

## Original Research Article

# Wortmannin exhibits anticancer activity in oral cancer cell line by targeting PI3K, AKT and mTOR pathway

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### Abstract

**Objective:** Although the molecular mechanism by which wortmannin exerts its anticancer properties in solid tumors is not fully understood, particularly in the context of oral cancer where research is scarce, this study seeks to explore how wortmannin disrupts the PI3K pathway, consequently affecting the proliferation and apoptosis of human oral cancer cells.

**Materials and Methods:** *In-silico* investigation included drug-likeness predictions, oral cancer and wortmannin targets, Protein-Protein Interactions (PPI), hub gene analysis, the top 10 KEGG pathways, Gene Ontology (GO), and molecular docking tests. *In vitro* experiments examined Viability Assay, apoptosis, cell cycle, Reactive Oxygen Species ROS and MMP levels, and gene expression.

**Results:** Twenty commonly expressed genes affect cell proliferation, apoptosis, the PI3K signaling system, and the cell cycle as a result of *in-silico* analysis. Top 10 genes include *mTOR*, *MAPK1*, *PIK3CA*, *PTGS2*, *MAPK8*, *AR*, *TERT*, *PIK3CB*, *PARP1*, and *PIK3CG*. Wortmannin may treat oral cancer by targeting the PI3K/AKT signaling pathway, which is linked to these genes. *In vitro* tests showed anti-proliferative effects ( $IC_{50} = 3.6 \pm 1 \mu M$  and  $IC_{25} = 1.8 \pm 1 \mu M$ ), late-stage apoptosis, reduced ROS, and MMP changes. Wortmannin downregulated *mTOR*, *PIK3CA*, *ERK*, *PTEN*, *STAT3*, and *AKT*. In addition, *BCL2* and *cMYC* levels decreased and *BAD* and *BAK* expression increased.

**Conclusion:** The *in-silico* strategy used in this study establishes the framework for cancer therapeutic research. This research has revealed wortmannin's ability to treat oral cancer in clinical settings. To validate *in-silico* and *in-vitro* findings, more assays and *in-vivo* research are needed.

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### Introduction

Oral cancer presents a considerable obstacle in the realm of oncology research, primarily owing to the complexities associated with its advanced stage detection and the constraints surrounding available treatment modalities. Oral cavity squamous cell carcinoma (OCSCC) represents the most prevalent histological type among malignant tumors of the oral cavity, accounting for more than 90% of cases. According to GLOBOCAN estimates (Sung et al. 2021), there are roughly 380,000 new cases of OCSCC globally, accompanied by an age-standardized mortality rate of 2.8 per 100,000 for men and 1.0 per 100,000 for women. The Global Cancer Observatory (GCO) (Bray et al. 2024), an interactive web-based platform curated by the International Agency for Research on Cancer (IARC), indicates that across the six continents, Asia exhibits the highest incidence of oral cancer (encompassing lip and tongue), accounting for 65.8% of all cases, with Europe following at 17.3% and North America at 7.3%. The efficacy of existing therapies for oral cancer exhibits variability, as responses differ among individuals. This pertains to the concept of genetic variability (Coletta et al. 2020). The management of oral cancer necessitates a collaborative approach involving a multidisciplinary team that will determine the most suitable treatment modality.

The PI3K/AKT/mTOR and RAF/MEK/ERK pathways are frequently stimulated by mutations and chromosomal translocations in critical targets. The PI3K/AKT/mTOR signaling pathway exhibits dysregulation across nearly all types of neoplasms, characterized by alterations in its components. RAF/MEK/ERK signaling cascades facilitate the transmission of signals from the cell surface to the nucleus, thereby influencing gene expression, regulating cell cycle processes, and orchestrating apoptosis. *RAS*, *B-Raf*, *PI3K*, and *PTEN* frequently serve as alternative upstream

sites. The two pathways engage in a reciprocal interaction that contributes to the process of tumorigenesis. Alterations in *PTEN* lead to a suppression of the RAF/MEK/ERK pathway activity through mechanisms involving *AKT* phosphorylation and the inhibition of *RAS*. The PI3K/Akt pathway plays a pivotal role in the development and progression of breast cancer, and there has been extensive investigation into the efficacy of PI3K inhibitors within this context (Bartholomeusz and Gonzalez-Angulo 2012).

The phosphoinositide 3-kinase (PI3K)/Akt signal transduction pathway plays a pivotal role in cell proliferation and differentiation, often displaying alterations in tumor cells (Xiu-Lan Huang, Guo-Hui Cui 2008, Wu and Huang 2007, Carnero 2010). Indeed, this pathway is crucial in preserving the biological attributes of malignant cells. Moreover, the PI3K/Akt signaling pathway may facilitate tumor cells in evading the detrimental effects induced by anti-cancer therapies (Xiu-Lan Huang, Guo-Hui Cui 2008, Wu and Huang 2007, Carnero 2010). Consequently, extensive research has been conducted on inhibitors of this pathway to assess their capacity to impede tumorigenesis.

Wortmannin serves as an example of a PI3K inhibitor. Wortmannin, a steroid metabolite classified within the viridin family, was isolated from *Penicillium wortmannii* in 1957. This compound selectively obstructs the catalytic function of PI3K via the p110 subunit, thereby impeding the activation of the PI3K/Akt pathway and consequently, inhibiting cellular proliferation (Wipf and Halter 2005). A multitude of studies has indicated that wortmannin exhibits extensive anti-fungal and anti-inflammatory properties (Hazeki 1995), with at least one investigation revealing its potential anti-tumor effects (Isbarn et al. 2008). Recent investigations have utilized wortmannin in the treatment of diverse advanced tumors, such as lung cancer, (Zhang et al. 2010)

and gastric cancer (Yan and Xu 2003, Zhang and Fan 2007), highlighting its promising role in oncological therapy.

Considering the lack of complete understanding of the molecular mechanism underlying wortmannin's anticancer effects in oral cancer, this study employed an innovative strategy integrating network pharmacology with molecular docking in order to identify and confirm the pharmacological mechanisms involved in the treatment of oral cancer with wortmannin. *In-vitro* validation was additionally utilized. The beneficial effects of wortmannin in preventing or treating oral cancers have not been thoroughly investigated however. Therefore, in order to find a potential therapeutic use for this illness, this study was planned to investigate the effects of wortmannin on the proliferation and death of oral cancer cells. In this study, we performed gene expression analysis and identified specific genes linked to the PI3K pathway.

## Materials and Methods

### Prediction of drug-like properties

In order to evaluate the drug-likeness of wortmannin, Lipinski's rule of five (RO5) was utilized as a framework for the screening of oral pharmaceuticals intended for human use. A multitude of parameters was meticulously assessed. The SMILES representation of wortmannin, CC12CCC(C(C1CCC(=C)C2CC=C3C(COC3=O)O)(C)CO), was submitted to the SwissADME server (<http://www.swissadme.ch>), a digital platform designed to compute various parameters such as absorption, distribution, metabolism, excretion (ADME), oral bioavailability (OB), and drug-likeness (DL) (Daina et al. 2017).

### Investigating target genes associated with wortmannin in the context of oral cancer

In order to ascertain the targets of wortmannin, the "Swiss Target Prediction

database

(<http://www.swisstargetprediction.ch/>)"

(Daina et al. 2019) was utilized. The DisGeNet database was employed to extract the genes pertinent to oral cancer, which were subsequently analyzed in relation to the genes linked with wortmannin. The prevalent genes were then discerned and chosen for additional examination.

### Functional gene ontology and KEGG pathway enrichment analysis

In conducting Gene Ontology (GO) and Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway enrichment analysis, we employed two distinct tools: the Database for Annotation, Visualization, and Integrated Discovery (DAVID, <https://david.ncifcrf.gov/>, ver. 6.8) for the GO analysis, and the ShinyGO database (ShinyGO,

<http://bioinformatics.sdstate.edu/>) for the pathway enrichment analysis. DAVID serves as a multifaceted instrument for the annotation and interpretation of gene lists, whereas ShinyGO is dedicated to the analysis of Gene Ontology and pathway enrichment. KEGG serves as an extensive repository of pathways, offering visual depictions of biochemical processes (Ladke et al. 2022, Kanehisa et al. 2021). Gene Ontology serves as an essential resource for functional genomics, providing comprehensive definitions and classifications of gene functions (Ashburner et al. 2000). In order to present and analyze the data, we constructed enrichment charts and histograms utilizing the Bioinformatics cloud platform (<http://www.bioinformatics.com.cn/>), an online resource specifically tailored for data processing and visualization.

### Construction of a network for protein–protein interactions

Protein-protein interactions (PPI) are fundamental to biological processes and are vital for comprehending the intricate systems that operate within living cells (Lin

and Lai 2017). The analysis of the target gene cluster for mapping the PPI network was conducted utilizing the Search Tool for the Retrieval of Interacting Genes (STRING) database (<http://string-db.org/>; version 11.5). The examination centered on "Homo sapiens" as the species, employing a threshold of  $>0.9$  to guarantee the reliability of the information presented. Following this, the PPI network was established utilizing Cytoscape (<https://cytoscape.org/>; version 3.9.1), a prominent bioinformatics tool renowned for its capabilities in data visualization and integration (Shannon et al. 2003). The Cytoscape plugin cytoHubba (<https://apps.cytoscape.org/apps/cytohubba>; version 0.1) was utilized to discern clusters or regions of high interconnectivity within the PPI network.

