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Therapeutic Potential of *Hibiscus rosa sinensis* Leaves in Targeting Lung Cancer via Modulation of EGFR Signalling

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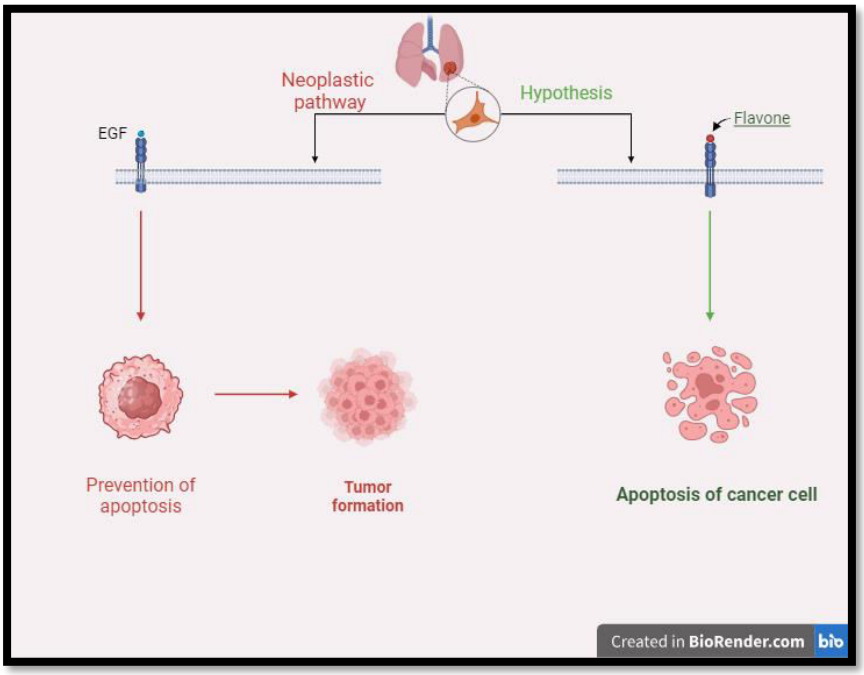
Purpose: To investigate the potential of *Hibiscus rosa sinensis* (HRS) leaves as a therapeutic agent for treating lung cancer, specifically targeting the Epidermal Growth Factor Receptor (EGFR) signalling pathway. The focus was on evaluating the effects of methanol extracts of HRS leaves on A549 lung cancer cells. **Method:** Methanol extracts of HRS leaves were prepared and tested on A549 lung cancer cells. The half-maximal inhibitory concentration (IC50) was determined to assess the extract's potency. Apoptosis was evaluated 24 hours after treatment using AO/EtBr staining. Cell migration assays were conducted to examine the impact of the extract on cell movement. Gene expression analysis was performed to measure the levels of EGFR, BCL2, and BAX. In silico docking studies were carried out to determine the binding affinity of flavone, a key phytochemical in HRS, with EGFR. **Results:** The study found that the methanol extract of HRS leaves significantly inhibited the growth of A549 lung cancer cells, with an IC50 value of 24.68 µg/ml. Apoptosis was confirmed 24 hours post-treatment, as evidenced by AO/EtBr staining. The migration assays demonstrated a reduction in cell movement in treated cells. Gene expression analysis revealed a downregulation of EGFR and the apoptotic marker BAX, while the anti-apoptotic marker BCL2 was upregulated. The in silico docking results showed that flavone had a binding affinity of -6.8 kcal/mol with EGFR.**Conclusion:** The findings suggest that *Hibiscus rosa sinensis* leaves have significant potential as a novel treatment option for lung cancer. The extract effectively inhibits EGFR signalling, reduces cell proliferation, and induces apoptosis in lung cancer cells. These results indicate that HRS leaves could be developed into a therapeutic agent for lung cancer treatment.

