantly ($p < 0.05$) and dose-dependently decreased the viability of MG-63 cells, with an IC_{50} value of 40 µg/mL. Cell morphology study revealed cellular shrinkage and reduced cell density. The scratch assay revealed anti-migratory activity, while gene expression studies indicated pro-apoptotic effects, including significant ($p < 0.05$) upregulation of BAX and down-regulation of BCL-2 and GSK3β. Bonding interactions were also observed with GSK3*β* and atomic contact energy of -5.69 kcal/mol.

Conclusion: According to the current study findings, hinokitiol prevented Morphological study of the effects of hinokitiol on osteosarcoma cells from proliferating, migrating, and induced apoptosis by upregulating BAX (a pro-apoptotic signal) expression and downregulating BCL-2 (anti-apoptotic signal) expression in osteosarcoma cells. *In silico* findings of hinokitiol showed a significant bonding interaction with GSK3β and its downregulated gene expression probably prevented cancer cell survival.

Keywords: Hinokitiol, osteosarcoma, MTT assay, *in silico*, glycogen synthase kinase 3*β*

INTRODUCTION

Osteosarcoma is a rare malignant condition and is the most prevalent bone cancer. Osteosarcoma primarily affects youths and adolescents.[1] The treatment of osteosarcoma has advanced significantly in the modern era. The treatment includes radiation, chemotherapy, and even surgery. Currently, individuals with osteosarcoma have a 5-year survival rate of approximately 60-70%. Ifosfamide and methotrexate are some of the chemotherapy drugs used. Various combinations and other cytotoxic substances like etoposide have also been

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proposed in the literature.[2] However, taking these medications can cause several difficulties and adverse effects, such as neutropenia, mouth fissures, exhaustion, severe diarrhea, nausea, and emesis. The most notorious culprit may be anthracyclines, which cause acute reactions such as chest pain and dyspnea, underscoring a significant drawback of chemotherapy.[3] Chemoresistance is another issue posed by contemporary therapies. These therapeutic limitations have inspired researchers to explore new avenues, such as finding new targets and understanding their mechanisms, to identify cutting-edge treatments for a variety of cancers, including osteosarcoma. The most popular cell lines for osteosarcoma are MG-63 cells, which were generated from young Caucasian patients and were derived from their fibroblastic or epithelial origins. MG-63 cells have a highly proliferative phenotype.[4] The intent of using this specific cell line was to improve its affordability and accessibility as well as be beneficial for experimental research.

Glycogen synthase kinase 3β (GSK3β) is an important protein kinase that regulates metabolism, apoptosis, inflammation, and cell differentiation.[5-7] Aberrant activation of GSK3β has been observed in neurodegenerative diseases, cardiovascular diseases, and some oncological conditions.[8,9] Osteosarcomas express more GSK3β than normal cells and tissues. According to several recent studies down-regulated GSK3β may inhibit cancer cell growth and trigger apoptosis in human osteosarcoma cells.[10,11] The enhancement of Wnt signaling and catenin signaling through the inhibition of GSK3β is also believed to limit osteosarcoma cancer cell survival and proliferation. Based on these findings, increased expression of GSK3β in cancer is a potential therapeutic target.

Hinokitiol, chemically known as 2-hydroxy-4-isopropylcyclohepta-2,4,6-trien-1-one, (Figure 1), belongs to the monoterpenoid class and has a range of medicinal properties, including neuroprotective,[12] anti-tyronase,[13] anti-inflammatory[14] and anti-proliferative.[15] Hinokitinol has been demonstrated in recent studies to be effective against lung adenocarcinoma,[16] melanoma,[17] and breast cancer[18] cell lines. It also interferes with signaling pathways and explains how protein expression stops cancer cells from proliferating, migrating, and metastasizing. The effectiveness of hinokitiol against various cancers has been studied *in vitro*, and its benefits for bone loss have been evaluated; however, its potential against bone cancer remains unexplored. In this study, its efficacy against osteosarcoma was evaluated to assess the anti-proliferative and pro-apoptotic effects of hinokitiol in osteosarcoma cells *via in vitro* and *in silico* targeting of GSK3*β*.

