

Original Article

Running Title: N-Nornuciferine Promotes A549 Apoptosis

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N-Nornuciferine Promotes Human Non-Small Cell Lung Cancer A549 Cells Apoptosis via miR-361-3p/TRAF2/JNK Axis

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Abstract

Background: N-Nornuciferine (N-NF) is an aporphine alkaloid in lotus leaf with a good resource for obtaining the biologically active substances with antioxidant properties. The purpose of this study was to explore the effect of N-NF on cell viability and apoptosis in A549 cells in vitro culture and to explore its mechanism of action.

Method: In this experimental study, we cultured A549 cells in vitro and added different concentrations of N-NF for intervention to explore cell apoptosis and its mechanism. Cell viability was detected by CCK-8, and cell apoptosis level was detected by FCM. The mechanism of action of N-NF was explored by regulating the expression of miR-361-3p. The data were analyzed using the one-way analysis of variance (ANOVA) technique in SPSS 22.0 software, with statistical significance set at a level of $P < 0.05$.

Results: The study results showed that N-NF could significantly inhibit the viability of A549 cells and promote apoptosis. Using dual-luciferase reporter gene and molecular biology detection experiments, it was found that N-NF inhibits the viability of A549 cells through inhibiting the expression of miR-361-3p and promoting the TRAF2/JNK pathway.

Conclusion: This study provides a basis for the development of N-NF as an anticancer drug and explains its mechanism of promoting apoptosis of A549 cells.

Keywords: MicroRNAs, Apoptosis, Carcinoma, Non-small-cell lung

Introduction

Many studies in recent years have shown that adding natural plants components in the treatment of cancer can have many beneficial effects on cancer treatment, such as reducing the side effects of radiotherapy and chemotherapy,¹ inhibiting drug resistance of cancer cells,² and directly acting on signaling pathways and kill cancer cells.³ Lotus leaf was widely used in traditional Chinese medicine.⁴ A network pharmacology analysis and review of the nuciferine component extracted from lotus leaf revealed extensive anti-obesity, anti-inflammatory and anti-

tumor effects.⁵ Lotus leaf flavonoids extracted from lotus leaves could promote the apoptosis of human lung cancer A549 cells through the ROS/p38 MAPK pathway.⁶ Lotus leaf was often added as one of the main active ingredients in traditional Chinese medicine to treat cancer.^{7,8} Lotus leaf ethanol extract has strong antioxidant, anti-inflammatory and cancer cell-specific cytotoxicity characteristics, showing strong MCF-7 and Jurkat cell toxicity.⁹ N-Nornuciferine (N-NF) were an aporphine alkaloid in lotus leaf¹⁰ and were a good resource for obtaining the biologically active

substances with antioxidant properties.¹¹ A pharmacokinetic study showed that after oral administration and injection of Nuciferine (NF) and N-NF to SD rats, the drug distribution and half-life in the plasma and brain of rats were observed.¹² NF and N-NF were found to have a relatively wide volume of distribution and slow elimination half-life. It was found that N-NF has an effective inhibitory effect on CYP2D6 isoenzyme.¹³ Natural small molecule compounds with high affinity to Traf2 and Nck-interacting kinase (TNIK) exert tolerance to CYP2D6, suggested that related substances that inhibit CYP2D6 may exert pharmacological effects through the TRAF2 pathway.¹⁴

The tumor necrosis factor receptor-associated factor 2 (TRAF2) was a second messenger adaptor protein that plays an essential role in propagating TNF- α -mediated signaling pathways.¹⁵ In recent years, several studies have found that TRAF2 plays an important role in the occurrence and development of lung cancer;¹⁶ also, it was found to be a key specific receptor that plays an important role in canonical NF- κ B and JNK MAPK signaling.¹⁷ CSTMP induced apoptosis of A549 cells were through IRE1 α -TRAF2-ASK1 complex-mediated ER stress, JNK activation, and mitochondrial dysfunction.¹⁸ Through high-throughput sequencing and bioinformatics analysis, researchers found that miR-361-3p may be involved in the regulation of TRAF2 expression and affect the downstream NF- κ B pathway, thereby affecting cell apoptosis.¹⁹ Researchers used in vitro experiments to construct an LPS-induced HMC3 cell apoptosis and inflammation model and found that miR-361-3p can inhibit the transcription and translation of TRAF2 by binding to TRAF2.²⁰ It was found that miR-361-3p has a binding relationship with both circ_0058357 and ABCC1, and inhibits the expression of ABCC1 by binding to ABCC1 3'UTR. miR-361-3p-mediated inhibition of

ABCC1 affects the growth, invasion, migration, apoptosis and sensitivity to DDP of H1299/DDP and A549/DDP cells. In addition, circ_0058357 can regulate the expression of ABCC1 by competitively binding to miR-361-3p.²¹ There may be an important regulatory role between miR-361-3p and TRAF2, but whether this role exists in the occurrence and development of non-small cell lung cancer has not yet been reported.

To the best of our knowledge, no study has yet addressed the impact of N-NF on the in vitro culture of lung cancer cells. In this study, we demonstrated the effect of N-NF on A549 cell apoptosis through in vitro cell experiments and explored the signaling pathways through which it may play a role, providing a reference for exploring the pharmacological mechanism of anti-tumor effects of N-NF.

Materials and Methods

Study design

In this experimental study, we directly added different concentrations of N-NF to the culture medium during the in vitro culture of non-small cell lung cancer cell line A549 cells in order to observe the effect of N-NF on A549 cell apoptosis. During the experiment, we also used molecular biology techniques to detect the apoptosis phenotype and the expression of related genes. The interaction between miR-361-3p and TRAF2 gene in A549 cells was detected using dual-luciferase reporter gene detection technology.

Cell culture

A549 cell were purchased from National collection of authenticated cell cultures, serial number: SCSP-503. The cell culture medium was F-12K (Gibco, 21127022), supplemented with 10% fetal bovine serum FBS (Gibco, 10099-141). Cells were cultured in vitro in a cell culture incubator, and the environmental conditions were 37.5°C, 5% CO₂, and saturated humidity.

