

Globularifolin Exerts Anticancer Effects on Human Lung Cancer Cells Via Regulation of ROS and Suppression of PINK1/Parkin Mitophagy Pathway

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Submitted: 17-Feb-2022

Revised: 14-Mar-2022

Accepted: 23-Apr-2022

Published: 23-Nov-2022

ABSTRACT

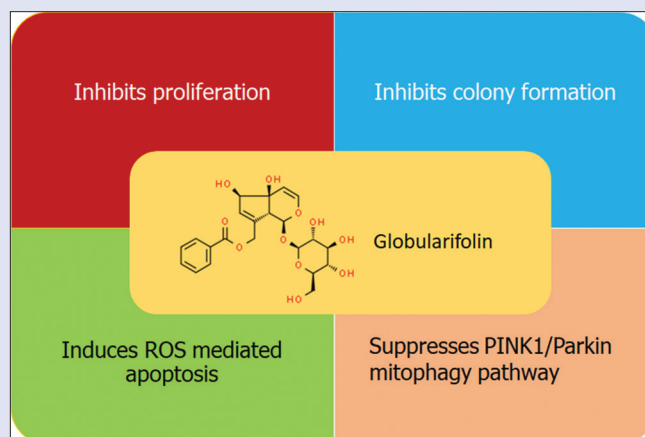
Background: Lung cancer exhibits extremely high mortality, and there is a pressing need to identify novel leads for the development of efficient chemotherapy for lung cancer. Thus, there is a need to identify novel and effective molecules for the management of lung cancer. **Objectives:** In the present study, the *in vitro* anticancer effects of globularifolin were evaluated against human lung cancer and attempts were made to explore the underlying molecular mechanisms. **Materials and Methods:** Cell viability and proliferation was determined by MTT, EdU, and colony formation assays. Cellular processes were detected by annexin V/PI, confocal microscopy and flow cytometry. Protein expression was detected by western blotting. **Results:** The results showed that globularifolin suppressed the growth and colony formation of the human lung cancer cells and exhibited an IC_{50} of 8 μ M against the HCC827 and SK-LU-1 lung cancer cell lines. However, the IC_{50} of globularifolin was found to be comparatively higher against the normal MRC-5 cells. The globularifolin-induced inhibition of human lung cancer cells were found to be due to the induction of apoptosis, which was associated with upregulation of Bax, caspase-3 and caspase-9, and downregulation of Bcl-2. Further investigation of the underlying mechanism revealed that inhibitory effects of globularifolin were mediated through apoptotic induction driven through inhibition of PI3K/Akt and PINK/Parkin signaling pathways driven respectively through reactive oxygen species (ROS) production and mitophagy. **Conclusion:** Taken together, the results show that globularifolin exerts anticancer effects on human lung cancer cells via regulation of ROS and suppression of PINK1/Parkin mitophagy pathway.

Key words: Apoptosis, flow cytometry, globularifolin, lung cancer, mitophagy, ROS

SUMMARY

- Globularifolin inhibits viability and proliferation of lung cancer cells.
- Globularifolin induces ROS mediated apoptosis in lung cancer cells.

- Globularifolin inhibits PINK1/Parkin mitophagy pathway in lung cancer cells.



Abbreviations used: EDU: 5-ethynyl-2'-deoxyuridine; MTT: (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide, DMSO: dimethyl sulfoxide.

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DOI: 10.4103/pm.pm_87_22

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INTRODUCTION

Of all of the lethal malignancies, lung cancer is ranked as one of the most common cancers across the globe.^[1] On the basis of its cellular origin, lung cancer has two broad categories: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC).^[2] More than 85% of lung cancer cases are represented by NSCLC.^[3] The higher mortality of lung cancer results from inefficient diagnostic tools and less effective treatment strategies. In most cases, the patients are detected with advanced stage of lung cancer at the time of diagnosis.^[3] Researchers are thus actively engaged in the search for novel and better treatment strategies against lung cancer. In connection with this, diverse plant-derived chemical molecules are being evaluated for their anticancer role against lung cancer. The phytochemicals belonging to the class of iridoids along with their glycosyl derivatives have also generated considerable attention, owing to their huge pharmacological potential.^[4] The iridoids are typically the monoterpenoids with cyclopentanopyran in

form.^[5] The iridoids are the biosynthetic products of 8-oxogeranial and are usually present as glucosyl-conjugates in the plant kingdom.^[6] These compounds form the active constituents of several medicinally important plants and have been shown to possess neuro-protective and anticancer effects.^[7] Belonging to the class of iridoids, globularifolin is an acylated glucosyl-iridoid present in only a few genera of plant family, *Plantaginaceae* which include *Globularia* and *Veronica*.^[8,9] However,

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Cite this article as: Li W, Lin C, Chen Z. Globularifolin exerts anticancer effects on human lung cancer cells via regulation of ROS and suppression of PINK1/parkin mitophagy pathway. *Phcog Mag* 2022;18:844-50.

its isolation is mainly performed from the plant species, *Globularia cordifolia*.^[10] The anticancer role of globularifolin was recognized recently.^[11] Consistently, the present study was designed to investigate the anticancer effects of globularifolin against the human lung cancer cells and to unveil the underlying molecular mechanisms.