Keywords: *Hibiscus rosa sinensis*, Lung cancer, anoikis, Growth factor

Graphical abstract



Introduction

Around the world, cancer is the second most prevalent cause of death. In men, Lung, prostate, colorectal, stomach, and liver cancers are the most common types of cancers, whereas breast, colorectal, lung, cervical, and thyroid cancers are the most common among women (Chiu et al., 2015).The eminent chronic proliferative property of cancer is imparted largely by growth factors. Growth factors maybe synthesized by cancer cells themselves or cancer cells may send signals to stimulate normal cells to secrete growth factor (Shrivastava & Bhadauria, 2016).Malignant transformation is due to the overexpression or hyperactivation of genes that promote cell survival and proliferation (oncogenes) or inactivation of tumor suppressor genes (TSGs) that control cell growth. In addition, overexpression of growth factors and/or their receptors leads to constant activation of downstream signalling pathways, promoting the growth and survival of cancer cells (Bei et al., 2012).Research suggests that growth factors can increase transcription of certain proto-oncogene (myc and fos)(Sotiriou et al., 2003) .The growth factors that are recognized as transforminggrowth factor (TGF), platelet-derivedgrowth factor (PDGF), hepatocytegrowth factor (HGF), fibroblastgrowth factor (FGF), vascular endothelial growth factor (VEGF), and insulin-like growth factor are among them can stimulate cancer cell and stromal cell proliferation, migration, and invasion thereby promoting tumor growth, angiogenesis, and metastasis(Hua et al., 2020)(Bach, 2018).In normal cell physiology, EGFR signalling controls cellular differentiation, proliferation, growth, and survival. During tumorigenesis, mutations in EGFR lead to increased kinase activity supporting survival, uncontrolled proliferation, and migratory functions of cancer cells (Halder et al., 2023).EGFR is

frequently amplified, overexpressed, and mutated in multiple cancers(Khaddour et al., 2021).

In many regions of the world, particularly India, traditional knowledge of using herbs as remedies for human disorders is rapidly vanishing. Even today, tribals and certain local communities in India still practice herbal medicine to cure a variety of diseases and disorders(Mahishi et al., 2005).Globally, plant research has received greater prominence in the last several decades. Plants are rich in several classes of bioactive compounds, being one of the most plentiful sources of new ingredients responsible for treating many diseases(dos Santos Nascimento et al., 2021)(Vasava et al., 2024).Biologically active molecules obtained from plant sources, mostly including secondary metabolites, have been considered to be of immense value with respect to the treatment of various human diseases(Wani et al., 2020)Phytochemicals and derivatives present in plants are promising candidates to improve treatment efficiency in cancer patients and decrease adverse outcomes. A number of these phytochemicals are naturally occurring biologically active compounds with significant antitumor potential.To develop a phytochemical-based anticancer medication that is both side-effect-free and effective, natural extracts from both wet and dry plant material are first tested for possible biological action against cancer. This is followed by by purification of active phytochemicals based on bioassay-guided fractionation and testing for in vitro and in vivo effects(Pratheeshkumar et al., 2015).Research has indicated that the Hibiscus genus of plants may yield physiologically active chemicals with anti-inflammatory and heart-protective properties. Thus, the Hibiscus genus could be an incredible natural resource for the creation of novel medications and could offer developing nations an affordable method of treating diseases like cancer.(El-Elaimy, 2012).

Studies have shown that the plants of the Hibiscus genus have the potential to provide biologically active compounds that act as antioxidants and cardio protective agents. Hence, Hibiscus genus may be a great natural source for the development of new drugs and may provide a cost-effective mean of treatment for cancer and other diseases in the developing world (El-Elaimy, 2012).

Material and Methods

Identification and collection of plant leaves The fresh leaves of HRS were identified and collected from the campus of P.G department of biosciences Sardar Patel University, Gujarat. The methanolic plant extract was prepared by 10 gm of powdered sample in 100 ml of methanol for 12 h and then filtered using Whatman filter paper. Extracts were used for different tests. The methanolic extract of leaves was subjected to phytochemical screening to determine secondary metabolites such as Tannin, Saponin, Flavonoid, Phenolic Acid, Alkaloid etc using standard procedures.

Cell culture

A549 cells were cultured in MEM (HiMedia) containing 10% fetal bovine serum and incubated at 37º C in a humidified atmosphere of 5% CO_2. During the experiments, the cultures were maintained in 1Xantibiotic solution.