IC50 calculation of N-Nornuciferine on A549 cells

By setting the N-Nornuciferine (N-NF) (MCE, HY-N2129) concentration gradient according to the compound description (0, 1, 2, 4, 8, 16 μM), after continuous intervention of A549 cells for 48 hours during in vitro culture, the CCK-8 reagent (Sigma, 96992) was used to detect cell viability. According to the effects of different concentrations of N-NF on the viability of A549 cells, the IC50 value was calculated using GraphPad Prism 8.0 software.

Experimental design

In order to investigate the effect of N-NF on A549 culture in vitro, based on the experimental results of IC50 (through experiments and calculations, we found that the IC50 concentration of N-NF on A549 cultured in vitro for 48 hours was 8.641 μM), we set the N-NF concentration gradient as N-NF(H): 16 μM , N-NF(M): 8 μM , N-NF(L): 4 μM . At the same time, we set 10 μM Cisplatin (CDDP, MCE, HY-17394) as a positive control group.²² To investigate the impact of regulating miR-361-3p on A549, we used hsa-miR-361-3p mirVana® miRNA mimic (miR-361-3p mimic, Gibco, 4464066) to up-regulate the expression level of miR-361-3p and use hsa-miR-361-3p mirVana® miRNA inhibitor (miR-361-3p inhibitor, Gibco, 4464084) to down-regulate the expression level of miR-361-3p.

Cell viability assay

After adjusting the A549 cell density to 2000 cells/well, we spread the cells into a 96-well cell culture plate, added CDDP and different concentrations of N-NF according to the experimental design, and then incubated at 37.5°C under 5% CO₂, for in vitro culture. At 0, 12, 24, 36, and 48 hours after the start of in vitro culture, the cell viability of each group were detected using the CCK-8 kit, and the calculated cell viability percentage data were plotted using GraphPad Prism 8.0 software.

PI and Hoechst 33258 staining

PI (Invitrogen, P1304MP) and Hoechst 33258 (Invitrogen, H1398) were used to perform fluorescent staining on A549 cells in each group to detect cell apoptosis. After staining according to the instructions of the kit, take pictures using an inverted fluorescence microscope.

Flow Cytometry (FCM) analysis

eBioscience™ Annexin V Apoptosis Detection Kit (Invitrogen, BMS500FI-300) were used to detect the apoptosis of A549 cells in each group. After collecting and staining cells according to the kit operating manual, use the Invitrogen Attune™ NxT flow cytometer for detection and data collection, and use FlowJo V10.0 software for data analysis.

RNA extraction, reverse transcription and real-time quantitative polymerase chain reaction detection (qPCR)

TaKaRa MiniBEST Universal RNA Extraction Kit (Takara, 9767) were used to extract total RNA from cells in each group. RNA concentration and purity were measured using the Thermo Scientific™ NanoDrop™ One microvolume UV-Vis spectrophotometer. Subsequently, the samples used for miRNA detection were subjected to reverse transcription and qPCR detection operations according to the instructions of the Mir-X miRNA First-Strand Synthesis Kit (Takara, 638315). Then, the full-length sequence of miR-361-3p (5'-UCCCCCAGGUGUGAUUCUGAUUU -3') was used as the forward primer for qPCR detection, and the reverse primer used the mRQ 3' Primer provided in the kit, and use the U6 forward and reverse primers provided in the kit to detect the expression level of the internal reference gene U6.²³ The total RNA samples used for gene mRNA expression detection were grouped and reverse transcribed to obtain double-stranded cDNA according to the requirements of PrimeScript™ RT Master Mix (Takara,

RR036). Then, prepare the qPCR reaction system according to the instructions of the TB Green® Fast qPCR Mix (Takara, RR430) kit. The name of the gene to be analyzed and the primer sequence information are listed in table 1. The primers used in qPCR were synthesized and quality controlled by Invitrogen. The qPCR detection process was performed on a Roche Lightcycler 480 II qPCR instrument. After obtaining the Ct value, the $2^{-\Delta\Delta Ct}$ method were used for data analysis. The housekeeping gene GAPDH was used as an internal control to normalize the relative gene.

Dual luciferase assay

The target gene of miR-361-3p were confirmed by 3'-untranslated region (UTR) luciferase, wherein the wide-type (WT, 5'-GCCCCCUACUGGUGUCUGGGGU -3'), and mutant (MU, 5'-GCCCCCUACUGGUGUGACCCCU -3') 3'-UTR of TRAF2 were cloned into the downstream of a luciferase reporter gene in the pmirGLO vector (Promega, USA). Cells were co-transfected with luciferase constructs and miR-361-3p mimic. After 48 hours, a dual-luciferase reporter assay system (Promega, USA) system was used to study the luciferase activity.

WesternBlot

All groups of cells were lysed in RIPA-PMSF protein extraction reagent (Invitrogen, 89900). The subsequent SDS-PAGE gel preparation, electrophoresis and membrane transfer processes refer to the experimental steps in our previously published paper. The secondary antibody used in the experiment were Anti-GAPDH antibody (Abcam, ab9485), Anti-JNK1 antibody (Abcam, ab199380), Anti-TRAF2 antibody (Abcam, ab126758). The secondary antibody used in the experiment was Goat Anti-Rabbit IgG H&L HRP (Abcam, ab6721). Then developed with ECL exposure solution, imaged and photographed in a multi-function imager. ImageJ 7.0 software was used to read

the band grey value, and GAPDH was used as the internal reference genes to normalize the target protein expression.

Statistical analysis

The statistical analysis of all data in the present study were performed using SPSS 22.0 software. The obtained experimental data were expressed as standard mean deviation (mean \pm SD), and a single-factor ANOVA were employed for inter-group comparisons, while the Scheffe's post-hoc test was used for pairwise comparison. Statistical significance was determined at a P -value ≤ 0.05 .

