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In silico and In vitro Evaluation of Cytotoxic Potential of Hinokitiol against Osteosarcoma by targeting Glycogen Synthase Kinase 3β

Cheriyan et al. Cytotoxic Potential of Hinokitiol against Osteosarcoma

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Abstract
Objective: The current study aimed to assess the anti-proliferative and pro-apoptotic effects of hinokitiol on osteosarcoma cells by *in vitro* and *in silico* targeting of glycogen synthase kinase 3β.
Material and Methods: The MTT assay was used to evaluate the cytotoxic potential of hinokitiol in osteosarcoma cells, Hinokitiol was utilized in a variety of concentrations (5, 10, 20, 40, 60, 80µg/ml), and inhibitory concentration IC_{50} dose were calculated. Cell morphology, migration (scratch assay), and gene regulation for expression (RT-PCR) for pro-apoptosis study were also conducted and IC_{50} dose was considered for the aforesaid studies. The role of hinokitiol's anti-proliferative effects on glycogen synthase kinase-3β was also examined using *in silico* and its gene expression methods.
Results: Hinokitiol dose-dependently decreased the viability of MG-63 cells with an IC_{50} of 40µg/ml. Cell morphology study finding revealed cellular shrinkage and reduced cell density, Scratch assay revealed it had anti-migratory activity, and pro-apoptotic property of target genes was revealed in the gene expression study. Bonding interactions were also observed with glycogen synthase kinase-3β and atomic contact energy observed was -5.69kcal/mol
Conclusions According to the current study's findings, Hinokitiol prevented MG63 cells from proliferating, migrating and induced apoptosis effect via upregulation of BAX (a proapoptotic signal) expression down-regulation of BCL-2 (antiapoptotic signal) expression, in osteosarcoma cells. *In silico* findings of hinokitiol showed significant bonding interaction with glycogen synthase kinase 3 β and its downregulated gene expression probably preventing cancer cell survival.
Keywords: Hinokitiol, Osteosarcoma, MTT assay, *in silico*, Glycogen synthase kinase 3β

INTRODUCTION

Osteosarcoma is regarded as a rare malignant condition, being the most prevalent bone cancer. Osteosarcoma primarily impacts youths and adolescents [1]. Treatment for osteosarcoma has advanced significantly in the modern era. It includes radiation, chemotherapy, and even surgery. Currently, people with osteosarcoma have a 60-70% 5-year survival rate. Ifosfamide and methotrexate are some of the chemotherapy drugs used. Various combinations and other cytotoxic substances like etoposide have also been proposed in the literature [2]. However, taking these medications might cause several difficulties and adverse effects, such as neutropenia, mouth fissures, exhaustion, severe diarrhoea, nausea, and emesis. The most infamous culprits may be anthracyclines

causing chest pain and shortness of breath, among the acute reactions demonstrating how these significant side effects are a key disadvantage of using chemotherapy[3]. Chemoresistance is another issue of contemporary therapies. These therapeutic limitations have inspired researchers to start looking in 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 which was generated from young Caucasian patients, and derived from their fibroblastic or epithelial origins. MG-63 cells are highly proliferative phenotype [4]. The intent of using this specific cell line was its affordability and accessibility as well as being beneficial for experimental research.

Glycogen synthase kinase 3β (GSK3β) is an important protein kinase, that regulates, metabolism, apoptosis, cell differentiation, inflammation, and cell differentiation [5, 6, 7]. An aberrant activation of GSK3β has been demonstrated in neurodegenerative diseases, cardiovascular diseases, and some oncological conditions. [8,9]. Osteosarcomas express more GSK3β than normal cells and tissues do, 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 signalling and catenin signalling through the inhibition of GSK3β is also said to limit osteosarcoma cancer cell survival and proliferation. Based on these findings, increased expression of GSK3β in cancer is thought to be a therapeutic target.

Hinokitiol, chemically known as 2-hydroxy-4-isopropylcyclohepta-2,4,6-trien-1-one, (Figure 1) belongs to a member of the monoterpenoids class and unveils a range of medicinal properties comprising neuroprotective[12], anti-tyronase[13], anti-inflammatory [14] and anti-proliferative[15.] Hinokitinol has been demonstrated in recent research to be effective against lung adenocarcinoma [16], melanoma [17], and breast cancer [18] cell lines. It also interferes with signalling pathways and explains how the expression of proteins stops cancer cells from proliferating, migrating, and metastasizing. The effectiveness of Hinokitiol against various types of cancers has been studied in *in vitro*. The benefit of it, in bone loss has been evaluated, but not in bone cancer. In this study, its efficacy against osteosarcoma was evaluated by *in vitro* apoptosis and migration tests in the MG-63 cell line, as well as *in silico* GSK3β molecular binding capacity.