Cytotoxicity assay

MTT assay was done to determine the anticancer activity of samples on A549. l50 µL of cell containing medium was added per well in 96-well plates. For the next 24 h, cell plates were incubated at 5 % CO_2 incubator. 100 µl of the samples were added with varied concentrations at 5% CO_2 incubator for 24 h. The sample solution was removed and followed by adding 100 µl/well (5 mg/ml) of MTT in PBS. 150 µl of DMSO was added after 4 h of incubation. The viable cells were obtained and the absorbance was read at 590 nm. The IC50 values of the compound were determined through graph where the increase in concentration reduced the cell viability percentage by 50%.

The percentage (%) of cell viability, which is represented by the given formula: Percentage of cell viability = (A590 of treated cells /A590 of control cells) *100

Scratch Assay: Confluent monolayer of A549 cells was wounded with 100µl pipette tip. Cells were incubated for 24 h with HRS methanolic extract. The migrated distance of cells was measured and three randomly chosen fields were analyzed for each well to observe the anti-migratory and anti-proliferative potency of HRS methanolic extract.

Cell death analysis (Morphological analysis): A549 cells were treated with HRS methanolic extract at 50% confluency for 24 h. Cells were fixed in methanol and stained with Acridine orange (AO)/ Ethidium bromide (EtBr) staining (a mixture of stain containing 100 µg/mL AO and 50 µg/mL EtBr) for 10 minutes at room temperature in dark. Cell morphology was examined under a fluorescent microscope (Fluorescence Inverted Microscope).

Gene expression analysis: Cells were exposed to PPP for 24 h and used for RNA isolation. After assuring the quality and quantity of RNA, it was used for cDNAsynthesis (Bio-Rad iScript cDNA)**.** qPCR was performed with triplicate assays using the SYBR Green mix. GAPDH was used as an internal control. Data analysis was done by CT value and fold change expression was calculated by the $2^{-\Delta\Delta C\ T}$ method. Primers EGFR, BAX, BCL2 and GAPDH were designed using Primer 3.0 software from NCBI.

***in silico* analysis**

Molecular docking of the ligand Flavone with EGRF was conducted using AutoDockTools 1.5.7 and PyMOL 2.5.8 software. First, the target protein structure was downloaded in PDB format from the RCSB Protein Data Bank. The ligand was obtained in SDB format from the PubChem database. PyMOL software was used to

create the binding modes in 'pdbqt' format and record all non-bonded interactions. The grid box dimensions were defined, positioning the ligand binding site in the center of the grid box of the protein, with spatial dimensions specified along the XYZ axis. AutoDock's command line interface was then utilized for docking. A log file was generated, listing the binding modes and their corresponding binding energies.

Statistical analysis: All data are expressed as Mean ± SEM. Data was analyzed on GraphPad prism software using student's t-test. Statistical significance was set at $p< 0.05$. * denotes $p \leq 0.05$, ** denotes $p \leq 0.01$, *** denotes $p \leq 0.001$ and **** denotes $p \leq 0.0001$.

Resultand Discussion

The preliminary phytochemical analysis of the leaves of *HRS* demonstrated the presence of flavonoids, saponins, and tannins, while alkaloids were absent. These findings are detailed in Table 1.

Sr. No.	Phytochemical	Results
1.	Tannin	++
2.	Saponin	+
3.	Flavonoid	++
4.	Phenolic acid	+
5.	Alkaloid test	-

Table:1 Represent the Phytochemical screening of Methanolic extract of HRS leaves.Results shows++ Significantly present, + Trace and - absent

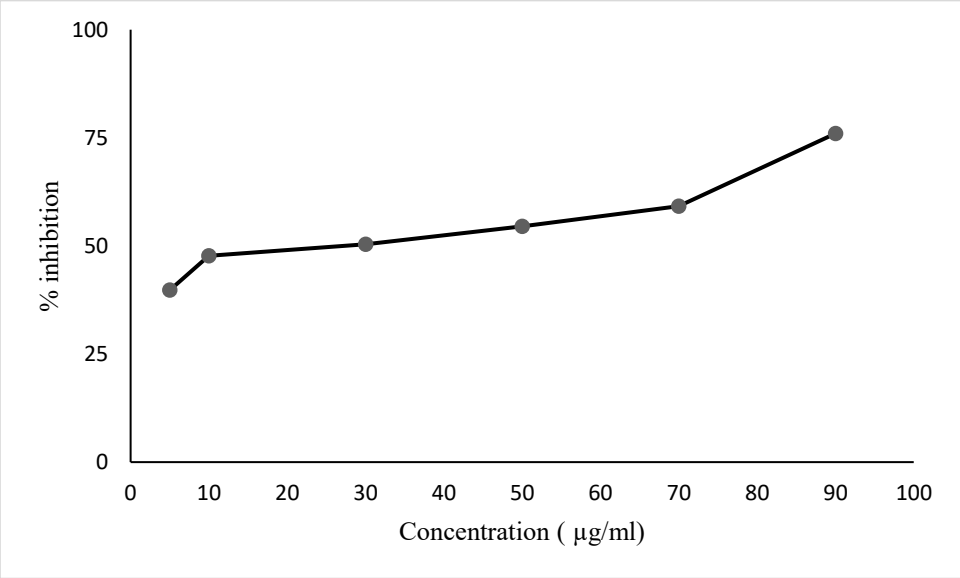
Total phenolic content.