Results

Effect of N-Nornuciferin on A549 cell activity

The results showed that the IC50 concentration of N-NF was 8.641 μ M (Figure 1B). The cell activity monitoring results showed that with CDDP as compared with the two groups, there was no significant difference ($P > 0.05$) in cell viability between the N-NF(M) and the N-NF(H) groups (Figure 1C, Table 2).

N-Nornuciferine promotes A549 cell apoptosis by activating TRAF2/JNK pathway

We detected the expression of PI to determine the apoptosis of A549 cells in each group, and found that the number of PI-positive cells was higher in the CDDPCDDP and N-NF(H) groups (Figure 2A). It was found that in comparison with the control group, the proportion of cells in the CDDPCDDP, N-NF(H) and N-NF(M) groups significantly increased ($P < 0.05$), but there was no significant difference in the N-NF (L) group ($P > 0.05$) (Figure 2B, C). qPCR results showed that in comparison with the Control group, cell apoptosis-related genes (COX2, Caspase-3, BAX, JNK and TRAF2) were significantly up-regulated ($P < 0.05$) (Figure 2D, Table 3). WesternBlot results showed that as compared with the Control group, the

expression levels of TRAF2 and JNK proteins in the CDDPCDDP and N-NF(H) groups significantly increased ($P < 0.05$) (Figure 2E).

Effect of N-Nornuciferine on miR-361-3p and TRAF2/JNK pathway

The qPCR results showed that as compared with the Control group, the expression level of miR-361-3p in the CDDP, N-NF(H) and N-NF(M) groups were significantly down-regulated ($P < 0.05$) (Figure 3A). Dual-luciferase detection results showed that miR-361-3p can bind to 7 base sites in the 3'UTR region of TRAF2 and inhibit TRAF2 expression (Figure 3B). The results showed that as compared with the miR-361-3p mimic group, the expression level of miR-361-3p in the miR-361-3p mimic+N-NF group was significantly down-regulated ($P < 0.05$). As compared with the Control group, the expression levels of miR-361-3p in the N-NF group and the miR-361-3p inhibitor group were significantly down-regulated ($P < 0.05$) (Figure 3C). As shown in figure 3D, WB detection found that as compared with the Control group, adding miR-361-3p mimic significantly down-regulated the protein expression levels of TRAF2 and JNK ($P < 0.05$). The protein expression level of TRAF2 in the miR-361-3p mimic+N-NF group was significantly higher than that in the miR-361-3p mimic group ($P < 0.05$), but there was no significant difference from the Control group ($P > 0.05$).

Discussion

Our findings demonstrated a significant inhibitory effect of N-NF on the proliferation of A549 cells coupled with an induction of apoptotic processes. Through the employment of dual-luciferase reporter gene assays and molecular biology methodologies, it was found that the underlying mechanism of N-NF's antiproliferative action on A549 cells entails the suppression of miR-361-3p expression and the facilitation of the

TRAF2/JNK signaling cascade. This investigation furnishes foundational insights into the potential utilization of N-NF as a therapeutic agent against cancer and elucidates its apoptotic mechanisms targeting A549 cells.

In our study, we found that N-NF can also effectively promote the apoptosis of A549 cells in vitro, with an IC₅₀ concentration value of 8.641 μ M after 48 hours of action. By setting a concentration gradient, we found that 8 μ M and 16 μ M N-NF exerted almost the same effect as CDDP on inhibiting A549 cell activity (Figure 1). And in the study of the effect of N-NF on A549 cell apoptosis, we found that there was no significant difference between 16 μ M N-NF and 10 μ M CDDP in inducing apoptosis of A549 cells ($P > 0.05$). As compared with the control group, although 8 μ M N-NF can also significantly increase the apoptosis level of A549 cells ($P < 0.05$), it was still less efficient than the 16 μ M N-NF and the 10 μ M CDDP groups ($P < 0.05$). The results of qPCR targeting apoptosis-related genes also confirmed the results of FCM (Figure 2B, C, D). Natural products, as substances widely found in nature, have made significant contributions to the treatment of diseases in the history of human development.²⁴⁻²⁶ With the continuous advancement and development of natural product purification and analysis technology, more and more new natural products are being re-recognized.²⁷ These results indicated that N-NF can only exert its apoptosis-inducing effect at higher concentrations, and at the IC₅₀ concentration, it can only inhibit the activity of A549 cells but cannot significantly induce cell apoptosis.

In order to analyze the mechanism and pathway of N-NF-induced apoptosis in A549 cells from a deeper level, we chose the TRAF2/JNK pathway for study. Our results showed that in comparison with the control group, the expression level of TRAF2 protein was significantly up-regulated under the

intervention of 16 μM N-NF ($P < 0.05$), but the expression level of JNK only showed an up-regulation and did not show significant difference ($P > 0.05$) (Figure 2E). In previous studies, researchers only found that N-NF has an effective inhibitory effect on CYP2D6 isoenzyme.¹³ The CYP2D6 gene encodes the CYP2D6 enzyme. The CYP2D6 enzyme is an important member of the cytochrome P450 system and a key enzyme in the metabolism of various drugs. Its polymorphism has a significant regulatory effect on the body's drug metabolism rate.²⁸ Due to the mechanism of action of N-NF in inhibiting CYP2D6, during previous studies, researchers have discovered a mutually exclusive relationship between CYP2D6 and the IRE1 α /TRAF2/NF- κ B pathway,²⁹ These results suggest that the function of N-NF in inducing cell apoptosis may be closely related to its function in regulating TRAF2. In recent years, studies have found that the TRAF2/JNK pathway plays a key role in the process of apoptosis. It was found that inhibiting IRE1 α /TRAF2/JNK can effectively protect neurons from damage caused by cerebral ischemia-reperfusion.³⁰ These results indicated that N-NF can promote the expression of TRAF2 protein, but its downstream JNK regulatory effect showed no significance. Our results showed that 16 μM and 8 μM N-NF and 10 μM CDDP can significantly downregulate the expression level of miR-361-3p. Combined with the analysis of TRAF2 expression, we speculated that there is a mutually exclusive relationship between miR-361-3p and TRAF2 in A549 cells. The results of the dual-luciferase assay confirmed our conjecture and were consistent with the results of previous researchers (Figure 3A, B).^{19,20}