MATERIALS AND METHODS

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

Cell line maintenance

From NCCS in Pune, osteosarcoma cell lines (MG-63) were purchased. The cells were grown in T25 culture flasks containing 10% Fetal bovine serum (FBS) and 1% antibiotics added to Dulbecco's Modified Eagle Medium (DMEM). Cells were housed in a humid atmosphere with 5% CO_2 at 37 degrees. Once confluence was reached, the cells were trypsinized and passaged.

Cell viability (MTT) assay

Cytotoxicity (loss of viable cells) was assessed using the MTT test [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 foetal bovine serum was added and kept for 24 hours in an incubator. The cells were then exposed to various doses of hinokitiol in triplicate (5, 10, 20, 40, 60, 80µg/ml) at 5% CO_2 at 37°C for 24 hours. After that, cells were added with MTT reagent and incubated for 4 hours (Sigma, MO, USA). The same amount of time was also spent incubating untreated (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 Dimethyl sulfoxide DMSO (100µl) and incubated in the dark for an hour. A 96-well image reader was used to detect the absorbance at 570 nm. The measure of viable cells was signified as a percentage of control cells established in a serum-free medium. The control medium with no treatment was considered as 100% cell viable. The cell viability is calculated using the formula: % cell viability = [A570 nm of treated cells/A570 nm of control cells] × 100.

Cell morphology study

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

Cell migration analyzed by scratch wound healing assay

Osteosarcoma cell (MG63) was planted onto a six-well culture plate at a density of 2×10^5 and was kept overnight. The incubated cells were then washed using DPBS and a sterile micropipette tip of 200µl, was used

to make a scratch [20]. The detached cells and other cellular debris were washed with Dulbecco's phosphate-buffered saline (DPBS.) The cells were then treated with an optimal dose of hinokitiol (40µg/ml) for 24h while the 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 PCR

Using real-time Polymerase Chain Reaction (PCR), the gene expression of pro-apoptotic and anti-apoptotic cells was examined, and Trizol Reagent (Sigma) was used to separate the total Ribo Nucleic acid (RNA). Briefly, 1-2µg of total RNA was transformed to cDNA using, PrimeScript, 1st strand cDNA synthesis kit (Takara, Japan) in accordance with the manufacturer's instructions [21]. Primers were designed to amplify targeted genes specifically. 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'. The PCR reaction was performed with iTaq, Universal SYBR green supermix (Bio-Rad, USA), which contains SYBR green dye and all the PCR components [22]. Stratagene's MX3000p PCR machine was used to conduct real-time PCR. The results were analyzed using the comparative CT method and $2^{-\Delta\Delta C_T}$ and the Schmittgen and Livak 2CT method was utilized to calculate the fold change.

Molecular docking study

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 format 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) was used to create grid boxes and pdbqt files for the generation of proteins and ligands. ADT was used to modify the native GSK3β structure by adding polar hydrogen's, unified atom Kollman charges, solvation parameters, and fragmental volumes. Auto Grid was used to construct the grid maps that represented the proteins throughout the actual docking process. The x, y, and z axes' dimensions were set to 100, 100, and 100 respectively, while the grid spacing was set to 0.403. The 100 docking conformers were carried out 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 looked for both particular and non-specific interaction residues throughout 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 were analyzed by one-way ANOVA followed by Student's-t-test using SPSS version 20, represented as mean ± SD for triplicates. Statistical significance was determined at a level of $p<0.05$.

RESULTS AND DISCUSSION

Hinokitiol significantly Reduces MG-63 Cell Viability

Cellular survival following exposure to hinokitol at various doses (5, 10, 20, 40, 60, and 80µ g/ml) was determined based on absorbance readings obtained from the MTT assay. Results were compared to corresponding negative controls (untreated cells) after 24 hours of incubation and expressed as percentage viabilities. Hinokitiol dose-dependently decreased the viability of MG-63 cells with an IC_{50} of 40 µg/ml. (Figure 2)

Morphological study of Hinokitiol on osteosarcoma (MG63) cell line

The decrease in the cell population was seen with the hinokitiol incubation. As can be seen in (Figure 3), the cells with IC_{50} dose of 40 µg/mL of hinokitiol revealed cellular shrinkage and reduced 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 (×10) using an inverted phase contrast microscope

Hinokitiol decreases wound closure in MG-63 cells

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 totally prevented MG63 cell migration after 24 hours of incubation. (Figure 4)

Gene expression profiles induced by hinokitiol

Hinokitiol treatments modulated the apoptosis marker genes in MG-63 cells. In order to know the apoptosis mechanism caused by the treatment of hinokitiol on osteosarcoma cell line (MG-63) an mRNA expression study of three genes mainly considered for its involvement in apoptotic pathways regulation such as BAX, BCL-2, and GSK3β were studied. Hinokitiol treatment decreased the expression of BCL-2, an apoptosis inhibitor in cells and GSK3β, a kinase when compared to the untreated cells. An up regulation of BAX gene expressions

and down regulation of BCL-2 and GSK3β was significantly observed with hinokitiol treated group as compared with control group (Figure 5 & 6).

