

Xanthohumol inhibits proliferation via increasing reactive oxygen species and inducing G1/S phase cell cycle arrest in NG108-15 cells

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Article

Keywords:

Posted Date: November 10th, 2023

DOI: <https://doi.org/10.21203/rs.3.rs-3553380/v1>

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Additional Declarations: No competing interests reported.

Abstract

Xanthohomul (XN), a naturally prenylated flavonoid, is extracted from hops (*Humulus lupulus* L.; Cannabaceae). XN treated NG108-15 cells significantly decreased cell viability through increased ROS and arrested the cell cycle at G1/S phase. Notably, XN treatment did not induce apoptotic responses. Further research revealed XN-treatment increased reactive oxygen species (ROS) levels which was alleviated by N-acetyl-L-cysteine (NAC). Moreover, XN triggered the phosphorylation of the estrogen-regulated kinase (ERK1/2) and p38 mitogen activated protein kinase (MAPK). Intriguingly, our observations indicated that only ERK inhibitor could partly relieve XN-induced G1/S cycle arrest and proliferation inhibition. Hence, XN emerges as a prospective candidate for an anti-neurotumor pharmaceutical agent.

Introduction

Gliomas are among the most prevalent brain cancers, accounting for 81% of malignant (cancerous) brain tumor cases. In 2021, the WHO classified gliomas changed from Roman numerals (I, II, III, IV) to Arabic numerals (1, 2, 3, 4) to classify and categorize glioma subtypes. According to the new stipulating molecular criteria, Glioblastoma is still classified as CNS WHO grade 4 due to its high malignancy¹. Glioblastoma, characterized by a notably short median overall survival of approximately 16 to 18 months, predominantly appears in elderly patients. The traditional clinical management for glioblastoma entails surgical resection, followed by chemotherapy and radiation, alongside innovative approaches that encompass immunotherapeutic modalities. Unfortunately, tumor recurrence post-treatment is still the major cause of poor prognosis in glioma patients². The increasement of glioma patients contributes to a substantial burden on individuals, families, and society. Therefore, it is critical to develop novel anti-tumor drugs for glioblastoma treatment³. In the last decade, following maximal safe surgical resection, glioma patients commonly conjunct treat by radiation with temozolomide (TMZ) (Temodar ®) and chemotherapy with TMZ. Although TMZ is the first-line agent for patients with glioma, the vast majority of patients inevitably develop resistance to the drug⁴. Thus, finding innovative pharmaceutical drug with antitumor properties to combat glioblastoma multiforme is urgent.

Nowadays, to explore novel chemotherapeutic drugs, researcher has paid more attention to the natural products due to its limited side effect. Xanthohumol (XN, chemical formula: $C_{21}H_{22}O_5$), a prenylated flavonoid predominantly sourced from *Humulus lupulus*, has been used for treatment of diverse conditions such as inflammation, diabetes, aging, and cancer. Increasing studies have testified the remarkable antitumor activity of XN in various cancers⁵. To date, XN inhibits the growth and proliferation via elevating reactive oxygen species (ROS), promoting apoptosis and inducing cell cycle arrest. XN-upregulated miR-204-3p expression induces apoptosis via inhibiting IGFBP2/AKT/Bcl2 pathway in glioblastoma U87 MG cells⁶. Moreover, increased ROS levels and perturbation of the MAPK pathways have been implicated in XN-induced apoptotic responses in human glioblastoma cell lines T98G and U87-MG⁷. XN treatment has also been shown to augment ROS levels and effect G1 phase cell cycle arrest in

human acute myeloid leukemia HL-60 cells⁸. Additionally, XN's influence extends to the initiation of AIF pathway-mediated apoptosis through the induction of mitochondrial stress in rat glioma C6 cells⁹. However, the anti-glioma efficacy of XN remains incompletely understood. Herein, we utilize NG108-15 cell to investigate the impact of XN on glioma cells and its in-depth anticancer mechanism.

In this study, we demonstrated that XN effectively inhibited proliferation while apoptosis was not significantly involved in this process. Additionally, our investigations have delved into the intricate machinery encompassing ROS modulation, activation of the ERK1/2 pathway, and the imposition of cell cycle arrest as essential contributors to XN-induced glioma cell death. Consequently, Xanthohumol emerges as a promising therapeutic candidate for the treating of glioblastoma.

Results

Xanthohumol inhibits cell viability in NG108-15 cells

To evaluate the anti-glioma effects of XN, NG108-15 cells were subjected to Xanthohumol (approximately 0–60 μM) for 24 hours. Initially, Varied concentrations of XN were treated to observe the morphology of NG108-15 cells by phase-contrast microscopy. We discovered that while high doses of XN (20–60 μM) dramatically blocked the proliferation of NG108-15 cells, low doses of XN (0–10 μM) had no effect on cell growth (Fig. 1A). This result suggested that high doses of XN might have an anti-proliferation effect on the NG108-15 cells. Subsequently, in order to get the cytotoxicity of XN on NG108-15 cells in vitro, cell viability was measured by the CCK-8 and cell mortality was determined through Trypan Blue assay after 24 hours XN treatment, respectively (Fig. 1B and 1C). The findings indicate a dose-dependent inhibitory impact of XN-treatment on the proliferation of NG108-15 neuro cancer cells. Moreover, the IC_{50} value approximated 40 μM . Consequently, this specific XN concentration of 40 μM was selected for continued experimentation.

Xanthohumol triggers ROS generation and cell cycle arrest in NG108-15 cells

Intracellular reactive oxygen species (ROS) are crucial in regulating tumor cell proliferation. The fluorescent probe DCFH-DA was used to assess the impact of XN on ROS level in NG108-15 cells. XN-treatment group (40 μM , 1h) of ROS levels were significantly higher than that in control group (Fig. 2A). Notably, anti-oxidant N-acetyl-Lcysteine (NAC) pretreatment efficiently reduced ROS generation by XN (Fig. 2B). Furthermore, CCK-8 results indicated XN-treatment decreases cell proliferation, while the pretreatment of NAC was observed to alleviate this suppressive effect (Fig. 2C). To elucidate the fundamental mechanisms underpinning the inhibitory effects of XN on cell proliferation, Cell cycle analysis was performed. XN treatment (40 μM , 24 h) indicated around 30% increase in the G1 phase of NG108-15 cells whereas 20% decrease in the S phase (Fig. 2D). These results suggested that XN exerts its inhibitory influence on cell viability was mediated by both ROS activation and G1/S cell cycle arrest.

