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Lactobacillus casei enhances the apoptosis inducing effect of geniposide on U87 human glioma cells *in vitro*

Fei CHEN[1], Zhipeng TENG[1], Xing PENG[1], Haibo WU[1], Wenwu WAN[1], Haibo LONG[1*]

Abstract

The goal of this work was to clarify the mechanism of action of Lactobacillus casei on genipin in triggering glioma apoptosis. DPPH and ABTS free radicals could be successfully eliminated by geniposide, LC-NBRC101979 (Lactobacillus casei), and geniposide + LC-NBRC101979. Geniposide and LC-NBRC101979 had effects that were stronger when combined than when taken alone. Geniposide, LC-NBRC101979, and Geniposide + LC-NBRC101979 did not have any detrimental effects on healthy epithelial cells HBE, but they greatly decreased the growth of U87 glioma cells, according to cell studies. Additionally, the combination of geniposide and LC-NBRC101979 had a stronger inhibitory impact than either drug alone. Results from qPCR revealed that in U87 cells, geniposide, LC-NBRC101979, and eniposide + LC-NBRC101979 could up-regulate caspase-3, COX-2, p53, c-myc, p21 mRNA expression and down-regulate Bcl-2, NF-κB expression. According to the results of the study on the effects of oxidative stress on cells, geniposide, LC-NBRC101979, and geniposide combined with LC-NBRC101979 can decrease the levels of MDA, LDH, and NO in normally damaged epithelial cells caused by oxidative stress and increase the levels of CAT, SOD, GSH-Px, and T-AOC. According to HPLC data, LC-NBRC101979 was able to convert the majority of geniposide into geniposide. It is clear that LC-NBRC101979 may enhance geniposide's inhibitory impact on glioma.

Keywords: Gardenia jasminoides; gardenia; genipin; Lactobacillus casei; glioma.

Practical Application: Gardenia is a health food that has been consumed as a beverage and in cooking for centuries in China. Although *Gardenia jasminoides* contains the active ingredient geniposide, its effects are not the same as those of geniposide derivatives. In order to increase the action of geniposide, *Lactobacillus casei* can hydrolyze it. According to this study, *Lactobacillus casei* can prevent glioma by producing more genipin. The study's findings presented a fresh strategy for *Gardenia jasminoides* to create premium health food.

1 Introduction

Rubiaceae includes the species *Gardenia jasminoides*. *Gardenia jasminoides* as a food can be used in cooking, used to stew soup, can also be used as a kind of tea drinks (Figure 1, Chan et al., 2022). *Gardenia jasminoides* is employed in functional foods and possesses liver-protecting, cholagogic, soothing, hemostasis-inducing, detumescence, etc. properties. The herb *Gardenia jasminoides* is frequently used to treat diabetes, hypertension, sprains, and icteric hepatitis (Hu, 2021). The primary active ingredient in *Gardenia jasminoides*'s therapeutic properties is geniposide, a kind of iridoid glycoside that is readily soluble in water. Depending on the source, it contains somewhere between 3% and 8% of the total (Chan et al., 2022). The enzyme-glucosidase has the ability to dissolve the glycosidic link in geniposide and hydrolyze it into geniposide. Studies have revealed that geniposide significantly affects disorders of the cardiovascular, central neurological, and digestive systems (Qian et al., 2018). Geniposide also contains some anti-inflammatory properties and can be used to treat soft tissue injuries. Geniposide is hydrolyzed into geniposide by the enzyme of β-glycosidase, which *Lactobacillus casei* may make in high quantities (Hua et al., 2018). To boost the biological activity of geniposide, *Lactobacillus casei* may increase the rate at which geniposide is converted to geniposide by this mechanism.