### Analysis of molecular docking interactions between wortmannin and hub genes

In order to explore the mechanistic relationship between candidate proteins (hub genes) and wortmannin, molecular docking was performed to evaluate the interactions between the hub targets and wortmannin. The molecular docking process was carried out using CB-Dock, a tool that efficiently identifies active sites within a protein, assesses their centres and dimensions, and allows for customization of the grid box size in accordance with the query ligands (Liu et al. 2020). The Protein Data Bank (<http://www.rcsb.org>) served as the source for obtaining crystal structures of the target proteins. The 3D structure of wortmannin was sourced from the PubChem compound database (<https://pubchem.ncbi.nlm.nih.gov/>). The protein and ligand structures served as inputs for CB-Dock, facilitating a docking analysis that examined the binding interactions between the proteins and wortmannin. The Discovery Studio Visualizer software (Accelrys Software Inc.) was utilized for the visualization and analysis of the docking results.

### Expression levels of central hub genes

The study used Gene Expression Profiling Interactive Analysis (GEPIA; <http://gepia2.cancer-pku.cn/>) to confirm that the hub genes were expressed differently in oral cancer tissues compared to normal oral tissues. The Genotype-Tissue Expression (GTEx) and Cancer Genome Atlas (TCGA) databases are utilized by the GEPIA web server which provides interactive and modifiable functionalities. In addition, GEPIA made it easy to analyze these genes according to pathological phases, which shed light on how they are expressed at various points in the cancer progression (Tang et al. 2017).

### Comprehensive survival analysis of central genes

The Kaplan-Meier Plotter (<http://kmplot.com/analysis/index.php?p=service>) (Nagy et al. 2021), a cancer genomics dataset, was employed to examine the influence of hub targets on the overall survival (OS) of patients with oral cancer. This dataset facilitates the evaluation of the prognostic relevance of genes on survival outcomes. Patients with oral cancer were classified into two groups according to the expression levels of the hub genes: low and high expression. A Kaplan-Meier survival plot was created to compare the survival outcomes of the two groups. Furthermore, hazard ratios (HR) together with their respective 95% confidence intervals and log-rank P-values were computed for additional statistical significance evaluation.

### Analysis of wortmannin's anticancer effects

#### Cell culture and maintenance

The KB cell line, associated with oral cancer, was procured from the National Centre for Cell Sciences (NCCS) located in Pune. The cells were cultivated in DMEM (Gibco™) medium at a controlled temperature of 37°C. The culture medium was enriched with 10% Fetal Bovine Serum

(FBS, Gibco™) and 1% antimycotic-antibiotic solution (Gibco™). The cells were sustained in an environment enriched with CO<sub>2</sub> at a concentration of 5% and at 37 degrees Celsius to facilitate their growth and ensure their viability.

### Formulation of stock solutions

To conduct cytotoxicity testing on the KB cell line, wortmannin (Sigma-Aldrich) was formulated as a stock solution at a concentration of 10 mg/ml by dissolving 10 mg of wortmannin in 1 ml of dimethyl sulfoxide (DMSO). The stock solution was subsequently stored at -80°C. A working solution with a concentration of 1 mg/ml was prepared from the stock solution by diluting it in complete medium and subsequently filtering it through a sterile 0.22 µm filter to guarantee sterility. Compounds were diluted in complete culture medium to achieve concentrations of 0.2, 0.4, 0.8, 1.6, 3.2, and 6.4 µM. The final concentration of DMSO utilized in the assays was confirmed to be below 0.01%. Doxorubicin (DOXO), recognized for its proven effectiveness in cancer treatment, was utilized as a reference chemotherapeutic agent.

### Assessment of the cytotoxic effects of wortmannin

The MTT conversion assay was utilized to evaluate cell cytotoxicity in the context of wortmannin. KB cells were placed in a 96-well plate at a concentration of  $5 \times 10^4$  cells/ml. Different concentrations of wortmannin, from 0.2 to 6.4 µM, were administered to the cells. Following the treatment, 20 µl of a 5 mg/ml MTT solution was introduced to each well and incubated for a duration of 4 hr. Following this, 100 µl of DMSO was introduced to each well to facilitate the dissolution of the formazan crystals. The absorbance at 570 nm, which reflects the quantity of purple formazan crystals generated and signifies viable cells, was quantified using the Multi-scanGo Thermofischer Scientific ELISA plate reader. The IC<sub>50</sub> values were determined

through the utilization of a Microsoft Office Excel worksheet (Kumbhar *et al.* 2024).

### Analysis of apoptosis

The apoptosis assay was conducted utilizing the FITC Annexin V/Dead Cell Apoptosis Kit (Invitrogen-Molecular Probes®). The protocol outlined by the manufacturer was adhered to meticulously. Flow cytometry was employed to analyze these stained cells at emission wavelengths of 530 and greater than 575 nm (Wyreogonekbska *et al.* 2013).

### Analysis of the cell cycle

KB cells, irrespective of whether they underwent treatment with wortmannin, were collected 24 hr subsequent to washing with 1X PBS and trypsinization. A mixture comprising 25 µL of RNase A (20 mg/ml Invitrogen), 2 mM MgCl<sub>2</sub> (Sigma), and 5-10 µL of 100 µg/ml propidium iodide (Invitrogen) was introduced to the collected cells. The cells were subsequently incubated at ambient temperature for a duration of 10 to 15 min, followed by analysis utilizing a FACS Caliber instrument from BD Bioscience (Kumbhar *et al.* 2024, Changade *et al.* 2024).

### Assessment of the production of intracellular reactive oxygen species

Flow cytometry was utilized to evaluate the generation of reactive oxygen species, employing DCFHDA as described by Brandt and Keston in 1965 (Brandt and Keston 1965). In this experiment, KB cells were cultivated in 6-well plates for a duration of 24 hr. Upon achieving 70-80% confluence, the cells underwent treatment with wortmannin and DOXO at concentrations reflective of their respective IC<sub>50</sub> and IC<sub>25</sub> values. The fluorescence was measured using flow cytometry with the Beckman Coulter Cytomics FC 500 instrument, utilizing wavelengths of 495 nm and 520 nm (Kumbhar *et al.* 2024, Changade *et al.* 2024).

### Assessment of mitochondrial membrane potential ( $\Delta\Psi_m$ )

To evaluate the mitochondrial membrane potential ( $\Delta\Psi_m$ ) in KB cells, we utilized the MitoProbe™ DiIC1 (5) Assay Kit, following the guidelines provided by the manufacturer. This kit comprises DiIC1(5), a cyanine dye that responds to alterations in membrane potential, alongside CCCP, a disruptor of mitochondrial membrane potential utilized for research purposes. DiIC1(5) possesses the ability to efficiently penetrate the cell cytoplasm and subsequently accumulates within mitochondria exhibiting an active  $\Delta\Psi_m$ , resulting in a pronounced far-red fluorescence. The intensity of DiIC1(5) staining diminishes in correlation with a reduction in mitochondrial membrane potential (Kumbhar et al. 2024, Changade et al. 2024).

### Isolation of RNA and reverse transcription polymerase chain reaction (RT-PCR)

A total of  $5 \times 10^4$  cells were cultivated in 24-well plates for a duration of 24 hr prior to treatment. Subsequently, they were incubated for a period ranging from 4 to 24 hr in a fresh culture medium supplemented with fetal bovine serum and an antimycotic-antibiotic combination. The cultured cells were subjected to the specified concentrations of the drug. Subsequent to the incubation of cells with the drug, the growth medium was removed, and total RNA was extracted from the cells utilizing the TRIzol method (TRIzol™ Reagent, Invitrogen Cat No. 15596018), in accordance with the manufacturer's guidelines. The reverse transcription of RNA was conducted utilizing the High-Capacity cDNA Reverse Transcription Kit, (Applied Biosystems, Cat No. 4368814), in accordance with the manufacturer's guidelines for cDNA synthesis, and subsequently stored at -20 degrees Celsius for future applications. The primers utilized in the current investigation are detailed in Supplementary Table 1.

Table 1. Molecular docking scores of wortmannin and 10 hub target proteins determined by CBDock database are presented with details.

Receptor	PDB ID	Binding energy kcal/Mol
PARP1	1UK1	-10.1
PIK3CB	2Y3A	-9.4
PTGS2	3NTG	-9.2
PIK3CA	2RD0	-9
mTOR	3JBZ	-8.9
PIK3CG	2A4Z	-8.7
MAPK1	1TVO	-8.2
TERT	3DU6	-7.8
MAPK8	3PZE	-7.5
AR	1XQ3	-7.2

### Analysis of statistical data

The experiments were conducted in duplicates and triplicate accordingly, with results expressed as the mean  $\pm$  standard deviation (SD). Data analysis was performed utilizing GraphPad Prism 8. "Two-way ANOVA" was conducted, followed by the appropriate post-hoc tests which was determined and recommended by the software at \*\*\*\*p<0.0001, \*\*\*p<0.001, and \*\*p<0.01 to assess the statistical significance among the groups in comparison to the control.