MATERIALS AND METHODS

Cell lines and culture conditions

The lung cancer cell lines (HCC827 and SK-LU-1) and the normal lung epithelial cell line (MRC-5) were purchased from the American Type Collection Center (ATCC, USA). The cells were cultured in Dulbecco's modified eagle medium (DMEM) (Thermo Fisher Scientific) in supplementation with 10% fetal bovine serum (FBS, Hyclone) and penicillin-streptomycin (1% v/v, Thermo Fisher Scientific). The

cell culture was done in a humidified CO₂ incubator with a 5% CO₂ concentration at 37°C.

Cell viability assay

The viability of lung cancer cell lines along with the normal epithelial lung cell line, treated with varying concentrations of globularifolin, was determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] assay. The lung cancer cell lines (HCC827 and SK-LU-1) and normal lung epithelial lung cell line (MRC-5) were cultured in a 96-well plate in 100 µL DMEM at 37°C for 24 hrs with 2×10^4 cells per well. 100 µL culture medium with or without globularifolin was added to each well after 24 hrs to make final globularifolin concentration varying between 0 and 160 µM and incubation at 37°C was prolonged for 24 hrs again. Afterwards, 20 µL

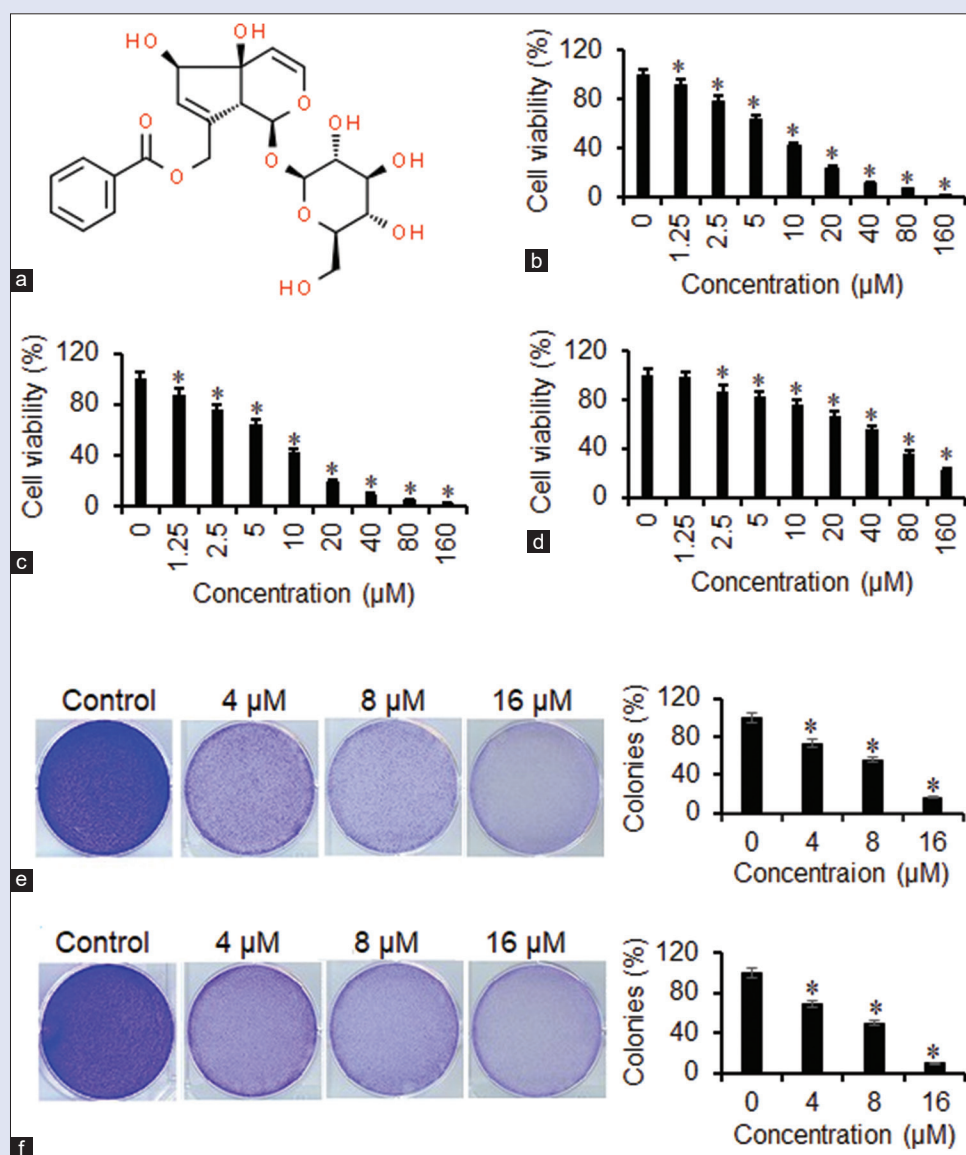


Figure 1: Globularifolin selectively inhibits lung cancer cell growth *in vitro*. (a) Chemical structure of globularifolin (b) MTT assay for determination of relative viability of HCC827 lung cancer cells administered with globularifolin (0–160 µM) for 24 hrs at 37°C (c) MTT assay for determination of relative viability of SK-LU-1 lung cancer cells administered with globularifolin (0–160 µM) for 24 hrs at 37°C (d) MTT assay for determination of relative viability of MRC5 normal lung epithelial cells administered with globularifolin (0–160 µM) for 24 hrs at 37°C (e) analysis of colony formation from HCC827 cancer cells administered with or without globularifolin (4 µM, 8 µM, or 16 µM) (f) analysis of colony formation from SK-LU-1 cancer cells administered with or without globularifolin (4 µM, 8 µM, or 16 µM). The experiments were performed in triplicate and expressed as mean ± SD (**P* < 0.05)