Docking Study

Molecular modelling is an application wherein 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 the binding energy in order to elucidate the probable mechanism of 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 out of 100 conformers.

The amino acids of isoleucine (ILE) 62, alanine (ALA) 83, aspartic acid (ASP) 133, tyrosine (TYR) 134, valine (VAL) 135, proline (PRO) 136, TYR 140, arginine (ARG) 141, Glycine (GLN) 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 investigation demonstrated that hinokitiol interacts with the ARG 113, TYR 134, LEU 81, VAL 135, and LYS 197 amino acids via hydrogen bonds and hydrophobic interactions within the binding cavity. Hinokitiol formed three strong hydrogen bonds between hydroxyl and carbonyl groups of the ligand with the side chains of ARG 113, TYR 134, and LEU 81. The bond distances of 2.9 Å, 2.1 Å, and 1.7 Å were noted respectively (figure 1a). 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 are engaged in the Van der Waals interactions: PRO 136, ASP 190, VAL 82 and GLU 80 (Figure 7).

Worldwide, osteosarcoma is a relatively prevalent malignancy that frequently affects children and teenagers. The osteosarcoma long-term survival rate has plateaued, although the prognosis for the disease has improved as a result of the introduction of novel therapeutic approaches. To enhance the long-term prognosis for patients with osteosarcoma, novel, inventive treatments must be developed. Finding effective anti-carcinogenic medicines for human osteosarcoma was the goal of the current investigation. Hinokitiol is a tropolone derivative compound occurring naturally in cupressaceous plants Hinokitiol demonstrated a wide range of medicinal activities, including neuroprotective [12], anti-enzymatic[13], and anti-inflammatory [14], anti-cancer [15] actions. Hinokitiol exhibited an anti-carcinogenic effect against several different cell lines. In lung adenocarcinoma cancer cells, it has been demonstrated to cause DNA damage[16]. In melanoma cell line(B16-F10) it have increased the process of apoptosis by interfering extracellular signal-regulated kinase and mitogen-activated protein kinase phosphatase[17] and in breast cancer cell line hinokitiol prevented the expression of heparanase, which in turn suppressed the proliferation of breast (4T1) cancer cells[18]. In this investigation, we looked at hinokitiol anti-carcinogenic effects 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 an excellent model cell line for the creation of innovative therapeutic therapies for osteosarcoma patients [23].

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay relies on living cells converting MTT into formazan crystals to detect mitochondrial activity, a typical method for assessing the metabolic activity of viable cell [19]. This method is universally used to assess the in vitro cytotoxic nature of drugs/ chemicals on cell lines as the overall mitochondrial activity of the majority of cell populations is correlated with the ratio of viable cells [19]. In the study we have found a dose-dependent decrement in the percentage of viable osteosarcoma (MG-63) cells as we increased the concentration of hinokitiol however we have selected hinokitiol (IC_{50}) dose of 40 µM for further research, The morphological analysis of the MG-63 cells was done following the exposure with the hinokitiol on osteosarcoma cell line by an inverted phase contrast microscope. The findings demonstrated that when compared to untreated cells, hinokitiol-treated cells underwent considerable morphological alterations characterized by decreased cell density, and cell shrinkage common feature seen for 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 with a scratch test as 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 (ECM) enter into the circulation attach to an unrelated location then extravasate to produce far-off foci [24]. Hinokitiol 40µM 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 studies, there are two primary apoptotic pathways: the intrinsic or mitochondrial pathway and the extrinsic pathway [25]. Most cancer drugs do follow the mitochondrial pathway and in the current study of cell viability, we have observed that hinokitiol has reduced the 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 the pro-apoptotic proteins into the cytoplasm [25]. The changes in mitochondria are controlled by proteins namely B-cell lymphoma protein 2 (BCL-2) and (BAX) proteins. BAX

protein promotes apoptosis by releasing cytochrome c from the mitochondria. This aids in the 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 will 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 apoptosis-related genes BAX and BCL-2 was evaluated. The BAX genes are crucial for controlling apoptosis. In the current study hinokitiol treatment reduced the expression of BCL-2, an apoptosis inhibitor in the cells. Significantly higher BAX gene (proapoptotic) expressions were seen in the hinokitiol-treated group.