The methanolic extract of HRS showed 119.7 µg/ml of phenolic content at 765 nm of absorbance. Among the four crude extracts, leaf contained the highest (146.64 ± 3.94 mg GAE/g) amount of total phenolic content compounds followed by stem (144.42 ± 16.05), root (89.81 ± 3.00) and then seed kernel (70.34 ± 10.59 mgGAE/g). (Sembiring et al., 2017)

DPPH radical scavenging activity HRS methanolic plant extracts exhibited scavenging activity with an increase in the concentration of the plant extracts. The IC50 values

were calculated to be 367.3 µg/ml. The IC_{50} values for extract, vitamin C, and TBHQ samples were 366.33 ± 10.5, 62.64 ± 3.5.(Ahmad Nejhad et al., 2023)

The MTT assay is performed using the A549 cells using HRS methanolic leaves extracts. The result obtained showed an IC50 value of 22.68 µg/ml as shown in graph:1 The methanolic extract of C.gigantea exhibit cytotoxic effect against A549 and MCF7 cell line.IC-50 value of extract in A549 (27.32 µg/mL) and MCF7 (43.65 µg/mL) cells.(Damodaran et al., 2019)



Graph:1Dose response curves of A549 cells treated with HRS. IC_{50} =24.68 µg/ml. **.** MTT assay was used to determine the IC_{50} of HRS on A549 cells. Cells were exposed to different concentration of HRS(5,10,30,50,70 and 90 µg/ml) for 24 h.

The A549 cell line were treated with 10 mg/ml of HRS methanolic extract and subjected to staining with AO and EtBr to detect apoptotic cells. Both Acridine orange (AO) and Ethidium bromide (EtBr) intercalate with DNA, and stains the cell nuclei. The results in Fig 1 indicated that HRS methanolic extract have effective apoptotic activity on A549 lung cancer cells and the number of apoptotic cells increased progressively after the treatment.After the treatment of HRS cells were in early stage and late apoptotic stage as compared to untreated cells.

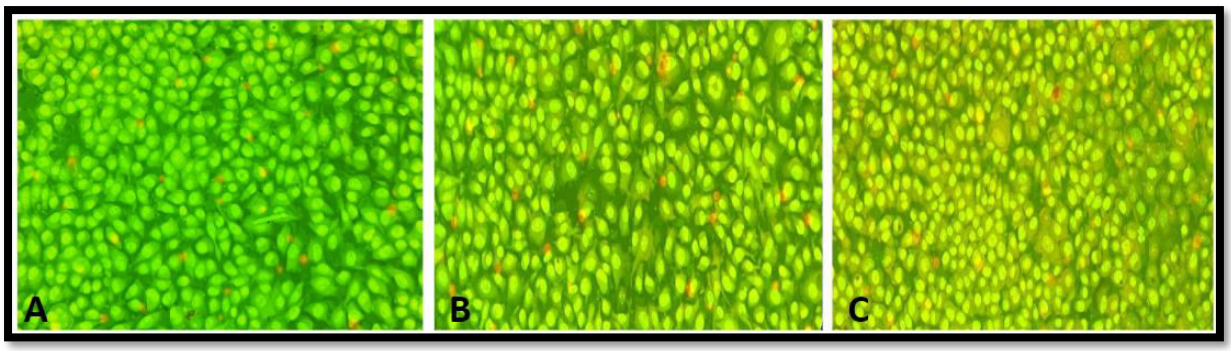


Fig:1Cell death analysis by AO/EtBr staining on A549 cells after the exposure of HRS for 24 h. (A) Control cells in normal condition. (B),(C)HRS treated cells undergoing apoptosis.Cell is in early apoptotic and late apoptotic stage.