of MTT solution (0.5%) was added to each well, and an additional incubation of 4 hrs at 37°C was followed. The medium was replaced by 150 μ L DMSO. Absorbance was recorded at 570 nm for each well using spectrophotometer to analyze the cell viability.

Colony formation assay

The colony formation from HCC827 and SK-LU-1 cancer cells was assessed with or without globularifolin, using colony formation assay. In brief, after being counted using a hemocytometer, 250 cancer cells were added to each well of a 6-well plate. The cells were administered with or without globularifolin (4 μ M, 8 μ M or 16 μ M) and then cultured for 12 days at 37°C. The colonies were then washed using phosphate-buffered saline (PBS) and fixed with 70% ethanol. Staining of colonies was performed using 0.1% crystal violet solution and colonies were finally examined and subsequently counted under light microscope.

EdU assay

The approximate estimation of the SK-LU-1 cancer cells undergoing DNA-synthesis was made using the EdU assay. After being administered with or without globularifolin (4 μ M, 8 μ M or 16 μ M) for 24 hrs at 37°C in 12-well plates, the cells were harvested by centrifugation and

then labeled with EdU (5-ethynyl-2'-deoxyuridine) solution from EdU labeling/detection kit (RiboBio, China), as per the manufacturer's instructions. The cells were fixed with methanol and incubated with glycine. Afterwards, the cells were washed with PBS and treated with anti-EdU solution. The cells were made permeable using 0.5% Triton X-100. The cell nuclei were stained using 0.5% DAPI solution. The cells were finally examined with fluorescent microscope to detect the relative percentage of EdU positive cells.

Annexin V/PI assay

The apoptosis of cancer cells under varying concentrations of globularifolin was studied with the help of FITC-Annexin V/PI Apoptosis detection kit (ThermoFisher Scientific Massachusetts, United States) following the manufacturer protocol in combination with flow cytometry.

Western blotting

The extraction of total proteins was performed by treating the cancer cells, administered with or without globularifolin, with RIPA lysis and extraction buffer (Thermo Fisher Scientific). The protein lysates with equal concentrations were resolved on 8%–10% SDS-PAGE gels. The