Time-dependent response of Xanthohumol on MAP kinase signaling pathway activation

The MAP kinase signaling pathway has been well-documented in its association with crucial cellular processes, encompassing proliferation, differentiation, apoptosis, cell cycle regulation, and stress responses¹⁰. In order to investigate the XN treatment on NG108-15 cells on MAP kinase signaling pathway, the total and phosphorylated MAPKs were analyzed using Western blot analysis after XN treatment. The cells were exposed to 40 μ M of XN for 15 min, 30 min and 60 min, respectively. Besides, the vehicle groups were given 3 μ l DMSO. Cells treated with XN for 30 min show significantly up-regulated phosphorylation of p38 when compared to the control and vehicle groups (Fig. 3A). Furthermore, XN treatment for 60 min markedly activated phosphorylation of Extracellular-signal-regulated kinases (ERK) in comparison to control and vehicle groups (Fig. 3B). Intriguingly, XN treatment on NG108-15 cells did not elicit activation in either the SAPK/JNK signaling pathway or c-Jun, the downstream transcriptional factor of JNK signaling pathway (Fig. 3C).

Xanthohumol exclusively arrests cell cycle via ERK pathway

In order to investigate whether XN induces cell cycle arrest through the ERK or p38 signaling pathway, the cells were pre-exposed with ERK inhibitor PD98059 (PD), p-p38 inhibitor SB203580 (SB) or a combination of both, each for a duration of 30 minutes, prior to XN treatment. The results suggest that XN induced G1/S cell cycle arrest in NG108-15 cells only through activation of the ERK signaling pathway by flow cytometry (Fig. 4A and 4B). Subsequently, to substantiate the pivotal role of the ERK signaling pathway in XN-induced inhibitory effects, cells were pre-treated with PD and subjected to CCK-8 assays. Our findings elucidate that XN-treatment decreases cell proliferation while the PD effectively abolished this effect (Fig. 4C).

Xanthohumol treatment did not induce apoptosis in NG108-15 cells

To further determine whether the reduction in cell viability induced by XN via apoptosis, the cell apoptosis was assessed through Annexin V-FITC/PI assays. The flow cytometric analyses revealed that XN treatment did not obviously affect the Annexin V-FITC stained cells or PI-positive cells (Fig. 5A). Furthermore, in order to investigate whether XN treatment induces caspase 3-dependent apoptosis through p38 signaling pathway, immunofluorescence was used to detect Caspase 3. Treatment with XN (40 μ M, 24 h) with or without p38 inhibitor SB showed no significant difference versus control group (Fig. 5B).

Discussion

Chemotherapy is acknowledged for its efficacy in eradicating rapidly dividing cells, yet its administration is frequently accompanied by considerable adverse reactions. Therefore, the pursuit of chemotherapeutic drugs capable of selectively targeting cancer cells with minimal side effects remains a central concern in cancer research. XN is a natural product that has been previously documented to exhibit anti-apoptotic and anti-proliferative properties in various cancer cell types, including prostate cancer, breast cancer, and colon cancer⁵. In this investigation, we discovered that XN inhibited the cell growths in NG108-15 cells and explored the potential signaling pathways and mechanisms of its effects on glioma cells.

XN exhibited a significantly a dose-dependent decrease in the survival rate of NG108-15 cells, concurrently increasing the cell death rate. It has been reported that ROS is involved in different signaling pathways to control cell stability. Pertinently, when ROS exceeds the antioxidant defense system, cell stress occurs. This redox imbalance is closely related to the occurrence of tumors^{11–13}. Previous experimental findings have demonstrated that XN, a flavonoid compound, exhibits antioxidant properties. Conversely, contrasting experimental evidence has indicated a pro-oxidative role for XN^{8, 14}. In this study, our data showed that XN significantly increased ROS levels. N-acetyl-L-cysteine (NAC) alleviated both the XN-induced ROS and antiproliferation effect of XN on NG108-15 cells. Hence, the pro-oxidative impact of XN assumes a crucial role in the anti-proliferation of NG108-15 cells.

Mutations of cell cycle regulatory genes and malfunction of checkpoints controls are two main points leading to cell cycle deregulation^{15, 16}. Therefore, many conventional chemotherapeutic agents target cell cycle checkpoint arrest in cancer cells, thus inhibiting proliferation of cancer cells. Our data demonstrated that XN treatment arrest cell cycle at the G1/S phase in NG108-15 cells. XN was observed to induce cell cycle arrests at distinct phases, including G1, S and G2/M stages in different tumor cells¹⁷. The divergence in cell cycle arrests induced by XN is likely associated with tumor heterogeneity. It has been reported that p38 MAP kinases play a vital role in the G2/M phase in response to UV irradiation and ERK have a significant role in the G1/S phase^{18–20}. Therefore, we ran western blotting to see whether there was/were any activation(s) of extracellular regulated kinase (ERK), c-Jun NH-2 terminal kinase (JNK) and p38 MAPK, which belong to MAPKs family²¹. ERK has long been recognized as a cell proliferation signal, and many anticancer drugs induce apoptosis of cancer cells by activating the ERK MAP kinase signaling pathway^{22, 23}. Treatment with XN activated both p38 and ERK in NG108-15 cells. In this research, we discovered that only ERK signaling pathway plays a vital regulatory function in XN-treatment arrest cells in the G1/S cycle. The different types of cancer cells with different basal MAPK levels, we can call it: MAPK fitness zone. Once this zone is broken, cells will have a stress response. The hyperphosphorylation of ERK was checked in many lung cancer cell lines, it was shown that XN inhibited ERK signaling pathway to suppress cell growth of lung adenocarcinoma H1563 cell line. Moreover, XN induced apoptosis through improving the levels of phospho-ERK1/2 and phospho-JNK1/2 in MM cell lines U266 and RPMI 8226. ROS can trigger the signaling pathways related to cell migration and proliferation, including members of Mitogen-activated protein kinases (MAPK) signaling pathway^{24–26}. More research is required to determine whether XN-induced ROS activates the MAPK signaling pathway to inhibit cell proliferation.

Previous studies have shown that XN induced apoptotic responses in diverse cancer cell lines, including those derived from colon and leukemia^{27, 28}. Our previous study showed that XN treatment induce paraptosis, a caspase-independent cell death, in HL-60 cells, while it did not trigger typical apoptosis in B16-F10 cells²⁹. In our study, XN triggered apoptosis was not found by Western blotting and immunofluorescence analyses. According to Philippe P. Roux and John Blenis, most stimuli active p38 also can activate JNK, however, in this research, only activate p38³⁰. This might be the possible reason why p38 showed activated in the western blot, however no significance in cell cycle analysis and immunofluorescence.

