Molecular Mechanism by which Diosbulbin B Regulates the TP63 Gene in Triple-Negative Breast Cancer

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Research

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Abstract

Background

Dioscorea bulbifera L. is mainly used for antitumor therapy in clinical practice. Studies have shown that the drug-containing serum obtained from *Dioscorea bulbifera* L. induces the apoptosis and inhibits the proliferation of rat breast cancer cells. However, the main active compounds and the molecular mechanism in triple-negative breast cancer are still unclear.

Methods

The TCMSP, GeneCards, GEO and TCGA databases were used to identify genes that intersect the target genes of *Dioscorea bulbifera* L., active *Dioscorea bulbifera* L. components and triple-negative breast cancer targets, and Kaplan-Meier and GSEA methods were used to analyze the survival and pathway enrichment of the intersecting genes, respectively. Subsequently, in vitro experiments were performed for verification.

Results

A total of 14 active components were screened, and the targets of the active components were combined with triple-negative breast cancer-related targets and differentially expressed genes obtained from the GeneCards database. Three genes (CCNB1, PGR and TP63) are related to the anti-breast cancer effects of *Dioscorea bulbifera* L., and TP63 ($P<0.05$) is related to breast cancer survival. The GSEA showed that the TP63 gene is related to the apoptosis pathway, and TP63 gene analysis in the TCGA clinical database showed the greatest expression difference in triple-negative breast cancer. The in vitro experiments showed that diosbulbin B, the main antitumor compound in *Dioscorea bulbifera* L., may inhibit the proliferation and migration of MDA-MB-231 breast cancer cells and induce their apoptosis by promoting the expression of the TP63 gene.

Conclusion

The present study fully elucidated the active components, potential targets and molecular mechanisms of *Dioscorea bulbifera* L. against triple-negative breast cancer and provided a new method for revealing the scientific basis and therapeutic mechanism of Chinese medicine in treating diseases.

Background

Breast cancer is the most common malignant tumor among women in the world. In the 2018, the Global Burden of Disease Study indicated that there were 18.1 million new cancer cases and 9.6 million cancer deaths. Among these cases, breast cancer accounted for 11.6%, which was second only to lung cancer and was the longest. The diagnosis of cancer is one of the leading causes of cancer death[1]. Triple-negative breast cancer (TNBC) is a subtype of breast cancer and is defined by the lack of expression of estrogen receptor (ER), progesterone receptor (PR) and estrogen receptor 2 (HER2); in addition, TNBC

accounts for 15-20% of breast cancer patients with relatively poor results compared to those with other breast cancer subtypes[2,3]. It is clinically recognized that the lack of molecular targets for TNBC therapy is the main reason for the poor outcomes[4]. Therefore, it is necessary to explore the molecular mechanism of triple-negative breast cancer and determine new treatment methods for triple-negative breast cancer.

Previous studies have demonstrated that traditional Chinese medicine, as an adjuvant therapy for radiotherapy or combined with chemotherapy, may enhance the therapeutic effect, reduce adverse reactions, improve the quality of life and prolong survival, and traditional Chinse medicine has increased in popularity in Western countries[5]. The traditional Chinese medicine derived from *Dioscorea bulbifera* L., is mainly used clinically for the treatment of tumors and thyroid diseases [6]. In vivo and in vitro experiments using rats gavaged with *Dioscorea bulbifera* L. and *Angelica sinensis* demonstrated that rat serum inhibits the proliferation of sarcoma cells and liver cancer cells, indicating that *Dioscorea bulbifera* L.- medicated serum may induce breast cancer cell apoptosis and inhibit cell proliferation [7]. Diosbulbin B is the main compound in the traditional Chinese medicine derived from *Dioscorea bulbifera* L., and has the greatest antitumor activity [8]. Studies have found that diosbulbin B downregulates the CDR1 as circRNA through the miR-7-5p/REGγ axis to inhibit gastric cancer cell proliferation and promote cell apoptosis, and studies have also reported that diosbulbin B combined with ferulic acid increases antitumor activity and prevents diosbulbin B-induced liver damage[9,10]. Although it has been reported that *Dioscorea bulbifera* L. and its main component, diosbulbin B, have antitumor activity, research on the target and molecular mechanism in triple-negative breast cancer has been contradictory.

Network pharmacology of Chinese medicine is a method that involves bioinformatics, and it is the basis for revealing the mechanism of Chinese medicine treatment and provides a new scientific basis for Chinese medicine research[11,12]. This method reveals the pharmacological mechanism of the complex components of traditional Chinese medicine from multiple angles and provides information about the relation between traditional Chinese medicine and disease. The relationship between each target and disease[13] provides a favorable basis for clinical TCM treatment programs. The present study mainly used the method of traditional Chinese medicine network pharmacology to explore the mechanism of *Dioscorea bulbifera* L. triple-negative breast cancer and explored the antitumor effects of *Dioscorea bulbifera* L. on MDA-MB-231 breast cancer cells through in vitro experiments. Follow-up pharmacological research and clinical trials will provide additional information.

Materials And Methods

Network pharmacology method

Prediction of *Dioscorea bulbifera* L. active components

Through the Chinese Medicine System Pharmacology Analysis Platform (TCMSP) (http://tcmspw.com/tcmsp.php) database, the chemical composition of *Dioscorea bulbifera* L. was

searched, and drug-like (DL) properties ≥ 0.18 and oral absorption availability (OB) ≥30% were used as the screening criteria. *Dioscorea bulbifera* L. has 14 chemical components that meet these criteria.

Prediction of target genes of *Dioscorea bulbifera* L. active components

The drug targets were retrieved from the TCMSP Analysis Platform (http://tcmspw.com/tcmsp.php) database.

Prediction of *Dioscorea bulbifera* L. target genes

The active component of *Dioscorea bulbifera* L. was combined with the target to in a Perl software program to obtain the target gene of the active ingredient, and the full name of the target gene was simplified using the UniProt database to annotate the target genes of Chinese medicine.

The set of disease targets in triple-negative breast cancer

Breast cancer-related gene targets were obtained through a GeneCards (https://www.genecards.org/) search, and breast cancer-related clinical data were downloaded from the TCGA database, which contained 103 noncancerous and 1109 tumor samples. Two triple-negative breast cancer datasets, GSE76250 and GSE61724, were retrieved from the GEO database.

Network construction of Huangyaozi and triple-negative breast cancer target genes

Through the prediction of the *Dioscorea opposita* active ingredient target genes and the set of triple-negative breast cancer disease targets, overlapping genes were identified.

Cell experiments

Cell culture

MDA-MB-231 human breast cancer cells were purchased from the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in DMEM containing 10% fetal bovine serum, 100 μg/mL ampicillin and 100 μg/mL streptomycin at 37°C in 5% CO2 and saturated humidity in an incubator.

CCK-8 detection of the effect of diosbulbin B on MDA-MB-231 cell proliferation

MDA-MB-231 cells in the logarithmic growth phase were seeded in 96-well plates at 3×10^3 cells/well and treated with different concentrations of diosbulbin B (0, 50, 100, 150, 200 and 250 μM) for 24 and 48 h. CCK-8 reagent (10 μl) was added to each well, and the cells were incubated for an additional 2 h to detect cell proliferation at 450 nm. GraphPad Prism 7.0 software was used to analyze and process the data.

Scratch detection of the effect of diosbulbin B on MDA-MB-231 cell migration

MDA-MB-231 cells in the logarithmic growth phase were seeded at 1×10^6 cells/well in a 96-well plate. After 24 h of attachment, the cell migration ability was determined using a scratch wound-healing assay.

Before adding diosbulbin B, it was sterilized with 1000 μl. The pipette tip was used to make a vertical scratch as a uniform wound area. After washing the monolayer twice with PBS, serum-free medium was added, and images were acquired at 0 h. After 24 h, different concentrations of diosbulbin B (100, 150 and 200 μM) were added. Images were taken under an inverted microscope at 48 h.

Flow cytometry analysis of the effect of diosbulbin B on MDA-MB-23 cell apoptosis

MDA-MB-231 cells were seeded in a 6-well plate. After attachment, the cells were treated with diosbulbin B at different concentrations (0, 100, 150 and 200 μM) for 24 h. Subsequently, the cells were collected according to the instructions of a Annexin V-FITC apoptosis detection kit (BMS500FI/100, Thermo Fisher), washed twice with binding buffer (1x), resuspended in 5 μl of Annexin V-FITC and 10 μl PI of staining solution in 190 μl of binding buffer (1x) and incubated at room temperature in the dark for 15 minutes. Finally, flow cytometry (Thermo Fisher) was used to detect cell apoptosis, and FlowJo 7.6 software (De Novo Software, Los Angeles, CA, USA) was used to analyze and process the data.

Hoechst staining for the detection of diosbulbin B-induced MDA-MB-231 cell apoptosis

MDA-MB-231 cells were seeded at 3×10^4 cells/well in 6-well plates. After attachment, the cells were treated with diosbulbin B at different concentrations (0, 100, 150 and 200 μM) for 24 h. Subsequently, the original culture medium was aspirated, and the cells were washed twice with PBS. Next, 500 μl of cell fixation solution was added to each well. After 15 minutes, the fixation solution was aspirated, and the cells were washed twice with PBS. Then, 500 μl of Hoechst was added, and the cells were washed twice with PBS. Images were acquired using an inverted fluorescence microscope.

QRT-PCR verification of the effect of diosbulbin B on TP63 gene expression in MDA-MB-231 breast cancer cells

MDA-MB-231 cells were treated with diosbulbin B at different concentrations (0, 100, 150 and 200 μM) for 24 h, and total RNA was extracted using TRIzol® reagent (US Patent No. 5,346,994). The RevertAidTM first-strand cDNA synthesis kit (Thermo, 1622) was then used to synthesize cDNA according to the kit instructions. SYBR Green Real-time PCR Master Mix and the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, USA) (TOYOBO) were used for the quantitative PCR amplification of TP63 with GAPDH as an internal control, and the relative expression of TP63 was evaluated by the $2^{-\triangle\triangle Ct}$ method. The primers used for qRT-PCR were as follows: forward 5'-GAAACCAGAGATGGGCAAGT-3' and reverse 5'-ATGCTATCTTCATCCGCCTTC-3'.

Western blot analysis verification of the effect of diosbulbin B on the expression of P63 protein in MDA-MB-231 breast cancer cells

MDA-MB-231 cells were treated with diosbulbin B at different concentrations (0, 100, 150 and 200 μM) for 24 h. Cell samples of each group were collected in 1.5-ml EP tubes. Then, 100 μl of cell lysate was added to each tube to extract total protein at 4°C followed by centrifugation at 12000 r/min for 15 minutes, and

the supernatant was collected. Protein samples (30 μg) were separated by polyacrylamide gel electrophoresis and then transferred to PVDF membranes. The membranes were blocked with 5% skim milk powder at room temperature for 1 h, and primary antibody (1:2000) was added and incubated overnight at 4℃. The membranes were then washed four times with TBST, and secondary antibody (1:5000) was added and incubated at room temperature for 1 h. The membranes were then washed four times with TBST. The membranes were developed using an ECL kit and visualized using a FluorChem M multicolor fluorescence and chemiluminescence imager.

Statistical analysis

The measurement data are expressed as the means ± standard deviation (SD), and SPSS 18.0 software was used for the data analysis. One-way analysis of variance and t-tests were used to determine the differences among multiple groups. $P<0.05$ was considered statistically significant.

Results

Data analysis

Screening of *Dioscorea bulbifera* L. Active Components

Drug-like (DL) properties ≥ 0.18 and oral absorption availability (OB) ≥ 30% were selected as the screening conditions for the search of the TCMSP database. A total of 14 chemical components of *Dioscorea bulbifera* L. met the conditions, and 14 active ingredients of *Dioscorea bulbifera* L. had good drug-like properties and oral absorption availability. The OB value (43.01%) and DL value (0.7) of diosbulbin B are shown in Table 1.

Table 1. Fourteen candidate components of Dioscorea bulbifera L. predicted according to OB and DL.

Molecular number	chemical compound	OB / %	DL
MOL000239	Jaranol	50.83	0.29
MOL000358	beta-sitosterol	36.91	0.75
MOL000422	kaempferol	41.88	0.24
MOL000449	stigmasterol	43.83	0.76
MOL000546	diosgenin	80.88	0.81
MOL000073	ent-Epicatechin	48.96	0.24
MOL007939	diosbulbin B	43.01	0.7
MOL000096	(-)-catechin	49.68	0.24
MOL009772	3,5,3'-trimethoxyquercetin	37.83	0.44
MOL009788	diosbulbin A	39.52	0.65
MOL009789	diosbulbin C	65.87	0.6
MOL009794	diosbulbin H	55.62	0.7
MOL000098	quercetin	46.43	0.28
MOL009800	kryptogenin	35.11	0.81

Dioscorea bulbifera L. Target gene/triple-negative breast cancer target network construction

Drug targets were retrieved from the TCMSP data platform. The GeneCards database retrieved 13,134 breast cancer-related targets and extracted each target gene. The TCGA database was used to download breast cancer-related clinical data, containing 103 noncancerous and 1109 tumor samples. The GEO database datasets of triple-negative breast cancer (GSE76250 and GSE61724) were obtained. Finally, the target genes of the active component, *Dioscorea bulbifera* L., and the target genes of triple-negative breast cancer were analyzed to obtain a Venn diagram (Figure 1). The results show that there were three genes (i.e., CCNB1, PGR and TP63) in this network (Figure 1).

Prognostic analysis of the CCNB1, PGR and TP63 genes

The prognostic evaluation of the three genes using the Kaplan-Meier survival curve online plotter showed that CCNB1, PGR and TP63 may affect the overall survival rate of patients with breast cancer and that TP63 was a significant factor for the overall survival rate of patients with breast cancer ($p<0.05$) (Figure 2).

TP63 GSEA

The TP63 gene was mainly enriched in pathways related to apoptosis in breast cancer ($p<0.01$) (Figure 3).

Experimental results

Diosbulbin B inhibits the proliferation of MDA-MB-231 breast cancer cells

The results of the CCK-8 analysis of MDA-MB-231 breast cancer cells treated with different concentrations of diosbulbin B (0, 100, 150, 200 and 250 μM) for 24 and 48 h are shown in Figure 4. Diosbulbin B significantly inhibited the viability of MDA-MB-231 cells in a dose- and time-dependent manner.

Diosbulbin B inhibits the migration of MDA-MB-231 breast cancer cells

Scratch experiments showed that, compared to the control group, the migration ability of MDA-MB-231 breast cancer cells was inhibited with different concentrations of diosbulbin B (0, 100, 150, and 200 μM) in a time- and dose-dependent manner after 24 and 48 h (Figure 5).

Diosbulbin B promotes apoptosis of MDA-MB-231 breast cancer cells

Flow cytometry was utilized to analyze the effect of diosbulbin B on MDA-MB-231 breast cancer cell apoptosis. After treatment with different concentrations of diosbulbin B (100, 150 and 200 μM) for 24 h, MDA-MB-231 cells were stained with Annexin V-FITC and PI, and the apoptosis rate was determined (Figure 6). After treatment with diosbulbin B, the percentage of apoptotic cells increased significantly in a dose-dependent manner, indicating that diosbulbin B induces MDA-MB-231 breast cancer cell apoptosis. Hoechst staining of the MDA-MB-231 breast cancer cells treated with different concentrations of diosbulbin B (0, 100, 150 and 200 μM) for 24 h were translucent and blue, and this color change occurred in a dose-dependent manner.

Diosbulbin B promotes TP63 gene expression in MDA-MB-231 breast cancer cells

After treating MDA-MB-231 cells with different concentrations of diosbulbin B (0, 100, 150 and 200 μM) for 24 h, qRT-PCR was performed to measure TP63 gene expression levels. As shown in Figure 7, TP63 gene expression gradually increased as the concentration of diosbulbin B increased.

Diosbulbin B promotes the P63 protein expression in MDA-MB-231 breast cancer cells

After treating MDA-MB-231 cells with different concentrations of diosbulbin B (0, 100, 150 and 200 μM) for 24 h, Western blot analysis was performed to measure the protein levels of P63. Compared to that of β-actin, which was the internal reference, the protein expression of p63 gradually increased with the increase in diosbulbin B concentration (Figure 8).