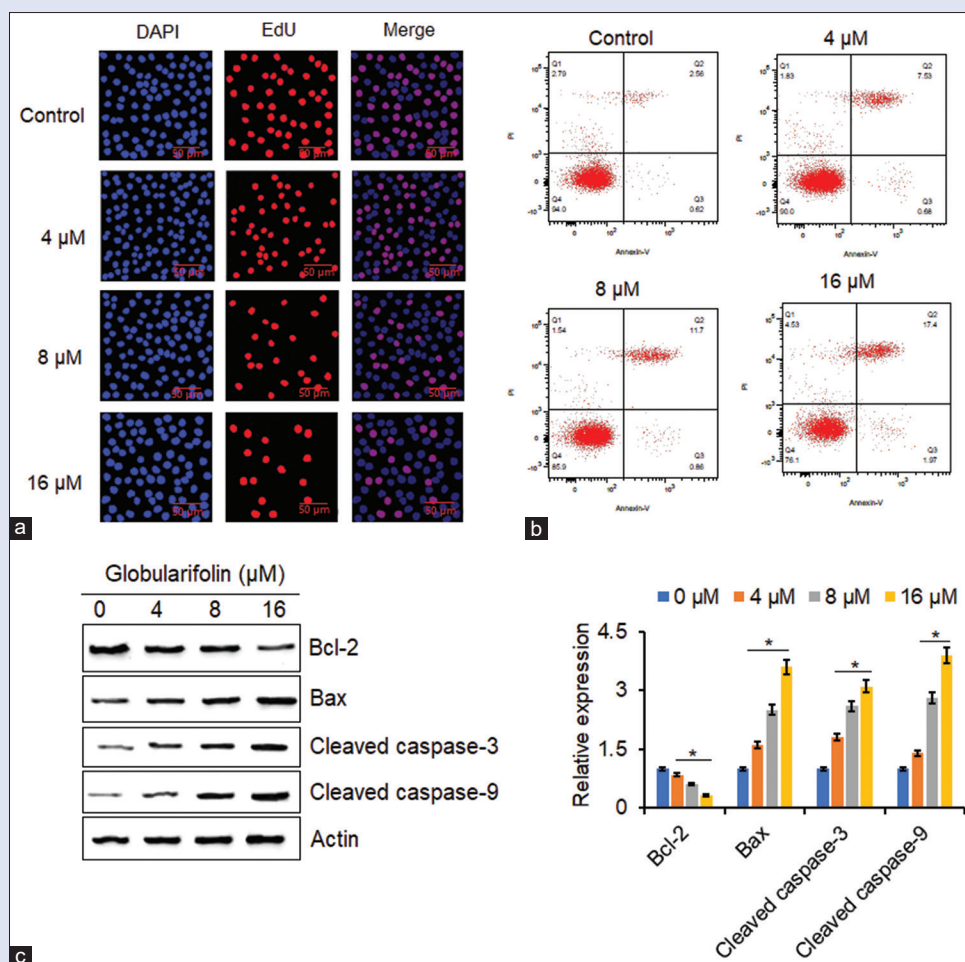


Figure 2: Globularifolin minimized proliferative viability and induced apoptosis in lung cancer cells. (a) EdU assay for the estimation of proliferative viability of SK-LU-1 cancer cells administered with or without globularifolin (4 μ M, 8 μ M, or 16 μ M) (b) flow cytometric analysis for the estimation of apoptosis of SK-LU-1 cancer cells administered with or without globularifolin (4 μ M, 8 μ M, or 16 μ M) (c) estimation of relative concentrations of Bax, Bcl-2, cleaved caspase-3 and cleaved caspase-9 from SK-LU-1 cancer cells administered with or without globularifolin (4 μ M, 8 μ M, or 16 μ M). The experiments were performed in triplicate and expressed as mean $\pm$ SD (*P < 0.05)