In conclusion, we have discovered that XN can effectively inhibit NG108-15 cells proliferation and its molecular mechanism is probably by promoting the expression of ROS and activating ERK signaling pathway to block cell cycle in G1/S phase(Fig. 6). This finding will provide a new experimental and theoretical basis for XN clinical treatment of glioma. Since XN is a natural product, fewer side effects will occur. However, more research is needed to be conclusive about XN specifically and selectively target only cancer cells, but less or no effect on normal cells³¹. Obviously, there is much possibility and potential for the application of XN in future glioma cancer treatment.

Materials and methods

Drugs and Materials

Xanthohumol, with a purity exceeding 98%, was provided by Yumen Technology Development Company in Gansu, China. It was dissolved in DMSO to create a stock solution at a concentration of 120mM. The XN stock solution was further diluted to achieve the required final concentrations using culture medium. CCK-8 was procured from Biosharp (China), Sodium Dodecyl Sulphate (SDS) and Dimethyl Sulfoxide (DMSO) were acquired from Sigma Chemical (St. Louis, MO, USA). Additionally, 0.25% trypsin, penicillin, and Trypan blue were obtained from Solarbio (China). NG-108 medium (DMEM) and fetal bovine serum (FBS) were sourced from Biological Industries in Israel. The extracellular signal-regulated kinase (ERK) inhibitor PD98059 and the P-38 MAPK inhibitor SB203580 were purchased from MedChemExpress in New Jersey, USA. primary antibody against total JNK, phosphor-JNK, total ERK, phosphor ERK, total p38 MAPK, phosphor-p38 MAPK, total c-Jun, phosphor c-Jun and caspase 3 were purchased from Cell Signaling Technology (Beverly, MA). All other chemicals used in the study were of analytical grade.

Cell line and cell culture

The NG108-15 mouse/rat neuroblastoma/glioma hybrid cell line were purchased from the Chinese Academy of Sciences Culture Preservation Committee Cell Bank. Many physiological properties of NG108-15 cells are similar to glial and neurons cells. These cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 0.1 mM hypoxanthine, 400nM aminopterin, 0.016mM thymidine, 10% activated FBS with 1 unit/mL penicillin and 1.5g/L sodium bicarbonate. The cell line was maintained at 37°C in a humidified incubator with a mixture of 95% air and 5% CO₂.

In vitro CCK-8 assay and Trypan blue assay

Cell viability was assessed using the CCK-8 assay, while cell death was determined through the Trypan Blue assay.

For the CCK-8 assay, exponentially growing cells were resuspended in 1% FBS medium until the cell density reached approximately 1×10^8 cells/L. The XN-containing cells were then transferred to 96-well plate microculture plates (Costar, Corning, USA) with 3 parallel samples per group. After 24 h, 10 μ l CCK-8 reagent was added to each well and incubated for 2h. At last, the absorbance value (OD value, wavelength is 450nm) of each cell hole was detected by enzymic labeling instrument, and the OD value was analyzed to quantify the influence of the drug.

Trypan blue is based on the only dead cells that can be dyed blue. Cells with exponential growth were inoculated into 6-well plates in 10% FBS medium and cultured for 24 h. Subsequently, the cells were exposed to different XN doses. After 24 hours, the cells were washed and resuspended in 1% FBS medium until the cell density reached about 1×10^8 cells/L. A cell suspension of 90 μ l was taken, and 10 μ l of Trypan Blue was added. The mixture was collected in an EP tube and gently pipetted to ensure thorough mixing. After 3–5 minutes, the cell suspension was loaded into a hemocytometer, and the blue-stained cells were counted.

Determination of ROS level with DCFH-DA

The intracellular levels of reactive oxygen species (ROS) were assessed using ROS Assay Kit (Beyotime). briefly, cells were loaded with 5 μ M DCFH-DA (florescent probe) for 1h, then treated with different doses XN for 1 h in DMEM without serum. Finally, after centrifugation, cells were resuspended in 500 μ L PBS and immediately subjected to flow cytometric analysis. The cells treated with XN but without DCFDA were designated as blanks. The ROS production was quantified by measuring the fluorescence intensity relative to untreated control cells.

DNA Cell cycle analysis Propidium iodine (PI) staining

The NG108-15 cells were exposed to XN or DMSO in 1% FBS medium for 24h. Then the cells were trypsinized, centrifuged and washed twice using pre-cold PBS. The cell pellet was subsequently resuspended in 70% ethanol and incubated at 4 °C for 24 h. After this incubation, the cells were centrifuged, the pellet was washed twice using pre-cold PBS again, then centrifuged and incubated with 100 μ l RNase A (1 mg/ml final concentration) 30 min at 37 °C. Subsequently, the cells were treated with 400 μ l of propidium iodide (at a final concentration of 50 μ g/ml) for 1 hour before being analyzed using flow cytometry. Flow cytometry analysis was conducted using FACSCalibur (Becton Dickinson, NJ), and at least 10,000 cells per group were collected.

Immunofluorescence

18×18mm Coverslips (fisher brand, USA) were put into the 6 well plate, each well per slip. Exponentially growing NG108 cells were trypsinized and centrifuged then equal allocated to the 6-well plate using 1%FBS medium. After incubating 24 h at 37 °C, adding XN or DMSO into the plate then incubating another day at 37 °C. The cells were briefly rinsed with PBS on the next day. Then PBS was aspirated, and the cells were covered with 2–4% formaldehyde in PBS to a depth of 2-3mm, fixing for 15 minutes at room temperature. After that, the formaldehyde was aspirated, and the cells were rinsed three times in PBS for 5 mins each. Following formaldehyde fixation, the cells were covered with ice-cold 100% DMSO and incubated in DMSO for 10 minutes at -20°C, followed by a rise in PBS for 5 minutes. Next, the cells were blocked in blocking buffer for 60 mins. Then the blocking solution was aspirated, and diluted primary antibody caspase 3 was applied, followed by an overnight incubation at 4°C. On the next day, the cells were rinsed three times in PBS for 5 mins each. The cells were then incubated in a fluorochrome-conjugated secondary antibody, diluted in antibody dilution buffer, for 1–2 hours at room temperature without light. Then the cells were rinsed three times in PBS for 5 min each again. Coverslips were placed onto the slides using Prolong Gold Antifade Reagent, and the slides were sealed by applying nail polish around the edges of the coverslips. After that, the slides were examined using Fluorescence microscope.