In order to confirm the inhibitory effect of N-NF on miR-361-3p, we overexpressed and inhibited the expression of miR-361-3p in A549 cells, and then used 16 μM N-NF for intervention after overexpressing miR-361-

3p. Our results showed that after overexpression of miR-361-3p, the expression levels of TRAF2 and JNK proteins were significantly inhibited ($P < 0.05$). However, when N-NF was added while overexpressing miR-361-3p, the expression level of miR-361-3p was significantly down-regulated ($P < 0.05$), and the expression level of TRAF2 were significantly up-regulated ($P < 0.05$) (Figure 3C, D). Our results showed that N-NF can significantly inhibit the expression of miR-361-3p in cells, and its inhibitory effect is equivalent to the level of miR-361-3p inhibitor ($P > 0.05$). As an important regulatory element in the TNF receptor-associated factors family, TRAF2 interacts with a variety of proteins and were closely related to the regulation of cell proliferation and apoptosis.³¹ Previous studies have shown that TRAF2 is susceptible to the regulation of miRNA, causing changes in its transcription level, thereby affecting its function.³² During our preliminary research, we found an interaction between miR-361-3p and TRAF2, so in the process of studying the pharmacological mechanism of N-NF, we selected miR-361-3p as a target to analyze the mechanism of N-NF regulating the TRAF2/JNK pathway. The above results confirmed that N-NF promotes the expression of TRAF2 by inhibiting the expression of miR-361-3p.

While our study confirmed that N-NF can induce apoptosis in A549 cells by suppressing the expression of miR-361-3p and activating the TRAF2/JNK pathway, we acknowledge that due to time constraints, we did not conduct animal experiments. Therefore, there are numerous unresolved issues awaiting resolution in the subsequent drug development and clinical trials of N-NF. Based on this study, our research team will continue to refine experimental designs and conduct more systematic pharmacological studies to elucidate the mechanism of action of N-NF.

Conclusion

We added different concentrations of N-NF during the in vitro culture of human non-small cell lung cancer cells A549 to explore its effect on the activity and apoptosis of A549 cells. The results showed that 8 μ M and 16 μ M N-NF could significantly inhibit the cell viability of A549 cells during 48 hours of intervention, and 16 μ M N-NF could significantly increase the apoptosis of A549 cells. By studying the relationship between N-NF and the miR-361-3p/TRAF2/JNK axis, it was found that the mechanism by which N-NF promotes apoptosis in A549 cells was by inhibiting the expression of miR-361-3p, thereby promoting the transcription of TRAF2. The significance of this study was to provide a reference for subsequent drug development based on N-NF.

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Authors' Contribution

Chengxiang Wang conceived and designed

the analysis and reviewed the manuscript. Lin Bai was responsible for performing the analysis and wrote the paper. All authors have read and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflict of Interest

None declared.

References

1. Sharma P, McClees SF, Afaq F. Pomegranate for prevention and treatment of cancer: An update. *Molecules*. 2017;22(1):177. doi: 10.3390/molecules22010177. PMID: 28125044; PMCID: PMC5560105.
2. Kubczak M, Szustka A, Rogalińska M. Molecular targets of natural compounds with anti-cancer properties. *Int J Mol Sci*. 2021;22(24):13659. doi: 10.3390/ijms222413659. PMID: 34948455; PMCID: PMC8708931.
3. Rajabi S, Maresca M, Yumashev AV, Choopani R, Hajimehdipour H. The most competent plant-derived natural products for targeting apoptosis in cancer therapy. *Biomolecules*. 2021;11(4):534. doi: 10.3390/biom11040534. PMID: 33916780; PMCID: PMC8066452.
4. Paudel KR, Panth N. Phytochemical profile and biological activity of nelumbo nucifera. *Evid Based Complement Alternat Med*. 2015;2015:789124. doi: 10.1155/2015/789124. PMID: 27057194; PMCID: PMC4710907.
5. Wan Y, Xia J, Xu JF, Chen L, Yang Y, Wu JJ, et al. Nuciferine, an active ingredient derived from lotus leaf, lights up the way for the potential treatment of obesity and obesity-related diseases. *Pharmacol Res*. 2022;175:106002. doi: 10.1016/j.phrs.2021.106002. PMID:

34826599.

6. Jia XB, Zhang Q, Xu L, Yao WJ, Wei L. Lotus leaf flavonoids induce apoptosis of human lung cancer A549 cells through the ROS/p38 MAPK pathway. *Biol Res.* 2021;54(1):7. doi: 10.1186/s40659-021-00330-w. PMID: 33653412; PMCID: PMC7923640.

7. Tong Y, Li Z, Wu Y, Zhu S, Lu K, He Z. Lotus leaf extract inhibits ER- breast cancer cell migration and metastasis. *Nutr Metab (Lond).* 2021;18(1):20. doi: 10.1186/s12986-021-00549-0. PMID: 33602253; PMCID: PMC7891157.

8. Li C, Zhou Z, Long X, Pan Y, Wang R, Chen X, et al. Inhibitory effect of lotus leaf-enriched flavonoid extract on the growth of HT-29 colon cancer cells through the expression of PI3K-related molecules. *Biomed Res Int.* 2022;2022:6770135. doi: 10.1155/2022/6770135. PMID: 35586809; PMCID: PMC9110183.

9. N'Guessan B B, Asiamah AD, Arthur NK, Frimpong-Manso S, Amoateng P, Amponsah SK, et al. Ethanolic extract of *Nymphaea lotus* L. (Nymphaeaceae) leaves exhibits in vitro antioxidant, in vivo anti-inflammatory and cytotoxic activities on Jurkat and MCF-7 cancer cell lines. *BMC Complement Med Ther.* 2021;21(1):22. doi: 10.1186/s12906-020-03195-w. PMID: 33413340; PMCID: PMC7791887.