## Results

### In-silico analysis

#### The molecular characteristics of wortmannin

Our findings indicate that wortmannin complies with Lipinski's Rule of Five (RO5). The molecular properties of wortmannin align with the RO5 criteria, suggesting that it exhibits advantageous characteristics typical of drug-like compounds.

### Analysis and identification of targets

The search term "Lip and oral Cavity carcinoma" yielded 734 targets linked to oral cancer screening. 100 wortmannin targets were found by searching the Swiss Target Prediction database. Twenty genes were found to be shared across the wortmannin targets and the oral cancer-related targets.

### Advancement of the protein-protein interaction network (PPI) and identification of critical hub targets

The prevalent targets of Wortmannin and oral cancer were incorporated into the STRING database for the construction of a protein-protein interaction network (Figure 1). The MCODE plugin was utilized to conduct cluster analysis. Three distinct clusters were delineated: Cluster 1 comprised 9 nodes and 15 edges, featuring the *PI3K* family and *mTOR*; Cluster 2 included 6 nodes and 7 edges, encompassing the *PARP1* and *MAPK* genes; and Cluster 3 consisted of 5 nodes and 4 edges, which included the *PTGS2* and *MMP13* genes. Utilizing three distinct algorithms—Degree, MCC, and MNC—within the CytoHubba software, this study successfully identified the ten most significant hub genes: *mTOR*, *MAPK1*, *PIK3CA*, *PTGS2*, *MAPK8*, *AR*, *TERT*, *PIK3CB*, *PARP1*, and *PIK3CG*. Proteins exhibiting the highest degree level rankings were recognized as central targets within the network. Among these hub genes, *mTOR* stood out as the most significantly active gene (Figure 2).

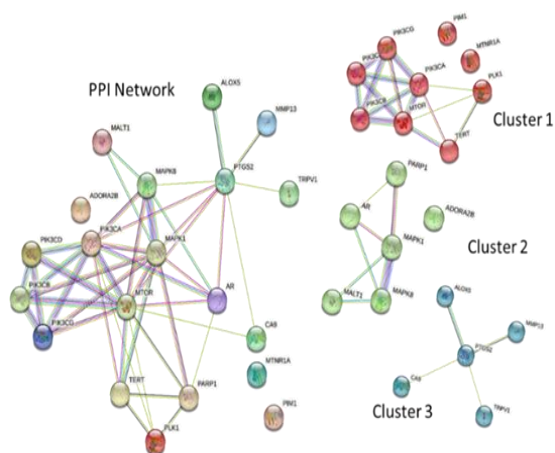


Figure 1. Protein-Protein Interaction network of 20 genes: Cluster 1: 9 proteins [nodes] and their association with each other [15 edges], (PPI enrichment p-value:  $1.86e-07$ ). Cluster 2: 6 proteins [nodes] and their association with each other [7 edges], (PPI enrichment p-value: 0.000399). Cluster 3: 5 proteins [nodes] and their association with each other [4 edges], (PPI enrichment p-value: 0.000106)

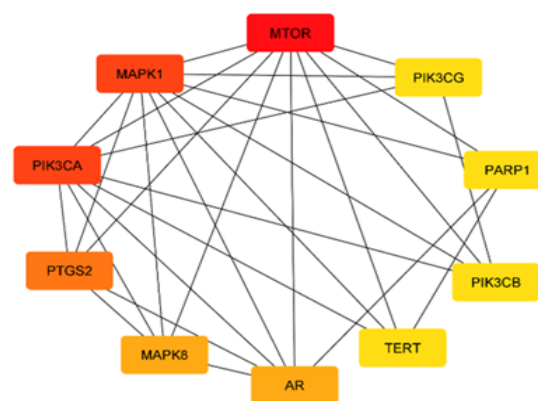


Figure 2. The illustration presents the leading 10 hub genes derived from a set of 20 common genes, as determined by the CytoHubba plugin within the Cytoscape framework. The hue of the colour signifies the significance of the Gene, arranged in a descending hierarchy.

### Gene ontology functional enrichment analysis

The GO enrichment analysis conducted on the 20 targets revealed a total of 95 GO terms identified. The findings indicate that these targets influence cell migration, angiogenesis, the PI3K-AKT1 pathway, and protein kinase B signaling. The results for the Cellular Component (CC) encompassed the cytoplasm, nucleoplasm, nucleus, and plasma membrane. The molecular function (MF) analysis indicates that the targets are primarily engaged in protein binding, ATP binding, and kinase activity (Figure 3).

### Analysis of KEGG enrichment pathways

The analysis of enriched genes indicates that the 26 significantly enriched pathways are likely to be the primary pathways involved in the treatment of oral cancer. The identified genes were primarily associated with cancer-related pathways, including those involved in microRNA regulation, apoptosis, insulin signaling, TNF signaling, PI3K-AKT signaling, and Erb signaling. This indicates that Wortmannin could be significant in the treatment of oral cancer via the pathways discussed, particularly highlighting the PI3K signaling pathway, which encompasses 20 potential targets, including the majority of the hub genes (Figure 4).

### Anti-cancer effects of Wortmannin in oral cancer

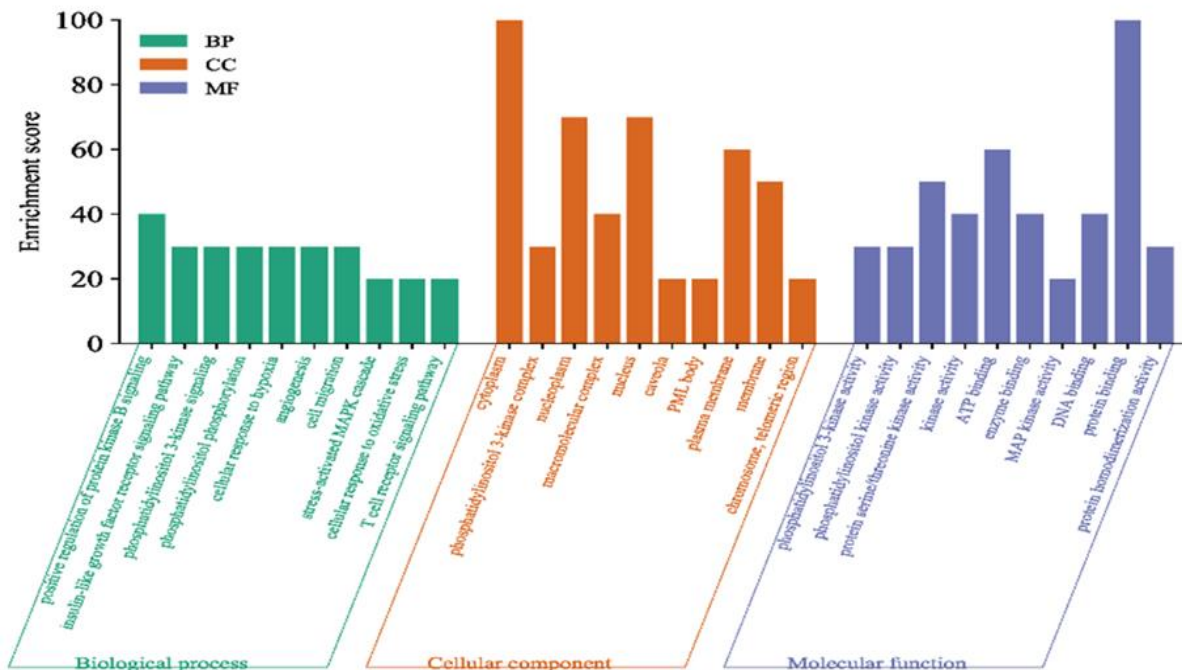


Figure 3. The GO enrichment analysis was conducted by selecting the top 10 biological processes (BP), molecular functions (MF), and cellular compartments (CC) from the DAVID Database concerning the targets associated with Wortmannin in the treatment of oral cancer. The X-axis represents the diverse Biological Process (BP), Cellular Component (CC), and Molecular Function (MF) Gene Ontology (GO) terms, while the Y-axis illustrates the enrichment score associated with each GO term.