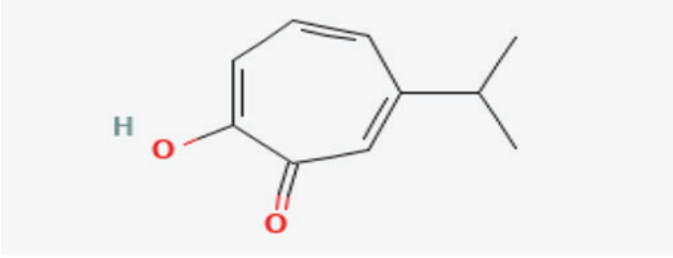

Figure 1. Structure of hinokitiol

MATERIALS AND METHODS

Hinokitiol was purchased from the Tokyo Chemical Industry to ensure material purity. The melting point of 51 ˚C and λmax (238, 320) were assessed using a melting point apparatus and ultraviolet absorption spectroscopy (Shimadzu 160 A) and found the same.

Cell line maintenance

From NCCS in Pune, MG-63 cells were purchased. The cells were grown in T25 culture flasks containing 10% fetal bovine serum (FBS) and 1% anti-biotics in Dulbecco's modified Eagle medium. Cells were housed in a humidified atmosphere with 5% CO_2 at 37 ˚C. Once confluence was reached, the cells were trypsinized and passaged.

Cell viability [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)] assay

Cytotoxicity (loss of viable cells) was assessed using the MTT assay.[19] This assay relies on the metabolic conversion of the soluble MTT salt, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, which symbolizes the normal function of mitochondrial dehydrogenase activity and cell viability, into an insoluble colored formazan product, which was estimated spectrophotometrically. The number of viable cells can be directly and proportionally estimated from the activity of mitochondria in living cells. MG-63 cells of $5X10^3$ density per well were coated on 96-well plates, and to this medium FBS was added and kept for 24 h in an incubator. The cells were then exposed to various doses of hinokitiol in triplicate (5, 10, 20, 40, 60, 80 µg/mL) at 5% CO_2 at 37 ˚C for 24 h. Subsequently, cells were added with MTT reagent and incubated for 4 h (Sigma, MO, USA). The same amount of time was also spent incubating untreated [dimethyl sulfoxide (DMSO)] cells. MTT solubilization solution (Sigma) was used to dissolve the formazan crystals after the incubation period; the formed formazan crystals were then made into a solution in DMSO (100 µl) and incubated in the dark for an hour. A 96-well image reader was used to detect absorbance at 570 nm. The percentage of viable cells was defined as the percentage of control cells established in a serum-free medium. The control medium without treatment was considered 100% viable. The cell viability was calculated using the following formula: % cell viability = [A570 nm of treated cells/A570 nm of control cells] x 100.

Cell morphology analysis

Based on the results of the MTT experiment, we selected the optimum dose (IC_{50} 40 µg/mL) for further research. Using a phase-contrast microscope, changes in cell morphology were analyzed. In a 6-well plate, MG63 cells were seeded at a density of 2×10^5 and were kept overnight. The cells were treated with an optimal dose of hinokitiol (40 µg/mL) for 24 h, and untreated cells served as the negative control. When the incubation period ceased, the medium was removed, and the cells were washed with phosphate buffer saline (pH 7.4) and examined under a phase-contrast microscope.

Cell migration was analyzed using the scratch-wound healing assay

MG63 cell line was planted onto a six-well culture plate at a density of 2 x 10^5 and was kept overnight. The incubated cells were then washed with Dulbecco's phosphate-buffered saline (DPBS), and a sterile micropipette tip of 200 µl, was used to make a scratch.[20] The detached cells and other cellular debris were washed with DPBS. The cells were then treated with an optimal dose of hinokitiol (40 µg/mL) for 24 h, and untreated cells served as the negative control. After incubation, the wells were washed and fixed in 4% paraformaldehyde. Photographs were taken using an inverted microscope (Euromex, The Netherlands).

RNA extraction and gene expression by real-time polymerase chain reaction (RT-PCR)

Using RT-PCR, the gene expression of pro-apoptotic and anti-apoptotic cells was examined, and Trizol Reagent (Sigma) was used to separate the total RNA. Briefly, 1-2 µg of total RNA was transformed to cDNA using, PrimeScript, 1st strand cDNA synthesis kit (Takara, Japan) by the manufacturer's instructions.[21] Primers were designed to specifically amplify targeted genes. The primer sequences BAX-Forward: 5'gctggacattggacttcctc3'; reverse: 5'ctcagcccatcttcttccag3'; BCL-2- forward: 5'gctggacattggacttcctc3'; reverse: 5'ctcagcccatcttcttccag3'. GAPDH- Forward: 5'cgaccactttgtcaagctca3'; reverse: 5' cccctcttcaaggggtctac3'. GSK3β- Forward: 5'ccgactaacaccactggaagct3' Reverse: 5'aggatggtagccagaggtggat3'. PCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad, USA), which contains SYBR green dye and all PCR components.[22] Stratagene's MX3000p PCR machine was used to perform real-time PCR. The results were analyzed using the comparative computed tomography method and $2^{-\Delta\Delta C}{}_T$ and the Schmittgen and Livak 2CT method was utilized to calculate fold changes.