10. Ouyang L. A quality evaluation method of lotus leaf based on its lipid lowering components using QAMS and chemometrics. *Heliyon.* 2023;9(12):e23009. doi: 10.1016/j.heliyon.2023.e23009. PMID: 38058446; PMCID: PMC796245.

11. Zhang H, Chen G, Zhang Y, Yang M, Chen J, Guo M. Potential hypoglycemic, hypolipidemic, and anti-inflammatory bioactive components in *Nelumbo nucifera* leaves explored by bioaffinity ultrafiltration with multiple targets. *Food Chem.* 2022;375:131856. doi: 10.1016/j.foodchem.2021.131856. PMID:

34942503.

12. Ye LH, He XX, You C, Tao X, Wang LS, Zhang MD, et al. Pharmacokinetics of Nuciferine and N-Nornuciferine, two major alkaloids from *nelumbo nucifera* leaves, in rat plasma and the brain. *Front Pharmacol.* 2018;9:902. doi: 10.3389/fphar.2018.00902. PMID: 30210336; PMCID: PMC6123365.

13. Bishayee A, Patel PA, Sharma P, Thoutireddy S, Das N. Lotus (*Nelumbo nucifera* Gaertn.) and its bioactive phytochemicals: A tribute to cancer prevention and intervention. *Cancers (Basel).* 2022;14(3). doi: 10.3390/cancers14030529. PMID: 35158798; PMCID: PMC8833568.

14. Ma L, Li R, Yao Z, Wang B, Liu Y, Liu C, et al. Computational study on new natural compound inhibitors of Traf2 and Nck-interacting kinase (TNIK). *Aging (Albany NY).* 2022;14(20):8394-410. doi: 10.18632/aging.204349. PMID: 36287174; PMCID: PMC9648813.

15. Li S, Wang D, Zhao J, Weathington NM, Shang D, Zhao Y. The deubiquitinating enzyme USP48 stabilizes TRAF2 and reduces E-cadherin-mediated adherens junctions. *FASEB J.* 2018;32(1):230-242. doi: 10.1096/fj.201700415RR. PMID: 28874458; PMCID: PMC5731130.

16. Wang B, Jie Z, Joo D, Ordureau A, Liu P, Gan W, et al. TRAF2 and OTUD7B govern a ubiquitin-dependent switch that regulates mTORC2 signalling. *Nature.* 2017;545(7654):365-9. doi: 10.1038/nature22344. PMID: 28489822; PMCID: PMC5695540.

17. Borghi A, Verstrepen L, Beyaert R. TRAF2 multitasking in TNF receptor-induced signaling to NF- κ B, MAP kinases and cell death. *Biochem Pharmacol.* 2016;116:1-10. doi: 10.1016/j.bcp.2016.03.009. PMID: 26993379.

18. Zhang J, Liang Y, Lin Y, Liu Y,

- YouYou, Yin W. IRE1 α -TRAF2-ASK1 pathway is involved in CSTMP-induced apoptosis and ER stress in human non-small cell lung cancer A549 cells. *Biomed Pharmacother.* 2016;82:281-9. doi: 10.1016/j.biopha.2016.04.050. PMID: 27470364.
19. Li Z, Sun J, Liu W, Wu J, Peng H, Zhao Y, et al. Changes in the circRNA expression profile of PC12 cells induced by TDCIPP exposure may regulate the downstream NF- κ B pathway via the Traf2 gene. *Chemosphere.* 2020;254:126834. doi: 10.1016/j.chemosphere.2020.126834. PMID: 32339792.
20. Zhou L, Huang X, Li H, Wang J, Lu Z. Triptolide improves Alzheimer's disease by regulating the NF- κ B signaling pathway through the lncRNA NEAT1/microRNA 361-3p/TRAF2 axis. *Exp Ther Med.* 2023;26(3):440. doi: 10.3892/etm.2023.12139. PMID: 37614428; PMCID: PMC10443046.
21. Chu D, Li P, Li Y, Shi J, Huang S, Jiao P. Identification of circ_0058357 as a regulator in non-small cell lung cancer cells resistant to cisplatin by miR-361-3p/ABCC1 axis. *Thorac Cancer.* 2021;12(21):2894-906. doi: 10.1111/1759-7714.14150. PMID: 34523261; PMCID: PMCPCMC8563160.
22. Chen YF, Pang YC, Wang HC, Wu PE, Chen ZJ, Huang D, et al. Identification of arnicolide C as a novel chemosensitizer to suppress mTOR/E2F1/FANCD2 axis in non-small cell lung cancer. *Br J Pharmacol.* 2024;181(8):1221-37. doi: 10.1111/bph.16281. PMID: 37926864.
23. Rice J, Roberts H, Rai SN, Galandiuk S. Housekeeping genes for studies of plasma microRNA: A need for more precise standardization. *Surgery.* 2015;158(5):1345-51. doi: 10.1016/j.surg.2015.04.025. PMID: 26094174.
24. Kim C, Kim B. Anti-cancer natural products and their bioactive compounds inducing ER stress-mediated apoptosis: A review. *Nutrients.* 2018;10(8):1021. doi: 10.3390/nu10081021. PMID: 30081573; PMCID: PMCPCMC6115829.
25. Islam MR, Akash S, Rahman MM, Nowrin FT, Akter T, Shohag S, et al. Colon cancer and colorectal cancer: Prevention and treatment by potential natural products. *Chem Biol Interact.* 2022;368:110170. doi: 10.1016/j.cbi.2022.110170. PMID: 36202214.
26. Tewari D, Patni P, Bishayee A, Sah AN, Bishayee A. Natural products targeting the PI3K-Akt-mTOR signaling pathway in cancer: A novel therapeutic strategy. *Semin Cancer Biol.* 2022;80:1-17. doi: 10.1016/j.semcancer.2019.12.008. PMID: 31866476.
27. Atanasov AG, Zotchev SB, Dirsch VM, Supuran CT. Natural products in drug discovery: advances and opportunities. *Nat Rev Drug Discov.* 2021;20(3):200-16. doi: 10.1038/s41573-020-00114-z. PMID: 33510482; PMCID: PMCPCMC7841765.
28. Cho CK, Kang P, Jang CG, Lee SY, Lee YJ, Bae JW, et al. PBPK modeling to predict the pharmacokinetics of venlafaxine and its active metabolite in different CYP2D6 genotypes and drug-drug interactions with clarithromycin and paroxetine. *Arch Pharm Res.* 2024;Epub ahead of print. doi: 10.1007/s12272-024-01495-0. PMID: 38664354.
29. Li XN, Zuo YZ, Qin L, Liu W, Li YH, Li JL. Atrazine-xenobiotic nuclear receptor interactions induce cardiac inflammation and endoplasmic reticulum stress in quail (*Coturnix coturnix coturnix*). *Chemosphere.* 2018;206:549-59. doi: 10.1016/j.chemosphere.2018.05.049. PMID: 29778080.
30. Xu B, Xu J, Cai N, Li M, Liu L, Qin Y, et al. Roflumilast prevents ischemic stroke-induced neuronal damage by restricting GSK3 β -mediated oxidative stress and IRE1 α /TRAF2/JNK pathway. *Free Radic Biol Med.* 2021;163:281-96. doi:

10.1016/j.freeradbiomed.2020.12.018.

PMID: 33359910.

31. Gu Y, Liang C. TRAIP suppressed apoptosis and cell cycle to promote prostate cancer proliferation via TRAF2-PI3K-AKT pathway activation. *Int Urol Nephrol*. 2024;56(5):1639-48. doi: 10.1007/s11255-

023-03890-w. PMID: 38100027.

32. Rudnicki M, Perco P, B DH, Leierer J, Heinzl A, Mühlberger I, et al. Renal microRNA- and RNA-profiles in progressive chronic kidney disease. *Eur J Clin Invest*. 2016;46(3):213-26. doi: 10.1111/eci.12585. PMID: 26707063.

Table 1. qPCR primers

Gene name	Primer forward	Primer reverse	Accession number
TRAF2	CGACCGTTGGGGCTTTGT	ACAGGTA CTTGGCTTCCAGC	NM_021138.4
JNK	CTGCGCTGCCGGTCTT	GCTTGCTTTTGT CAGGCACA	NM_001278548.2
BAX	GGGGAGCAGCCCAGAGG	CGATCCTGGATGAAACCCTG A	NM_001291428.2
Caspase-3	GGCGGTTGTAGAAGAGTTT CG	TCACGGCCTGGGATTTCAAG	NM_001354777.2
COX-2	GTTCCACCCGCAGTACAGA A	AGGGCTTCAGCATAAAGCG T	AY462100.1
GAPDH	AATGGGCAGCCGTTAGGAA A	GCCCAATACGACCAAATCA GAG	NM_001256799.3

qPCR: Quantitative polymerase chain reaction; TRAF2: TNF receptor associated factor 2; JNK: Mitogen-activated protein kinase 8; BAX: BCL2 associated X, apoptosis regulator; COX-2: Cytochrome c oxidase subunit II; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

Table 2. Cell viability

	Control	CDDP	N-NF(H)	N-NF(M)	N-NF(L)
0h	110.03 ± 6.60 ^a	104.75 ± 4.54 ^a	104.14 ± 18.81 ^a	112.17 ± 16.46 ^a	113.27 ± 9.48 ^a
12h	153.60 ± 10.12 ^a	79.99 ± 2.31 ^{bc}	75.65 ± 5.01 ^c	79.00 ± 2.57 ^{bc}	97.21 ± 13.92 ^{ab}
24h	137.31 ± 4.76 ^a	67.62 ± 6.18 ^{bcd}	51.68 ± 0.93 ^d	54.28 ± 4.32 ^{cd}	86.51 ± 1.92 ^b
36h	127.94 ± 7.96 ^a	38.89 ± 2.68 ^c	33.74 ± 5.54 ^c	41.14 ± 2.51 ^c	78.85 ± 5.24 ^b
48h	129.90 ± 4.18 ^a	21.20 ± 4.98 ^c	17.94 ± 0.69 ^c	32.56 ± 4.24 ^c	89.94 ± 6.00 ^b

Note: Different letters on the bar graph indicate significant differences between groups ($P < 0.05$); N-NF: N-Nornuciferine; CDDP: Cisplatin

Table 3. qPCR detection results of relative gene expression levels

	Control	CDDP	N-NF(H)	N-NF(M)	N-NF(L)
TRAF2	1.18 ± 0.09 ^c	2.60 ± 0.59 ^{ab}	2.87 ± 0.70 ^a	2.08 ± 0.78 ^b	1.17 ± 0.06 ^c
JNK	1.16 ± 0.15 ^b	2.50 ± 0.35 ^a	2.38 ± 0.77 ^a	2.08 ± 0.46 ^a	1.21 ± 0.35 ^b
BAX	1.06 ± 0.06 ^b	1.96 ± 0.68 ^a	2.07 ± 0.37 ^a	1.77 ± 0.72 ^a	1.52 ± 0.33 ^{ab}
Caspase-3	1.20 ± 0.04 ^{cd}	2.38 ± 0.35 ^a	2.24 ± 0.55 ^{ab}	1.77 ± 0.53 ^{bc}	0.78 ± 0.32 ^d
COX-2	1.03 ± 0.04 ^c	2.17 ± 0.41 ^a	2.02 ± 0.37 ^{ab}	1.55 ± 0.51 ^{bc}	1.00 ± 0.39 ^c

Note: Different letters on the bar graph indicate significant differences between groups ($P < 0.05$); qPCR: quantitative polymerase chain reaction; N-NF: N-Normuciferine; CDDP: Cisplatin; TRAF2: TNF receptor associated factor 2; JNK: Mitogen-activated protein kinase 8; BAX: BCL2 associated X, apoptosis regulator; COX-2: Cytochrome c oxidase subunit II