Discussion

With continuous in-depth research on the clinical use of integrated Chinese and Western medicine, traditional Chinese medicine has been utilized in breast cancer of all stages in the clinic, and the integrated use of both medicines has provided a variety of clinical treatments for breast cancer programs. This study analyzed the effective chemical components of *Dioscorea bulbifera* L. in triple-negative breast cancer and constructed a network diagram of *Dioscorea bulbifera* L. active chemical components, target genes and differentially expressed triple-negative breast cancer genes, and the results indicated that *Dioscorea bulbifera* L. has an anti-triple-negative breast cancer effect. The targets of *Dioscorea bulbifera* L. were analyzed, and diosbulbin B, the main antitumor component of *Dioscorea bulbifera* L., was selected for in vitro experimental verification, which provided a research basis for the molecular mechanism of *Dioscorea bulbifera* L in triple-negative breast cancer. The Venn diagram (Figure 1) indicated that the intersecting genes (CCNB1, PGR and TP63) may be the target genes of *Dioscorea bulbifera* L. in triple-negative breast cancer. Because the survival analysis of the target genes showed that (Figure 2) the TP63 gene ($p<0.05$) was statistically significant, TP63 was used for the GSEA. The TP63 gene is mainly enriched in pathways related to apoptosis in breast cancer ($p<0.01$) (Figure 3). Therefore, MDA-MB-231 breast cancer cells were selected for subsequent in vitro experiments.

According to the screening conditions (drug-like (DL) property ≥ 0.18 and oral absorption availability (OB) ≥ 30%), A total of 14 chemical components in *Dioscorea bulbifera* L. were identified. Among these, diosgenin had the highest DL property and OB value. The main component of *Dioscorea opposita* regulates the Cdc25C-Cdc2-cyclin B pathway in and the mitochondrial-mediated apoptosis of human breast cancer cell lines to induce G2/M phase arrest, and it may be a potential inhibitor of Skp2, with the ability to reduce cell viability and induce apoptosis[14,15]. In addition, diosgenin significantly inhibits actin polymerization, Vav2 phosphorylation and Cdc42 activation to inhibit the migration of MDA-MB-231 cells, the conferring antimetastatic potential[16]. However, the molecular mechanism of diosbulbin B, the main antitumor component of *Dioscorea bulbifera* L, in breast cancer remains unclear. Studies have reported that diosbulbin B downregulates the CDR1as circRNA through the miR-7-5p/REGγ axis to inhibit gastric cancer cell proliferation and promote cell apoptosis, and studies have also shown that diosbulbin B combined with ferulic acid increases antitumor activity and prevents diosbulbin B-induced liver injury[17,18].

The network pharmacology of Chinese medicine predicted that CCNB1, PGR and TP63 may be the target genes of *Dioscorea bulbifera* L. in breast cancer, and the survival analysis with respect to the TP63 gene ($p<0.05$) showed significance. CCNB1 may be an effective biomarker for the prognosis of patients with ER-positive breast cancer and for monitoring the efficacy of hormone therapy[17]. PGR is a progesterone receptor gene that is prone to mutation in metastatic ER-positive breast cancer; this mutation may cause the loss of PR protein expression due to clonal selection [19]. The TP63 gene is a member of the TP53 tumor suppressor gene family. Inducing cell apoptosis is the most important tumor suppressor function of p53. Studies have shown that full-length p63, similar to p53, has the ability to transactivate Bax and PUMA genes, thereby inducing tumor cell apoptosis[20-23]. Moreover, p63 is rarely mutated in human cancers. Because p63 is a transcription factor, small changes in p63 expression may play a decisive role in cell apoptosis and senescence. Studies have shown that TAp63 is a subtype of TP63 and that it plays

an important role in promoting apoptosis and tumor suppression[24,25]. The activity of TAp63 is related to the inhibition of the self-renewal ability of breast cancer cells. Tap63 inhibits tumor metastasis by regulating dicer and miRNAs, and knocking out TAp63 in mice leads to breast cancer metastasis[26,27]. Therefore, the expression of the TP63 gene is closely related to the occurrence and development of breast cancer.

The in vitro experimental results showed that the main antitumor compound of *Dioscorea bulbifera* L., diosbulbin B, inhibited the proliferation and migration of MDA-MB-231 breast cancer cells but induced apoptosis with increasing concentrations. In addition, diosbulbin B also promoted the expression of the TP63 gene and p63 protein in MDA-MB-231 breast cancer cells with increasing concentrations. Therefore, combined with the network pharmacology analysis of traditional Chinese medicine and research advancement with the TP63 gene in breast cancer, the main antitumor compound, diosbulbin B, from the traditional Chinese medicine, *Dioscorea bulbifera* L., may inhibit the proliferation and migration and induce the apoptosis of MDA-MB-231 breast cancer cells by promoting TP63 gene expression.

Conclusion

In this study, other active components and target genes may not have been included in our analysis because the public database is constantly updated. In addition, other active components (for example, quercetin, diosgenin, beta-sitosterol and diosgenin H) may also participate in the antitumor effect of *Dioscorea bulbifera* L. Therefore, it is necessary to further explore the molecular mechanism of *Dioscorea bulbifera* L. in breast cancer in vivo and in vitro.

Abbreviations

TCMSP: Traditional Chinese Medicine System Pharmacology; DL: Drug-like; OB: Oral absorption availability; TCGA: The cancer Genome Atlas; GEO: Gene Expression Omnibus; GSEA: Gene set Enrichment Analysis; TBST: Tris-bufered saline containing 0.1% Tween-20.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

All authors participated in the experimental design, analysis of the data, interpretation of the results, and review of the manuscript. LL performed the experiments; LSL analyzed the data, LL, LQF wrote the manuscript PWY reviewed and revised the article. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Figures

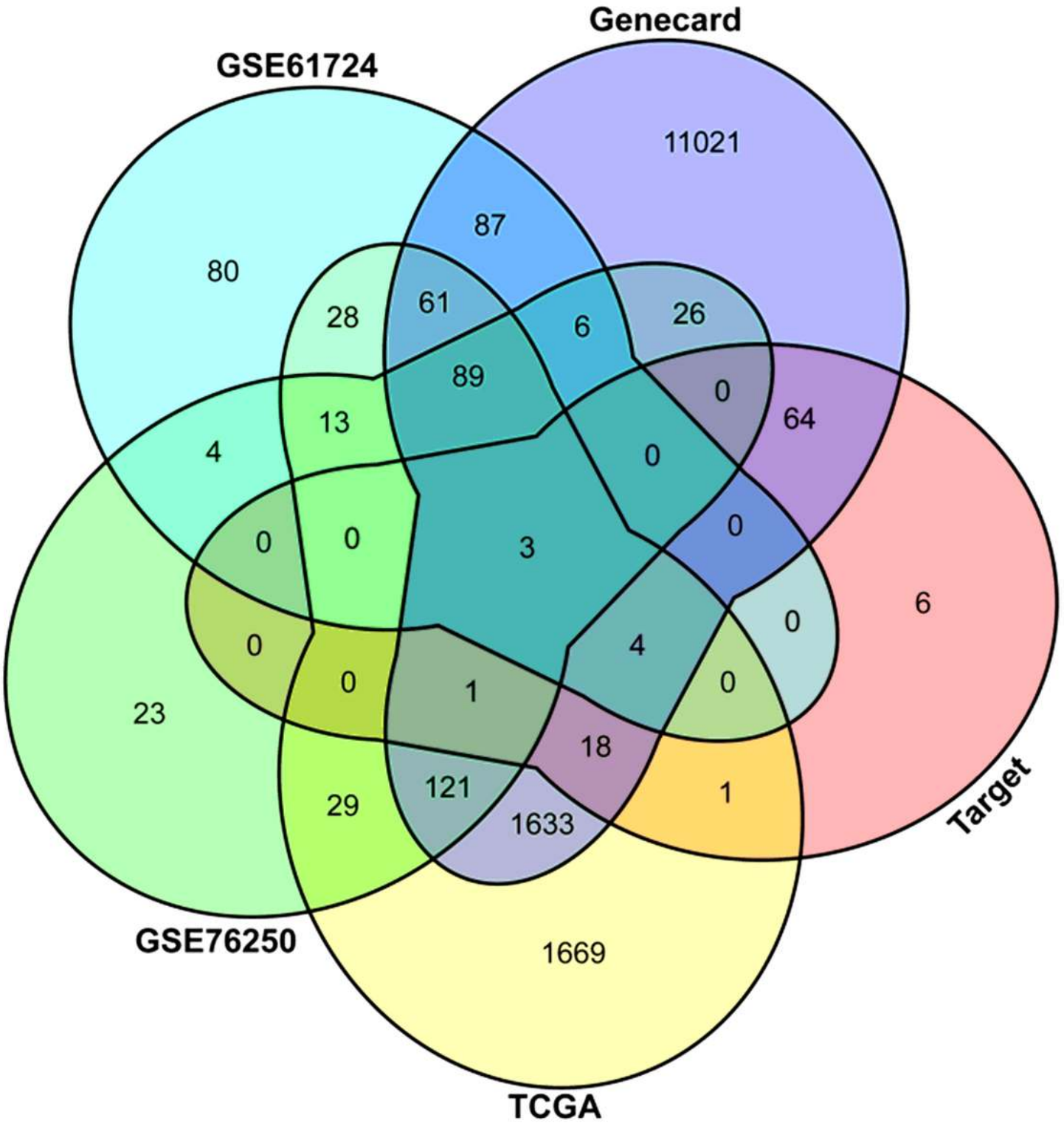
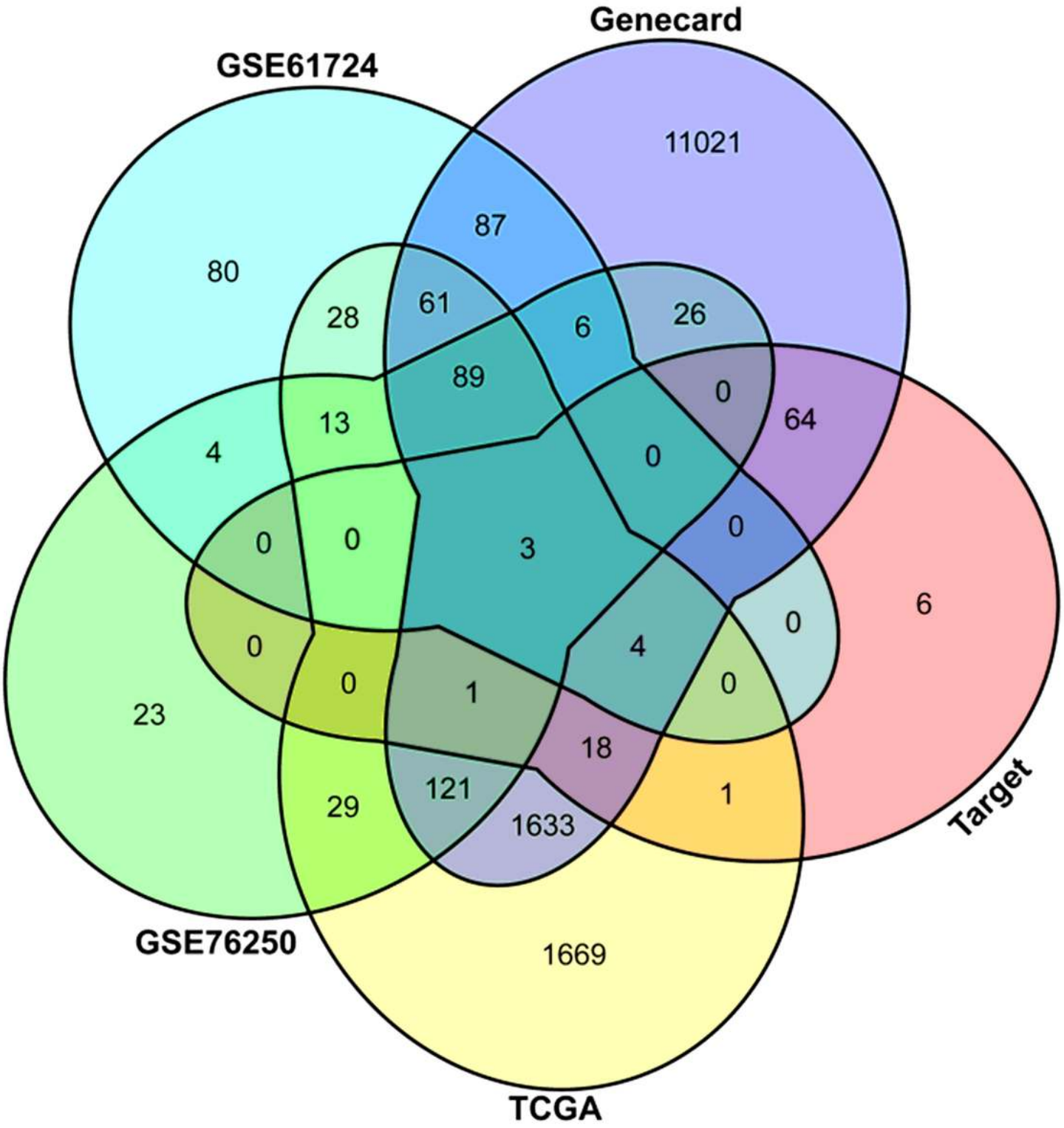


Figure 1

Intersection of Dioscorea bulbifera L. active component target genes, breast cancer disease-related target genes and differentially expressed genes.



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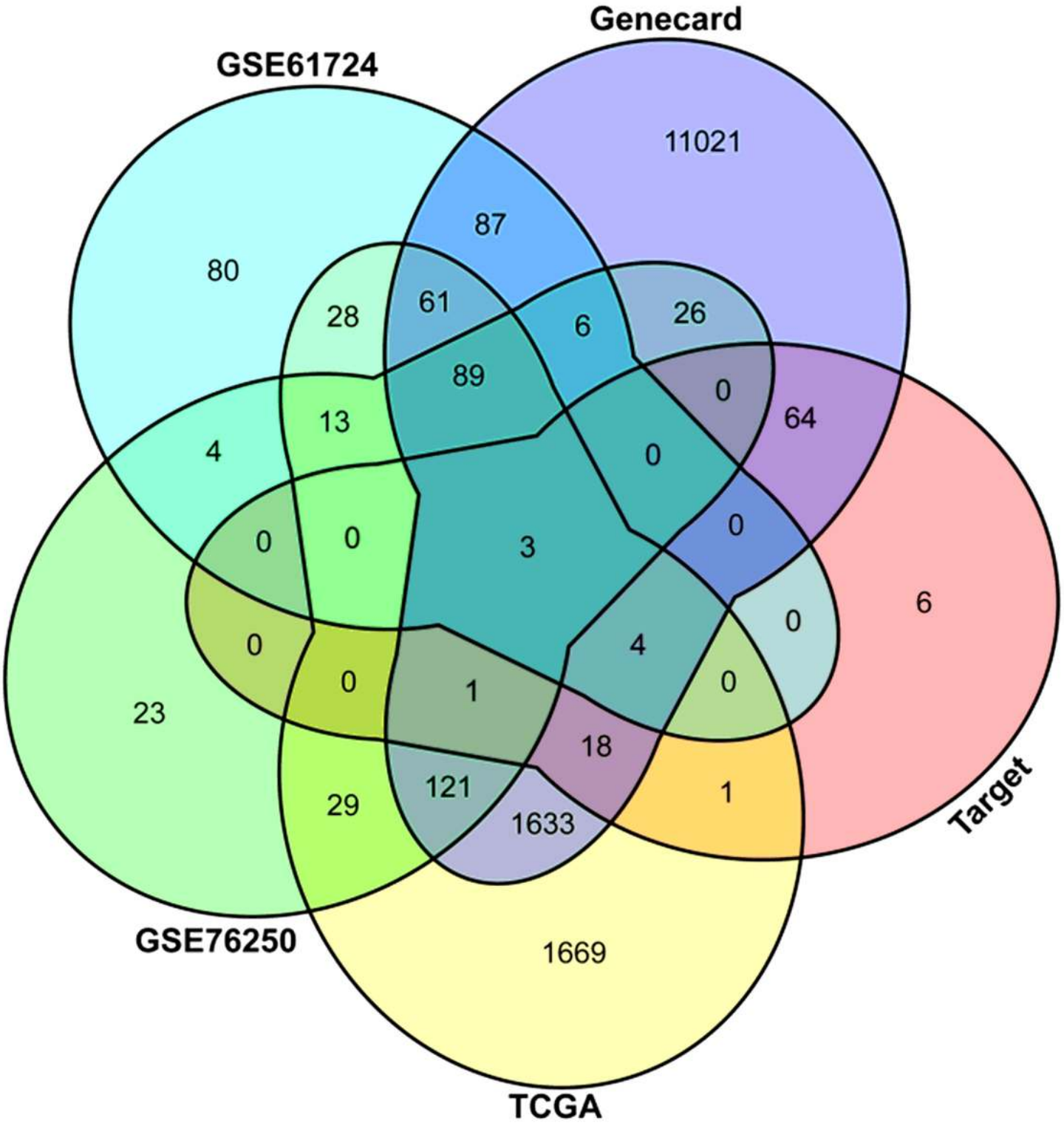