Glycogen synthesis kinase 3 β (GSK3β) is a protein that is highly relevant in different cancers because it plays a significant part in cell growth, proliferation, and migration [5,6,7] Serine/threonine protein kinase GSK3β has emerged as a crucial enzyme in controlling a number of crucial cellular signalling pathways by phosphorylating its substrates. By phosphorylating many oncogene proteins, such as β-catenin, carcinogenic transcription factors, and c-Myc, which causes their ubiquitin degradation and inactivation [27.] GSK3β adversely affects cell survival and proliferation under normal physiological settings. GSK3β is therefore commonly thought of as a "tumor-suppressor gene." According to Tang et al [11], osteosarcoma cells that overexpress GSK3β have a considerable positive impact on colony growth and tumour growth. Importantly, they showed how the osteosarcoma tumour was aided in growing by the aberrant activation of GSK3β. According to Cai et al.'s study, therapy with a GSK3β inhibitor reduces cell survival and proliferation rates, suggesting that GSK3β may be linked to the development of osteosarcoma [28]. In the current study, we have evaluated the role of hinokitiol on GSK3β by *in silico* models and it has revealed significant binding interaction. The GSK3β gene expression investigation also showed hinokitiol's potential to negatively regulate its function, thereby promoting cell death and mitigating cell survival. A downregulated GSK3β in osteosarcoma has reduced tumour cell viability and trigger apoptosis, according to recent research. 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 GSK3β and inhibiting the Wnt/-catenin pathway [29].

CONCLUSION

We used hinokitiol potent bioactive agent previously demonstrated anti-proliferative activity in various cell lines. In the current study we have evaluated the anti-proliferative activity of hinokitiol against the osteosarcoma cell line (MG-63) and showed significant cytotoxic action. A deep examination of the cell line after treatment with hinokitiol showed morphological changes such as cell shrinkage and reduced cell density. *In vitro* evaluation of wound healing assay showed an anti-migratory effect, a characteristic property of anticancer drugs. The study also analyzed the mRNA gene expression by RT-PCR in the MG-63 cell line, the pro-apoptotic BAX gene was upregulated and the anti-apoptotic gene BCL-2 and GSK3β was downregulated. The study also used *in silico* models to assess the binding energy of biomarker enzyme glycogen synthase kinase-3β regulated on numerous signalling pathways for cancer progression hinokitiol and showed effective interaction at various binding sites of GSK3β. Since the present study has been 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.

REFERENCES

1. Isakoff, M.S.; Bielack, S.S.; Meltzer, P.; Gorlick, R. Osteosarcoma: Current Treatment and a Collaborative Pathway to Success. J. Clin. Oncol. 2015, 33, 3029–3035
2. Kansara, M.; Teng, M.W.; Smyth, M.J.; Thomas, D.M. Translational biology of osteosarcoma. Nat. Rev. Cancer 2014, 14, 722–735.
3. Broder H, Gottlieb RA, Lepor NE. Chemotherapy and cardiotoxicity. Rev Cardiovasc Med. 2008 Spring; 9 (2) :75-83
4. Czekanska EM, Stoddart MJ, Richards RG, Hayes JS In search of an osteoblast cell model for in vitro research. Eur Cells Mater 2012, 24: 1473–2262.
5. Embi N, Rylatt DB, Cohen P. Glycogen synthase kinase-3 from rabbit skeletal muscle. Separation from cyclic-AMP-dependent protein kinase and phosphorylase kinase. FEBS J 2005, 107: 519–527.
6. Tejeda Muñoz N, Robles Flores M. Glycogen synthase kinase 3 in Wnt signaling pathway and cancer. IUBMB Life 2015, 67: 914–922.
7. Holmes T, O'Brien TA, Knight R, Lindeman R, Symonds G, Dolnikov A. The role of glycogen synthase kinase-3beta in normal haematopoiesis, angiogenesis and leukaemia. Curr Med Chem 2008, 15: 1493–1499.
8. Luo J. The role of glycogen synthase kinase 3β (GSK3β) in tumorigenesis and cancer chemotherapy. Cancer Lett 2009, 273: 194–200.
9. Domoto T, Pyko IV, Furuta T, Miyashita K, Uehara M, Shimasaki T, Nakada M, et al. Glycogen synthase kinase 3β is a pivotal mediator of cancer invasion and resistance to therapy. Cancer Sci 2016, 107: 1363–1372.