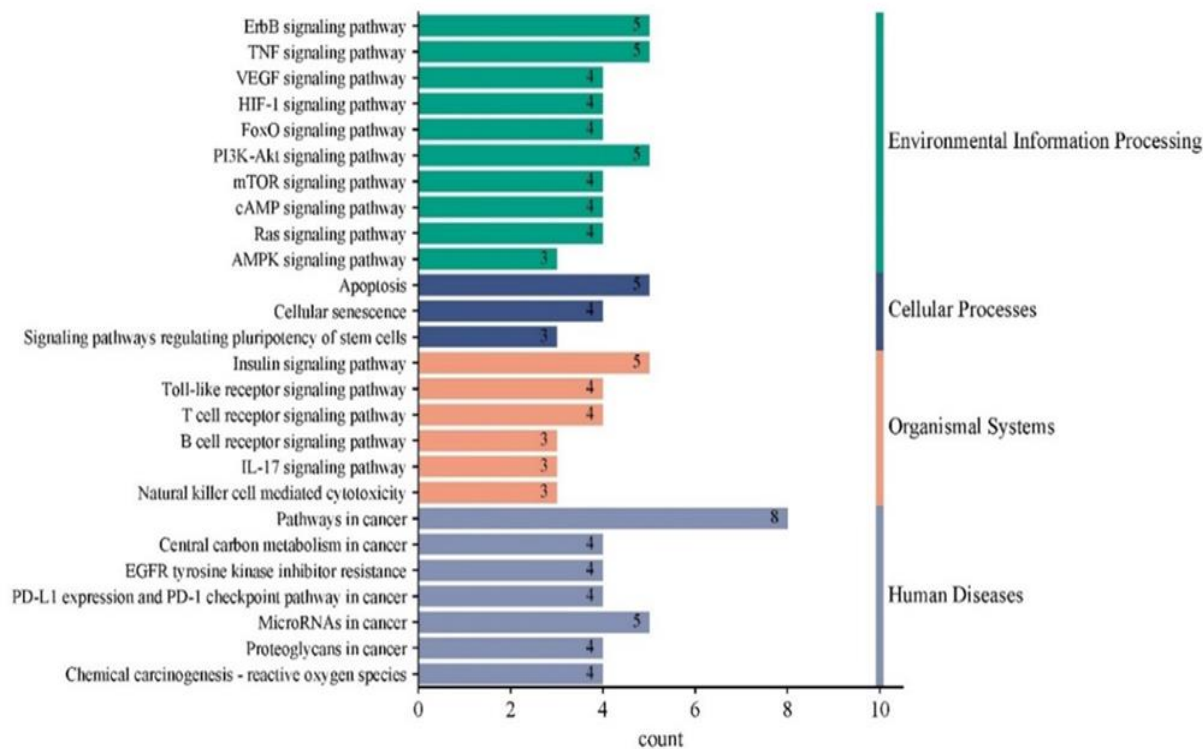


Figure 4. A comprehensive graph illustrating the foremost 26 signaling pathways associated with oral cancer. The X-axis denotes the count of genes, whereas the Y-axis illustrates an array of pathways categorized into specific biological processes. The pathways are classified according to their participation in various processes and systems.

### Validation of hub genes through molecular docking analysis

The ten hub proteins were chosen as targets for molecular docking to assess the reliability of the drug-target interactions. The structure of Wortmannin was submitted to CB-DOCK for the evaluation of its docking potential with *mTOR*, *MAPK1*, *PIK3CA*, *PTGS2*, *MAPK8*, *AR*, *TERT*, *PIK3CB*, *PARP1*, and *PIK3CG*. The prevailing notion is that a lower energy state, characterized by a stable conformation of the ligand binding to the

receptor, correlates with an increased likelihood of action. This study reveals that the binding energies of all core target proteins and *wortmannin* were below  $-7.0$ , suggesting that *wortmannin* exhibits superior binding activity with the core targets (Table 1). Among these, *PARP1* exhibits the highest activity with a binding energy of  $-10.1$  kcal/Mol, followed by *PIK3CB* at  $-9.4$  kcal/Mol and *PTGS2* at  $-9.2$  kcal/Mol. Figure 5 presents all the docking sketch maps of the target protein with wortmannin.

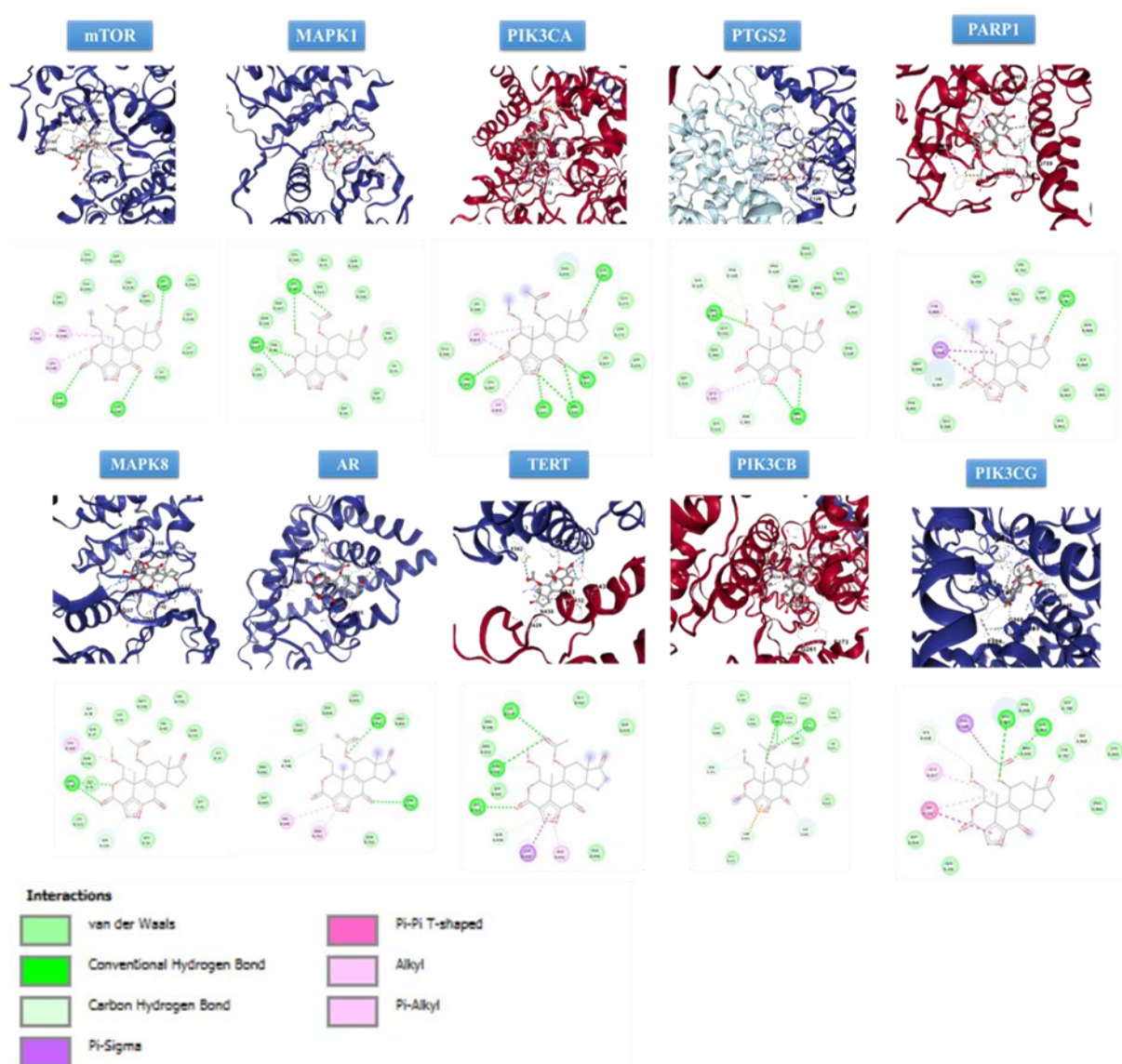


Figure 5. Illustrate and align diagrams depicting the molecular docking of wortmannin with the top ten hub genes [target proteins]. A comprehensive 3D and 2D representation elucidating the interactions between wortmannin and key hub genes, including *mTOR*, *MAPK1*, *PIK3CA*, *PTGS2*, *MAPK8*, *AR*, *TERT*, *PIK3CB*, *PARP1*, and *PIK3CG*.

## Anti-cancer effects of Wortmannin in oral cancer

### Validation of hub genes through external sources

#### Analysis of mRNA expression levels for hub genes

The GEPIA database was utilized to examine the differential expression of hub genes in oral cancer tissues compared to normal tissue. The mRNA levels of *mTOR*, *PIK3CA*, and *PARP1* were significantly elevated in oral cancer specimens compared to normal oral mucosa samples ( $p < 0.01$ ), as illustrated in Figure 6A. Furthermore, we

examined the correlation between the mRNA levels of hub genes and the pathological stages of oral cancer. The findings indicated that the levels of *PIK3CG* and *PTGS2* exhibited notable variations with respect to pathological stage, with a significant increase observed in stage III (refer to Figure 6B for details). The findings indicate a potential correlation between the expression levels of the aforementioned genes and the progression of oral cancer.