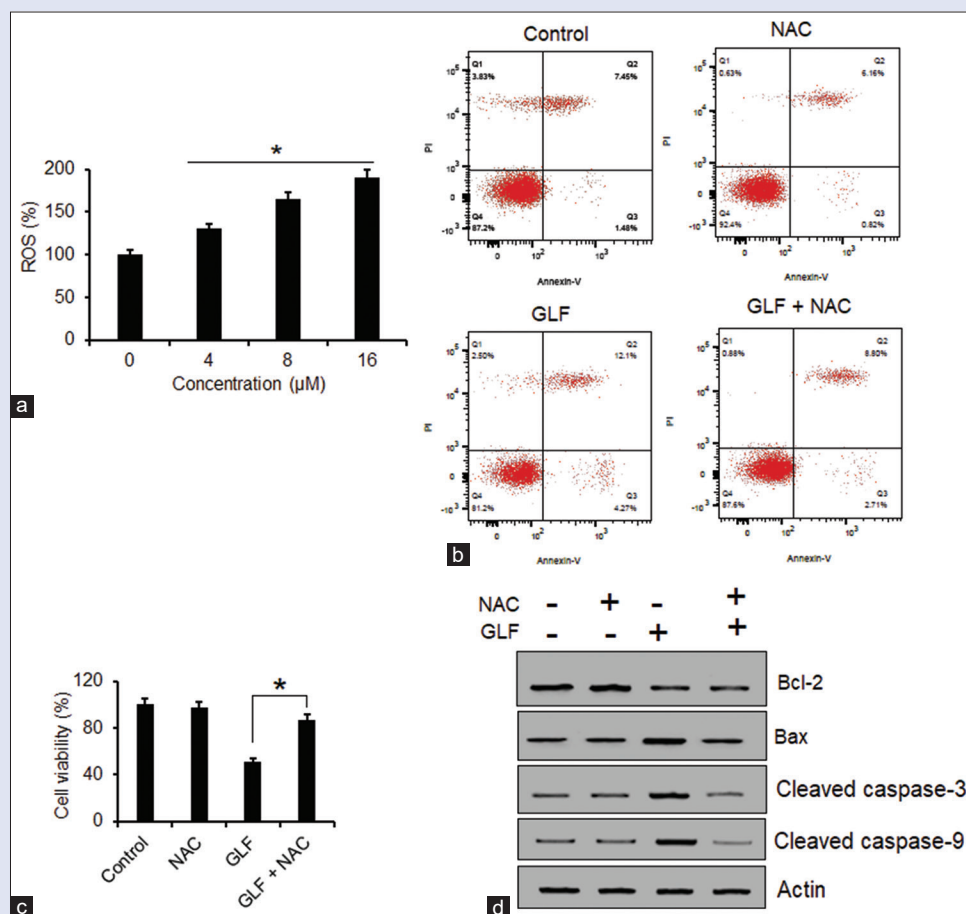


Figure 3: Globularifolin increased ROS production to induce apoptosis in lung cancer cells. (a) Determination of relative ROS count of SK-LU-1 cancer cells administered with or without globularifolin (4 μM, 8 μM, or 16 μM) (b) Flow cytometric analysis for the estimation of apoptosis of SK-LU-1 cancer cells administered with 12 nM NAC, 8 μM globularifolin (GLF), 12 nM NAC + 8 μM globularifolin (GLF) with reference to control cancer cells (c) determination of relative viability of SK-LU-1 cancer cells administered with 12 nM NAC, 8 μM globularifolin (GLF), 12 nM NAC + 8 μM globularifolin (GLF) with reference to control cancer cells using MTT assay (D) estimation of relative concentrations of Bax, Bcl-2, cleaved caspase-3 and cleaved caspase-9 from SK-LU-1 cancer cells administered with 12 nM NAC, 8 μM globularifolin (GLF), 12 nM NAC + 8 μM globularifolin (GLF) with reference to control cancer cells. The experiments were performed in triplicate and expressed as mean ± SD (**P* < 0.05)

gels were blotted to nitrocellulose membranes. The membranes were treated with 5% non-fat milk. This was followed by their exposure to specific primary antibodies Akt (sc-135829, Santa Cruz, CA, USA), phosphorylated (p)-AKT (sc-7985-R, Santa Cruz, CA, USA), PI3K (sc-136298, Santa Cruz, CA, USA), cleaved vaspase-3 (9661, Cell Signalling Technology), cleaved caspase-9 (20750, Cell Signalling Technology), Bax (sc-7480, Santa Cruz, CA, USA), Bcl-2 (sc-23960, Santa Cruz, CA, USA) and Actin (sc-58673, Santa-Cruz, CA, USA) at 4°C overnight. The membranes were then treated with horseradish peroxidase-conjugated secondary antibodies for 2 hrs at room temperature. The specific protein bands were detected using enhanced chemiluminescence substrate (ECS). The human β-actin served as the internal reference protein.

Determination of cellular ROS-levels

The relative ROS levels of SK-LU-1 cancer cells, treated with or without globularifolin (4 μM, 8 μM or 16 μM), were determined with the help of ROS assay kit (Abcam). To be precise, the SK-LU-1 cells were treated with or without indicated globularifolin concentrations for 24 hrs at 37°C. The cells were then washed using PBS and stained with DCFDA for 30 mins at 37°C. The stained cells were again washed with, lysed,

and re-suspended in PBS. The cells were analyzed with a fluorescent microscope under low-light conditions limiting photo-bleaching. FlowJo version 7.6 software was used for the analysis of mean fluorescence intensity.

Organelle fluorescent labelling and confocal microscopy

The SK-LU-1 cells were administered with or without 8 μM globularifolin at 37°C in petriplates. The cells were subsequently treated with MitoTracker Green (100 nM) for 35 mins for labeling the mitochondria. The lysosomes were labeled using LysoTracker Red (200 nM) for 15 mins at 37°C. The cells were then washed using PBS and ethanol fixed. Finally, the confocal microscope (Olympus) was used for organellar examination, and the results were analyzed using FV10 software (Olympus).

Statistics

All the experiments were performed using three replicates and the final results were presented as mean ± standard deviation (SD). The statistical tests like student's *t* test and one-way analysis of variance (ANOVA) were performed using GraphPad Prism 7.0 software. *P* < 0.05 was taken as statistically significant difference.

RESULTS

Globularifolin inhibited lung cancer cell proliferation

The effects of globularifolin [Figure 1a] on the viability of the HCC827 and SK-LU-1 lung cancer cells was determined by MTT assay. The results showed globularifolin significantly ($P < 0.05$) inhibited the growth of both the lung cancer cell lines and exhibited an IC_{50} of 8 μ M [Figure 1b and 1c]. In contrast, when the normal lung cell line (MRC-5) was administered with the same globularifolin dosages, the growth inhibitory effects were less prominent with an IC_{50} of 70 μ M [Figure 1d]. Besides, when HCC827 and SK-LU-1 cancer cells were treated with 0 μ M, 4 μ M, 8 μ M, or 16 μ M concentrations of globularifolin, the colony formation significantly ($P < 0.05$) declined for both the cell lines and followed dose dependence [Figure 1e and 1f]. The results thus specify that globularifolin exhibited potent growth inhibitory effects against the lung cancer cells, while the normal lung cells were least affected.