Molecular docking

Structure preparation

The crystal structure of GSK3β was downloaded from the Protein Data Bank (PDB) at (PDB_http://www.pdb.org/pdb/home/home CODE: 2O5K). The 3D structure of hinokitiol was downloaded from the Pubchem database for docking.

Molecular docking

Auto Dock 4.2 was used to perform docking calculations. Auto Dock Tools (ADT) were used to create grid boxes and .pdbqt files to generate proteins and ligands. ADT was used to modify the native GSK3β structure by adding polar hydrogens, unified Kollman charges, solvation parameters, and fragmental volumes. The Auto Grid was used to construct grid maps representing proteins throughout the docking process. The dimensions of the x, y, and z axes were set to 100, 100, and 100, respectively, and the grid spacing was set to 0.403. The 100 docking conformers were performed using the Lamarckian Genetic Algorithm (LGA), and the Auto Dock application was run with the following parameters; Maximum number of energy evaluations allowed is 250000; GA crossover mode is two points; GA population size is 150. Out of 100 LGA conformers, the conformer with the lowest binding energy was chosen for further analysis. We measured the binding energy and determined both particular and non-specific interaction residues of the entire molecule coupled to GSK3β. The software packages Pymol and Discovery Studio were used to visualize the docked conformations.

Statistical analysis

Data obtained from the study (Cell Viability and Gene expression) were analyzed by One-Way analysis of variance followed by Student's *t*-test using SPSS version 20 and represented as mean ± standard deviation for triplicate analyses. Statistical significance was determined at a level of $p < 0.05$.

RESULTS

Hinokitiol significantly reduces MG-63 cell viability

Cellular survival following exposure to hinokitiol at various concentrations (5, 10, 20, 40, 60, and 80 µg/mL) was determined using absorbance readings obtained from the MTT assay. Results were compared with the corresponding negative controls (untreated cells) after 24 h of incubation and expressed as percentages of viability. Hinokitiol significantly ($p < 0.05$) decreased MG-63 cell viability in a dose-dependent manner, with a half-maximal inhibitory concentration (IC_{50}) of 40 µg/mL (Figure 2)

Morphological study of the effects of hinokitiol on osteosarcoma (MG63) cells

A decrease in the cell population was observed following hinokitiol incubation. As depicted in (Figure 3) treatment with the IC_{50} dose of 40 µg/mL hinokitiol led to noticeable cellular shrinkage and a reduction in cell density, causing overall morphological alterations. MG-63 cells were treated with the test compound hinokitiol (IC_{50} 40 µg/mL) along with the control group for 24 h. The images were obtained at a magnification (x 10) using an inverted phase-contrast microscope.

Hinokitiol decreases wound closure in MG-63 cells

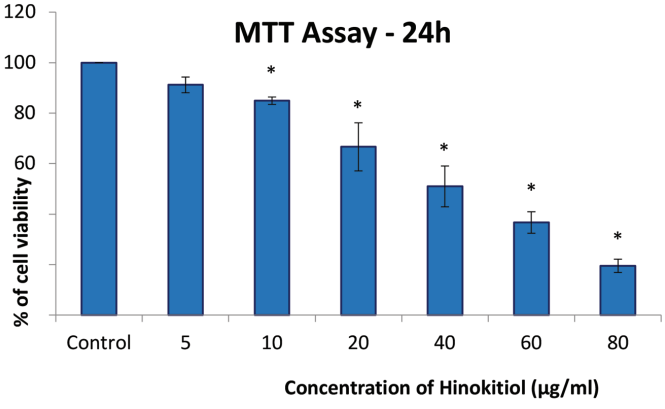

Figure 2. Cytotoxic effect of Hinokitiol on osteosarcoma cancer cells by MTT assay method. Data are shown as means ± SD (n= 3). *Compared with the control blank group, $p < 0.05$

SD: Standard deviation

At an IC_{50} of 40 g/ml, hinokitiol greatly reduced MG-63 cell motility in the wound-healing experiment, a common method for assessing cell migration and cell-cell contact. Hinokitiol virtually completely prevented MG63 cell migration after 24 hours of incubation (Figure 4).