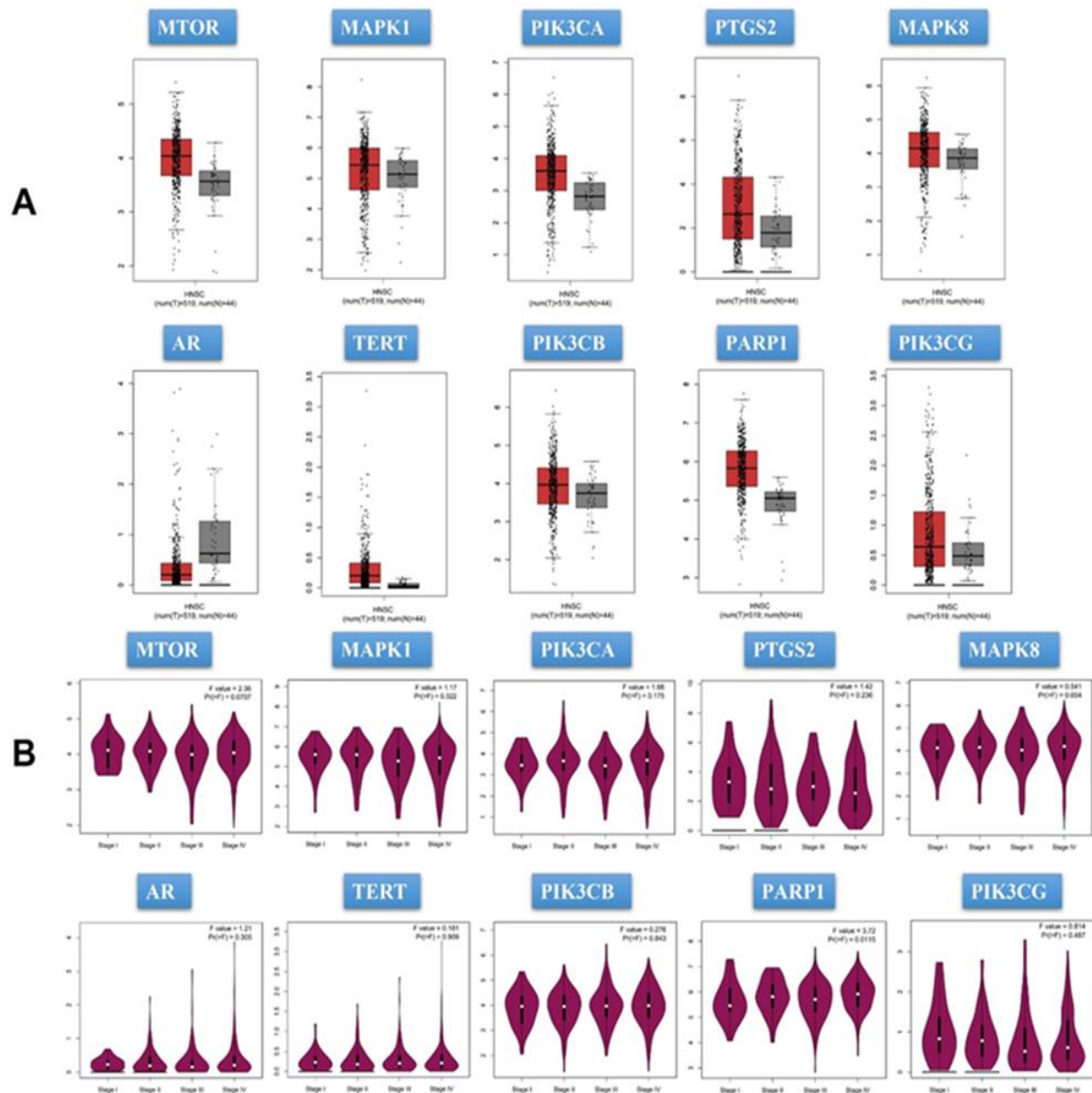


Figure 6. The mRNA expression levels of hub genes in The Cancer Genome Atlas (TCGA) and Genotype–Tissue Expression (GTEx) databases. (A) mRNA expression levels in the GEPIA database [Boxplot of hub genes]. Red depicts Oral cancer tissue, and grey depicts normal oral mucosa. (B) mRNA expression level and pathological stage in the GEPIA database [Stage plot of hub genes].

### Analysis of survival concerning the hub genes

Survival analyses were conducted for ten hub genes: *mTOR*, *MAPK1*, *PIK3CA*, *PTGS2*, *MAPK8*, *AR*, *TERT*, *PIK3CB*, *PARP1*, and *PIK3CG*. The findings indicated that all hub genes were significantly linked to the unfavourable prognosis of 875 oral cancer patients from the TCGA database ( $p < 0.05$ , as illustrated in Figure 7).

### Anticancer activity of wortmannin

#### Results of cytotoxicity

The experiment was conducted utilizing various concentrations of wortmannin (0.2, 0.4, 0.8, 1.6, 3.2, 6.4  $\mu\text{M}$ ), revealing that wortmannin displayed a significant cytotoxic effect on the KB cell line in a dose-dependent manner, evidenced by an IC50 value of  $3.6 \pm 1 \mu\text{M}$  and an IC25 value of  $1.8 \pm 1 \mu\text{M}$ .

### The influence of wortmannin on the cell cycle dynamics

In order to delve deeper into the anticancer properties of wortmannin, KB cells were subjected to treatment with two specific concentrations of wortmannin (IC25 and IC50) for a duration of 24 hours, followed by an analysis of cell cycle

distribution utilizing flow cytometry. The sub-G1 population, representative of apoptotic cells, was evaluated. The findings indicated a notable elevation in the sub-G1 peak when comparing the IC50 and IC25 concentrations of wortmannin to the untreated cells (Figure 8A, 8B).

### The influence of wortmannin on apoptotic processes

Subsequently, our objective was to assess the influence of wortmannin on apoptosis in KB cells, particularly as it manifested in the sub-G1 phase of cell cycle distribution. The proportion of apoptotic cells was quantified following incubation with wortmannin at the concentrations corresponding to IC25 and IC50. The findings indicated that wortmannin at concentrations of IC25 and IC50 led to a notable increase in both early apoptotic cell populations ( $21.60 \pm 3.513\%$  and  $21.200 \pm 2.069\%$ ,  $p < 0.0001$ ) and late apoptotic cell populations ( $32.875 \pm 3.185\%$  and  $54.550 \pm 4.639\%$ ,  $p < 0.0001$ ) when compared to the control (early apoptotic cells -  $6.188 \pm 0.848\%$  and late apoptotic cells -  $3.298 \pm 0.273\%$ ) (Figure 8C). Both concentrations IC25 and IC50 demonstrated a markedly late apoptosis in comparison to early apoptosis (Figure 8D).

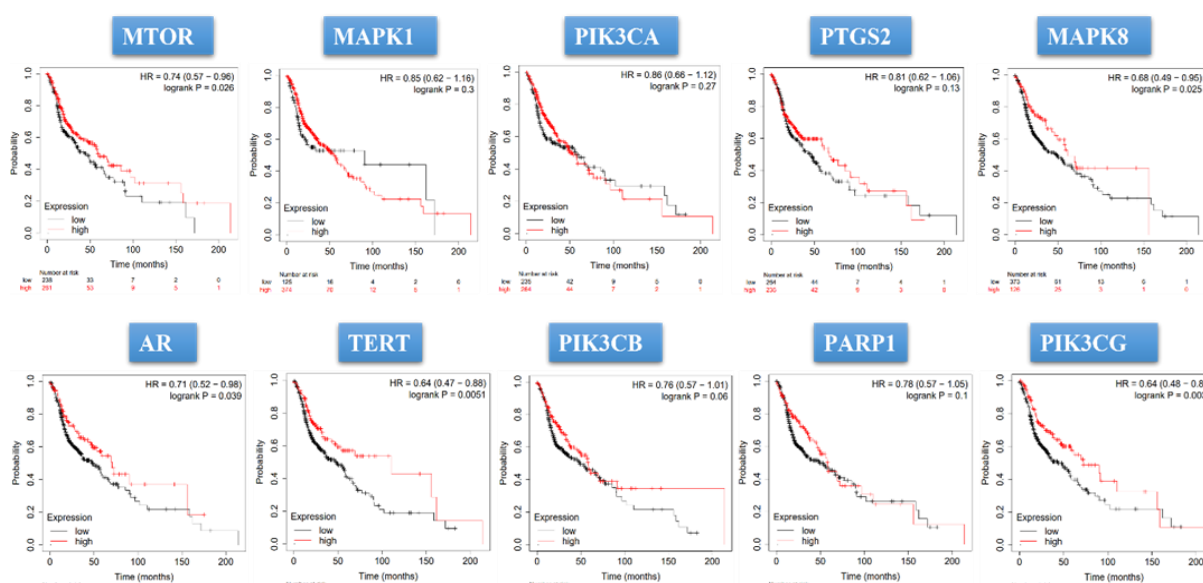


Figure 7. The Kaplan–Meier overall survival analyses of patients diagnosed with oral cancer, evaluated through the expression levels of the ten pivotal genes: *mTOR*, *MAPK1*, *PIK3CA*, *PTGS2*, *MAPK8*, *AR*, *TERT*, *PIK3CB*, *PARP1*, and *PIK3CG*. [HR=hazard ratio] (“[http://kmplot.com/analysis/index.php?p=service&cancer=pancancer\\_rnaseq](http://kmplot.com/analysis/index.php?p=service&cancer=pancancer_rnaseq)”).