Globularifolin induced apoptosis in lung cancer cells

To investigate the underlying reason for *in vitro* growth inhibitory effects of globularifolin against SK-LU-1 lung cancer cells, the EdU assay was performed. The results showed that the proportion of EdU-positive SK-LU-1 cancer cells declined proportionally with the increase in the globularifolin treatment dose [Figure 2a]. Overall, the relative number of EdU-positive cells was considerably lower for globularifolin-treated cells in comparison to untreated SK-LU-1 cancer cells. The flow cytometric study revealed that the number of apoptotic cancer cells increased under globularifolin administration, and the apoptotic induction was much evident when higher treatment concentrations were used [Figure 2b].

The western blotting of apoptosis marker proteins confirmed that globularifolin administration induced apoptosis in cancer cells. The expression of Bax, Caspase-3 and Caspase-9 (both cleaved) increased proportionally with increase in globularifolin dose, while that of Bcl-2 decreased [Figure 2c]. The findings are indicative of apoptosis induction in lung cancer cells by globularifolin treatment.

Globularifolin triggered apoptosis through ROS-driven selective inhibition of PI3K/Akt pathway

The estimation of the levels of reactive oxygen species (ROS) of SK-LU-1 cancer cells administered with or without 4 μ M, 8 μ M, or 16 μ M concentrations of globularifolin showed that the relative ROS levels were significantly higher ($P < 0.05$) in globularifolin-treated cancer cells and followed linear dose-dependence [Figure 3a]. Whether the generation of ROS molecules was responsible for triggering apoptosis in lung cancer cells, the ROS-scavenger N-acetyl cysteine (NAC, Sigma-Aldrich) was used. It was found that the pre-incubation of SK-LU-1 cancer cells with 12 μ M NAC significantly decreased ($P < 0.05$) the cell apoptosis under globularifolin [Figure 3b]. MTT assay also showed that the cell viability declined correspondingly [Figure 3c]. The results were also confirmed from the western blotting of apoptosis marker proteins [Figure 3d]. The study of AKT and p-AKT protein expression from SK-LU-1 cancer cells treated with or without 4 μ M, 8 μ M, or 16 μ M concentrations of globularifolin revealed that p-AKT protein expression decreased in a concentration-dependent manner [Figure 4a]. However, the expression of AKT protein remained unchanged. The results indicate that globularifolin inhibited PI3/AKT signalling pathway in lung cancer cells to exercise its anti-cancer effects. Interestingly, the pre-treatment of lung cancer cells with 12 μ M NAC attenuated the globularifolin-induced blockage of PI3K/AKT signalling pathway [Figure 4b]. It indicates

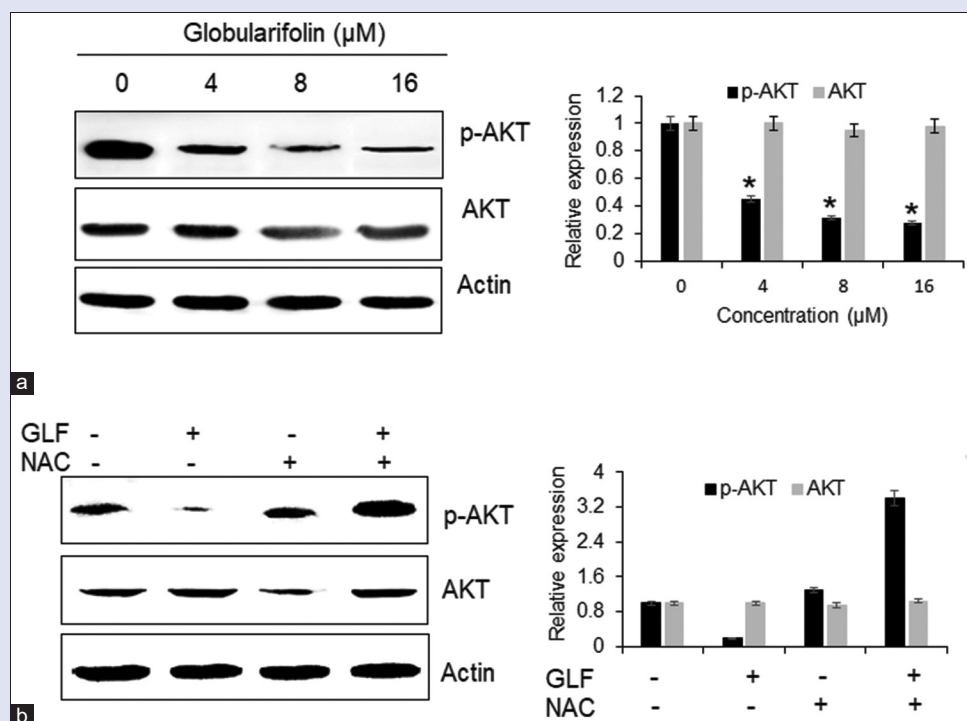


Figure 4: Globularifolin inhibited PI3K/AKT pathway via ROS production in lung cancer cells. (a) Western blotting of p-AKT protein from SK-LU-1 cancer cells administered with or without globularifolin (4 μ M, 8 μ M, or 16 μ M) (b) western blotting of p-AKT protein from SK-LU-1 cancer cells administered with 12 nM NAC, 8 μ M globularifolin (GLF), 12 nM NAC + 8 μ M globularifolin (GLF) with reference to control cancer cells. The experiments were performed in triplicate and expressed as mean $\pm$ SD (* $P < 0.05$)