Gene expression profiles of hinokitiol-induced genes

Hinokitiol treatment modulates apoptosis marker gene expression in MG-63 cells. To identify the mechanism of apoptosis caused by treatment with hinokitiol in osteosarcoma cell lines (MG-63), an messenger ribonucleic acid (mRNA) expression study of three genes mainly considered for their involvement in apoptotic pathway regulation, such as BAX, BCL-2, and GSK3β were studied. Hinokitiol treatment significantly ($p < 0.05$) decreased the expression of BCL-2, an apoptosis inhibitor in cells, and GSK3β, a kinase, compared with untreated cells. Additionally, a significant ($p < 0.05$) up regulation of *BAX* gene expression and downregulation of BCL-2 and GSK3β were observed in the hinokitiol-treated group compared to the control group (Figures 5, 6).

Docking study

Molecular modeling is an application in which molecular docking techniques are used to study how receptors interact with ligands. The Autodock 4.2 suite was used to visualize the binding affinities of the hinokitiol against target protein GSK3β (PDB CODE: 2O5K) based on binding energy to elucidate the probable mechanisms of the compounds. The best docking complex was obtained from 100 different conformers for further research based on the extent of hydrogen bonding, maximal occupancy of the binding pocket with the lowest binding energy, and other potential non-covalent interactions. The lowest binding energy (-5.69 kcal/mol) was observed among 100 conformers.

The amino acids of isoleucine 62, alanine 83, aspartic acid (ASP) 133, tyrosine (TYR) 134, valine (VAL) 135, proline (PRO) 136, TYR 140, arginine (ARG) 141, Glycine 185, asparagine ASN 186, LEU 188, ASP 200, and ARG 220 were noted as active site residues in the binding cavities of GSK3β *via* discovery studio visualizer. Interestingly, the current docking