Western blotting analysis and antibodies

Cells were plated in 6-well plates at a density of 3×10^5 cells per well and cultured overnight. Following the treatment of cells with either the vehicle (DMSO) or XN at specified times and concentrations, the cells were lysed in lysis buffer supplemented containing a protease and phosphatase inhibitor cocktail. Protein samples were checked using BCA protein assay kit (Servicebio) and denatured for 6 min at 98°C. The prepared samples were electrophoresed on a polyacrylamide gel (PAGE) and subsequently transferred to PVDF membranes (Millipore, USA). These membranes were blocked with 5% BSA for 1h, then incubated with corresponding primary antibodies at 4°C overnight. After washing three times by TBST buffer, the membranes were incubated with homologous secondary antibodies at room temperature and washed three times again. Western Bright ECL (Millipore, USA) and a multispectral imaging system (Protein Simple, USA) were employed for the development and exposure of the protein blots.

Apoptosis assay

NG108-15 cells were plated in 6-well dishes at a density of 3×10^5 cells per well and cultured overnight. Following this, the cells were treated with or without 40 μ M XN for 24 h. The cell suspension was meticulously formulated and subsequently subjected to staining using Annexin V-FITC and PI (Beyotime) for 30 minutes, adhering to the precise guidelines outlined by Beyotime Biotechnology manufacturers. The stained samples were analyzed by Becton Dickinson Fortessa SORP 14-color flow cytometer (USA).

Statistical analysis

All data are collected from different experiments and presented as the mean from independent experiments. The comparisons of data were used by ANOVA to assess statistical significant, $P < 0.05$ was considered significant.

Declarations

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Author contributions

C.H.: Investigation, formal analysis, writing original draft. J.T.: Investigation, methodology, funding acquisition. Writing review and editing. H.Y.: Resources. T.Z.: Resources. A.A.: Resources and supervision. X.M.: Conceptualization, project administration, Writing— review and editing, funding acquisition.

Funding

The work was supported by Shihezi University High-Level Talent Scientific Research Start-up Project (RCSX2018B01), Shihezi University High-Level Talent Scientific Research Project (KX018904), Shihezi University-level colleges independently support scientific research projects (ZZZC202127).

Competing interests

The authors declare no competing interests.

References

1. Louis, D. N.*et al.* The 2021 WHO Classification of Tumors of the Central Nervous System: a summary.*Neuro Oncol.* **23**(8), 1231-1251 (2021).
2. Weller, M.*et al.* European Association for Neuro-Oncology (EANO) guideline on the diagnosis and treatment of adult astrocytic and oligodendroglial gliomas.*Lancet Oncol.* **18**(6), e315-e329 (2017).
3. Miller, J. J.*et al.* Isocitrate dehydrogenase-mutant glioma: Evolving clinical and therapeutic implications.*Cancer.* **123**(23), 4535-4546 (2017).
4. Tripathi, S. K.*et al.* Emerging role of plumbagin: Cytotoxic potential and pharmaceutical relevance towards cancer therapy.*Food Chem Toxicol.* **125**, 566-582 (2019).
5. Harish, V.*et al.* Xanthohumol for Human Malignancies: Chemistry, Pharmacokinetics and Molecular Targets.*Int J Mol Sci.* **22**(9) (2021).
6. Chen, P. H.*et al.* The miR-204-3p-targeted IGFBP2 pathway is involved in xanthohumol-induced glioma cell apoptotic death.*Neuropharmacology.* **110**(Pt A), 362-375 (2016).
7. Festa, M.*et al.* Xanthohumol induces apoptosis in human malignant glioblastoma cells by increasing reactive oxygen species and activating MAPK pathways.*J Nat Prod.* **74**(12), 2505-13 (2011).
8. Wang, C. M.*et al.* Xanthohumol Induces ROS through NADPH Oxidase, Causes Cell Cycle Arrest and Apoptosis.*Oxid Med Cell Longev.* **2021**, 9877170 (2021).

9. Hou, S.*et al.* Xanthohumol-Induced Rat Glioma C6 Cells Death by Triggering Mitochondrial Stress.*Int J Mol Sci.* **22**(9) (2021).
10. Zhang, X. T.*et al.* Artematrolide A inhibited cervical cancer cell proliferation via ROS/ERK/mTOR pathway and metabolic shift.*Phytomedicine.* **91**, 153707 (2021).
11. Girisa, S.*et al.* Xanthohumol from Hop: Hope for cancer prevention and treatment.*IUBMB Life.* **73**(8), 1016-1044 (2021).
12. Moloney, J. N. and Cotter, T. G. ROS signalling in the biology of cancer.*Semin Cell Dev Biol.* **80**, 50-64 (2018).
13. Zhang, B.*et al.* Xanthohumol induces generation of reactive oxygen species and triggers apoptosis through inhibition of mitochondrial electron transfer chain complex I.*Free Radic Biol Med.* **89**, 486-97 (2015).
14. Liu, X.*et al.* Xanthohumol inhibits PRRSV proliferation and alleviates oxidative stress induced by PRRSV via the Nrf2-HMOX1 axis.*Vet Res.* **50**(1), 61 (2019).
15. Chen, M.*et al.* Capsaicin Inhibits Proliferation and Induces Apoptosis in Breast Cancer by Down-Regulating FBI-1-Mediated NF- κ B Pathway.*Drug Des Devel Ther.* **15**, 125-140 (2021).
16. Xie, H.*et al.* Ethanolic extract of Cordyceps cicadae exerts antitumor effect on human gastric cancer SGC-7901 cells by inducing apoptosis, cell cycle arrest and endoplasmic reticulum stress.*J Ethnopharmacol.* **231**, 230-240 (2019).
17. Tuli, H. S.*et al.* Xanthohumol: A Metabolite with Promising Anti-Neoplastic Potential.*Anticancer Agents Med Chem.* **22**(3), 418-432 (2022).
18. Shao, J.*et al.* Involvement of the arachidonic acid cytochrome P450 epoxygenase pathway in the proliferation and invasion of human multiple myeloma cells.*PeerJ.* **4**, e1925 (2016).
19. Wang, Z. Regulation of Cell Cycle Progression by Growth Factor-Induced Cell Signaling.*Cells.* **10**(12) (2021).
20. Keyvanloo Shahrestanaki, M.*et al.* Centaurea cyanus extracted 13-O-acetylsolstitialin A decrease Bax/Bcl-2 ratio and expression of cyclin D1/Cdk-4 to induce apoptosis and cell cycle arrest in MCF-7 and MDA-MB-231 breast cancer cell lines.*J Cell Biochem.* **120**(10), 18309-18319 (2019).
21. Huang, C.*et al.* JNK phosphorylates paxillin and regulates cell migration.*Nature.* **424**(6945), 219-23 (2003).
22. Forma, E. and Bryś, M. Anticancer Activity of Propolis and Its Compounds.*Nutrients.* **13**(8) (2021).
23. Zingales, V.*et al.* Sterigmatocystin-induced DNA damage triggers cell-cycle arrest via MAPK in human neuroblastoma cells.*Toxicol Mech Methods.* **31**(7), 479-488 (2021).
24. Cheng, S. C.*et al.* Quercetin Inhibits the Production of IL-1 β -Induced Inflammatory Cytokines and Chemokines in ARPE-19 Cells via the MAPK and NF- κ B Signaling Pathways.*Int J Mol Sci.* **20**(12) (2019).
25. Liu, X.*et al.* Xanthohumol chalcone acts as a powerful inhibitor of carcinogenesis in drug-resistant human colon carcinoma and these effects are mediated via G2/M phase cell cycle arrest, activation