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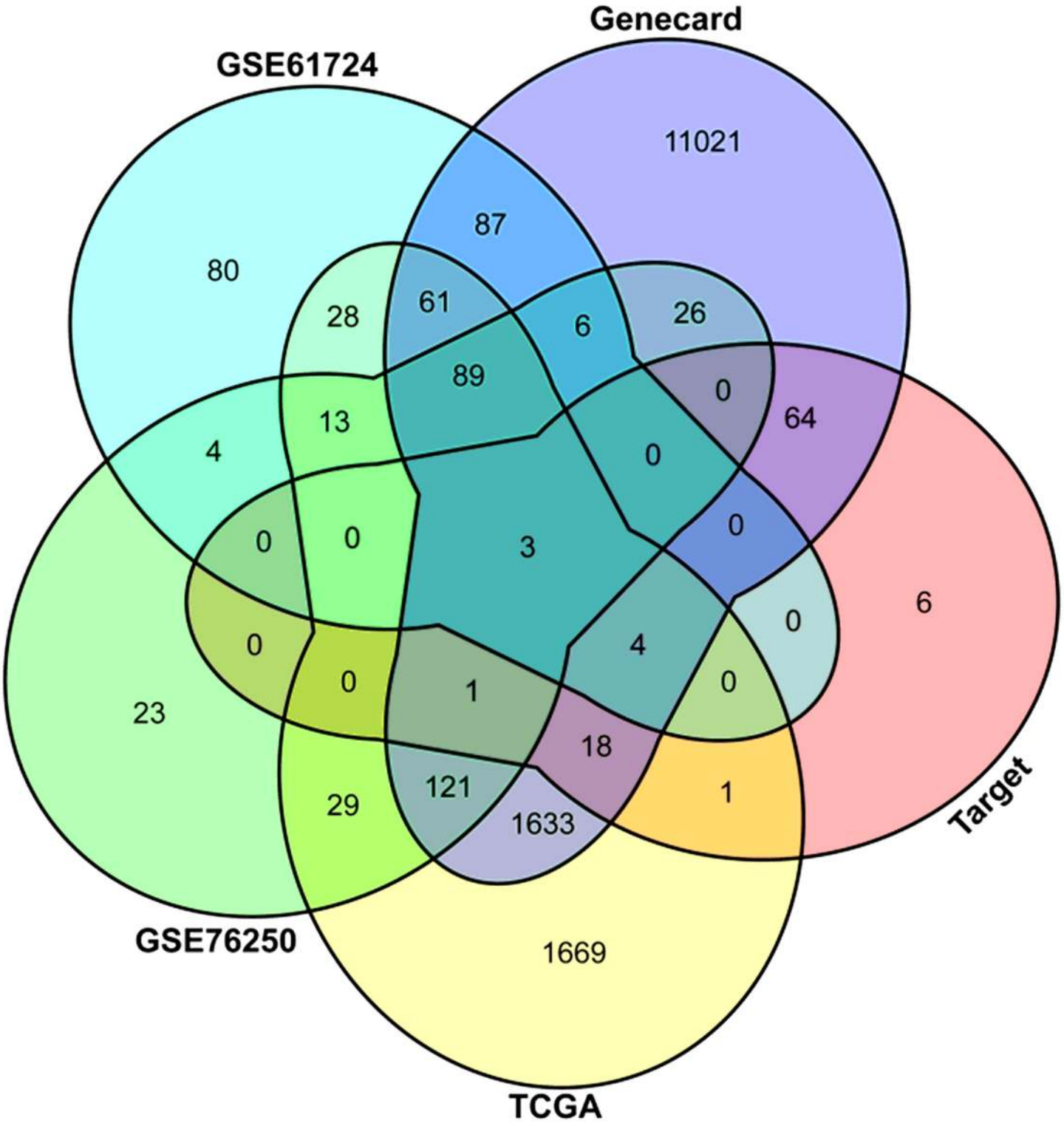

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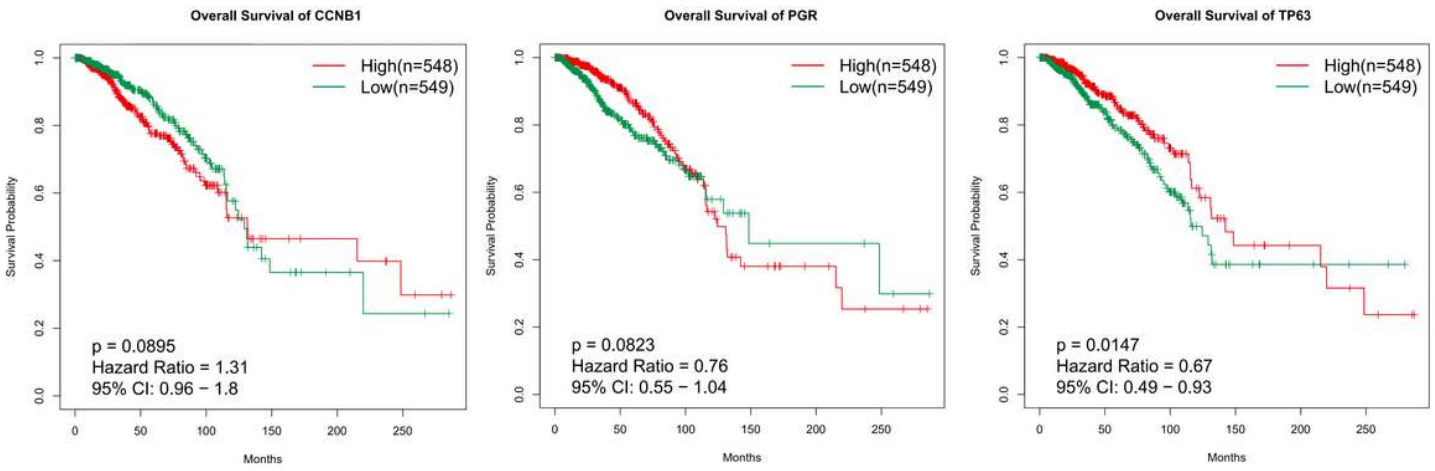
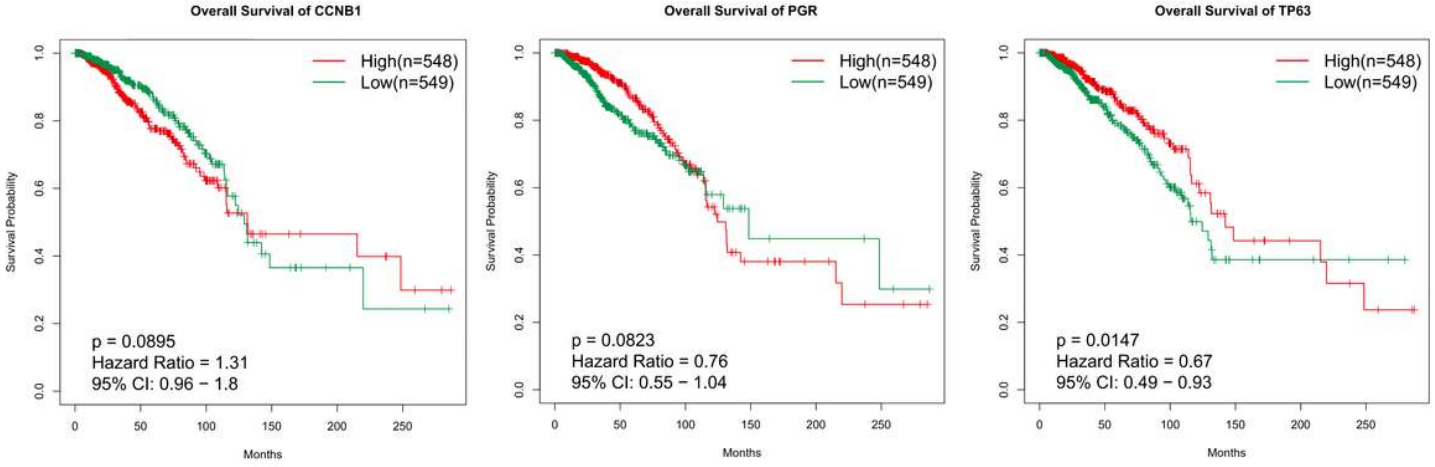


Figure 2

Overall survival rate of CCNB1, PGR and TP63 in breast cancer.



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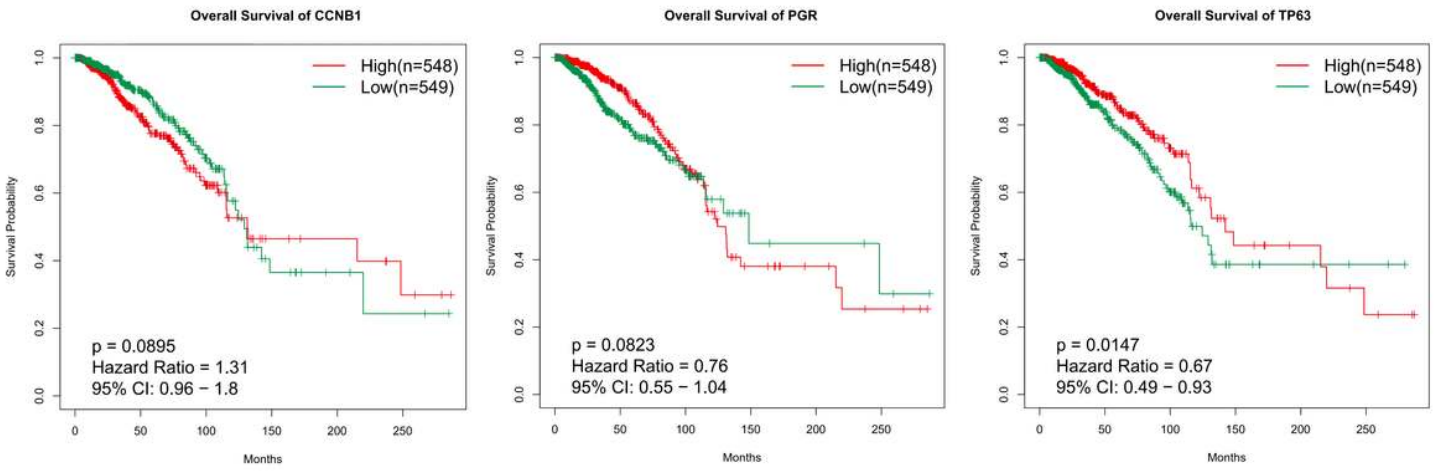

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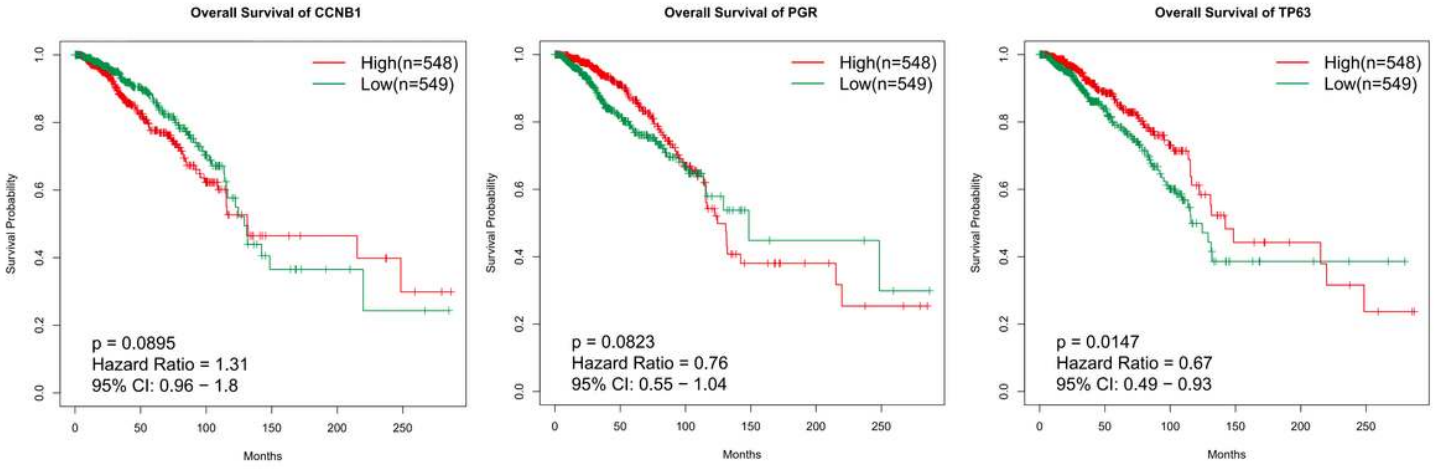

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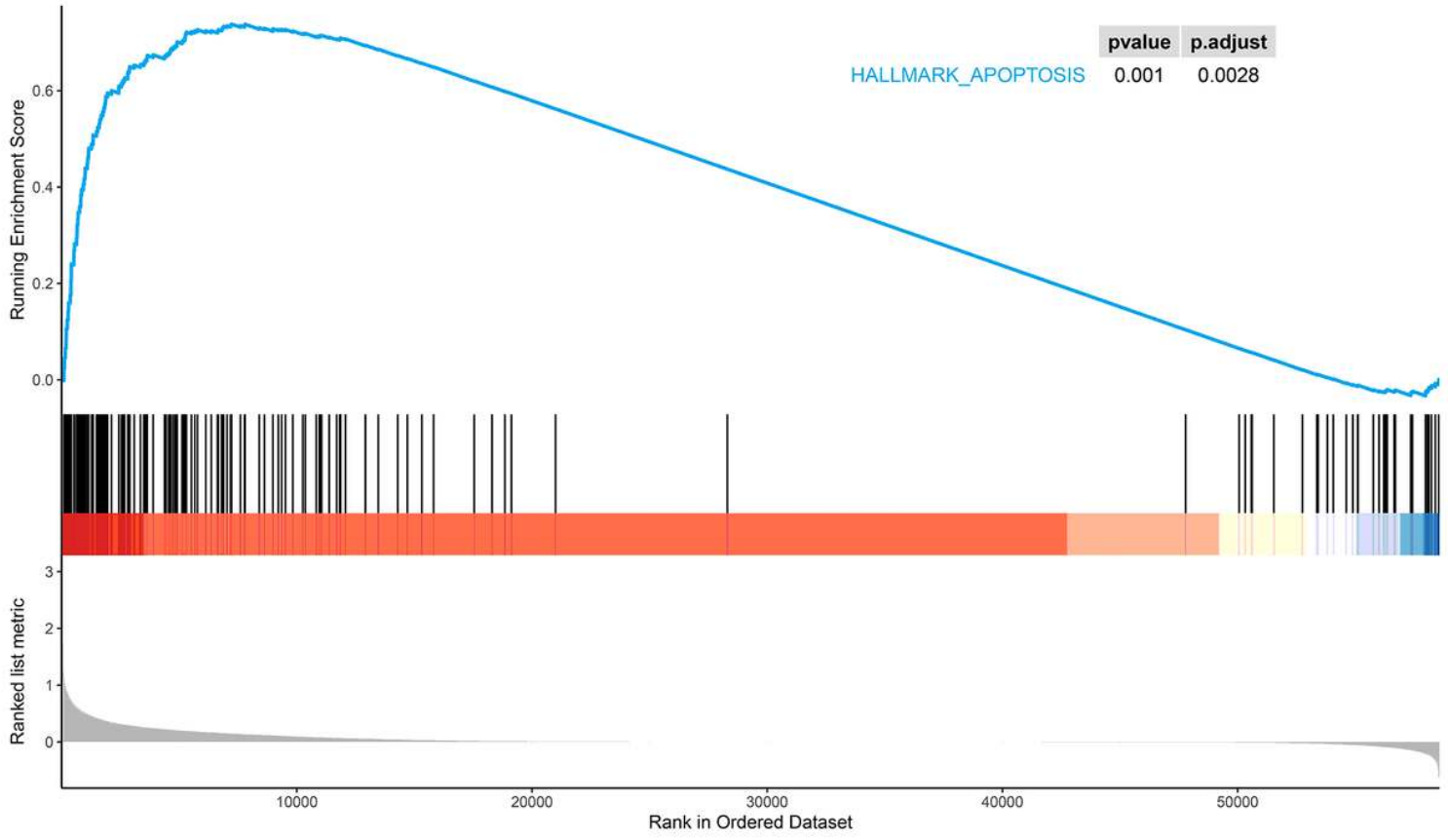