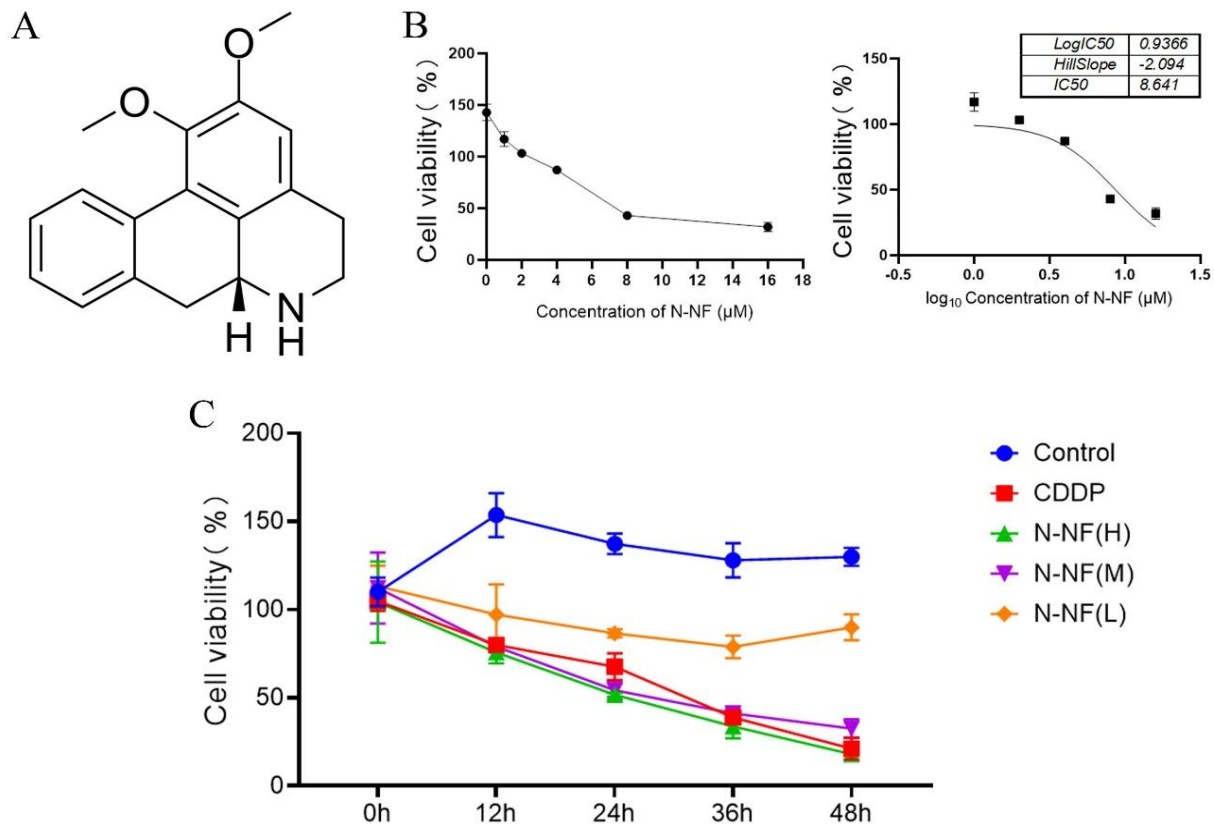


Figure 1. A: This image showed the chemical structure of N-NF. B: The CCK-8 kit was used to detect the effect of different concentrations of N-NF on A549 cell viability, and based on the results, the IC50 value was calculated to be 8.641 μM . C: Effects of CDDP and different concentrations of N-NF on the viability of A549 cells within 48 hours.