- of apoptotic pathways, caspase activation and targeting Ras /MEK/ERK pathway.*J buon.* **24**(6), 2442-2447 (2019).
26. Yue, J. and López, J. M. Understanding MAPK Signaling Pathways in Apoptosis.*Int J Mol Sci.* **21**(7) (2020).
 27. Grudzień, M.*et al.* The Effect of Xanthohumol Derivatives on Apoptosis Induction in Canine Lymphoma and Leukemia Cell Lines.*Int J Mol Sci.* **24**(14) (2023).
 28. Pan, L.*et al.* Xanthohumol induces apoptosis in cultured 40-16 human colon cancer cells by activation of the death receptor- and mitochondrial pathway.*Mol Nutr Food Res.* **49**(9), 837-43 (2005).
 29. Mi, X.*et al.* Xanthohumol induces paraptosis of leukemia cells through p38 mitogen activated protein kinase signaling pathway.*Oncotarget.* **8**(19), 31297-31304 (2017).
 30. Han, J.*et al.* An overview of mammalian p38 mitogen-activated protein kinases, central regulators of cell stress and receptor signaling.*F1000Res.* **9** (2020).
 31. Vesaghamedani, S.*et al.* Xanthohumol: An underestimated, while potent and promising chemotherapeutic agent in cancer treatment.*Prog Biophys Mol Biol.* **172**, 3-14 (2022).

Figures

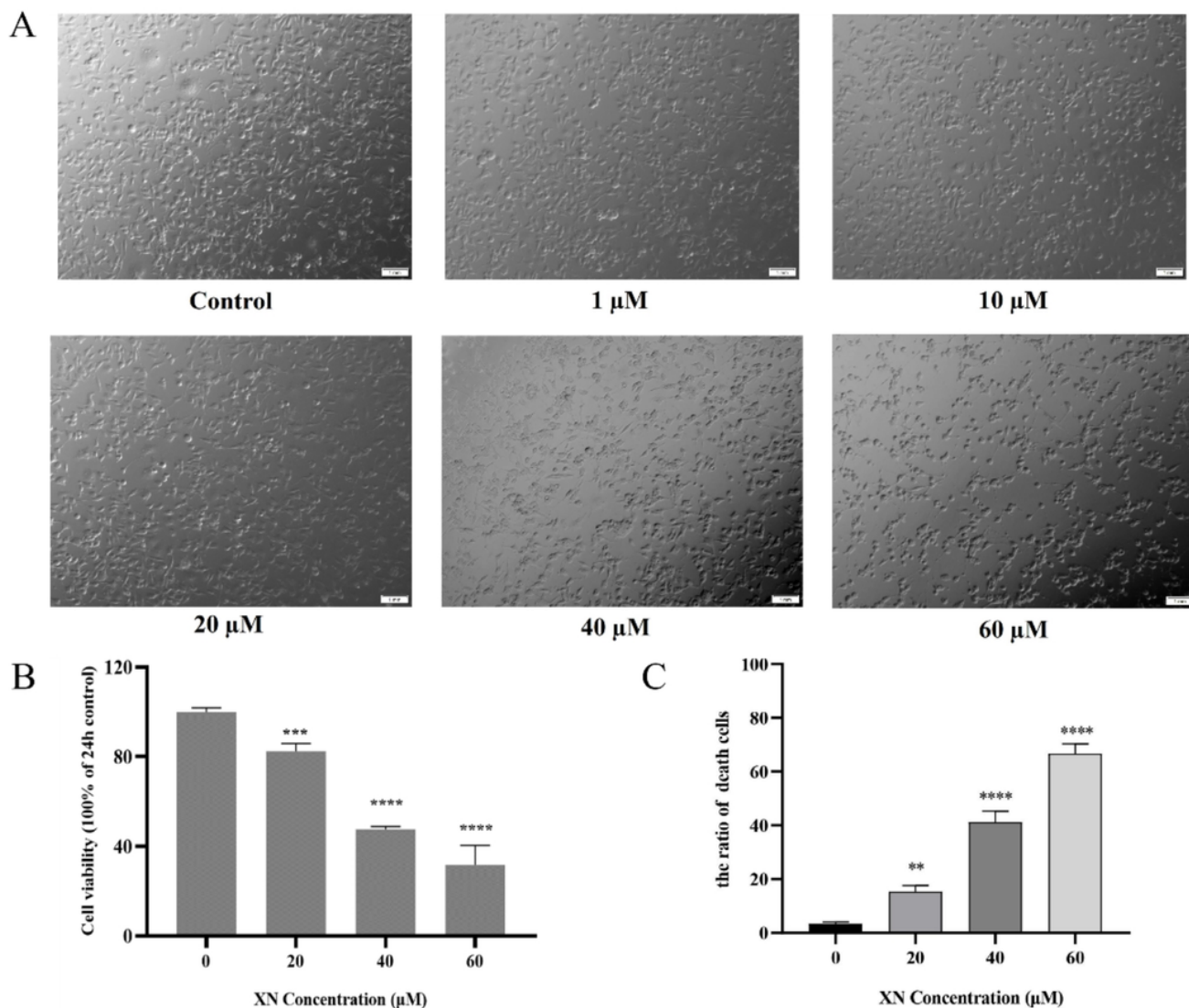


Figure 1

Effect of XN on cell viability. **(A)** The effect of XN on cell viability of NG108-15 cells after 24h treatment. The alterations in cellular morphology were examined under light microscope. **(B)** Cell viability was determined by Cell Counting Kit-8 assay. **(C)** Cell death was determined by using Trypan blue. Data is expressed as the mean $\pm$ S.E. from at least three experiments. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, compared with Control, one-way ANOVA test.