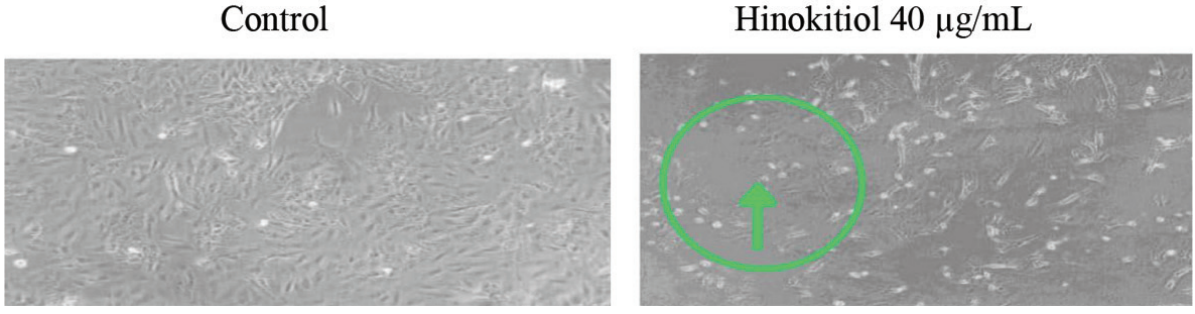

Figure 3. Morphological study of hinokitiol on osteosarcoma (MG63) cell line

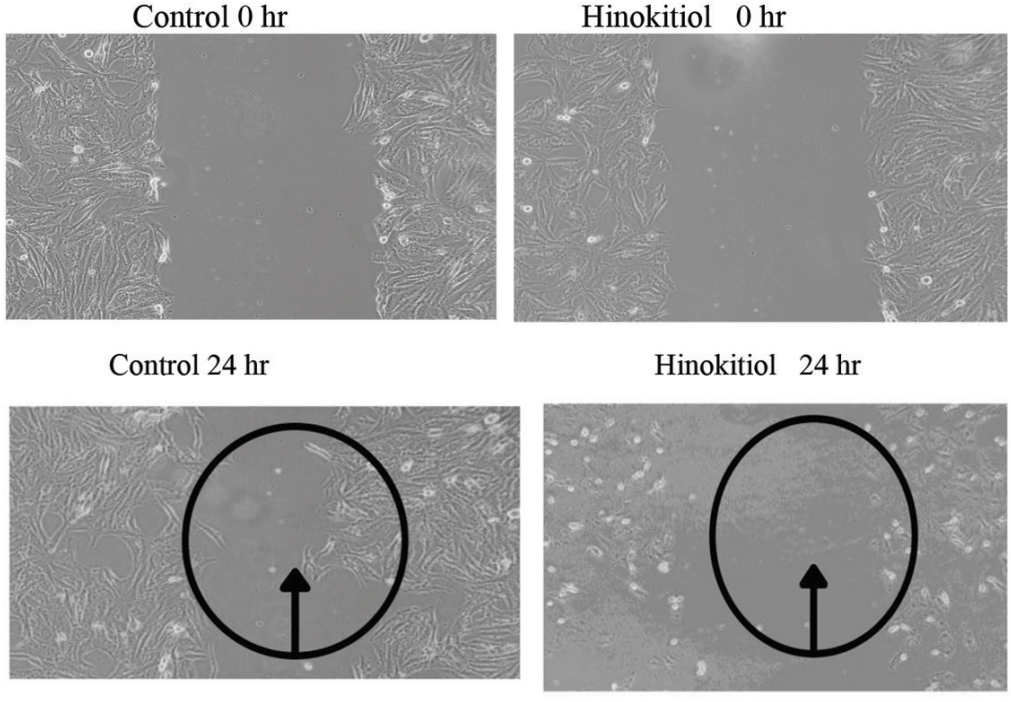

Figure 4. Cell Migration study of hinokitiol on osteosarcoma (MG63) cell line by scratch assay

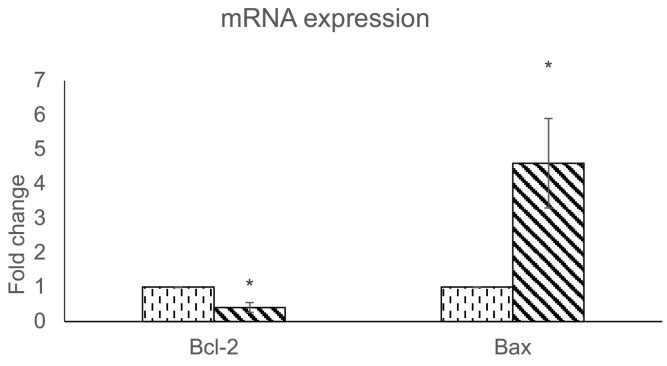

Figure 5. mRNA levels of target genes were analyzed by RT-PCR using GAPDH as a reference. Data are mean ± SEM of three independent tests with triplicate reactions. *$p < 0.05$ vs. control

RT-PCR: Real time-polymerase chain reaction, SEM: Standard error of mean

mRNA: Messenger ribonucleic acid

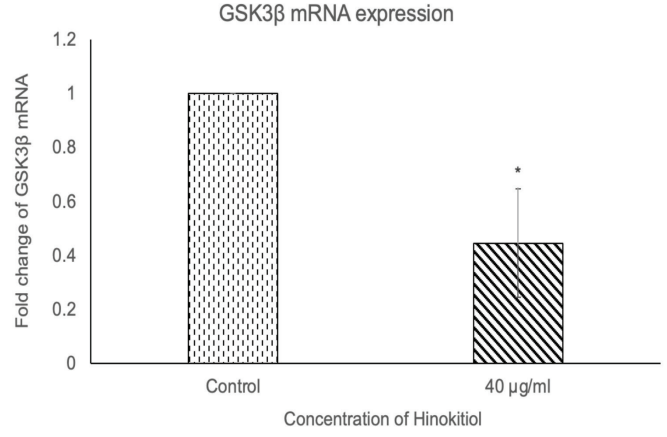

Figure 6. *GSK3β* gene expression is normalized to GAPDH mRNA expression and the results are expressed as fold change from control. Each bar represents mean + SEM of three independent observations. "*" represents statistical significance between control versus drug treatment groups at $p < 0.05$ level

SEM: Standard error of mean, mRNA: Messenger ribonucleic acid

investigation demonstrated that hinokitiol interacts with the amino acids ARG 113, TYR 134, LEU 81, VAL 135, and LYS 197 *via* hydrogen bonds and hydrophobic interactions within the binding cavity. Hinokitiol formed three strong hydrogen bonds between the hydroxyl and carbonyl groups of the ligand with the side chains of ARG 113, TYR 134, and LEU 81 (Figure 7B). The bond distances of 2.9 Å, 2.1 Å, and 1.7 Å were noted respectively. The one carbon-hydrogen bond was found in ASP 133. It was also observed to involve three alkyl hydrophobic interactions within the amino acids of VAL 135 and LYS 197. Furthermore, the following amino acids were involved in the van der Waals interactions: PRO 136, ASP 190, VAL 82, and GLU 80 (Figure 7).