The *in vitro* wound healing activity was observed through the migration assay to observe the invasive behaviour of the cells after being treated with dose. Fig 2 shows the wound healing activity of the A549 cells after being treated with the methanolic plants extract after 24 hrs of treatment. When compared with the control well it was clearly observed that the methanolic plant extract was able to arrest the further migration of the A549 cells with a very slow rate of migration potentials.

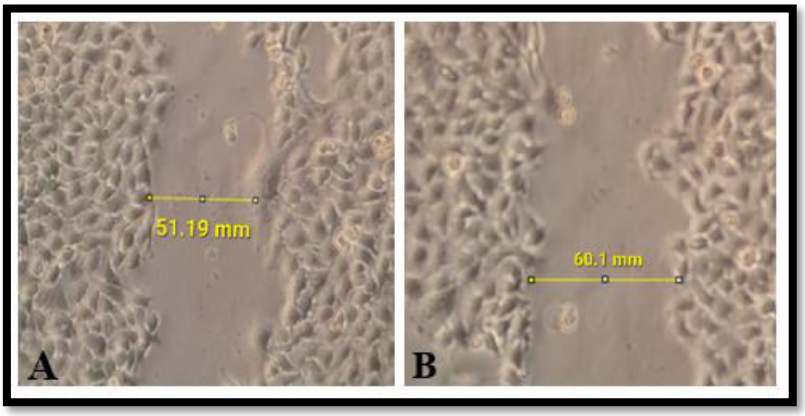
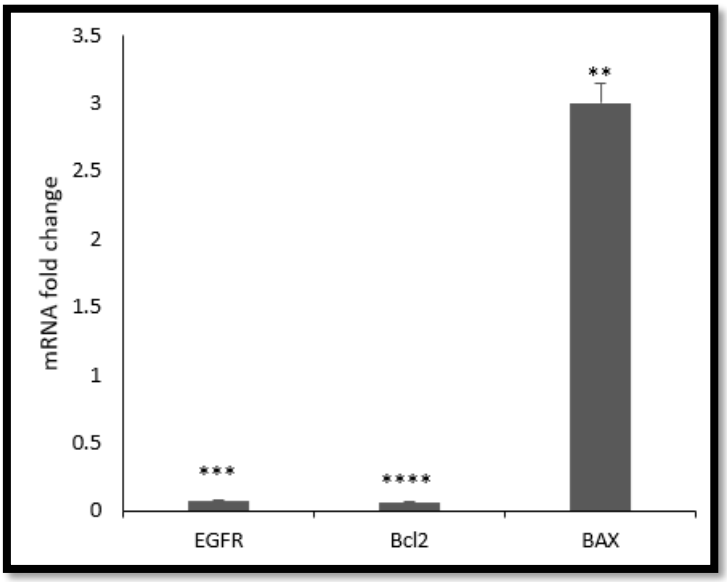


Fig:2Scratch assay of A549 cells treated with HRS. (A) At 24 h post scratching, cells without treatment have started migrating and covering the wound. Width of the remaining scratch is 51.19 mm. (B)HRS treated cells post 24 h of scratching. Width of the remaining scratch is 60.1 mm.

A549 cells were treated with methanolic extract of HRS and RNA was isolated and modulations in the gene expression were observed. Results from real time PCR suggests changes in the gene expression. As shown in fig 3 expression of EGFR was found to be lowered in treated group as compared to untreated group. mRNA fold change of EGFR was 0.07 after the exposure of HRS for 24 h. During tumorigenesis, mutations in EGFR lead to increased kinase activity supporting survival, uncontrolled

proliferation, and migratory functions of cancer cells (Halder et al., 2023).mRNA transcript level of Bcl2 and Bax were analysed.Bcl2 transcript level was 0.058 lowered and Bax was upregulated 3-fold higher due to treatment of methanolic extract that was given to the A549 cells.Bcl2 and Bax are the proteins whichmaintains the mitochondrial membrane permeability.Bcl2 is antiapoptotic protein in cancer; its high expression makes cells resistant against cell death.Many of the chemotherapyformulations work on down regulation of Bcl2 to escort the cells for apoptosis. Treatment ofextracts down regulates the expression of mRNA level of Bcl-2. Fold decreased expressionwas noticed of Bcl 2 gene. The mRNA level of Bax was increased significantly compared tothe control group after treatment of HRS methanolic extract. On the exposure of methanolic extract of HRS to in the cells gene alteration was observed and change in apoptotic marker was observed