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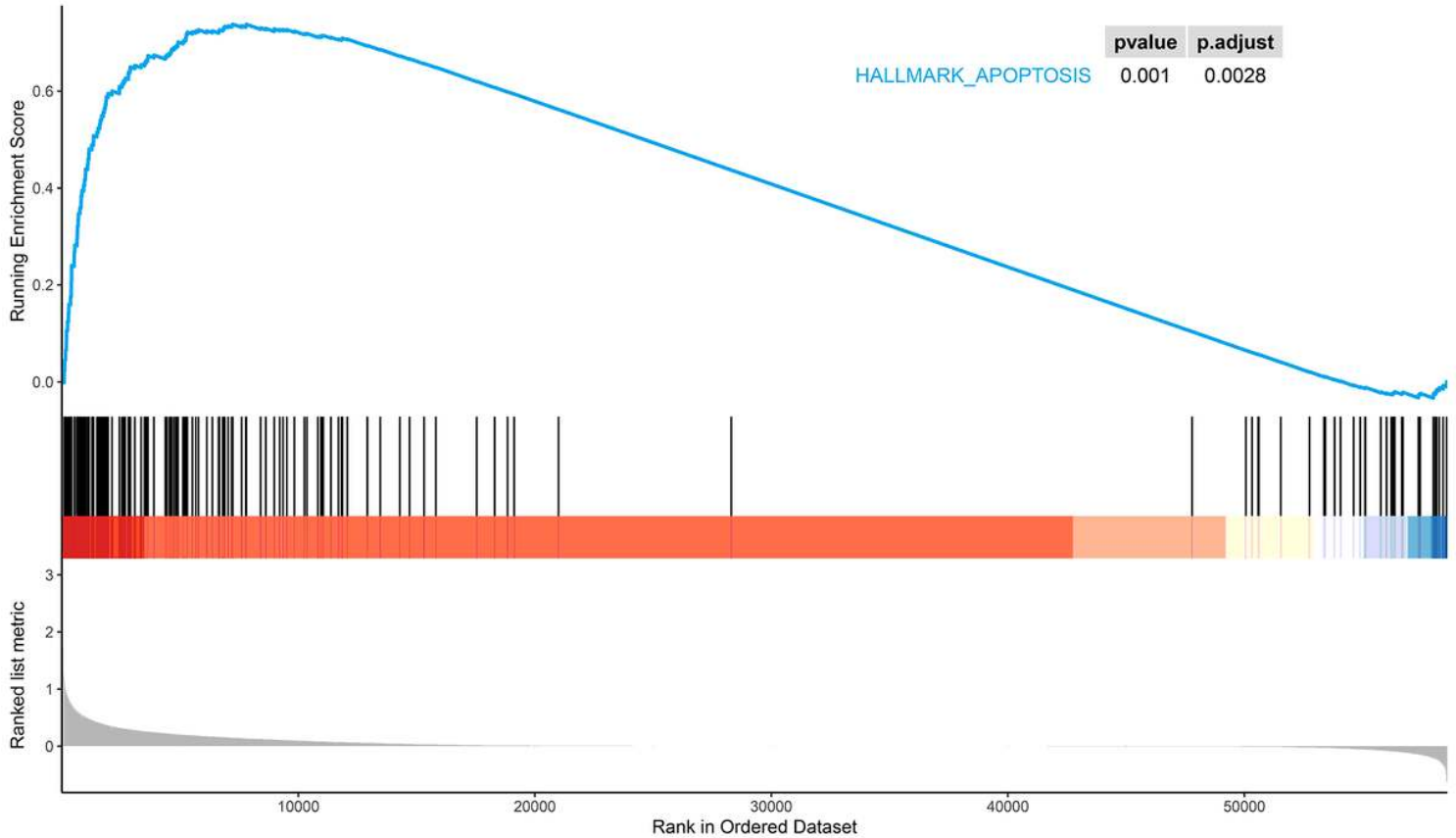
TP63 GSEA.



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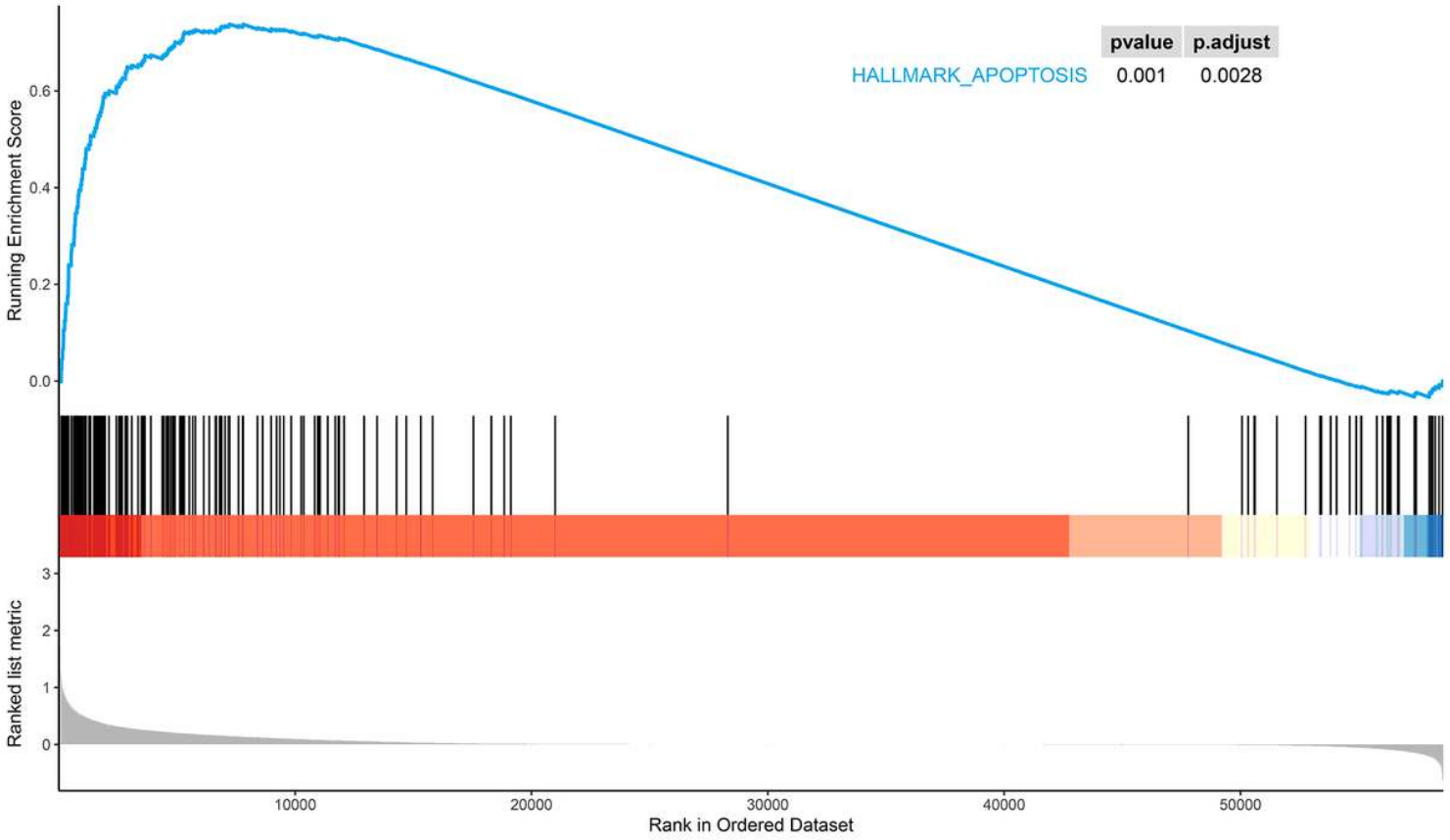

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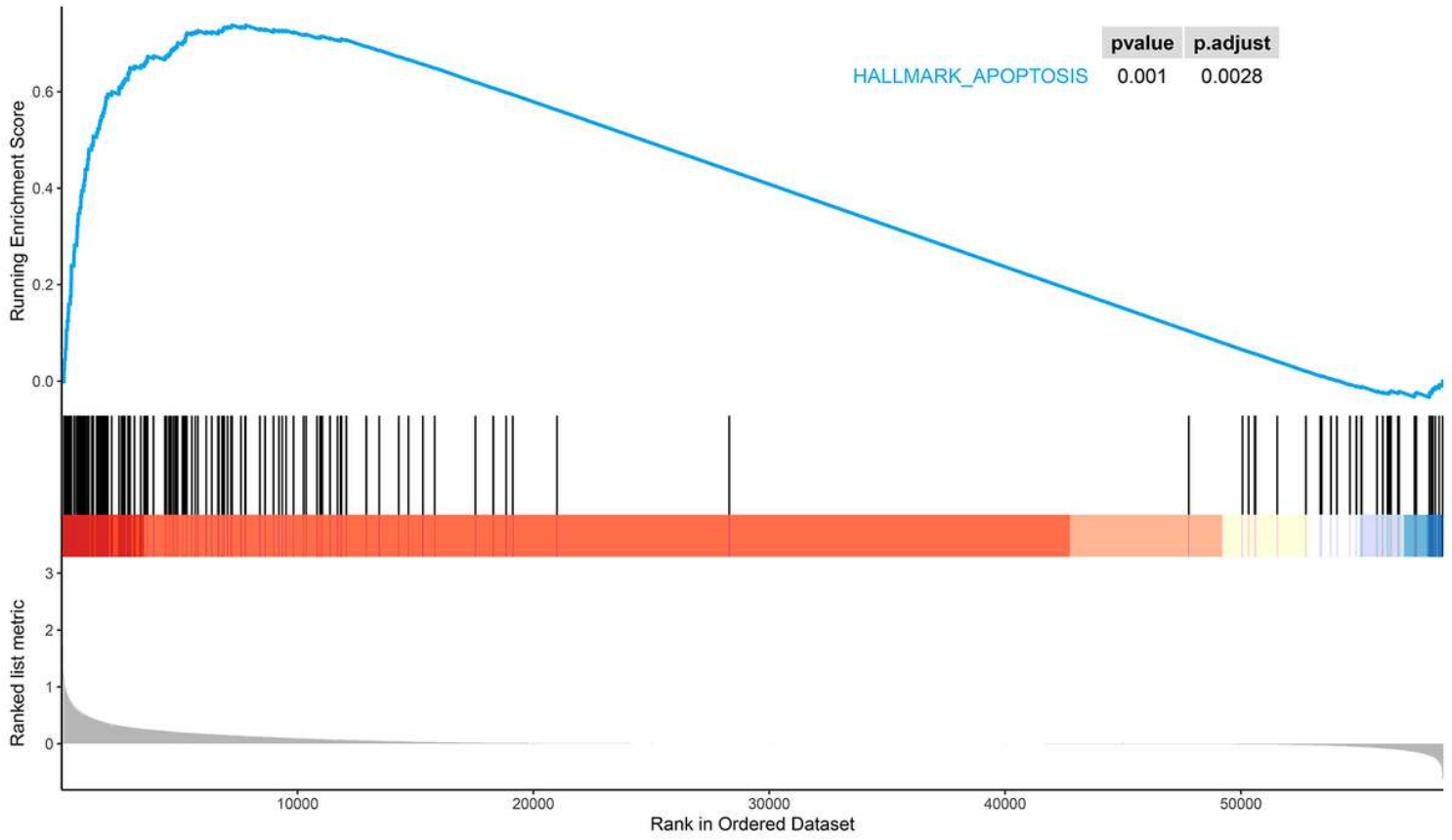