## Anti-cancer effects of Wortmannin in oral cancer

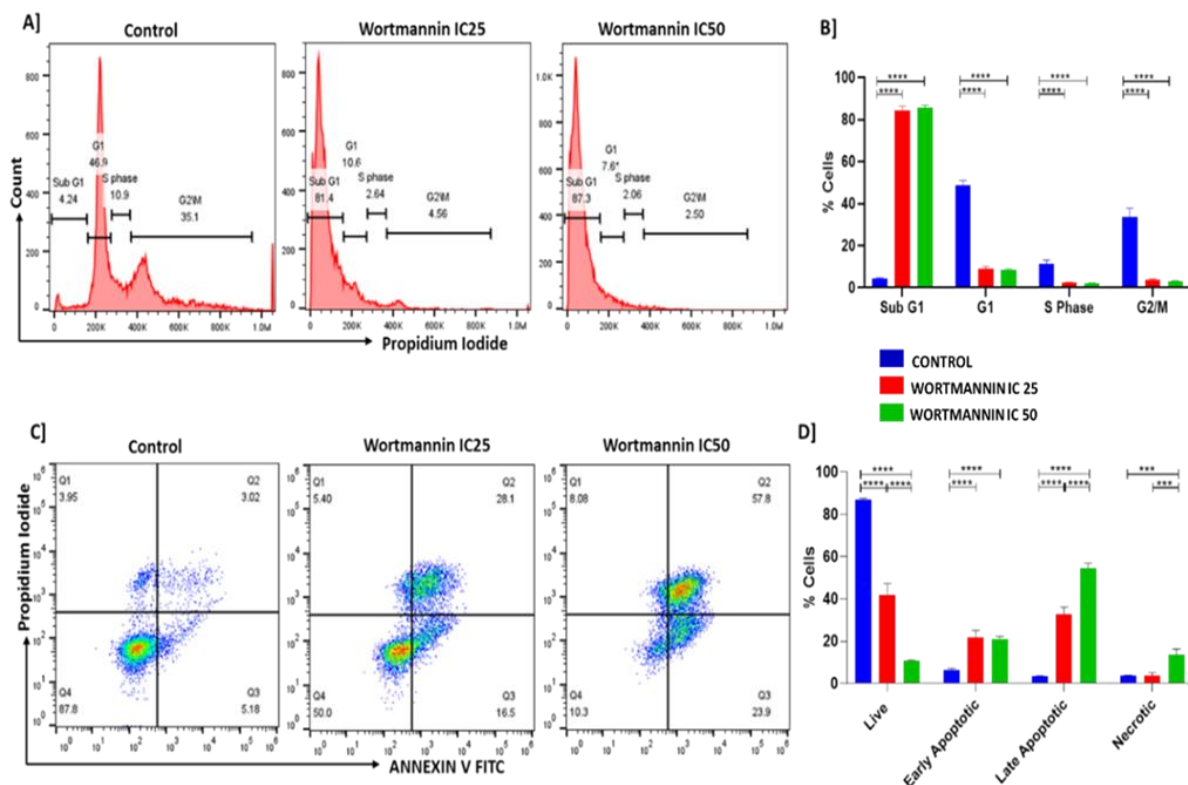


Figure 8. Effect of wortmannin on cell cycle and apoptosis in KB cells A) Flow cytometry histograms represent different stages of the cell cycle in KB cells in control (left) and after treatment of wortmannin with concentrations of IC25 and IC50 (right) for 24 hrs. using propidium iodide. B) The bar graph shows the percentages of cells in sub-G1, G1, S, and G2/M phases in control (blue) and after treatment of wortmannin with concentrations of IC25 (red) and IC50 (green) for 24 hrs. C) Flow cytometry dot plot indicating the percent cell populations in the apoptotic and necrotic quadrant in control (left) and after treatment of wortmannin with concentrations of IC25 and IC50 (right) for 24 hrs. using propidium iodide and Annexin-V FITC. D) The bar graph shows the percentages of live, early apoptotic, late apoptotic, and necrotic cells in control (blue) and after treatment of wortmannin with concentrations of IC25 (red) and IC50 (green) for 24 hours. Error bar shows mean with standard deviation. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ .

### Wortmannin diminishes the production of reactive oxygen species and alters the mitochondrial membrane potential ( $\Delta\Psi_m$ )

In order to deepen our understanding of the impact of wortmannin on reactive oxygen species production, we employed flow cytometry to quantify ROS levels. Figures 9A and 9B illustrate that the application of differing concentrations of wortmannin (IC25:  $3.038 \pm 0.31\%$  and IC50:  $1.3 \pm 0.37\%$ ) on KB cells over a 24-hour period led to a notable reduction in ROS levels when juxtaposed with untreated cells ( $74.23 \pm 1.38\%$ ). Variations in ROS levels effect cancer cells. Increased ROS

levels in cancer cells cause cellular instability and treatment resistance. Thus, reducing ROS may increase cell stability and chemosensitivity. Thus, reducing ROS production leads to cellular apoptosis and making cell chemosensitive. Verifying this requires a detailed investigation. Subsequently, we investigated the influence of wortmannin on the mitochondrial membrane potential within KB cells. The administration of wortmannin at the IC50 concentration resulted in a significant decrease in mitochondrial membrane potential (MFI:  $275.3 \pm 2.08$ ) when compared with the IC25 concentration (MFI:  $541.7 \pm 13.65$ ) and control cells

(MFI:  $545.7 \pm 20.01$ ) (Figure 9C and 9D). Interestingly, the mitochondrial membrane potential in cells subjected to the IC25 concentration of wortmannin exhibited a resemblance to that seen in control cells. During programmed cell death, a reduction in  $\Delta\Psi_m$  is a key event, often associated

with the release of cytochrome c from mitochondria. This indicates Pro-apoptotic property of wortmannin through MMP activity. Alterations in  $\Delta\Psi_m$  can influence ROS production, potentially causing oxidative damage, which subsequently lead to apoptosis.

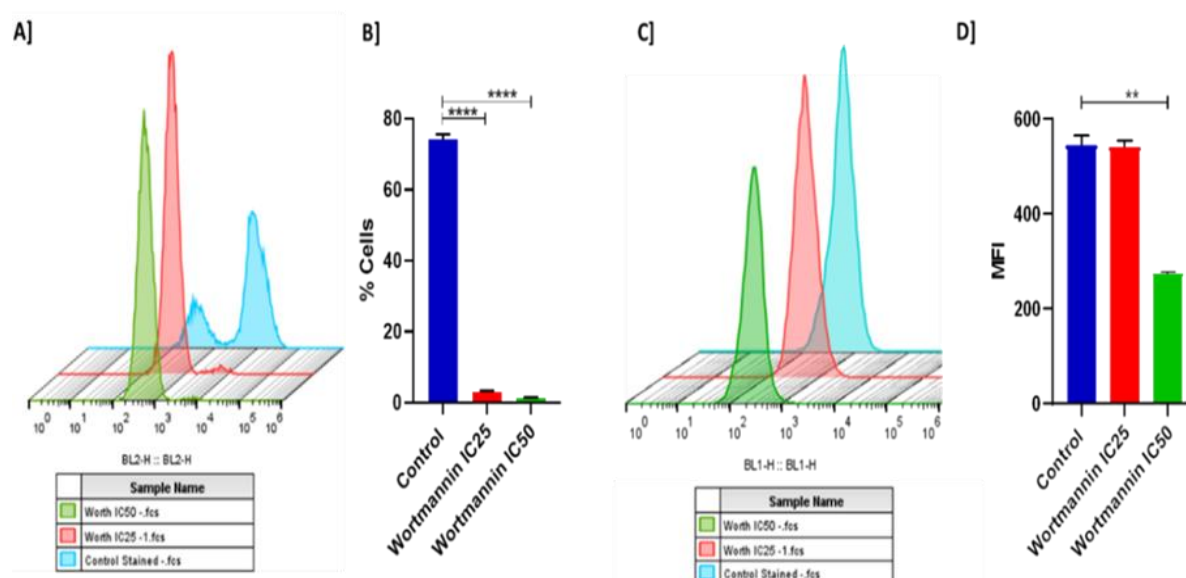


Figure 9. Effect of wortmannin on ROS production and membrane mitochondrial potential in KB cells A) Flow cytometry histogram shows the extent of reactive oxygen species (ROS) production before (blue) and after treatment of wortmannin with concentrations of IC25 (red) and IC50 (green) for 24 hrs. B) The bar graph shows the quantitative analysis of ROS production before (blue) and treatment of wortmannin with concentrations of IC25 (red) and IC50 (green) for 24 hrs. C) Flow cytometry histogram shows the membrane mitochondrial potential ( $\Delta\Psi_m$ ) before (blue) and after treatment of Wortmannin with concentrations of IC25 (red) and IC50 (green) for 24 hrs. D) The bar graph shows the quantitative analysis of membrane mitochondrial potential before (red) and after treatment of wortmannin with concentrations of IC25 (red) and IC50 (green) for 24 hrs. Error bar shows mean with standard deviation. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ .