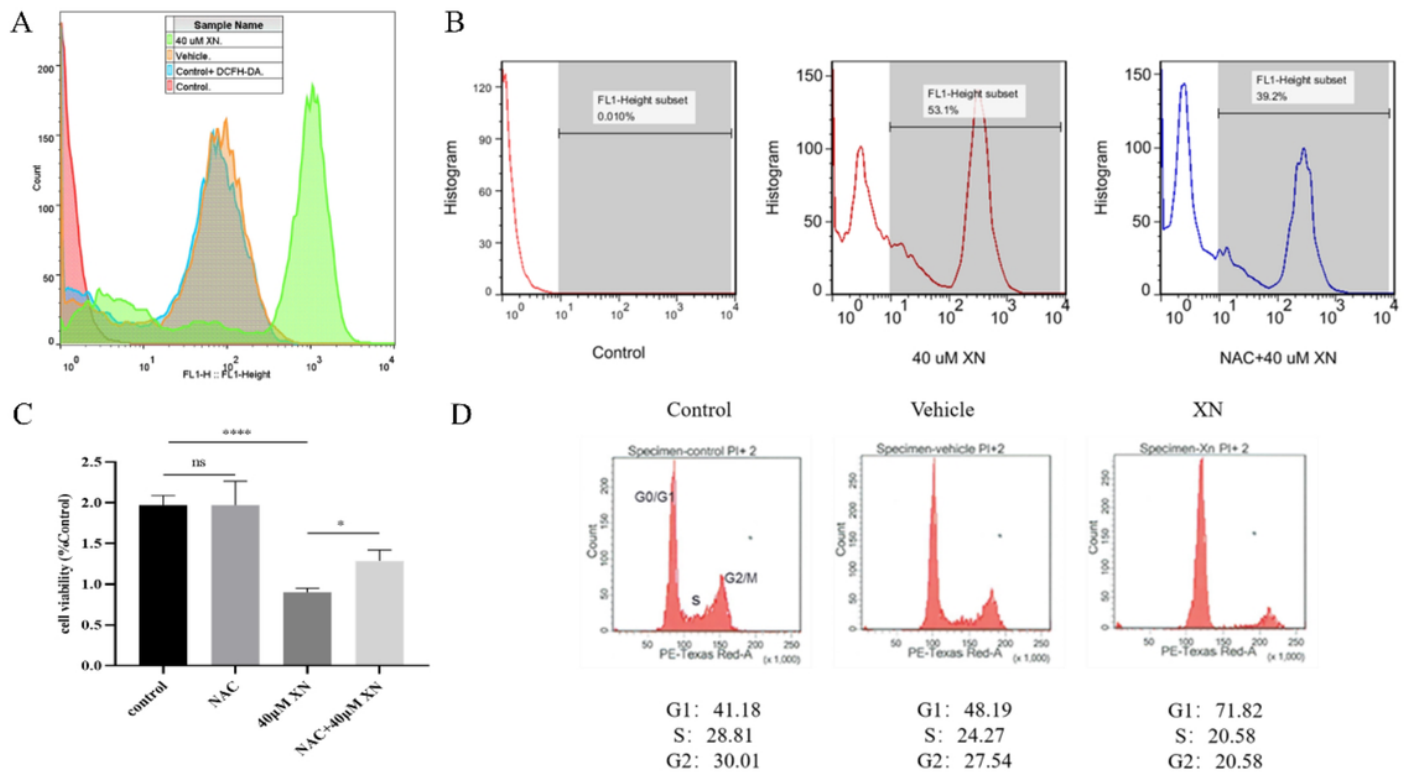


Figure 2

Effect of XN on the level of ROS and cell cycle in NG108-15 cells. **(A)** Intracellular ROS levels were measured by fluorescent probe DCFH-DA after 1 h treatment with DMSO or XN (40 μ M). Flow cytometry histograms showed ROS generated in NG108-15. **(B)** The effect of NAC (10 μ M) on ROS after treated with XN (40 μ M) for 0.5 h. **(C)** NG108-15 cells were pretreated with NAC (10 μ M), then added XN (40 μ M) for 24h. The percentage of viable cell counted using CCK-8. **(D)** Cells treated with DMSO or XN (40 μ M, 24h), were collected and stained with propidium iodide after incubation with RNaseA. Analysis was done by flow cytometry on a FACSCaliber. All data are presented as the mean $\pm$ S.E. from at least three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, compared with Control, one-way ANOVA test.