10. Mai W, Miyashita K, Shakoori A, Zhang B, Yu ZW, Takahashi Y, Motoo Y, Kawakami. K, Minamoto. T, Detection of active fraction of glycogen synthase kinase 3beta in cancer cells by nonradioisotopic in vitro kinase assay. Oncology 2006, 71: 297–305.
11. Tang QL, Xie XB, Wang J, Chen Q, Han AJ, Zou CY, Yin JQ, Liu DW, Liang Y, Zhao ZQ, Yong BC, Zhang RH, Feng QS, Deng WG, Zhu XF, Zhou BP, Zeng YX, Shen JN, Kang T. Glycogen synthase kinase-3β, NF-κB signaling, and tumorigenesis of human osteosarcoma. J Natl Cancer Inst. 2012 ;104 (10):749-63
12. Koufaki, M.; Theodorou, E.; Alexi, X.; Nikoloudaki, F.; Alexis, M.N. Synthesis of Tropolone Derivatives and Evaluation of Their in Vitro Neuroprotective Activity. Eur. J. Med. Chem. 2010, 45, 1107–1112.
13. Yoshimori, A.; Oyama, T.; Takahashi, S.; Abe, H.; Kamiya, T.; Abe, T.; Tanuma, S. Structure–Activity Relationships of the Thujaplicins for Inhibition of Human Tyrosinase. Bioorg. Med. Chem. 2014, 22, 6193–6200.
14. K.C. Chiu, Y.H. Shih, T.H. Wang, W.C. Lan, P.J. Li, H.S. Jhuang, et al. In vitro antimicrobial and antipro-inflammation potential of honokiol and magnolol against oral pathogens and macrophages J Formos Med Assoc, 2021, 120. 827-837
15. Zhang, G.; He, J.; Ye, X.; Zhu, J.; Hu, X.; Shen, M.; Ma, Y.; Mao, Z.; Song, H.; Chen, F. β-Thujaplicin Induces Autophagic Cell Death, Apoptosis, and Cell Cycle Arrest through ROS-Mediated Akt and P38/ERK MAPK Signaling in Human Hepatocellular Carcinoma. Cell Death Dis. 2019, 10, 1–14.
16. Li, L.-H.; Wu, P.; Lee, J.-Y.; Li, P.-R.; Hsieh, W.-Y.; Ho, C.-C.; Ho, C.-L.; Chen, W.-J.; Wang, C.-C.; Yen, M.-Y. Hinokitiol Induces DNA Damage and Autophagy Followed by Cell Cycle Arrest and Senescence in Gefitinib-Resistant Lung Adenocarcinoma Cells. PLoS ONE 2014, 9, e104203
17. Huang, C.-H.; Lu, S.-H.; Chang, C.-C.; Thomas, P.A.; Jayakumar, T.; Sheu, J.-R. Hinokitiol, a Tropolone Derivative, Inhibits Mouse Melanoma (B16-F10) Cell Migration and in Vivo Tumor Formation. Eur. J. Pharmacol. 2015, 746, 148–157.
18. Wang, W.-K.; Lin, S.-T.; Chang, W.-W.; Liu, L.-W.; Li, T.Y.-T.; Kuo, C.-Y.; Hsieh, J.-L.; Lee, C.-H. Hinokitiol Induces Autophagy in Murine Breast and Colorectal Cancer Cells. Environ. Toxicol. 2016, 31, 77–84.
19. Van Meerloo J, Kaspers GJ, Cloos J. Cell sensitivity assays: the MTT assay. Methods Mol Biol. 2011;731:237-245
20. Felice F, Zambito Y, Belardinelli E, Fabiano A, Santoni T, Di Stefano R. Effect of different chitosan derivatives on in vitro scratch wound assay: A comparative study. Int J Biol Macromol. 2015; 76:236–241.
21. K.J. Huang, C.H. Kuo, S.H. Chen, C.Y. Lin, Y.R. Lee Honokiol inhibits in vitro and in vivo growth of oral squamous cell carcinoma through induction of apoptosis, cell cycle arrest and autophagyJ Cell Mol Med, 2018, 22. 1894-1908
22. Morrison TB, Weis JJ, Wittwer CT. Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. Biotechniques.1998;24:954-958.
23. Lee, C.M.; Lee, J.; Nam, M.J.; Choi, Y.S.; Park, S.-H. Tomentosin Displays Anti-Carcinogenic Effect in Human Osteosarcoma MG-63 Cells via the Induction of Intracellular Reactive Oxygen Species. Int. J. Mol. Sci. 2019, 20, 1508.
24. Sundaram, G.M., Quah, S. and Sampath, P. (2018), Cancer: the dark side of wound healing. FEBS J, 285: 4516-4534
25. Jürgensmeier JM, Xie Z, Deveraux Q, Ellerby L, Bredesen D, Reed JC. Bax directly induces release of cytochrome c from isolated mitochondria. Proc Natl Acad Sci U S A. 1998 28 ; 95(9):4997-5002
26. Hardwick JM, Soane L. Multiple functions of BCL-2 family proteins. Cold Spring Harb Perspect Biol. 2013 ;5 2 :a008722.
27. Dang CV. MYC on the path to cancer. Cell. 2012;149(1):22-35.
28. Cai X1, Li M, Vrana J, Schaller MD. Glycogen synthase kinase 3-beta and extracellular signal-regulated kinase-dependent phosphorylation of paxillin regulates cytoskeletal rearrangement. Mol Cell Biol. 2006; 26: 2857–2868.
29. Lee IH, Im E, Lee HJ, Sim DY, Lee JH, Jung JH, Park JE, Shim BS, Kim SH. Apoptotic and antihepatofibrotic effect of honokiol via activation of GSK3β and suppression of Wnt/β-catenin pathway in hepatic stellate cells. Phytother Res. 2021 Jan;35(1):452-462.