### Dose-dependent effects of wortmannin on the expression of genes associated with apoptosis and proliferation

In order to delve deeper into the implications of wortmannin treatment on the proliferation and apoptosis of KB cells, we assessed the expression levels of pivotal genes associated with apoptosis, as well as those implicated in cellular growth and proliferation via the PI3K-AKT pathway. *BAD* and *BAK* demonstrate a distinct dose-dependent elevation in expression, exhibiting markedly increased levels at Wortmannin IC50 relative to wortmannin IC25 and the control, thereby highlighting their responsiveness to elevated doses. In comparison, *BAX* and *BCL-2* demonstrate

moderate expression levels when exposed to both wortmannin IC25 and IC50, indicating a constrained sensitivity to variations in dosage. In the interim, *cMYC* exhibits a pronounced sensitivity to both treatments, revealing persistently low expression levels in response to wortmannin IC25 and IC50, which signifies a robust suppression by these interventions (Figure 10A). The IC25 and IC50 concentration in the Apoptosis pathway demonstrated a varied notable significance concerning the expression of pro-apoptotic and anti-apoptotic genes. Furthermore, an experiment was conducted on the genes associated with cellular proliferation. The expression of the *AKT* gene exhibited no

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notable alterations at either the IC 25 or IC 50 concentrations. The treatment with wortmannin IC 50 predominantly resulted in a notable downregulation of genes such as *ERK-1*, *ERK-2*, *mTOR*, and *PTEN*. The *STAT3* gene exhibited a notable downregulation following treatment with wortmannin at an IC 25 concentration in comparison to the IC 50 value. In comparison to IC25, the IC50 treatment demonstrates a marginally elevated expression of both *STAT-3* and *AKT* (Figure

10B). The results suggest that distinct genes exhibit diverse sensitivities to wortmannin, with certain genes demonstrating upregulation at elevated doses, whereas others remain consistently suppressed. The overall IC 25 concentration of wortmannin has demonstrated a notable difference in gene expression in cellular proliferation mechanism when compared to IC 25. Whereas in apoptosis the IC50 and IC25 values showed different activity on Pro-apoptotic and Anti-apoptotic genes.

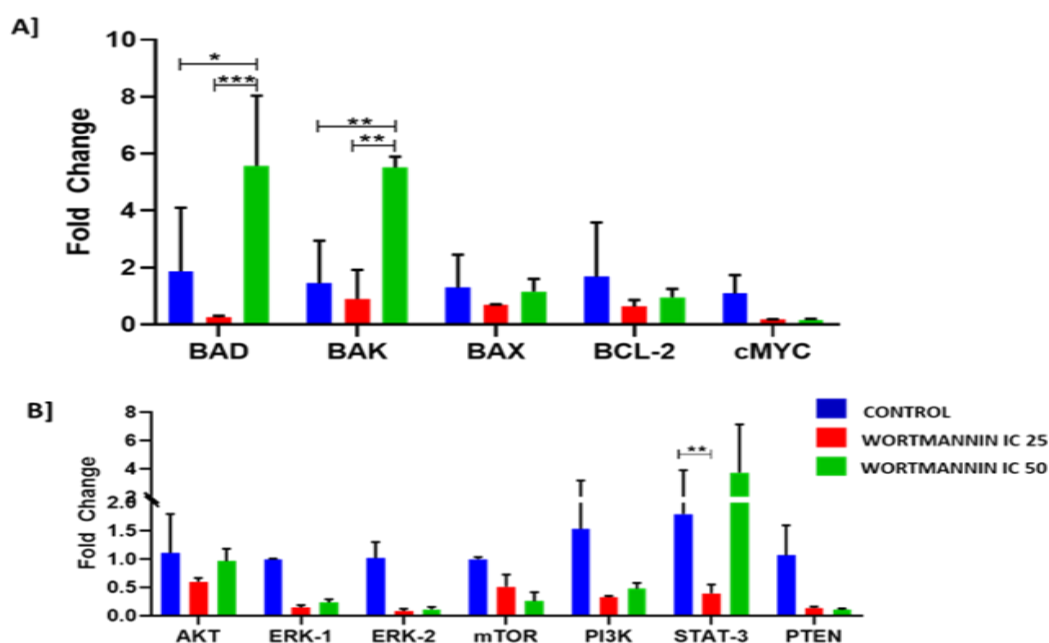


Figure 10. Dose-Dependent Effects of wortmannin on the Expression of Apoptosis and Proliferation-Related Genes in KB Cells-A] The bar graph shows fold change in the gene expression levels of *BAD*, *BAK*, *BAX*, *BCL-2* and *cMYC*, before (Blue) and after treatment of wortmannin with concentrations of IC25 (red) and IC50 (green) for 24 hrs. B] The bar graph shows fold change in the gene expression levels of *AKT*, *ERK-1*, *ERK-2*, *mTOR*, *PI3K*, *STAT-3* and *PTEN* respectively, before (Blue), and after treatment of wortmannin with concentrations of IC25 (red) and IC50 (green) for 24 hrs. Error bar shows mean with standard deviation. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001.

## Discussion

In spite of continuous research efforts and advancements in treatment modalities, the clinical outcomes and overall survival rates for HNSCC have exhibited only marginal improvement in recent decades, with a disheartening 5-year survival rate plummeting to as low as 50% (Argiris et al. 2004, EE et al. 1993). In light of the inadequate outcomes and considerable

toxicity linked to existing treatment modalities for head and neck cancer, contemporary investigations are focused on the pursuit of alternative therapies that promise diminished toxicity. Complementary and alternative medicine has garnered significant interest as a potentially valuable approach for cancer management, resulting in an intensified examination of these options in recent years.

Recently, more than 3000 anti-cancer products originating from plants have been introduced, and it is noteworthy that they generally exhibit considerably fewer side effects in comparison to traditional chemotherapy agents (Koehn and Carter 2005).

Wortmannin (C<sub>23</sub>H<sub>24</sub>O<sub>8</sub>) is a cell-permeable antifungal-antibiotic agent akin to the viridian group. It serves as a potent and irreversible inhibitor of *PI3K*, effectively blocking the PI3K-Akt signaling pathway, which plays a crucial role in cell cycle progression and apoptosis (Priulla *et al.* 2007, Wang *et al.* 2010). Numerous modifications and conjugations involving wortmannin have been documented, confirming its efficacy as an antitumor agent (Zhu *et al.* 2006, Yuan *et al.* 2006).

Our previous investigation revealed Wortmannin as a notable bioactive compound derived from *Glycyrrhiza glabra*, utilizing the principles of network pharmacology (Ladke *et al.* 2022). Consequently, this compound has been employed in the present study for additional analysis and validation. The present investigation employed an in-silico approach to identify anti-cancer genes that are influenced by wortmannin. The in-silico findings are corroborated by a range of assays conducted in an oral cancer cell line. Within the domain of in-silico research, the targets linked to oral cancer and wortmannin were carefully identified and scrutinized, culminating in the discovery of a common set of 20 genes through comparative analysis. Upon the establishment of the PPI networks and their subsequent integration into the cytoHubba software, the foremost 10 hub genes were identified: *mTOR*, *MAPK1*, *PIK3CA*, *PTGS2*, *MAPK8*, *AR*, *TERT*, *PIK3CB*, *PARP1*, and *PIK3CG*. The genes in question serve as the primary focal points for wortmannin within the framework of oral cancer therapy. The examination of gene ontology and pathways uncovered apoptosis, cellular proliferation, and

various signaling pathways, notably highlighting the PI3K-Akt pathway as a crucial mechanism at play. Through a thorough examination of the KEGG pathways, it became evident that the pathways most prominently linked to oral cancer encompassed pathways in cancer, the PI3K-Akt signaling pathway, the TNF signaling pathway, and the ErbB signaling pathway. The PI3K-Akt pathway is acknowledged as an essential element in the treatment strategy for oral cancer. The molecular docking process revealed advantageous interactions between wortmannin and the central hub genes, notably *PRAP* and *PIK3CA*. Wortmannin has been examined in relation to multiple cancer types, with studies suggesting that the compound promotes apoptosis via the PI3K-AKT signaling pathway (Wang *et al.* 2010, Priulla *et al.* 2007, Akter *et al.* 2012). Nevertheless, the body of research pertaining to oral cancer remains relatively sparse. A multitude of researchers have suggested that an aberrant or dysregulated PI3k-Akt signaling pathway plays a crucial role in breast malignancies associated with different types of chemotherapeutic resistance (Knuefermann *et al.* 2003, Liang *et al.* 2003, Zhou *et al.* 2005, Bachman *et al.* 2004). The RAS/RAF/MEK/ERK (MAPK) pathway plays a crucial role in various cellular processes, including the proliferation, survival, and invasion of tumor cells. The alteration of elements like *RAS*, *RAF*, and *MEK* leads to an imbalance in the pathway across different cancer types (Lauth 2011). Reports indicate that the PI3K/Akt and MEK/ERK pathways work in concert to facilitate tumor growth (Niederst and Engelman 2013). Signaling pathways exhibit intricate interactions, whereby the amplification of one pathway may either bolster or suppress another pathway. For example, the impact of PI3K/mTOR inhibitors on tumor cells is obstructed by the inhibition of *MEK* or the knockout of *ERK*, and the simultaneous inhibition of *MEK* and PI3K/Akt/mTOR

signaling consequently hinders tumor cell proliferation (Toulany et al. 2014).