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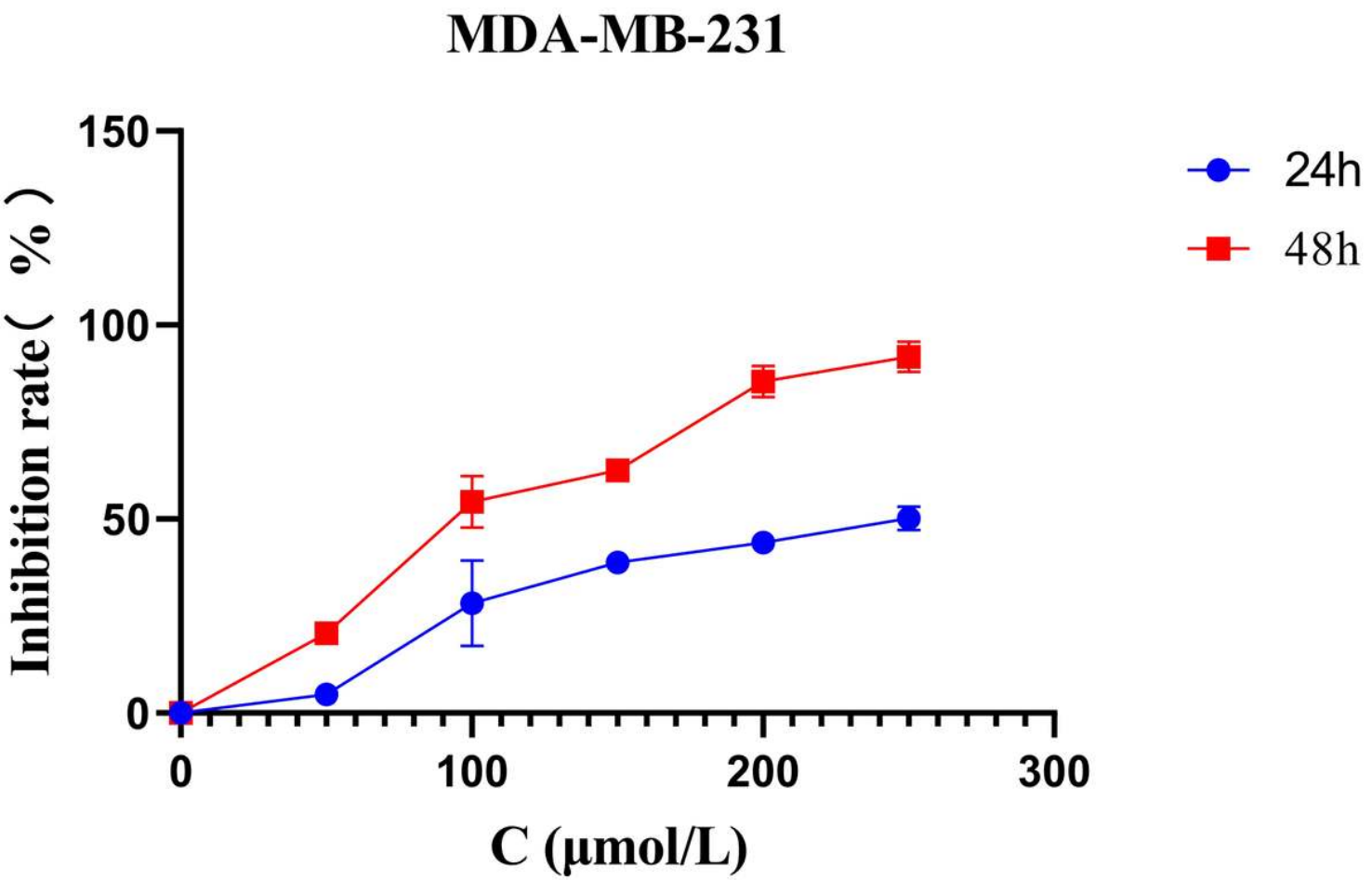
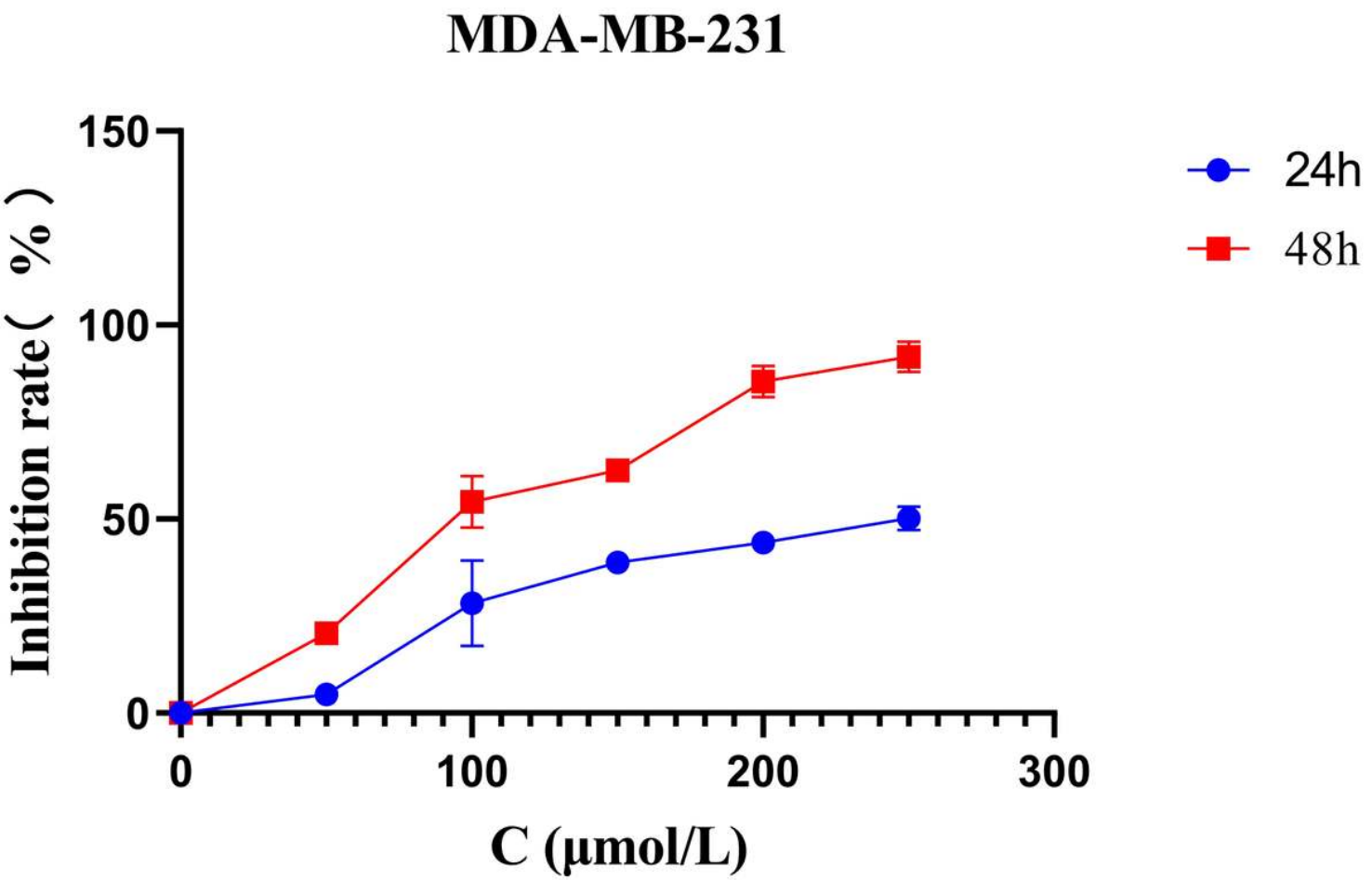


Figure 4

CCK-8 analysis of the MDA-MB-231 breast cancer cells. Treatment with different concentrations of diosbulbin B for 24 and 48 h inhibited the viability of these cells. The data are expressed as the mean ± SD of at least three independent experiments.



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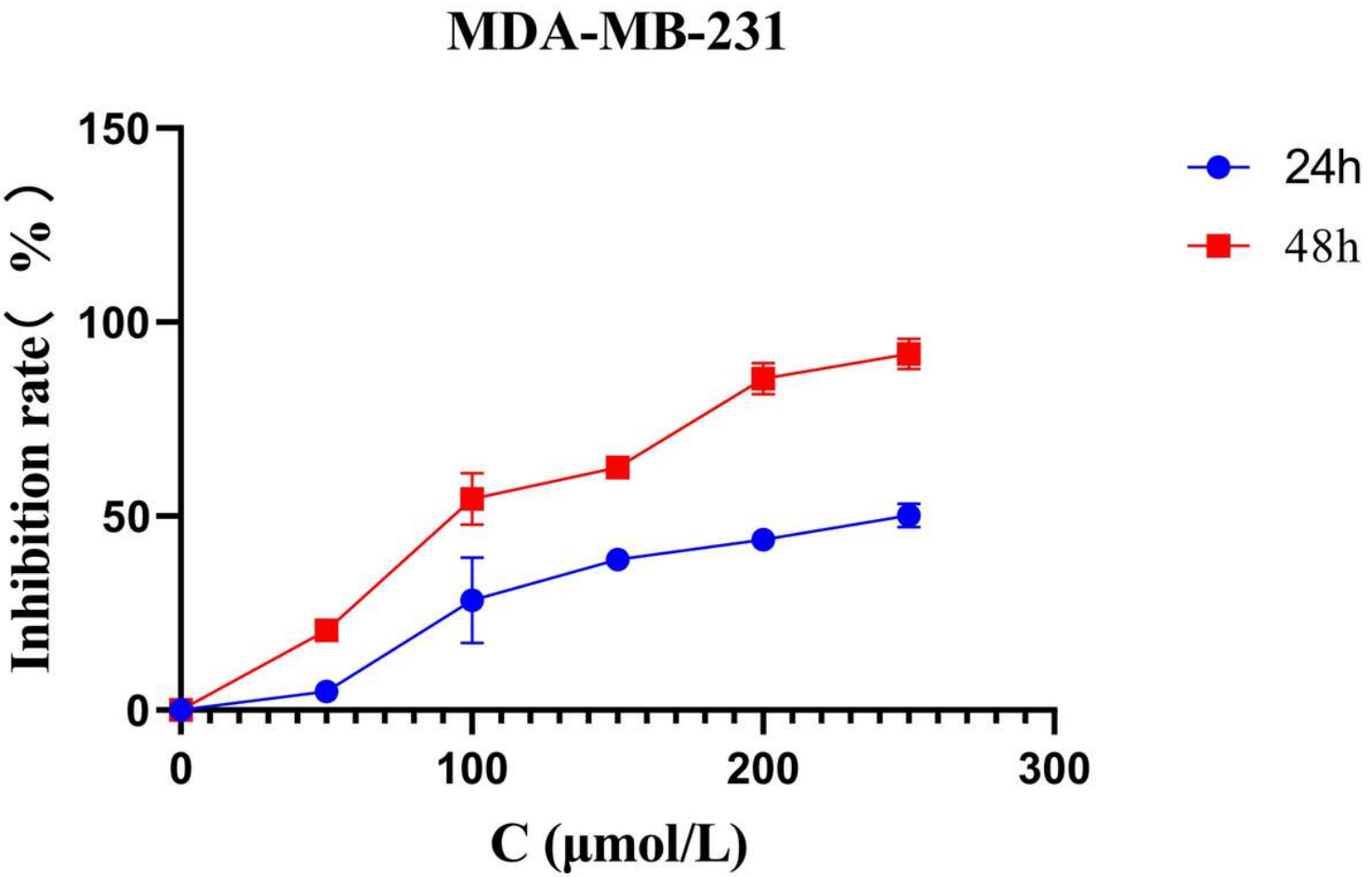

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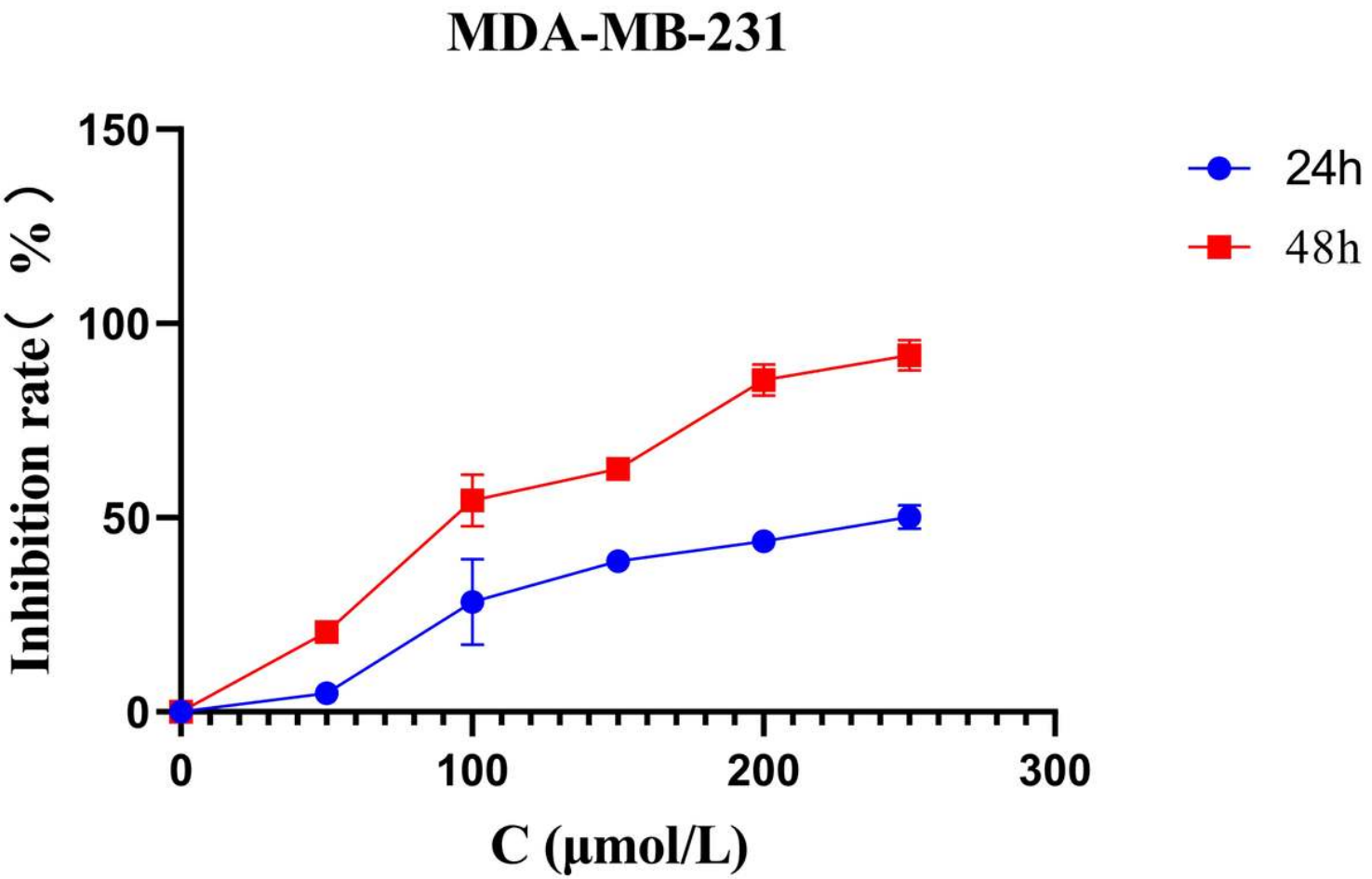

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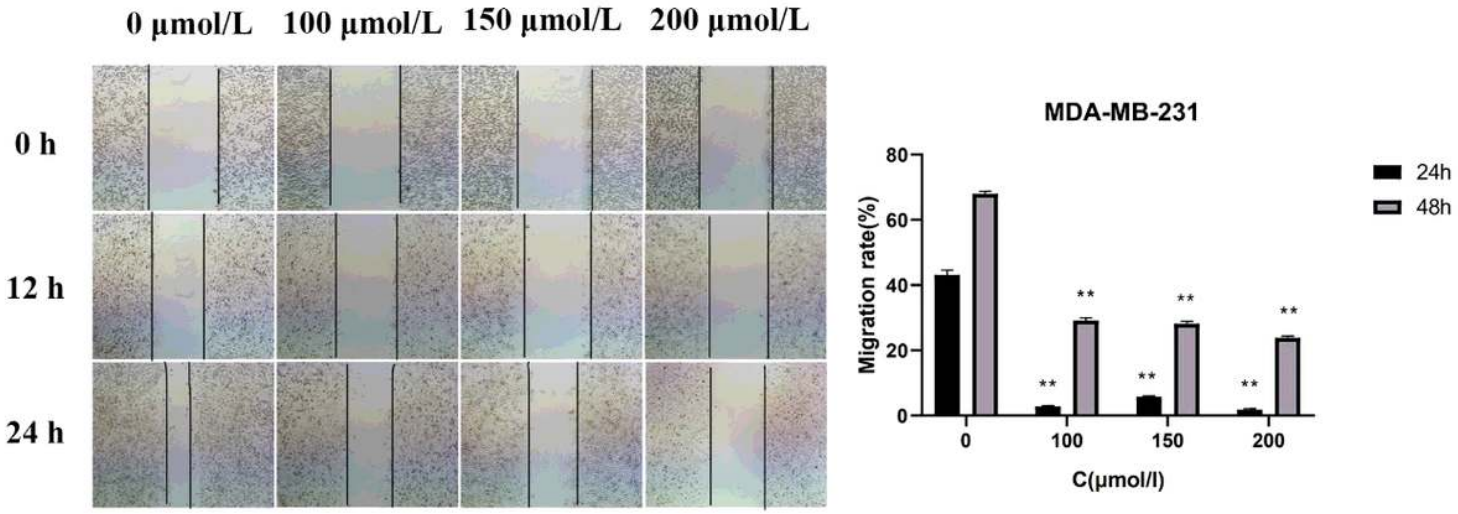

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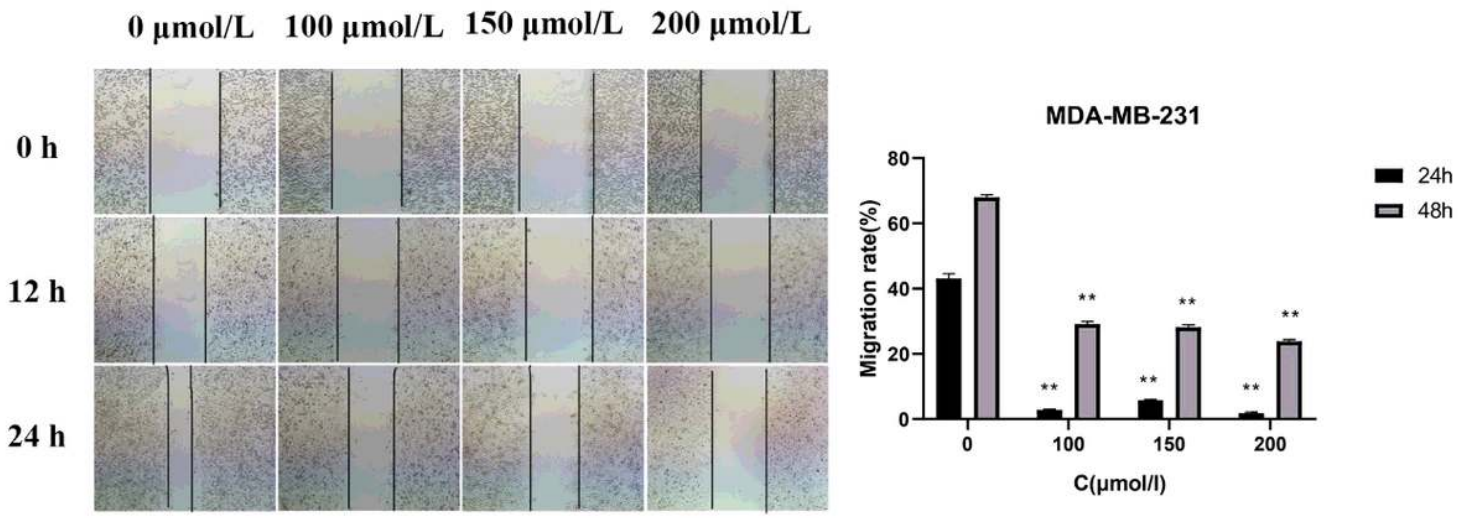
The effect of different concentrations of flavone B on cell migration. The percentage of migration rate is expressed as the mean ± SD of three independent experiments (** P <0.01 compared to the control group).