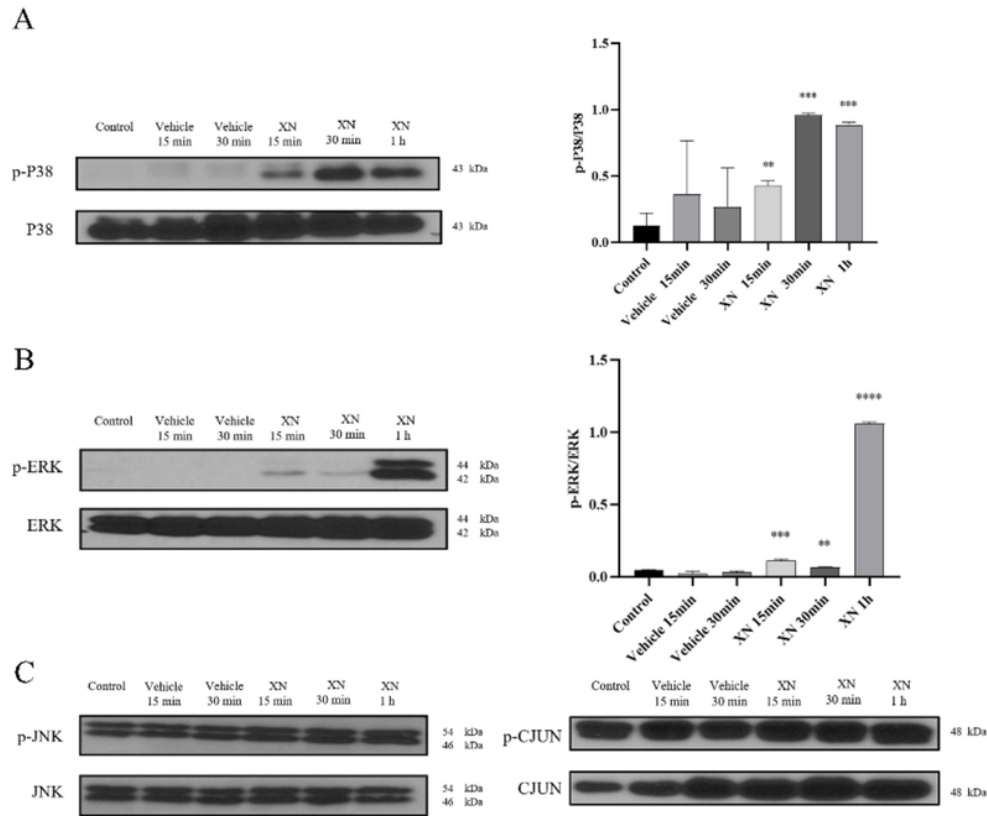


Figure 3

Effect of XN on MAPK in NG108-15 cells. **(A)** Cells were treated for 15 min, 30 min and 60 min with XN at a dosage of 40 μ M then harvested. The western blots shown are representative of p-p38 with mean $\pm$ S.E. Bar graphs show relative p-p38 levels. **(B)** Cells were treated for 15min, 30min and 60min with XN at a dosage of 40 μ M, then harvested. The western blots shown are representative of p-ERK with mean $\pm$ S.E. Bar graphs show relative p-ERK levels. **(C)** Cells were treated for 15min, 30min and 60min with XN at a dosage of 40 μ M then harvested. The western blots shown are representative of p-JNK and p-CJUN with mean $\pm$ S.E. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, compared with Control, one-way ANOVA test.

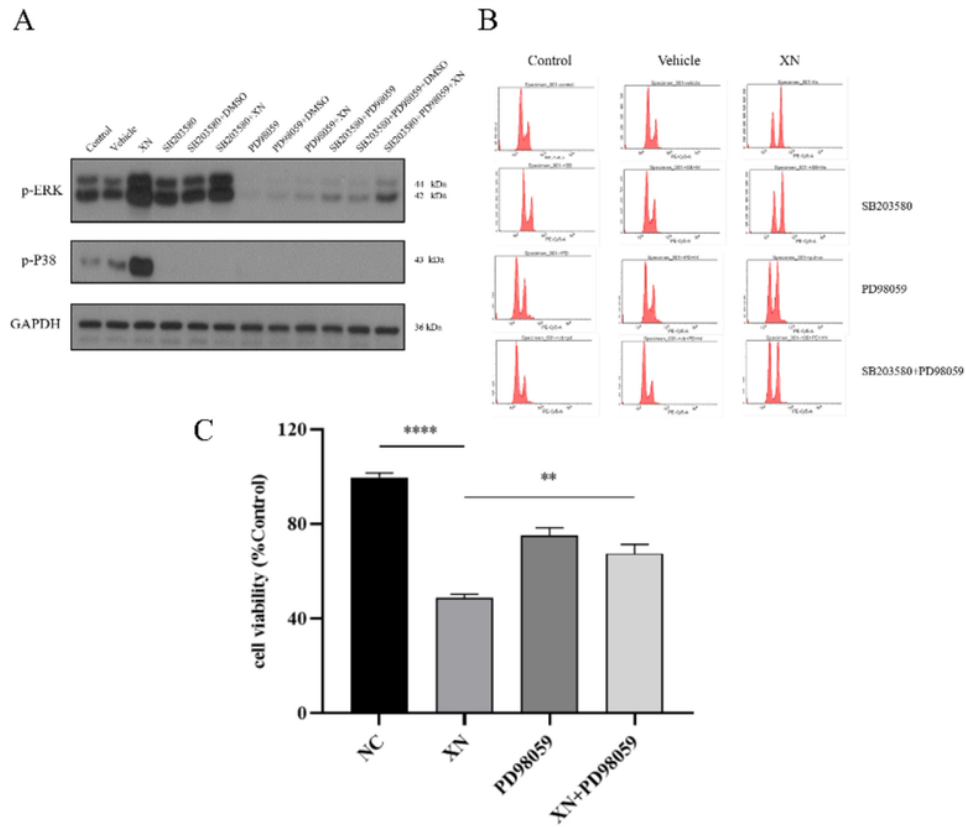


Figure 4

Effect of XN and inhibitors on NG108-15 cell cycle distribution. **(A)** Cells were pretreated for 30min with p38 inhibitor SB203580, P-ERK inhibitor PD98059 or both for 30min, then treated with DMSO or XN at a dosage of 40 μ M then harvested. **(B)** Cells were pretreated for 30min with ERK inhibitor PD98059 or p38 inhibitor SB203580 for 30min, then treated with DMSO or XN (40 μ M, 24h) were collected and stained with propidium iodide after incubation with RNaseA. Analysis was done by flow cytometry on a FACSCalibr and **(C)** the cell viability of NG108-15 cells was measured by trypan blue staining. All data are presented as the mean $\pm$ S.E. from at least three independent experiments. * P < 0.05, ** P < 0.01, **** P < 0.0001, compared with Control, one-way ANOVA test.