N-NF: N-Normuciferine; CDDP: Cisplatin

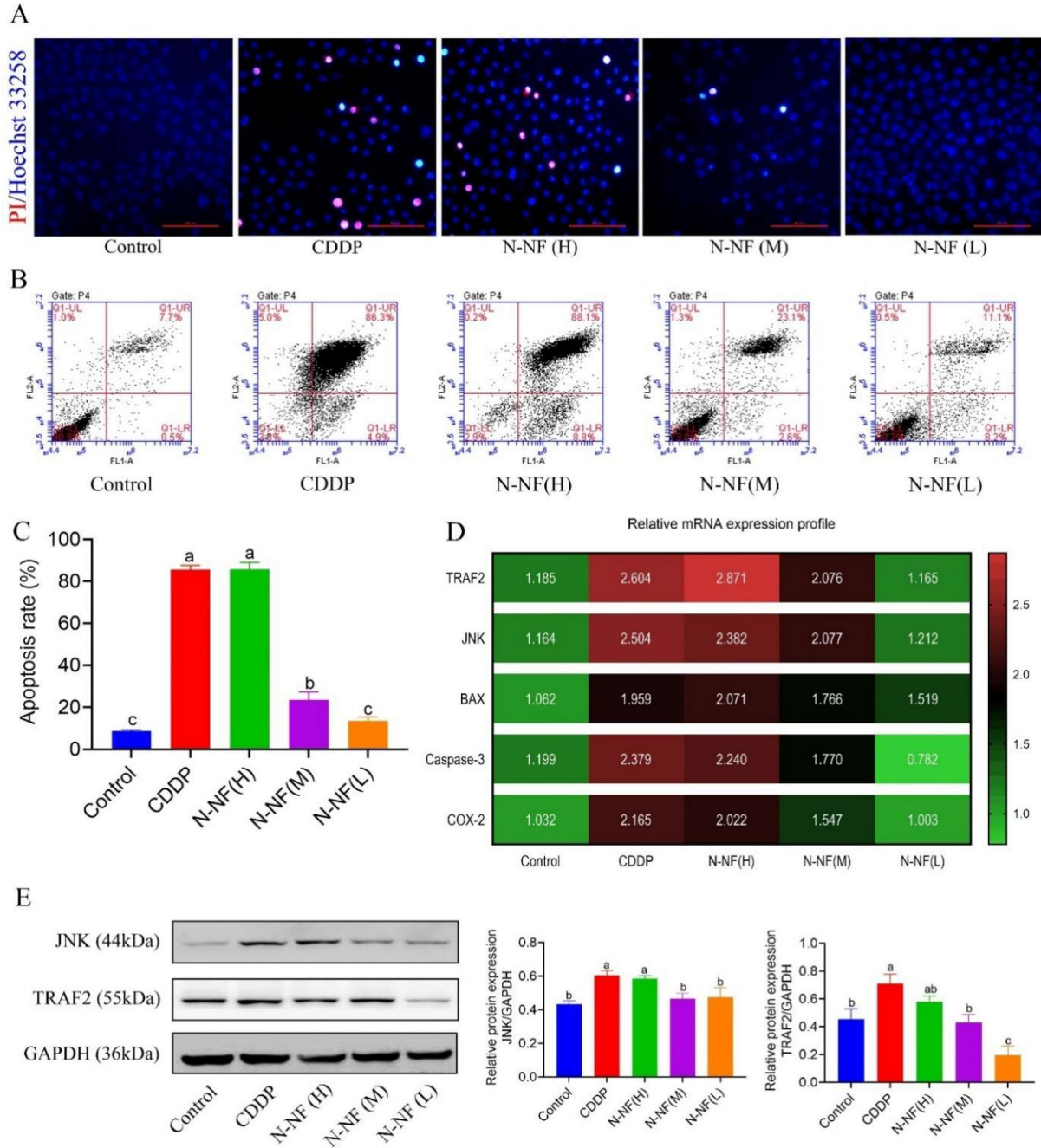


Figure 2. A: Fluorescence images co-stained with PI plus Hoechst 33258 to demonstrate the apoptosis of A549 cells. B, C: After AnnexinV-FITC/PI staining, flow cytometry was used to detect and quantitatively analyze the apoptosis of A549 cells. D: qPCR technology was used to detect the mRNA expression levels of TRAF2, JNK, BAX, Caspase-3 and COX-2 genes in A549 cells in each group. E: WesternBlot technology were used to detect and quantitatively analyze the JNK and TRAF2 protein expression of A549 cells in each group. Different letters on the bar graph indicate significant differences between groups ($P < 0.05$) (Scale bar = $50\mu\text{m}$).

qPCR: Quantitative polymerase chain reaction; N-NF: N-Normuciferine; CDDP: Cisplatin; TRAF2: TNF receptor associated factor 2; JNK: Mitogen-activated protein kinase 8; BAX: BCL2 associated X, apoptosis regulator; COX-2: Cytochrome c oxidase subunit II; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

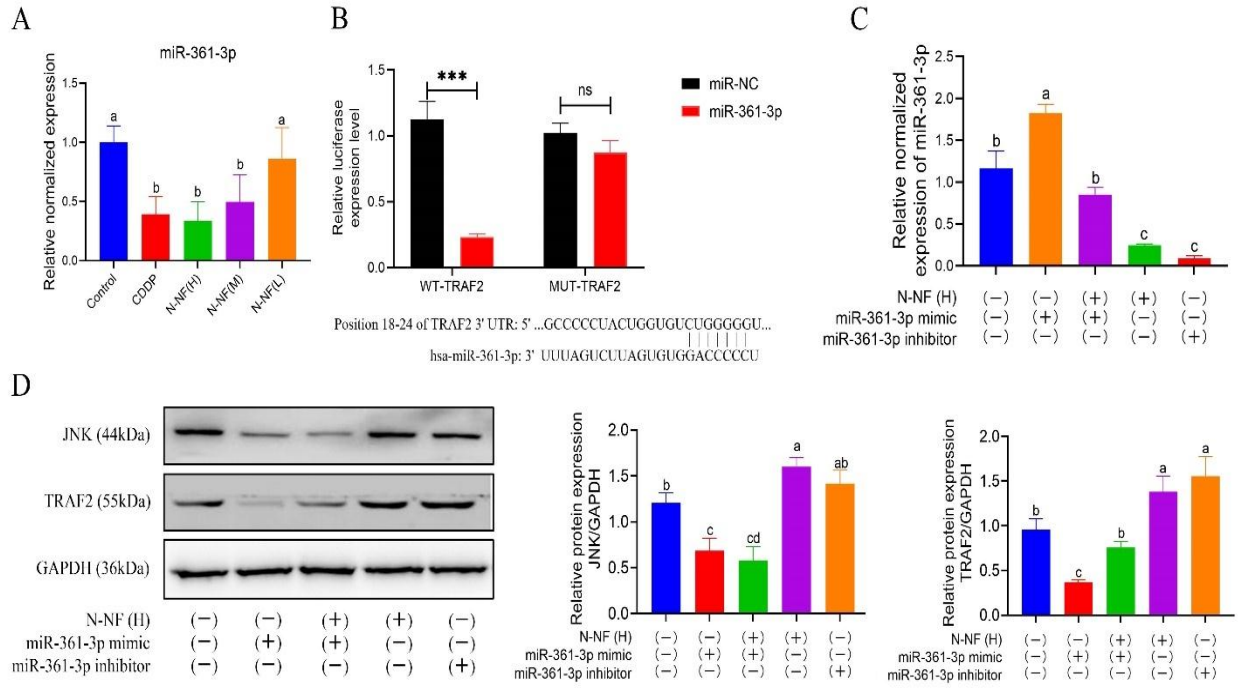


Figure 3. A: qPCR technology was used to detect the expression level of miR-361-3p in A549 cells in each group. B: Dual-luciferase assay technology was used to detect the interaction between miR-361-3p and TRAF2. C: After using mimic and inhibitor to regulate the expression level of miR-361-3p, the changes in the expression level of miR-361-3p in cells in each group. D: WesternBlot technology were used to detect and quantitatively analyze the JNK and TRAF2 protein expression of A549 cells in each group. Different letters on the bar graph indicate significant differences between groups ($P < 0.05$).

qPCR: Quantitative polymerase chain reaction; TRAF2: TNF receptor associated factor 2; JNK: Mitogen-activated protein kinase