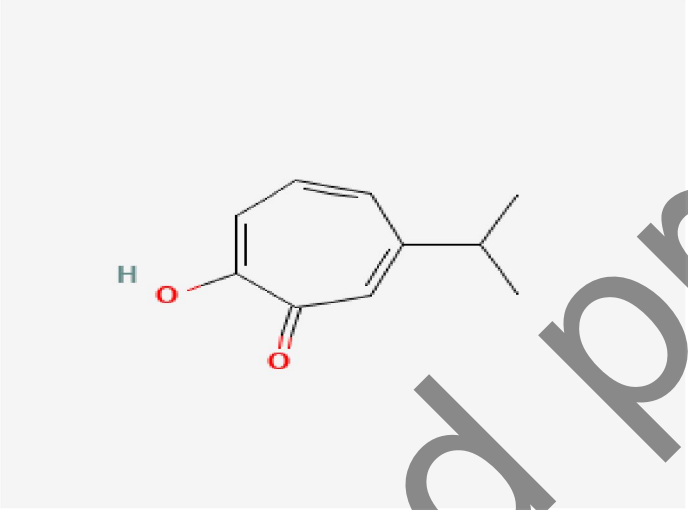

Figure 1 Structure of Hinokitiol

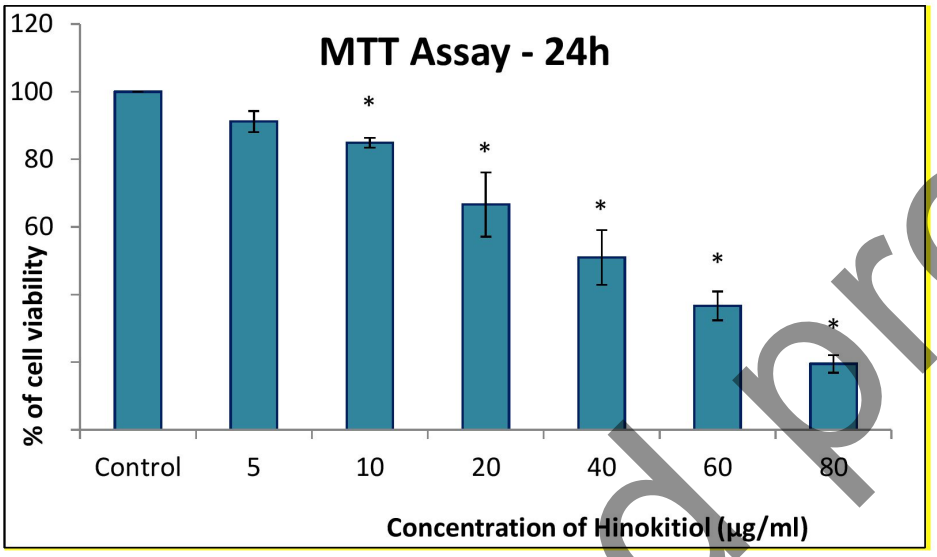

Figure 2 Cytotoxic effect of Hinokitiol on osteosarcoma cancer cells by MTT Assay method. Data are shown as means ± SD (n = 3). * compared with the control blank group, $p < 0.05$.