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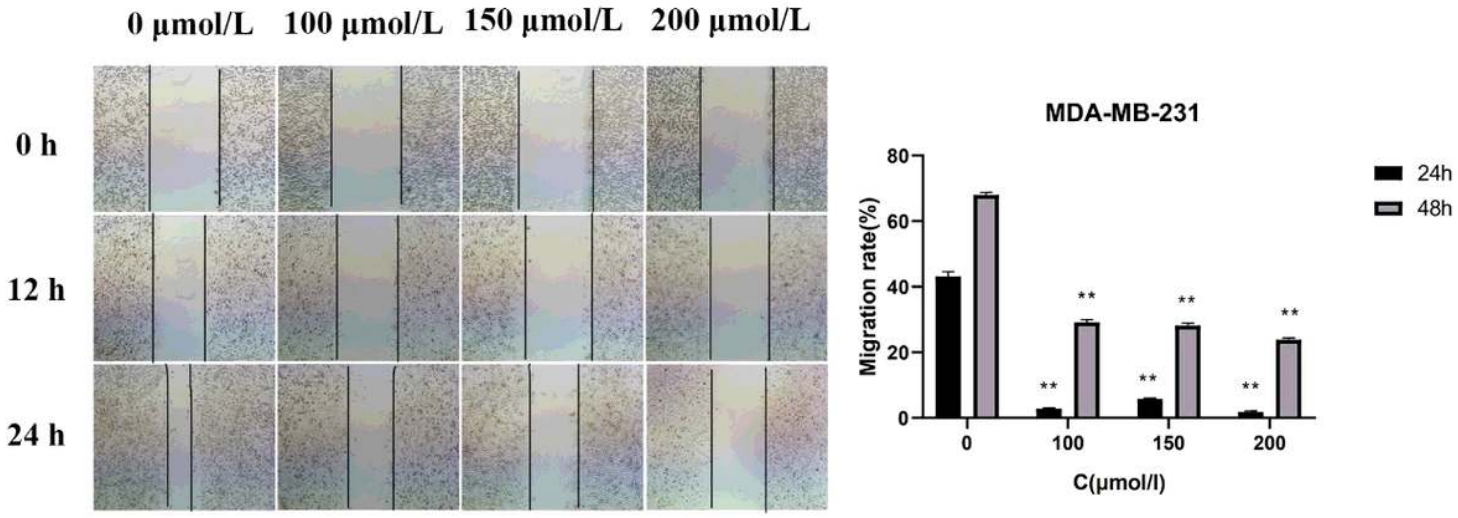

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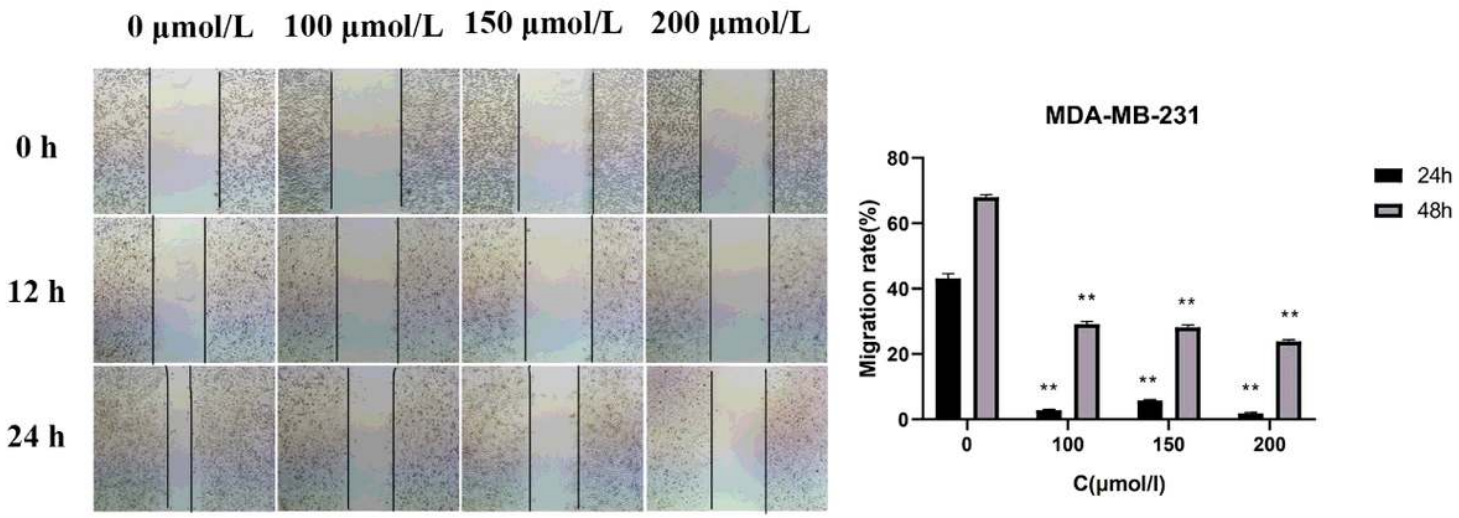

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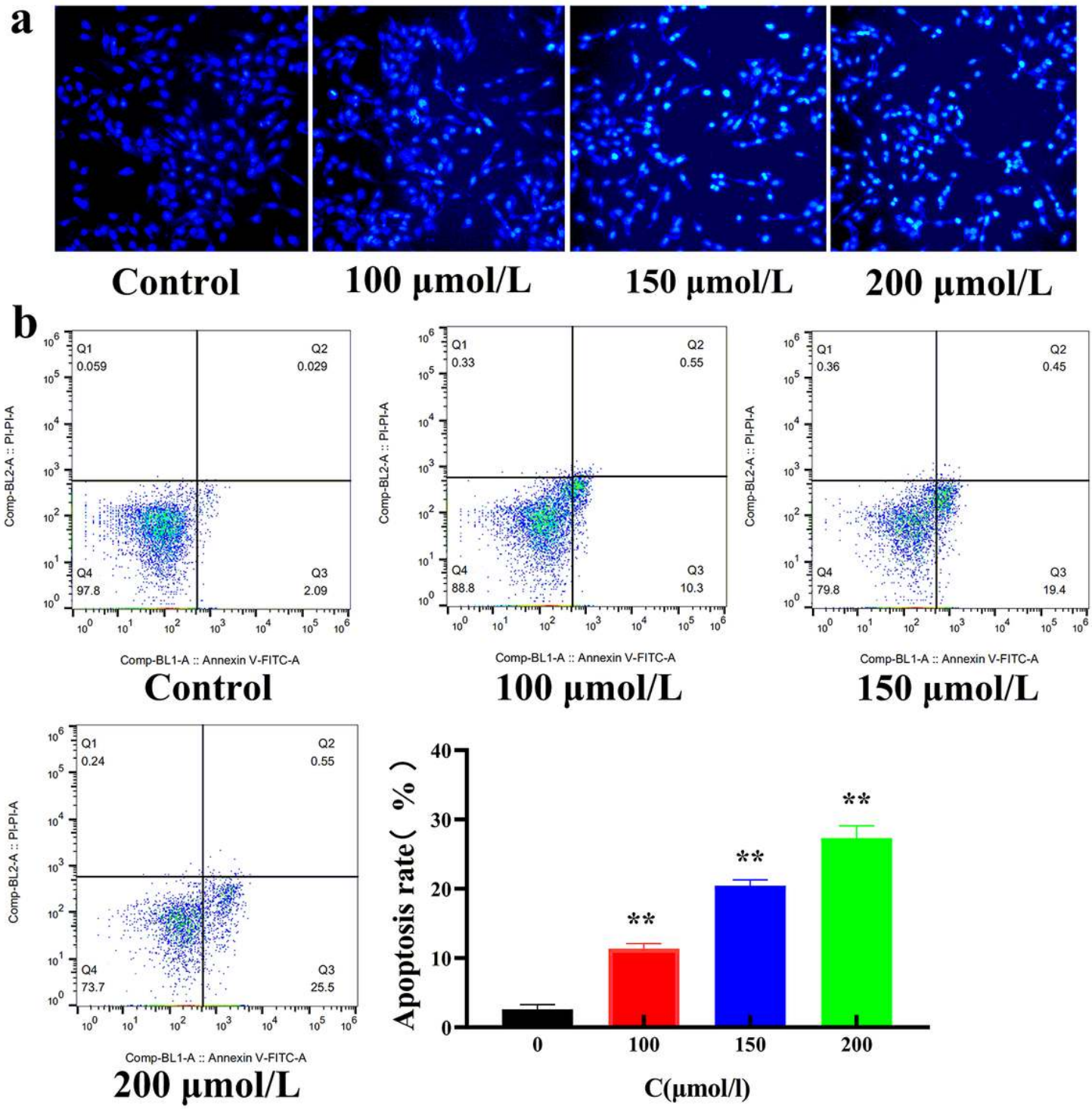

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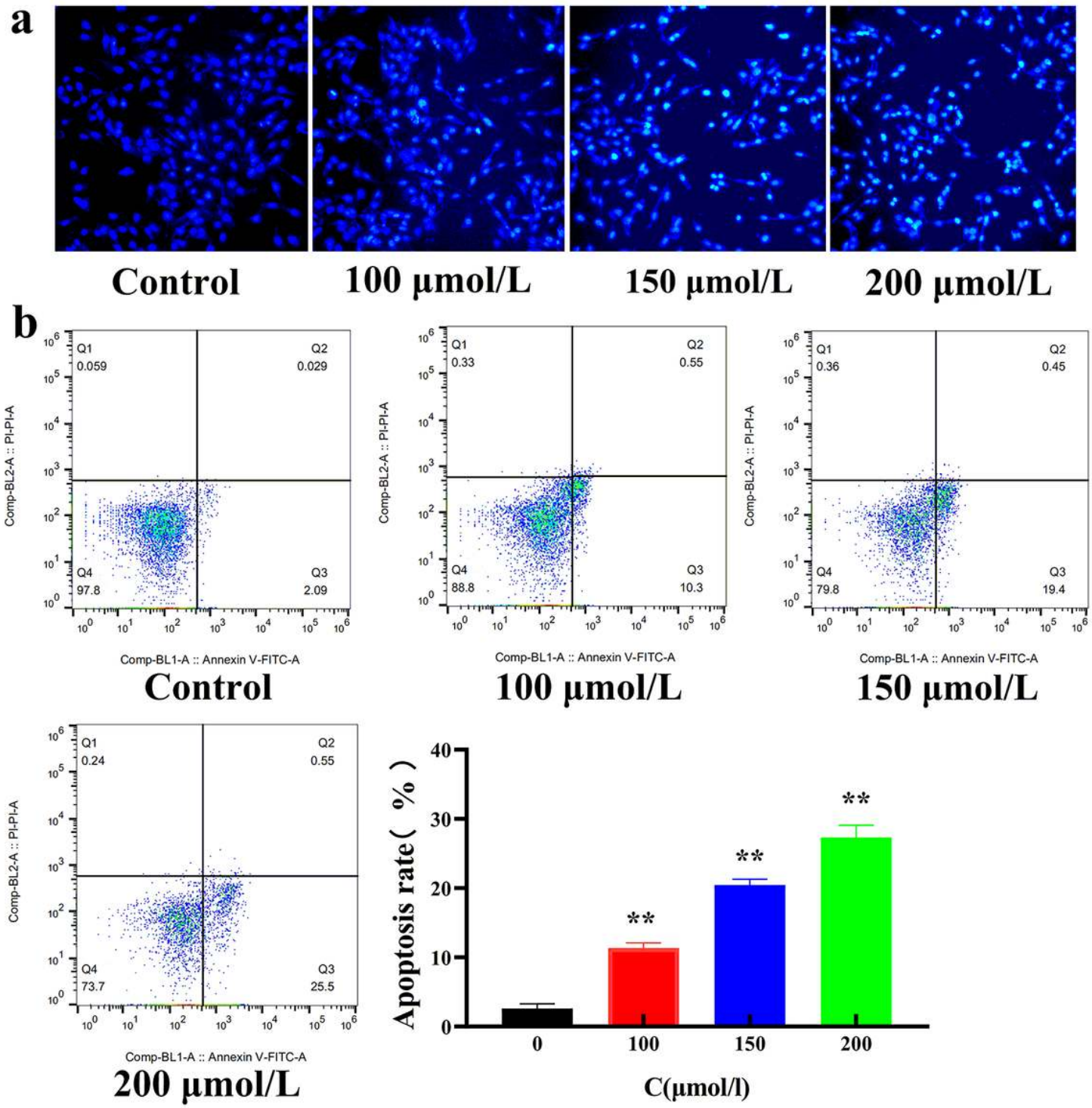
Apoptosis analysis after treatment of cells with different concentrations of diosbulbin B. a Hoechst staining revealed the morphological characteristics of apoptosis. b Annexin V-FITC/PI staining was used to analyze cell apoptosis via flow cytometry. The percentage of apoptotic cells is expressed as the average ± SD of three independent experiments (** $P < 0.01$ compared to the control group).



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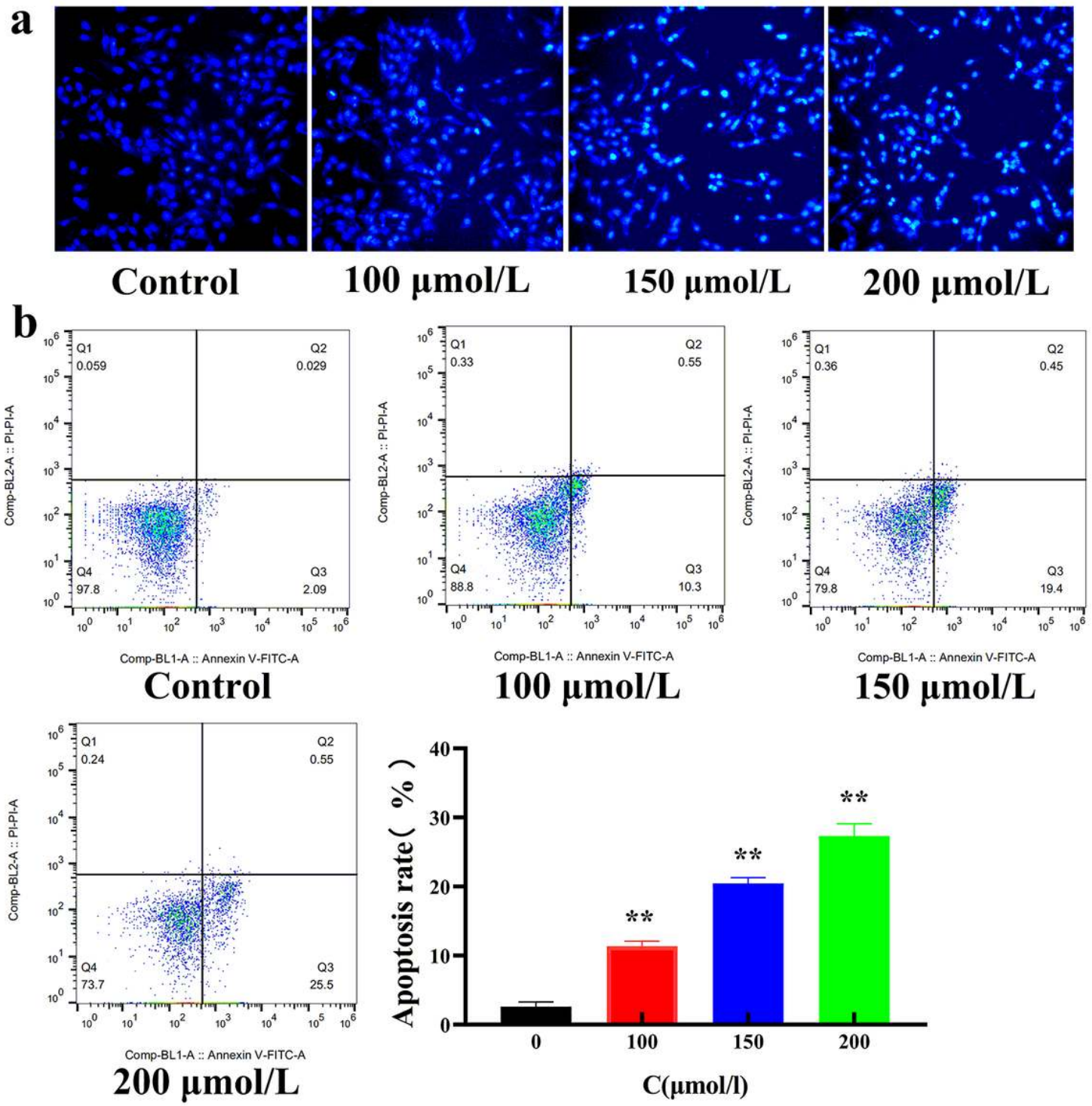