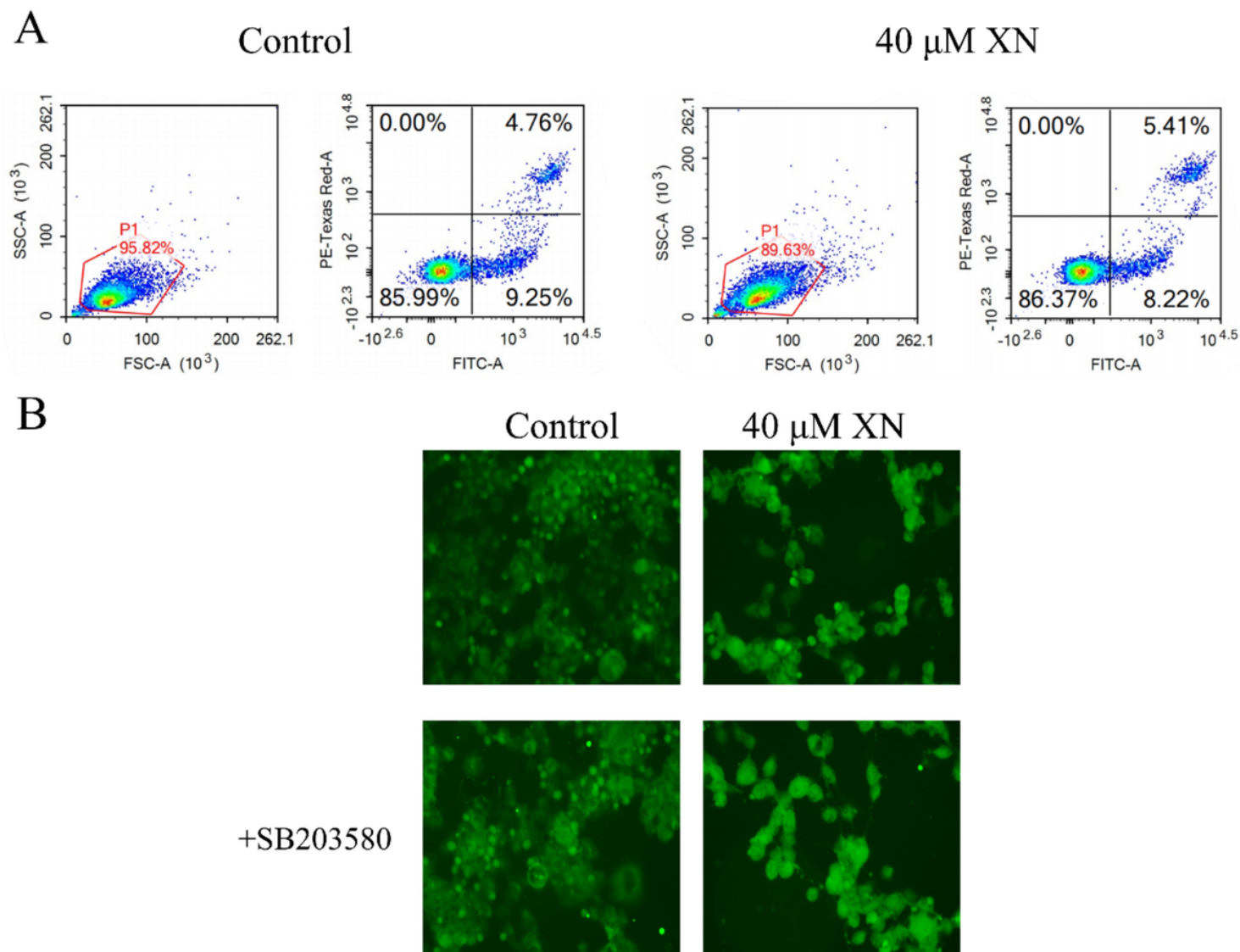


Figure 5

Effect of XN on apoptotic responses in NG108-15 cell. **(A)** Apoptosis in NG108-15 cells was assessed using flow cytometry after 24 h treatment with XN. **(B)** Cells were treated with DMSO or XN (40 μ M, 24h). The first line treated without p38 inhibitor; the second line treated with p38 inhibitor (SB203580). Caspase 3 was used as the primary antibody in order to see the apoptosis. Immunofluorescence was used to determine XN-induced apoptosis. All data are presented as the mean $\pm$ S.E. from at least three independent experiments.

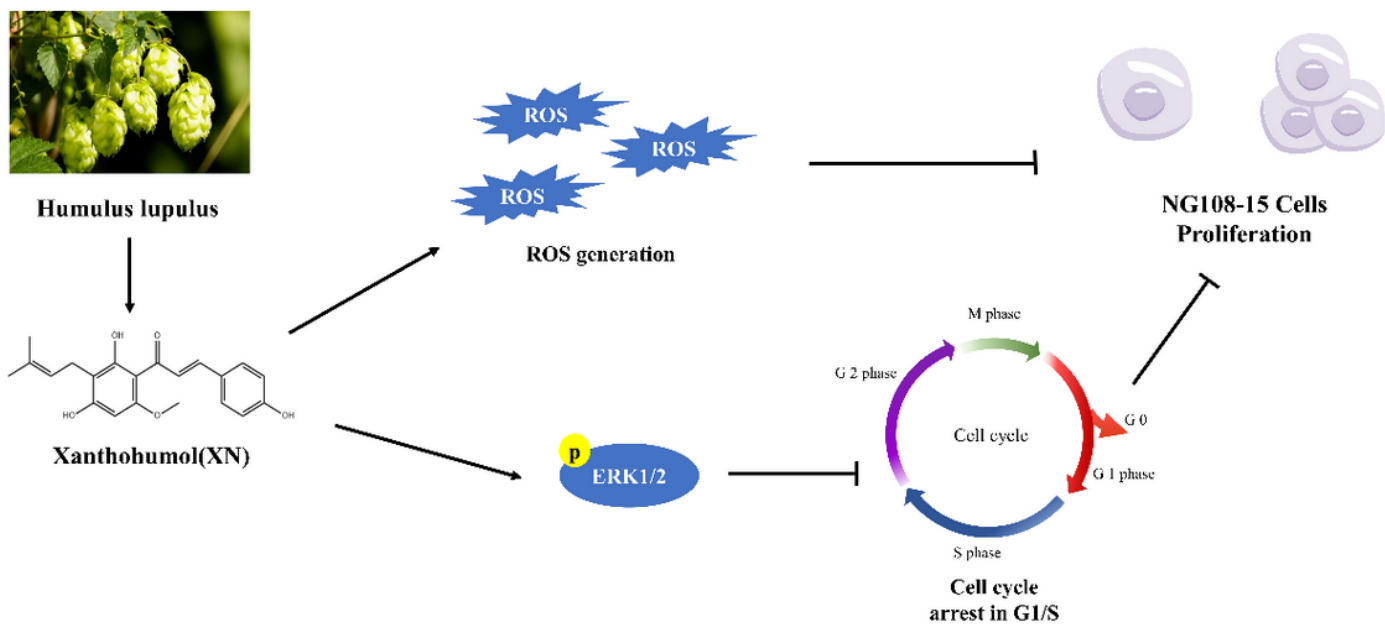


Figure 6

The proposed model of XN- treatment inhibits the proliferation of NG108-15 cells. XN-treatment inhibits cell proliferation via increasing reactive oxygen species (ROS) levels. XN triggered the phosphorylation of ERK1/2, which leads to G1/S cell cycle arrest.