Gliomas, the most prevalent primary intracranial tumors and together known as neuroepithelial neoplasms, account for 40%–50% of brain tumors. The incidence rate for the year is 38 people per 100,000 people (Li et al., 2020). Due to its space-occupying impact, glioma can produce headache, nausea, vomiting, seizures, impaired vision, and other symptoms. Additionally, because it affects the way the local brain tissue functions, it may also result in additional patient symptoms, substantially impairing therapy and jeopardizing life (Hagemann et al., 2012). Damage to tissues and cells, such as ischemia and inflammation, can result from oxidative stress. Oxidative cell injury results in an increase in neutrophil inflammatory infiltration, protease release, and the production of many oxidation intermediates (Li et al., 2014). Additionally, several studies have demonstrated that inflammation is crucial to the emergence, growth, and spread of malignancies. Inflammation causes the production of many cytokines and chemical agents at the early stages of tumor growth, which is more likely to result in malignant transformation of cell proliferation. At the molecular level, cells

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[1]*Department of Neurosurgery, Chongqing Traditional Chinese Medicine Hospital, Chongqing, China*
*Corresponding author: Haibol_spine@126.com

Figure 1. Application of *Gardenia jasminoides* as food.

undergo a malignant change due to inflammation and other causes (Ma et al., 2022; Levine et al., 2022). Proinflammatory cytokines, which are essential for the conversion of healthy cells into cancer cells, are produced in greater quantities when NF-κB levels are activated (Barberi et al., 2018). A crucial step in the malignant development of tumors is the p53 gene mutation, which can activate cyclooxygenase to send inflammatory signals (COX-2) (Wu & Zhang, 2021). Cancer and conditions associated to cancer frequently include loss of p53 activity. The p53 gene or the p53-regulated network can be altered to influence genomic stability, tumor development, anticancer treatment, and angiogenesis (Tian et al., 2022). The expression levels of target genes implicated in cell cycle arrest, apoptosis, senescence, and anti-angiogenesis can be increased in response to cellular stress programs and activation of the p53 pathway, preventing the transformation of malignant tumors. The cell's potential death program, apoptosis, is signaled to be activated by p53 in cases when metabolic abnormalities or genetic damage are too severe to correct (Blagih et al., 2020). One of the most effective inhibitors of the growth and spread of cancer is p53. A therapeutic target for cancer has been identified as the p53 signaling pathway. As a result, the p53 signaling pathway may be used as a therapeutic target for cancer (Wu & Zhang, 2021).

Lactobacillus casei can exert its biological activity and make fermented food have a good health functions (Rafiq et al., 2020, Hernández-Olivas et al., 2020; Balthazar et al., 2021). The purpose of this work was to examine the mechanism by which genipin increased the impact of *Lactobacillus casei* NBRC101979 (LC-NBRC101979) on glioma by inducing apoptosis in U87 human glioma cells when combined with *Lactobacillus casei* (*Lactobacillus casei* NBRC101979). The findings of this study will serve as a theoretical basis for the continued development of *Gardenia jasminoides* as a raw ingredient in health foods.

2 Materials and methods

2.1 Antioxidant capacity determination

The geniposide was dissolved in purified water in a concentration of 100 μg/mL solution. For 6 hours, the LC-NBRC101979 solution (10^6 CFU/mL, Xinyang Zhongjian Metrological Biotechnology Co., Ltd, Xinyang, Henan, China) was put in filtered water at 37 °C. The geniposide + LC-NBRC101979 solution (100 μg/mL geniposide and 10^6 CFU/mL LC-NBRC101979) was incubated for 6 hours in purified water at 37 °C. The antioxidant capabilities of geniposide, LC-NBRC101979, and geniposide + LC-NBRC101979 solutions were determined for 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis [3-ethylbenzothiazoline-6-sulphonic acid] (ABTS) (Lin & Zhao, 2010).