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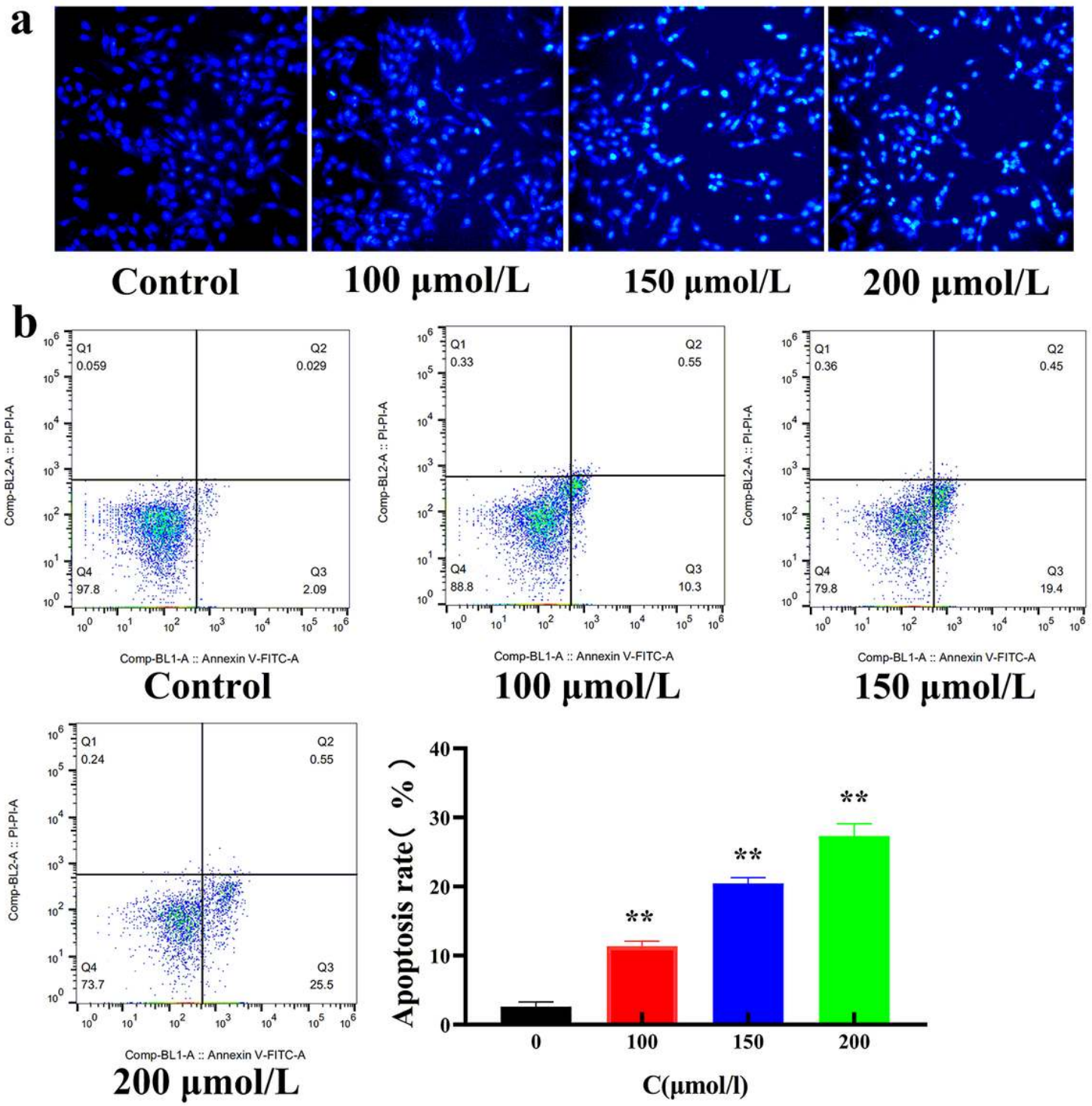

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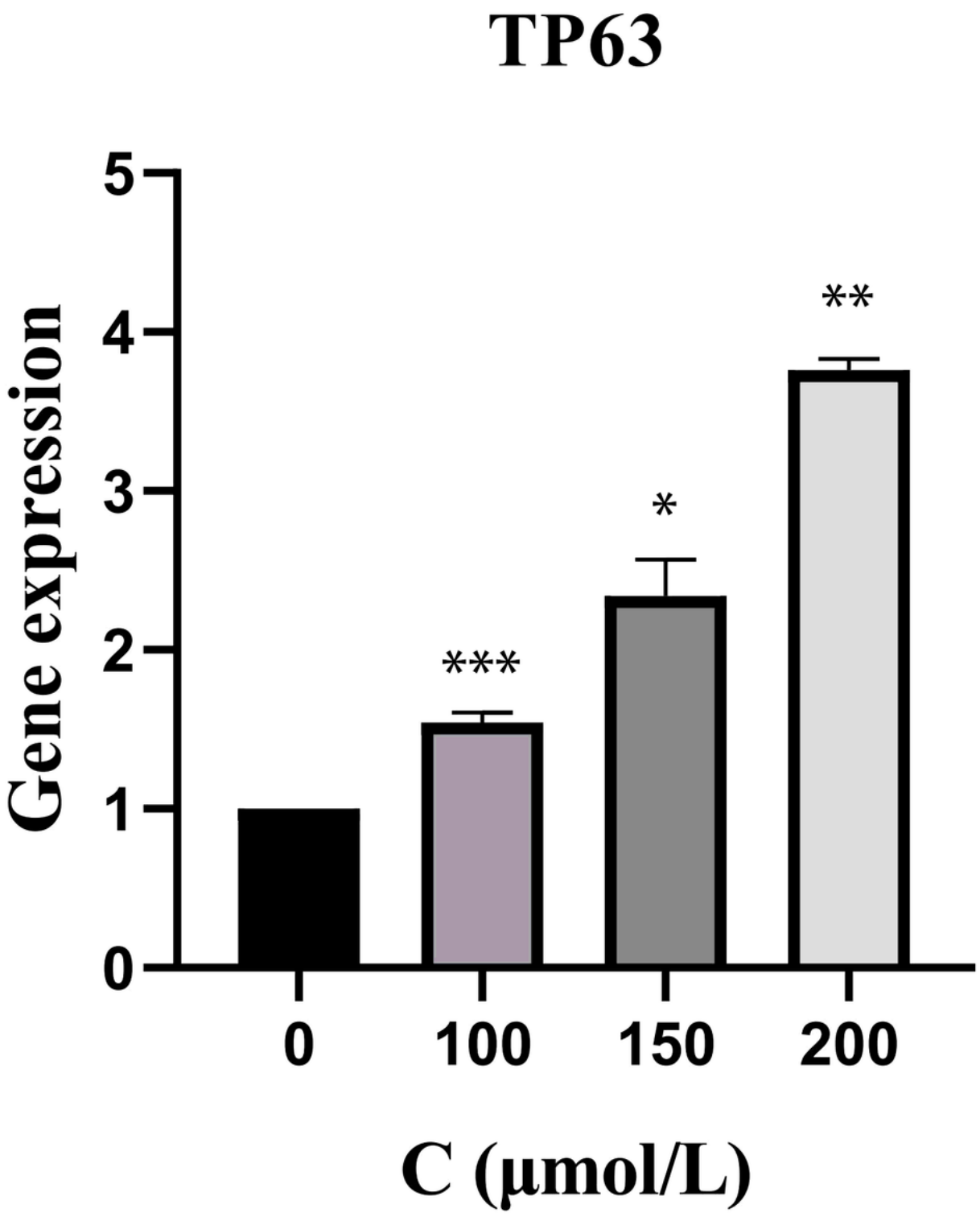

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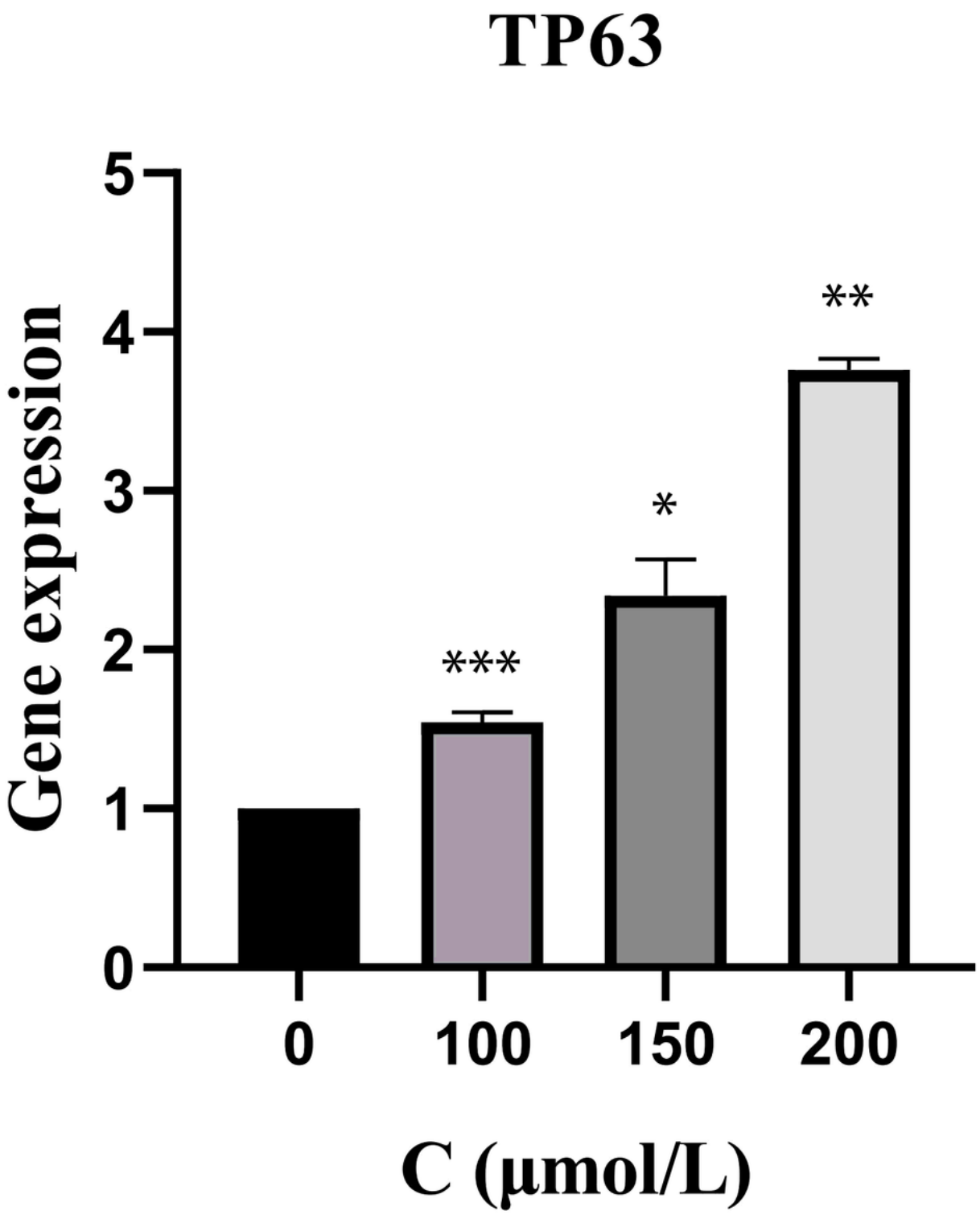
TP63 gene expression in cells treated with different concentrations of diosbulbin B ((***P<0.001**P<0.01 and *P<0.05 compared to the control group).



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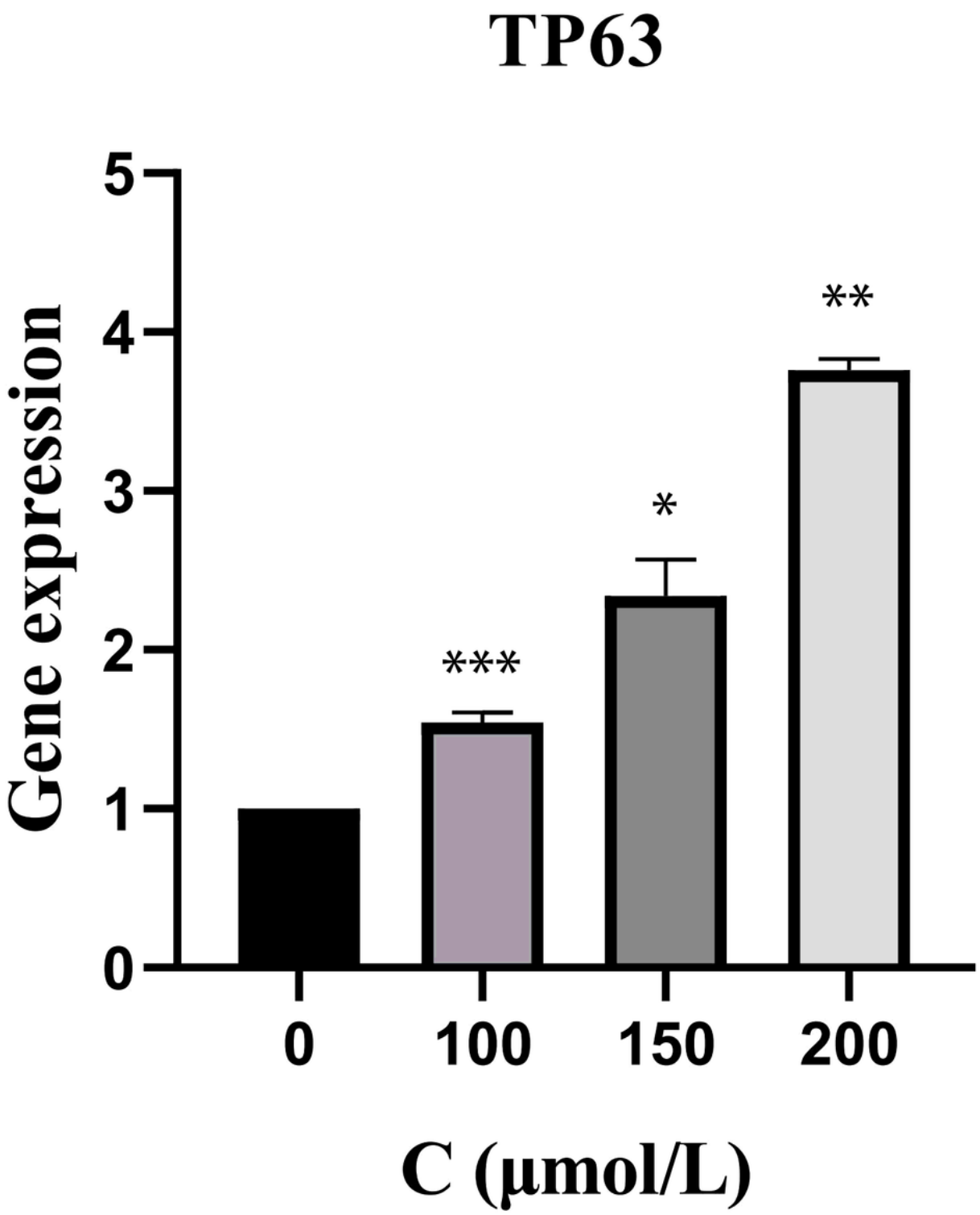