Graph:2HRS treatment alters gene expression of EGFR and apoptotic makers in A549 cells. Transcript fold change of *EGFR, BAX and BCL2* upon HRS treatment for 24 h in A549 cells. Expression of transcripts were normalized to *GAPDH*. All values as expressed as Mean ± SEM. ****, $p \leq 0.0001$.

The molecular docking study revealed that flavone binds to the epidermal growth factor receptor (EGFR) with a binding energy of -6.8 kcal/mol shown in figure 3. This negative binding energy indicates a favorable interaction, suggesting that flavone may effectively inhibit EGFR activity. Such interactions are significant for the design of anticancer therapies, as EGFR plays a crucial role in the proliferation and survival of cancer cells.Studies have shown that compounds like sorafenib bind to VEGFR with a binding affinity around -7.4 kcal/mol and sunitinib to PDGFR with approximately -8.5 kcal/mol(Jayaraman, 2021).

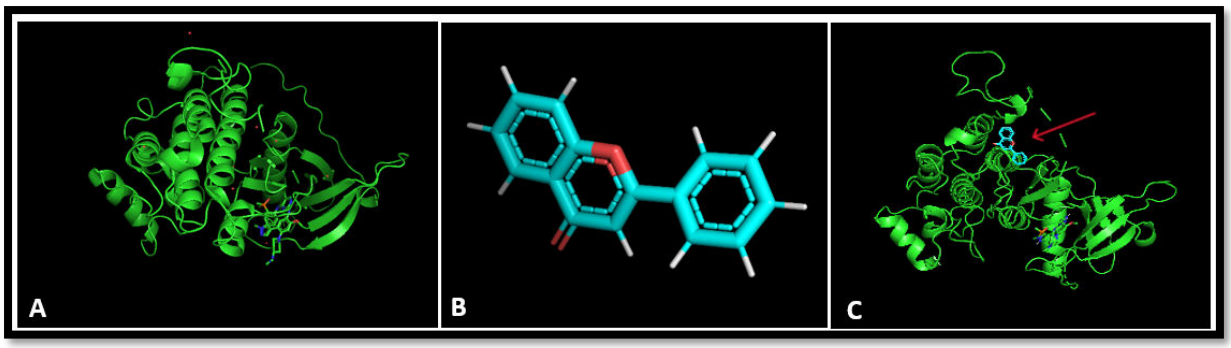


Figure 3: *insilico* analysis a)3D structure of EGFR **b)**3D structure of Flavone **c)**Molecular docking of Flavone and EGFR with binding affinity of -6.8 kcal/mol

Conclusion

The current work aims to provide an in vitro method for understanding how EGFR interacts with flavone. To attempt to confirm our findings, we treated lung cancer cells with extract of HRS leaves for a 24 hrs, and after that, we observed that the IC-50 value was 24.68 µg/ml. An investigation on gene expression revealed that after exposing HRS for 24 h, there was an overexpression of Bax and a downregulation of EGFR and Bcl2. A scratch experiment was used to monitor migration, and the results show that treated cells have covered a smaller surface area and travelled less distance than untreated cells.

Treatment for lung cancer involves the use of highly hazardous medications, as the disease is incredibly diverse. There are other adverse effects associated with chemotherapy and surgery. Flavone has the ability to suppress EGFR expression in lung cancer, as demonstrated by this study. A higher chance of survival and fewer adverse effects are possible when treating lung cancer with phytochemicals. In considering this, targeting EGFR by treating it with HRS may cause tumor cells to undergo apoptosis, which may result in the regression of lung adenocarcinoma and offer a new therapeutic approach for the treatment of NSCLC.

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Notes

The authors declare no competing financial interests.

Data Availability

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request. The raw data from the in vitro assays, including Cytotoxicity assay, migration assay and Gene expression analysis have been stored in storage system.

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