DISCUSSION

In this investigation, we examined the anticarcinogenic effects of hinokitiol on human osteosarcoma MG-63 cells, which were not previously reported. Due to their well-known lack of functioning p53 (p53 null status), MG-63 cells are excellent models for the development of innovative therapeutic therapies for patients with osteosarcoma.[23]

The MTT assay relies on living cells converting MTT into formazan crystals to detect mitochondrial activity, which is a typical method for assessing the metabolic activity of viable cells.[19] This method is universally used to assess the *in vitro* cytotoxic nature of drugs and chemicals in cell lines because the overall mitochondrial activity of the majority of cell populations is correlated with the ratio of viable cells.[19] In the study, we found a dose-dependent decrease in the percentage of viable MG-63 cells as we increased the concentration of hinokitiol; however, we have selected a hinokitiol (IC_{50}) dose of 40 µM for further research. Morphological analysis of the MG-63 cells was done following exposure to the hinokitiol in the osteosarcoma cell line using an inverted phase-contrast microscope. The findings demonstrated that compared with untreated cells, hinokitiol-treated cells underwent considerable morphological alterations characterized by decreased cell density and cell shrinkage, which is a common feature of apoptotic cells. The scratch-wound healing assay examines the capability of cells to drift and consequently heal the wound created in a confluent plate of cells. Cell migration can be easily measured using the scratch test because the metastatic process is a major contributor to cancer patient deaths.[24] Cancer cells propagate and proliferate throughout the body. They traverse through the extracellular matrix, enter the circulation, attach to an unrelated location, and then extravasate to produce far-off foci.[24] Hinokitiol 40 µM treatment significantly reduced cell movement in the osteosarcoma migration assay. The results of our study were consistent with tomentosin-induced toxicity on MG-63 cell lines.[23] The efficacy of cancer medication therapy depends on its capacity to cause cancer cells to undergo programmed cell death. According to previous studies, there are two primary apoptotic pathways: intrinsic or mitochondrial pathway and extrinsic pathway.[25] Most cancer drugs follow the mitochondrial pathway, and in the current study of cell viability, we observed that hinokitiol reduced mitochondrial activity. In the mitochondrial pathway, the process of apoptosis starts with the generation of intracellular impulses that eventually result in the opening of the inner membranes of mitochondria and the gradual release of pro-apoptotic proteins into the cytoplasm.[25] Changes in mitochondria are controlled by B-cell lymphoma protein 2 (BCL-2) and (BAX) proteins. BAX protein promotes apoptosis by releasing cytochrome C from the mitochondria. This facilitates subsequent activation of caspases, which eventually results in cell death.[25] According to theory, BCL-2 limits the activation of the apoptotic machinery downstream by preventing BAX from releasing cytochrome C. Consequently, cells survive, although BCL-2 is also engaged in relocating proliferating cells back to the resting phase of the cell cycle.[26] Thus, the impact of hinokitiol on the apoptosis-related genes