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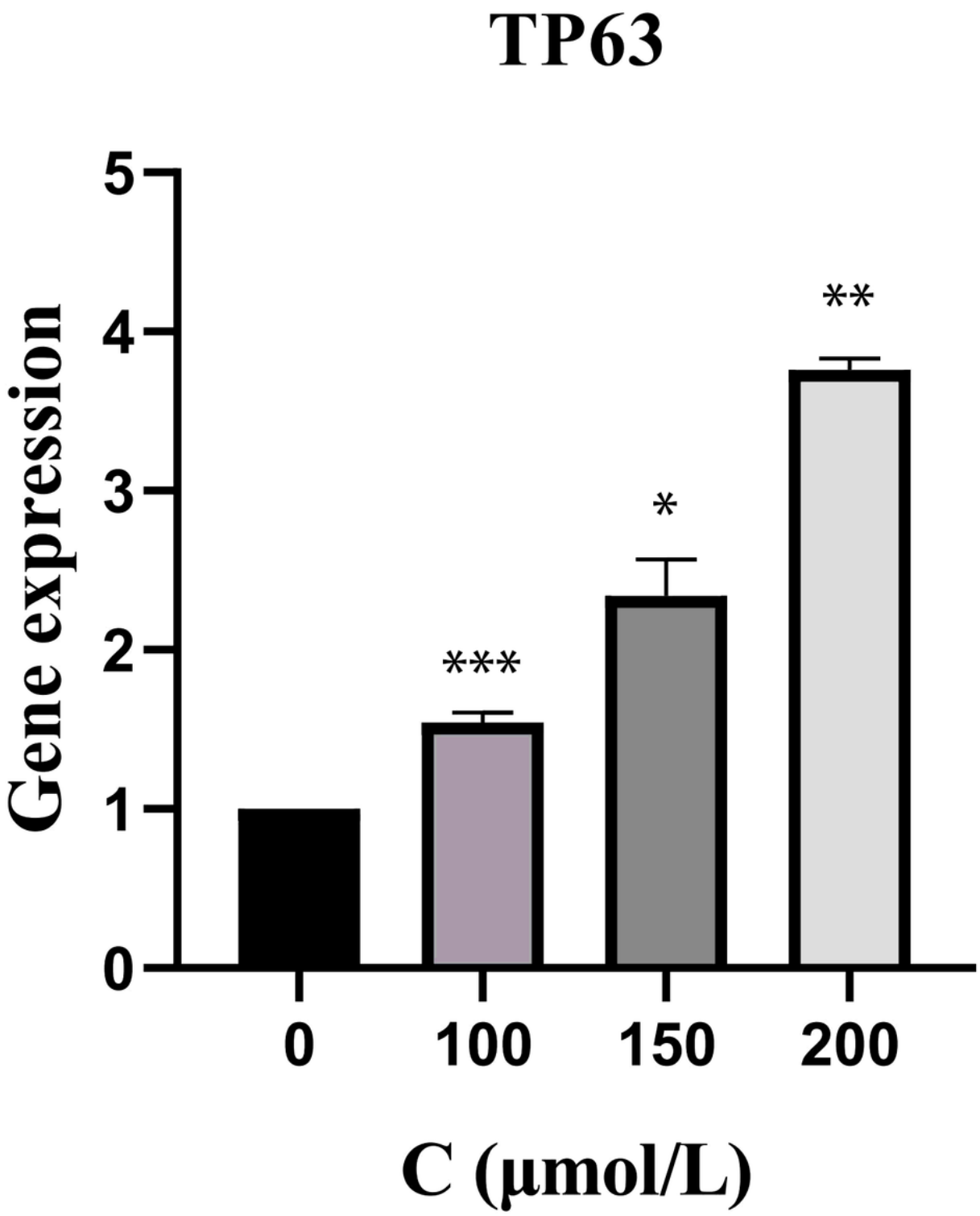

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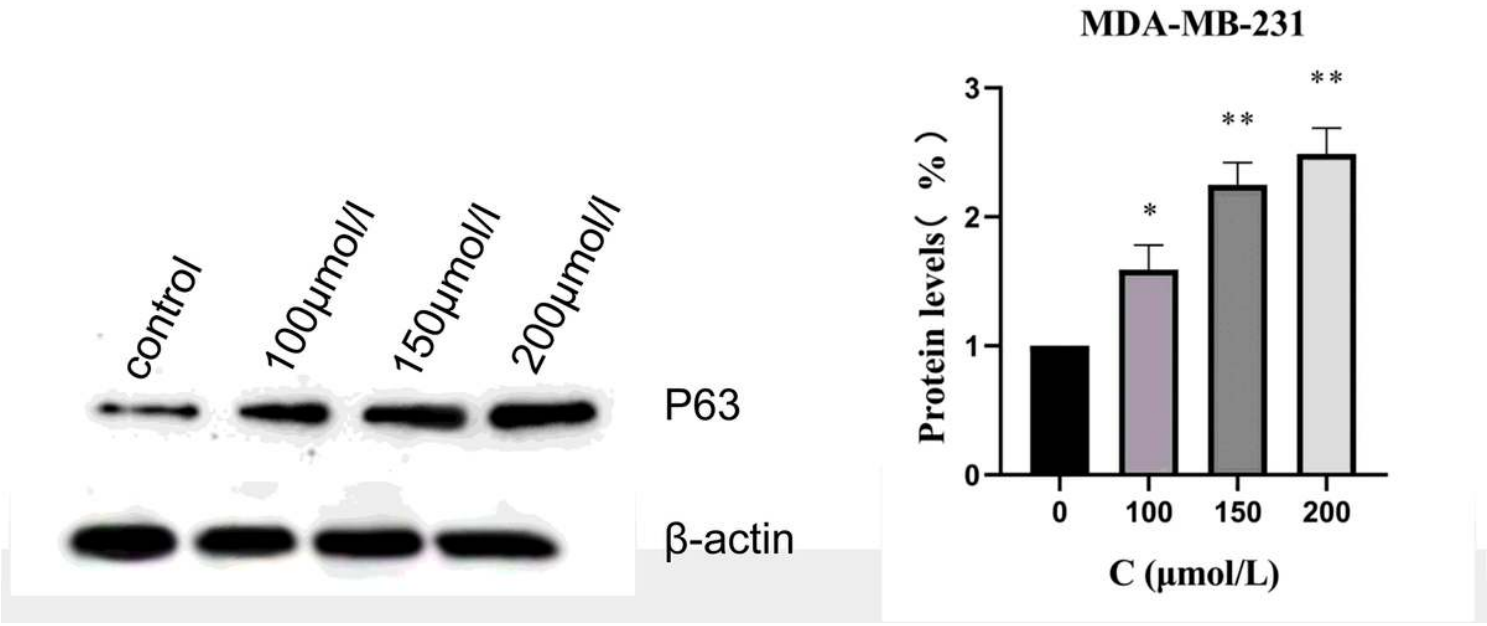

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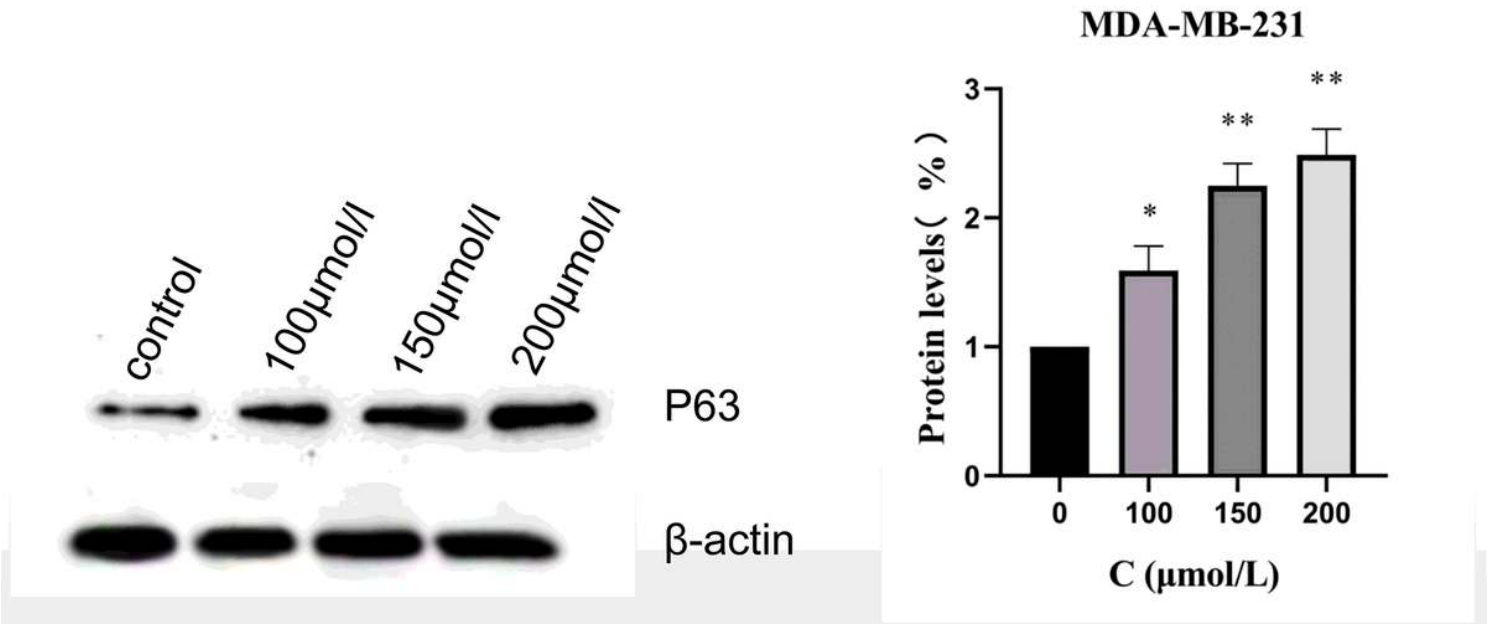

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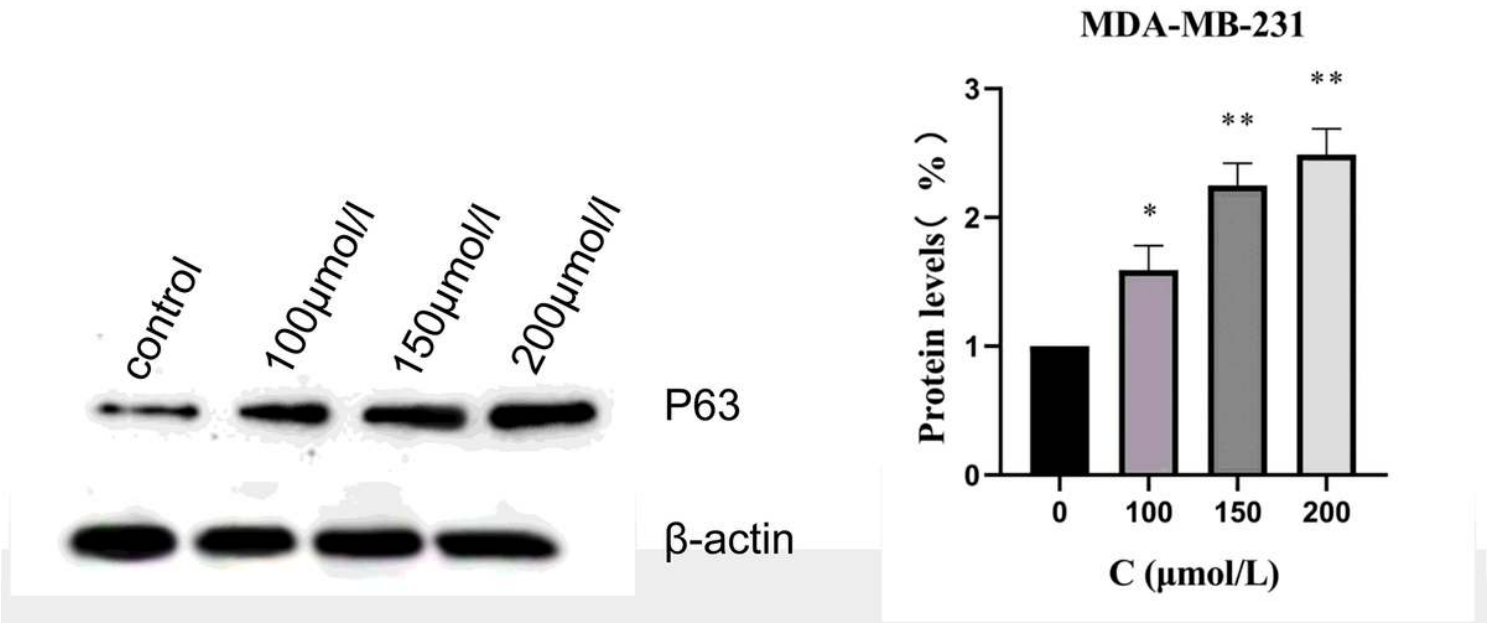

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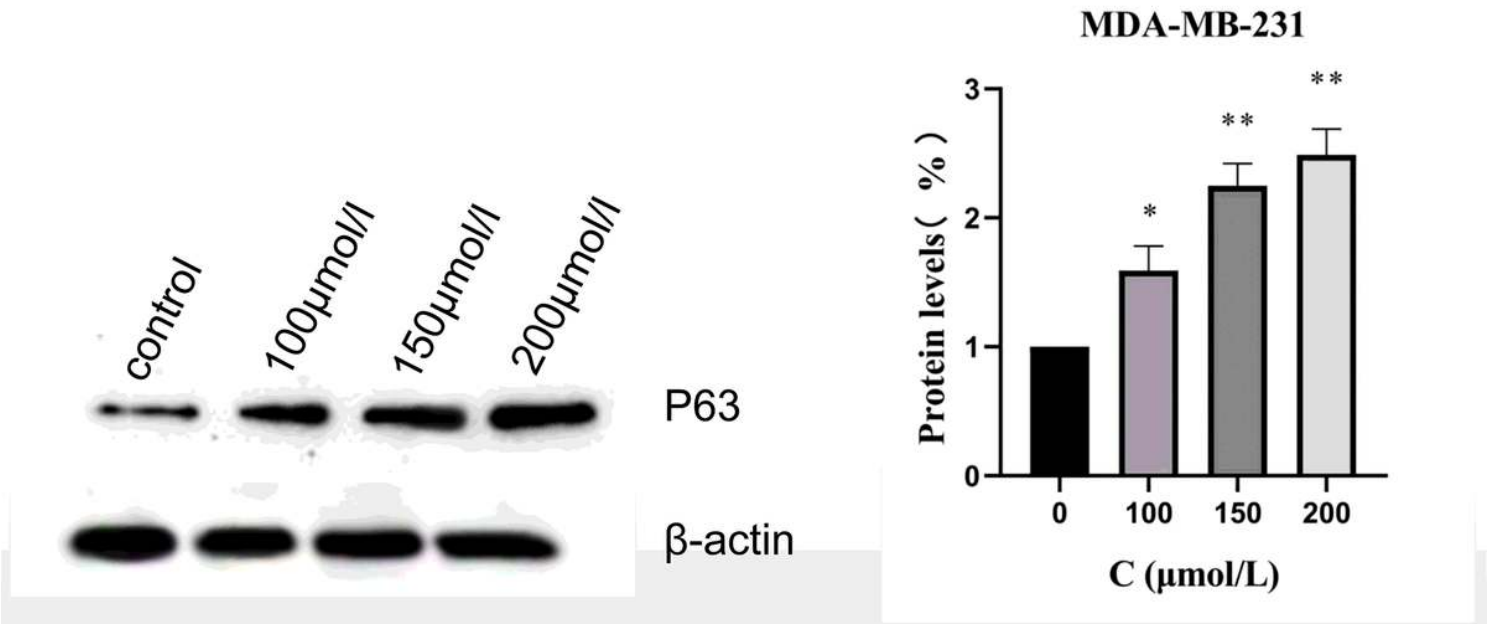

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