that inhibition of PI3/AKT signalling pathway in lung cancer cells by globularifolin was driven through intracellular ROS production. Collectively, the results suggest that administration of lung cancer cells with globularifolin led to a significant increase in ROS production, blocking PI3K/AKT signalling pathway, and thus induced cancer cell apoptosis evident as a decline in cell growth.

Globularifolin-induced apoptosis was partly mediated through mitophagy inhibition via PINK1/Parkin pathway

As indicated by the research investigations, mitophagy has a crucial involvement in mediating the cell apoptosis. The mitophagy was studied through fluorescent labelling of mitochondria and lysosomes with the employment of confocal microscopy. It was seen that the lysosomal vesicles were less prominent under the administration of lung cancer cells with IC_{50} concentration of globularifolin [Figure 5a]. The western blotting of PINK1 and Parkin proteins indicated that the expression levels of both of these mitophagy-regulatory proteins considerably decreased under globularifolin administration in concentration dependent manner [Figure 5b]. For the final confirmation of the involvement of mitophagy in exerting anti-cancer effects of globularifolin, mitochondrial division inhibitor-1 (Mdivi-1, Sigma-Aldrich) was used. Administration of SK-LU-1 cancer cells with 12 mM mitophagy inhibitor, Mdivi-1

increased the relative percentage of apoptotic cells as under globularifolin, and the level of apoptosis was significantly higher when dual treatment of Mdivi-1 and globularifolin was used in comparison to Mdivi-1 or globularifolin treatments alone [Figure 5c]. The MTT assay also indicated that cell viability was significantly lower under Mdivi-1 and globularifolin co-administration in comparison to either Mdivi-1 or globularifolin treatment alone [Figure 5d]. Taken together, the results are suggestive that administration of lung cancer cells with globularifolin inhibited the PINK1/Parkin mitophagy regulatory signaling pathway to partially induce apoptosis, manifesting as a decline in cancer cell growth and viability *in vitro*.

DISCUSSION

Lung cancer is the most prevalent and highly destructive human malignancy, accounting for the highest number of cancer-related deaths worldwide.^[1,12] Statistical data suggest that over the past 10 years, the incidence of lung cancer has lessened slightly; nevertheless, it still remains as the most dominant human cancer in terms of both prevalence rates and mortality.^[13] Efforts are continuously being made towards the discovery of better therapeutic strategies against lung cancer so as to improve the survival of lung cancer patients. A number of studies have indicated that globularifolin exhibited considerable anticancer effects against human cancer cells including the lung cancer.^[11,14,15] It was deduced that globularifolin has selective growth inhibitory potential