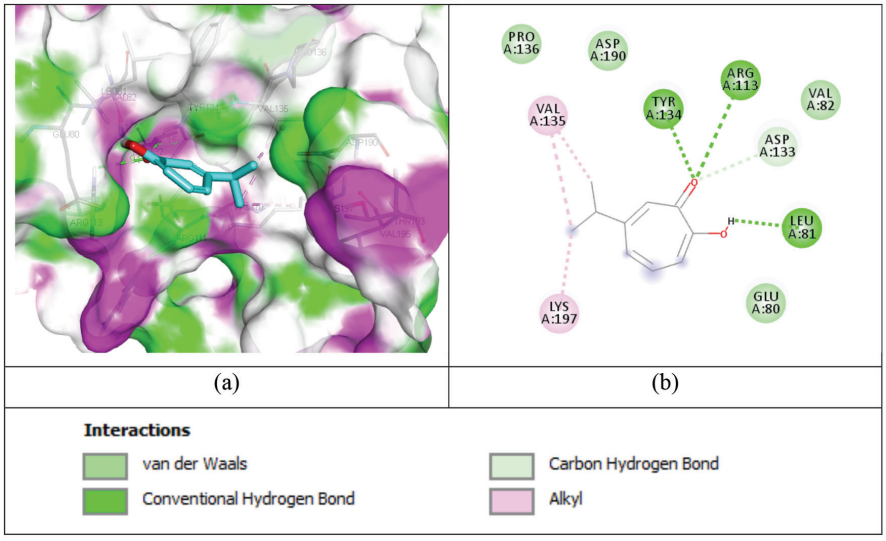

Figure 7. Three-dimensional (3D) and two-dimensional (2D) binding interactions poses of hinokitiol in the binding pocket of GSK3*β* (PDB CODE: 2O5K). Hydrophobic interactions and hydrogen bond and are indicated by pink and green dashed lines

BAX and *BCL-2* was evaluated. *BAX* genes are crucial for controlling apoptosis. In the present study, hinokitiol treatment reduced the expression of BCL-2, an apoptosis inhibitor. Significantly higher *BAX* gene (proapoptotic) expression was observed in the hinokitiol-treated group.

GSK3*β* is a protein that is highly relevant in different cancers because it plays a significant role in cell growth, proliferation, and migration.[5-7] The serine/threonine protein kinase GSK3*β* has emerged as a crucial enzyme in controllinof several crucial cellular signaling pathways by phosphorylating its substrates. Phosphorylating many oncogene proteins, such as β-catenin, carcinogenic transcription factors, and c-Myc, causes their ubiquitin degradation and inactivation.[27] GSK3*β* adversely affects cell survival and proliferation in normal physiological settings. GSK3*β* is therefore commonly thought of as a "tumor-suppressor gene". According to Tang et al.,[11] osteosarcoma cells that overexpress GSK3*β* have a considerable positive impact on colony growth and tumor growth. Importantly, the findings showed that osteosarcoma tumors were aided in growth by the aberrant activation of GSK3*β*. According to Cai et al.,[28] therapy with a GSK3*β* inhibitor reduces cell survival and proliferation rates, suggesting that GSK3*β* may be linked to the development of osteosarcoma. In the current study, we evaluated the role of hinokitiol on GSK3*β* by *in silico* models, and the results revealed a significant binding interaction. The *GSK3β* gene expression analysis also showed hinokitiol's potential to negatively regulate its function, thereby promoting cell death and mitigating cell survival. A downregulated GSK3*β* in osteosarcoma has reduced tumor cell viability and triggered apoptosis, according to recent researches. The results of our study were further substantiated by earlier findings that hinokitiol has an apoptotic and anti-hepatofibrotic impact on hepatic stellate cells *via* activating GSK3*β* and inhibiting the Wnt/-catenin pathway.[29]

CONCLUSION

We used a potent bioactive agent known to have anti-proliferative activity in various cell lines. In the current study, we evaluated the anti-proliferative activity of hinokitiol against the osteosarcoma cell line showed significant cytotoxic activity. A deep examination of the cell line after treatment with hinokitiol revealed morphological changes such as cell shrinkage and reduced cell density. *In vitro* evaluation of the wound healing assay revealed anti-migratory effects, which are characteristic properties of anti-cancer drugs. The study also analyzed *mRNA* gene expression by RT-PCR in the MG-63 cell line, and the pro-apoptotic *BAX* gene was upregulated and the anti-apoptotic gene *BCL-2* and GSK3*β* was downregulated. The study also used *in silico* models to assess the binding energy of the biomarker enzyme GSK3*β* regulated on numerous signaling pathways for cancer progression hinokitiol, and showed effective interactions at various binding sites of GSK3*β*. Because the present study was carried out only in the osteosarcoma MG-63 cell line, further research on other osteosarcoma cell lines will substantiate the claim of hinokitiol as a strong candidate drug for ameliorating bone cancer.

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Ethics

Ethics Committee Approval: There is no ethical problem related to the study.

Informed Consent: This study does not involve human participants or animal subjects requiring consent.

Authorship Contributions

Concept: B.V.C., Design: B.V.C., Data Collection or Processing: P.S, G.J., Analysis or Interpretation: K.K.K, P.B.S, Literature Search: A.K.S, A.R., Writing: P.S., G.J., K.E.

Conflict of Interest: The authors have no conflicts of interest to declare.

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