Kapoor et al. (2016), undertook a comprehensive evaluation of the antitumor properties of 6-gingerol on human oral (SCC4, KB) and cervical cancer (HeLa) cell lines, examining the effects both with and without the presence of wortmannin, rapamycin, and cisplatin. The administration of 6-gingerol resulted in G2-phase arrest in KB and HeLa cells, while simultaneously inducing S-phase arrest in SCC4 cells. The administration of 6-Gingerol, wortmannin, and rapamycin led to an almost two-fold enhancement in the expression of caspase 3 in all cell lines studied. The results indicate that 6-gingerol, when given alone or in combination with a PI-3 K inhibitor and cisplatin, may produce improved therapeutic results in the management of oral and cervical carcinoma (Kapoor et al. 2016). In their 2006 study, Gen Kondo and colleagues noted that the PI 3-K inhibitors, wortmannin and LY294002, markedly reduced the phosphorylation of *Akt* while promoting Fas-mediated apoptosis in OSCC cells. Moreover, it has been established that the PI3-K/Akt signaling pathway serves as a significant strategy for tackling cancer cells that demonstrate resistance to conventional treatments, including chemotherapy and ionizing radiation, alongside novel therapeutic approaches via death receptors like Fas and TRAIL (Kondo et al. 2006). In 2007, Masayasu Iwase and his colleagues conducted a study that revealed the significant inhibition of Akt phosphorylation in OSCC cells by the two PI3-K inhibitors, wortmannin and LY294002. The utilization of PI3-K inhibitors in OSCC cells significantly elevated the apoptosis levels triggered by cisplatin, 5-fluorouracil, or docetaxel. The results suggest that the suppression of PI3-K heightens the susceptibility of OSCC cells to apoptosis triggered by anticancer agents, facilitated by the modulation of expression and post-translational

alterations of both pro- and antiapoptotic proteins (Iwase et al. 2007). Poh et al. (Poh and Pervaiz 2005), undertook a study examining the impact of wortmannin on the PI3K/Akt pathway. Their findings indicate that the compound imposes a negative regulatory effect on various elements of the pathway, including *PI3K* and *mTOR*, which results in the dephosphorylation of Akt and a consequent reduction in its activity. Considering that both *PI3K* and *mTOR* are part of the same PIKK superfamily of kinases, which exhibit similar structural isoforms and responses, investigations into mTOR inhibitors have revealed compounds that simultaneously inhibit both *PI3K* and *mTOR* targets (Wu et al. 2022). The RAS/RAF/MEK/ERK (MAPK) pathway is integral to numerous cellular functions, encompassing the proliferation, survival, and invasion of tumor cells. The modification of components such as *RAS*, *RAF*, and *MEK* results in a disruption of the pathway observed in various cancer types (Lauth 2011). Evidence suggests that the PI3K/Akt and MEK/ERK pathways collaborate to promote tumor growth (Niederst and Engelman 2013). Signaling pathways demonstrate complex interactions, in which the enhancement of one pathway can either support or inhibit another pathway. The influence of PI3K/mTOR inhibitors on tumor cells is impeded by the inhibition of *MEK* or the knockout of *ERK*, and the concurrent inhibition of *MEK* alongside PI3K/Akt/mTOR signaling ultimately restricts tumor cell proliferation (Toulany et al. 2014).

Following exposure to IC<sub>50</sub> and IC<sub>25</sub> concentrations of Wortmannin, we noted a marked downregulation of the principal genes involved, thus confirming the significance of the PI3K-Akt pathway. Subsequent analyses of genes including *ERK1*, *ERK2*, *mTOR*, and *STAT3* demonstrated reduced levels in relation to IC<sub>50</sub> and IC<sub>25</sub> values, suggesting a possible involvement of the MAPK and JAK/STAT pathways as well. The results of

this investigation indicate that Wortmannin might influence its effects not solely through the PI3K-Akt pathway, but also possibly through the MAPK and JAK/STAT pathways. It is conceivable that an interaction may occur among these three pathways. The results of this investigation suggest that wortmannin has the potential to induce cellular apoptosis and modify intracellular levels of reactive oxygen species and mitochondrial membrane potential at both IC50 and IC25 concentrations.

The overall activity of the wortmannin on oral cancer cell line has been proposed and presented as seen in Figure 11. The findings indicate that employing IC25 concentration could be more advantageous than subjecting the cells to IC50 values of the drug. The IC25 concentration may be regarded as more efficacious, as it can yield

results comparable to those achieved with IC50 in case of genes associated with cellular proliferation. While this study provides valuable insights, it is important to acknowledge certain limitations that exist. The public databases utilized for the identification of wortmannin and oral cancer-related target genes may exhibit incompleteness, thereby potentially neglecting other target genes relevant to our investigation. In light of the compelling in vitro evidence supporting the chemopreventive and chemotherapeutic attributes of this medication, it is evident that further in vivo investigations employing a variety of tumor models are essential moving forward. It is imperative that the safety concerns related to wortmannin be addressed before proceeding with the clinical trials.

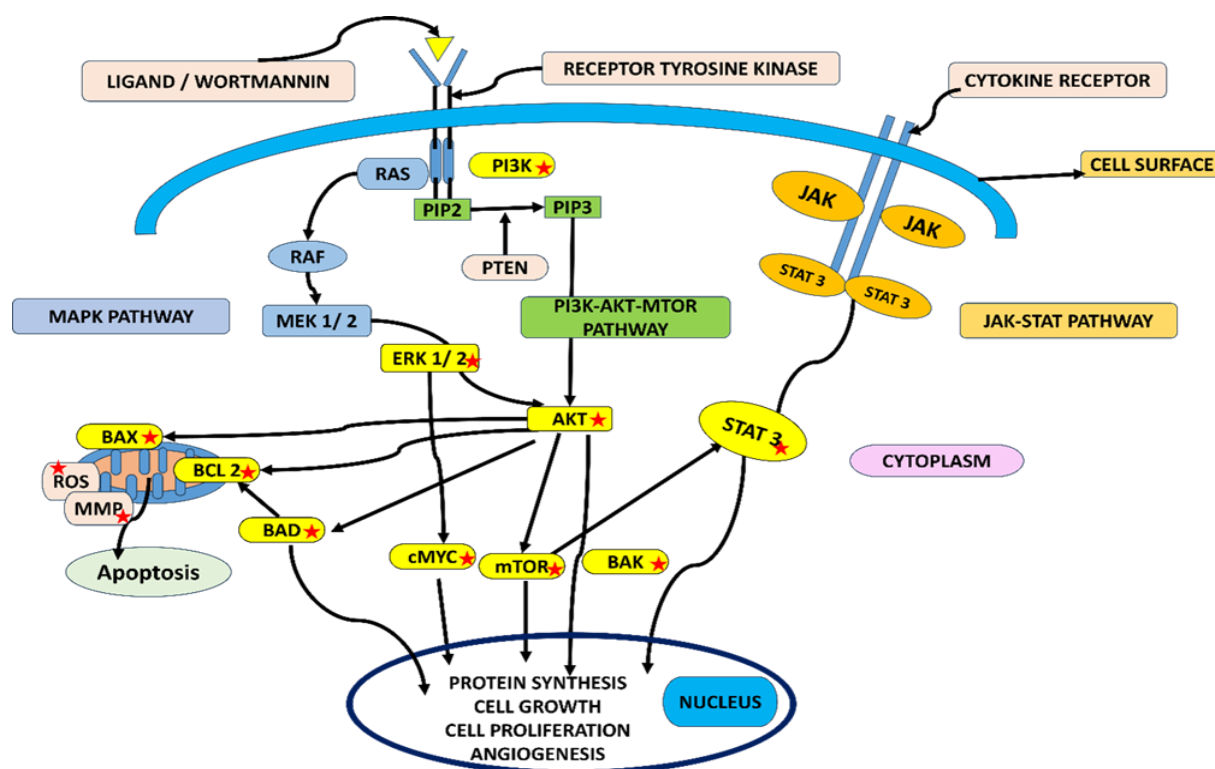


Figure 11. The apoptotic and anti-proliferative mechanism of wortmannin in Oral cancer cells as an inhibitor of *PI3K*, *AKT*, and *mTOR*. The inhibition of these genes affects the cellular proliferation. In addition, *BAD* and *BAK* promotes apoptosis. Wortmannin showed activity on ROS and MMP also causing apoptosis of the cell. Inhibition of *ERK 1/2* and *STAT3* genes lead to the inhibition of cellular proliferation which may have cross-talk with PI3K-AKT pathway. Cell cycle progress is arrested at the Sub G1 phase resulting in the inhibition of cell proliferation. [Genes present in yellow-coloured boxes are the genes used in the present study. indicates activity of wortmannin on the genes]

The study's findings indicated a downregulation of certain genes associated with the STAT and MAPK pathways, including *ERK1*, *ERK2*, and *STAT*. It also demonstrated alterations in gene expressions such as *BAK*, *BAD*, and *cMYC*, which are implicated in cellular apoptosis and proliferation. These alterations have the potential to impede the proliferation and differentiation of tumor cells, as well as diminish their invasiveness through the suppression of the PI3K/Akt signaling pathway. In conclusion, exposure to wortmannin markedly suppresses the proliferation and triggers apoptosis in oral cancer cells. The findings indicate that wortmannin may hold promise for targeted anti-tumor therapy and drug screening in the context of oral cancer. Given the persuasive in vitro evidence demonstrating the anticancer properties of this medicine, it is clear that additional in vivo studies utilizing diverse tumor models are necessary for future progress. Addressing the safety risks associated with wortmannin is essential prior to forward with the clinical trials.

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### Conflicts of interest

There are no conflicts of interest

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### Authors' Contributions

GK: Methodology, Formal analysis, Investigation, Software, Writing—original draft. PS: Formal analysis, Investigation, Visualization. VL: Conceptualization, Methodology, Funding acquisition, Supervision, Validation, Visualization, Writing—original draft, Writing—review & editing.

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