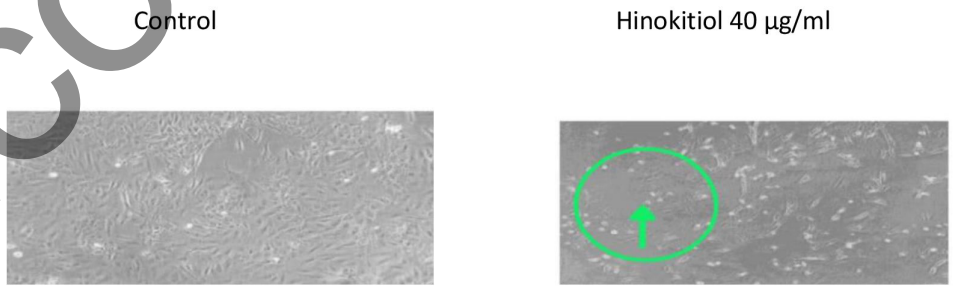

Figure 3 Morphological study of Hinokitiol on osteosarcoma (MG63) cell line

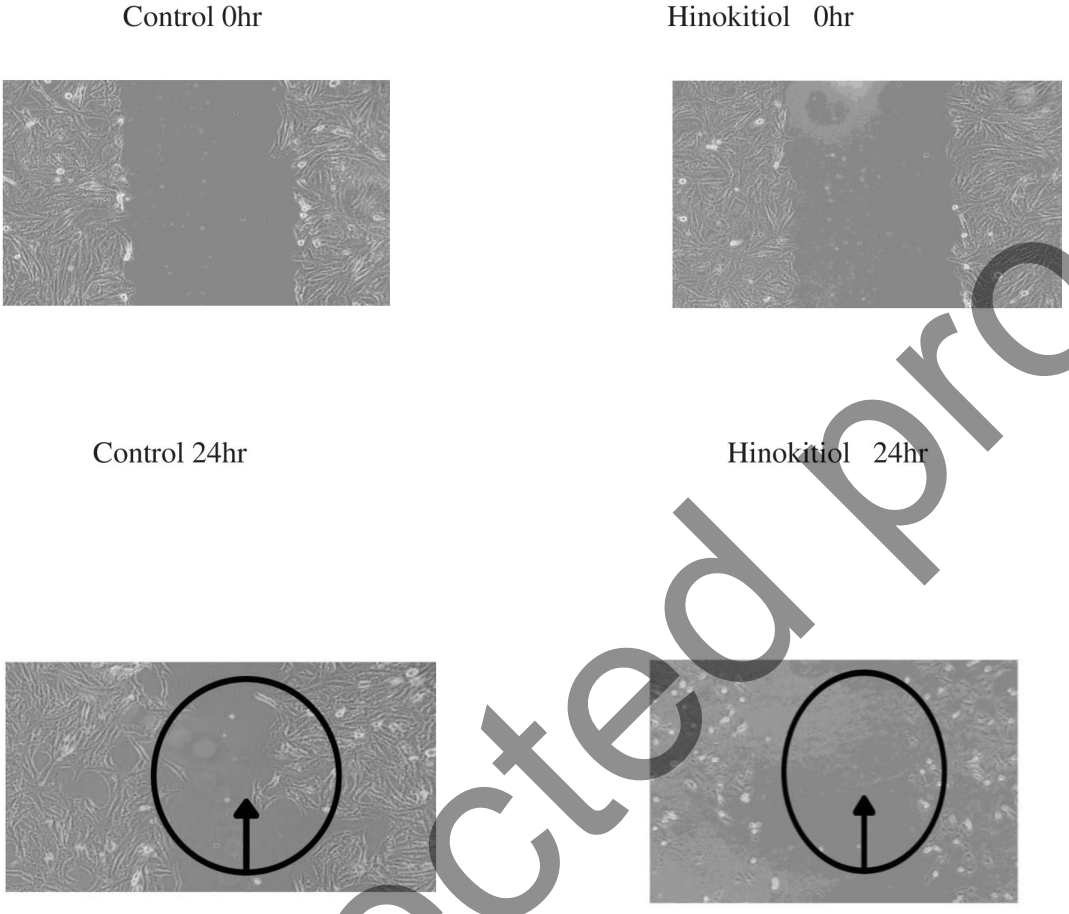

Figure 4 Cell Migration study of Hinokitiol on osteosarcoma (MG63) cell line by scratch assay

Figure 5 The mRNA level of the target genes was determined using total RNA isolation and RT-PCR normalised to GAPDH as reference genes. Each experiment included three replicate reactions. Each bar displays the mean and SEM of three separate tests.