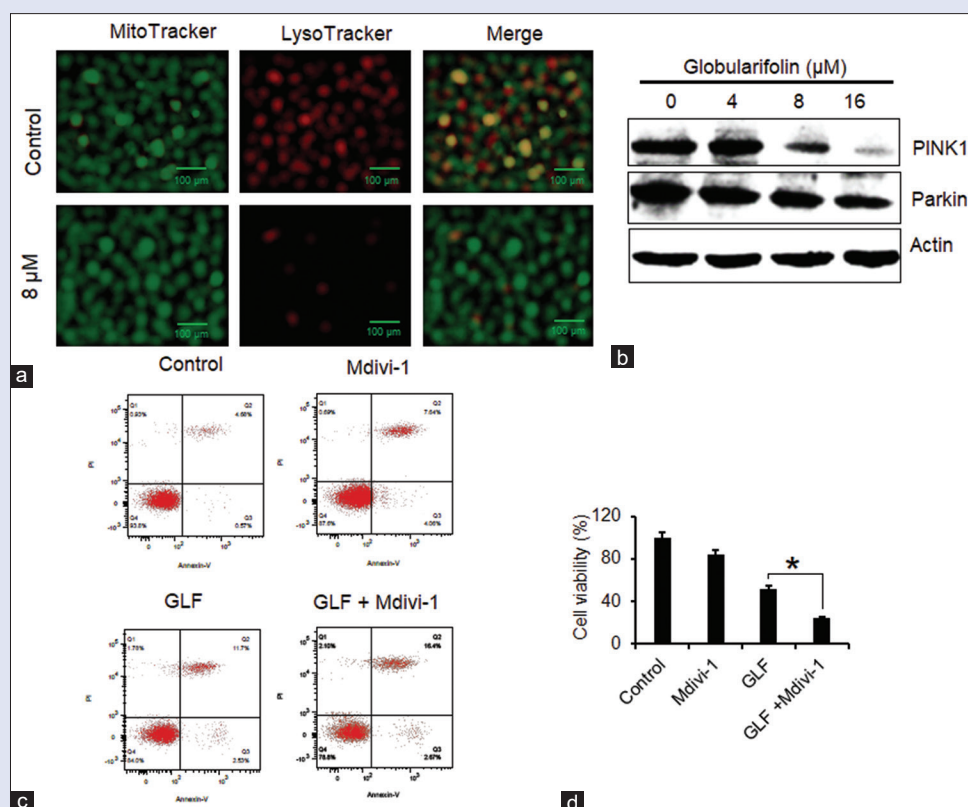


Figure 5: Globularifolin induced lung cancer apoptosis partly through mitophagy inhibition. (a) Confocal microscopic examination of lung cancer cell mitochondria and lysosomes from SK-LU-1 lung cancer cells treated with or without 8 μM globularifolin; (b) estimation of relative concentrations of PINK1 and Parkin proteins from SK-LU-1 cancer cells administered with or without globularifolin (4 μM, 8 μM, or 16 μM); (c) flow cytometric analysis for the estimation of apoptosis of SK-LU-1 cancer cells administered with 12 mM Mdivi-1, 8 μM globularifolin (GLF), 12 mM Mdivi-1 + 8 μM globularifolin with reference to control cancer cells (D) determination of relative viability of SK-LU-1 cancer cells administered with 12 mM Mdivi-1, 8 μM globularifolin, 12 mM Mdivi-1 + 8 μM globularifolin with reference to control cancer cells. The experiments were performed in triplicate and expressed as mean ± SD (* $P < 0.05$)

against the cancer cells. A similar observation was made from the current study. The globularifolin administration significantly inhibited growth and viability of lung cancer cells *in vitro* while minimal growth inhibitory effects were noticed against the normal lung cells. The reason for the higher activity of globularifolin against lung cancer cells as compared to the normal cells could be due to the fact that several signalling pathways are activated in lung cancer cells and globularifolin might be targeting one of those.^[11] The induction of apoptosis is regarded as one of the primary factors responsible for the inhibition of *in vitro* cancer cell growth.^[16] Cell apoptosis is a highly complex process and regulated by a set of signalling pathways like ERK/JNK, JAK/STAT, and PI3K/AKT to name a few, together with the involvement of cues of ROS signals.^[17] Herein, we deduced that being highly susceptible to intracellular ROS levels, the PI3K/AKT pathway was inhibited through ROS generation in lung cancer cells when administered with globularifolin. This is in confirmation with a previous study wherein globularifolin was shown to induce ROS production in adenoid cystic carcinoma cells.^[14] Moreover, the induction of apoptosis in lung cancer cells was partly exerted partly through inhibition of PINK1/Parkin regulated mitophagy. The mitophagy involves the selective mitochondrial degradation through autophagy and is necessary for restraining the mitochondrial count and maintaining a healthy cellular state.^[18] The impairment in the mitophagy leads to the accumulation of defective mitochondria, which are detrimental not only to cells but to mitochondria themselves.^[19] The PINK1/Parkin signalling pathway is best characterized by the regulatory node discriminating the healthy mitochondrial population and detrimental mitochondria.^[20] The PTEN-induced kinase 1 (PINK1) is involved in the recruitment and phosphorylation-mediated activation of Parkin protein and the latter functions in selective ubiquitination of proteins of outer mitochondrial membrane and allows passive mitochondrial degradation by lysosomes through mitophagy.^[21] The impairment in the PINK1/Parkin pathway leads to the accumulation of damaged mitochondrial populations causing enrichment of pro-apoptotic proteins like caspases resulting in cell apoptosis.^[22] The present study clearly indicated that globularifolin administration inhibited the PINK1/Parkin signalling pathway to partially block mitophagy driving the induction of apoptotic cell death. A similar mechanism of action has been observed for other chemical compounds against human cancer cells.^[23] Therefore, to conclude, the study proposes the potential of globularifolin to act as a lead therapeutic molecule against lung cancer. However, more studies involving the use of *in vivo* models are required to further validate the present findings. Moreover, the mechanism by which globularifolin targets PINK1/Parkin mitophagy pathway needs to be studied in more detail.

CONCLUSION

Taken together, the results of the current study are indicative of the anticancer effects of globularifolin against lung cancer through attenuation of PI3K/AKT and PINK1/Parkin signalling cascades. Globularifolin might thus be used as a lead molecule for the development of chemotherapy for lung cancer.

Acknowledgements

Authors acknowledge the Department of Respiratory and Critical Medicine, Central People's Hospital of Huizhou, Huizhou City, Guangdong Province, China for providing laboratory facilities for conduction of experiments

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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