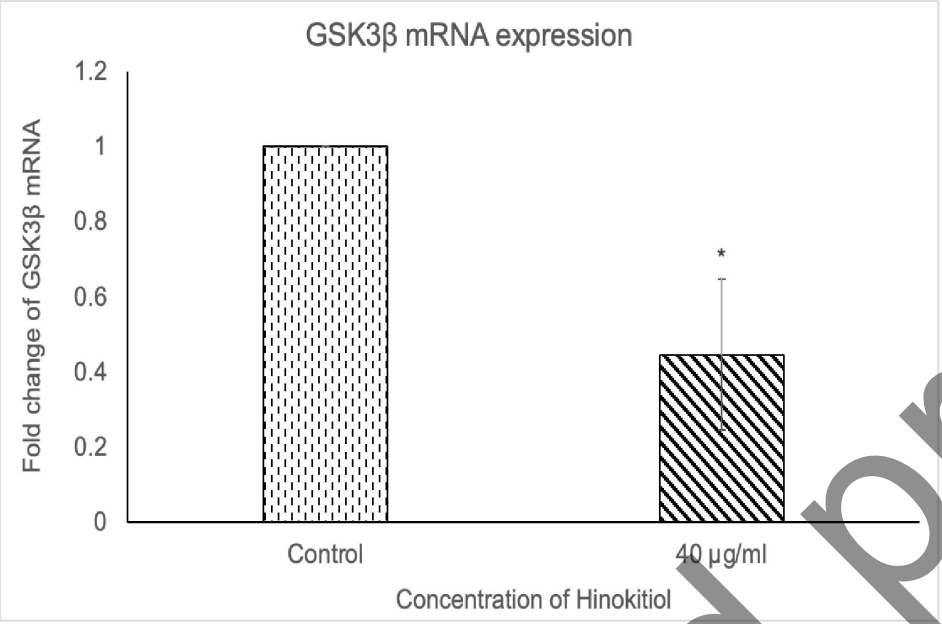

Figure 6 GSK3β gene expression is normalized to GAPDH mRNA expression and the results are expressed as fold change from control. Each bar represents mean ± SEM of three independent observations. '*' represents statistical significance between control versus drug treatment groups at $p<0.05$ level. .

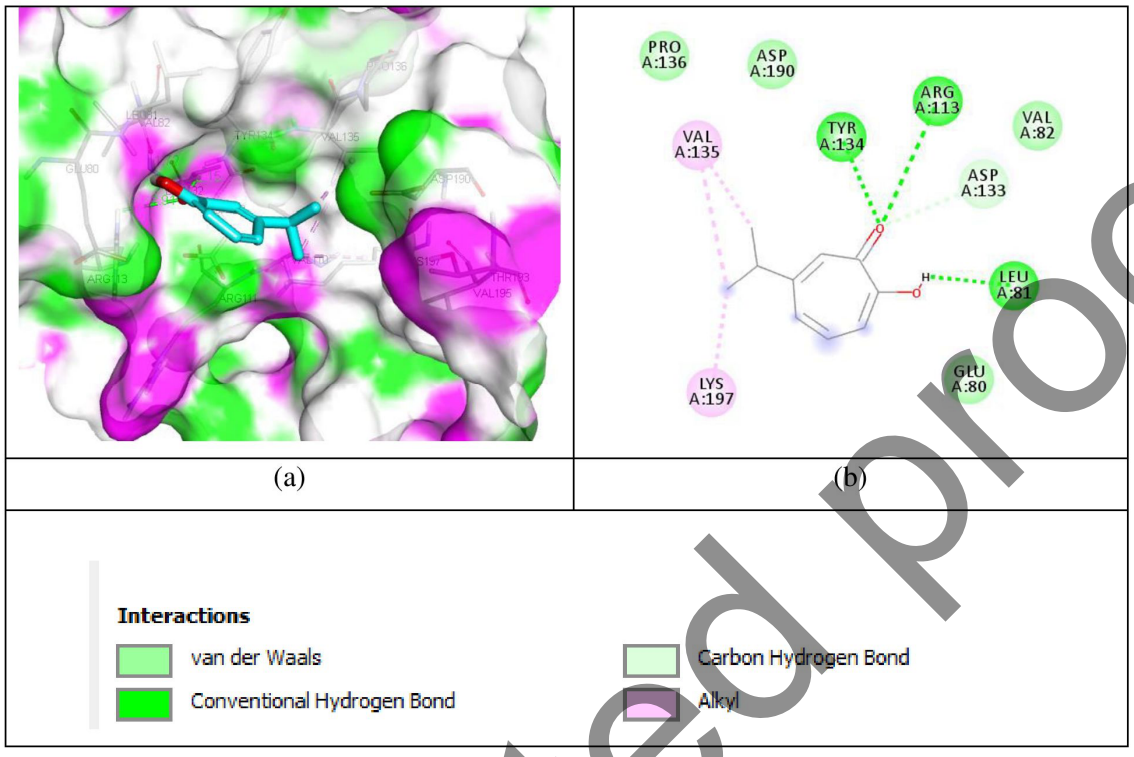

Figure 7 Three-dimensional (3D) and two-dimensional (2D) binding interactions poses of hinokitiol in the binding pocket of GSK3β (PDB CODE: 2O5K). Hydrophobic interactions and hydrogen bond and are indicated by pink and green dashed lines