2.2 Viability testing and cell culture

U87 human glioma cells were grown in Dulbecco's modified Eagle's medium (DMEM, Solarbio Life Sciences, Beijing, China), and HBE normal human bronchial epithelial cells in DMEM (H) media (Solarbio Life Sciences, Beijing, China). All media included 10% fetal bovine serum and 1% penicillin/streptomycin, and the cells were kept in a 37 °C incubator with 5% CO_2. A Cell Counting Kit-8 (CCK-8) test (Solarbio Life Sciences, Beijing, China) was used to measure cell viability. U87 and HBE cells were seeded onto 96-well cell culture plates and allowed to adhere for 24 hours at 37 °C with 5% CO_2. When the cell plating rate reached 80%, the media was aspirated, and the test chemicals were added to the cell culture medium at the final concentrations requested. After 24 hours, the media containing the geniposide, LC-NBRC101979, and geniposide + LC-NBRC101979 solutions was aspirated, and the cells were treated with 100 μL of new medium containing 10 μL CCK-8 buffer. After 4 hours of incubation at 37 °C, absorbance values at 450 nm were determined using a microplate reader (Nano-300, Hangzhou Allsheng Instruments Co., Ltd., Hangzhou, Zhejiang, China). The reading represented the number of viable cells and was displayed as a percentage of the control's viable cells.

2.3 RNA extraction and real-time quantitative PCR

Using an RNA extraction kit (BaiMaiKe Technologies, Beijing, China), total RNA was isolated from fresh cells and reverse transcribed into cDNA (Yeasen Technologies, Shanghai, China). Hieff™ qPCR SYBR® Green Master Mix was used to quantify the

target genes (Yeasen Technologies, Shanghai, China). The $2^{-\Delta\Delta Ct}$ technique was used to examine the results. Table 1 shows the primer sequences employed in this investigation (Hu et al., 2022; Long et al., 2022).

2.4 Assay for oxidative damage

HBE cells were seeded at 10^5 cells/well in 96-well cell culture plates, incubated overnight at 37 °C, and treated for 24 hours in triplicate with 100 µL culture media containing geniposide solution, LC-NBRC101979 solution, and geniposide + LC-NBRC101979 solution. The culture medium was then changed with H_2O_2 medium at a concentration of 100 µmol/L in 100 µL. To harvest cells, culture medium was aspirated. To harvest cells, culture medium was aspirated. Lactate dehydrogenase (LDH), malondialdehyde (MDA), nitric oxide (NO), glutathione peroxidase (GSH-Px), oxidized glutathione (GSSH), catalase (CAT), superoxide dismutase (SOD), and total anti-oxidation capability (T-AOC) levels were measured according to the manufacturer's instructions for the appropriate kits (Solarbio Life Sciences, Beijing, China).

Table 1. Primer sequences for qPCR in this experiment.

Gene	Primer Sequence
Actin	F: 5'-TCAAGAAGGTGGTGAAGCAGG-3'
	R: 5'-AGCGTCAAAGGTGGAGGAGTG-3'
p53	F: 5'-CTTTGAGGTGCGTGTTTGTGC-3'
	R: 5'-GGTTTCTTCTTTGGCTGGGGA-3'
p21	F: 5'-TGTCCGTCAGAACCCATGC-3'
	R: 5'-AAAGTCGAAGTTCCATCGCTC-3'
c-myc	F: 5'-TGGAACGTCAGAGGAGAAACGA-3'
	R: 5'-CTTGAACGGACAGGATGTAGGC-3'
Caspase-3	F: 5'-CATGGAAGCGAATCAATGGACT-3'
	R: 5'-CTGTACCAGACCGAGATGTCA-3'
COX-2	F: 5'-CTGGCGCTCAGCCATACAG-3'
	R: 5'-CGCACTTATACTGGTCAAATCCC-3'
Bcl-2	F: 5'-ATGTGTGTGGAGAGCGTCAACC-3'
	R: 5'-CAGAGACAGCCAGGAGAAATCAA-3'
NF-κB	F: 5'-GAAGCACGAATGACAGAGGC-3'
	R: 5'-GCTTGGCGGATTAGCTCTTTT-3'

2.5 HPLC (high-performance liquid chromatography) analysis

A standard reference solution with a concentration of 0.1 mg/mL was created. The geniposide solution and geniposide + LC-NBRC101979 solution were filtered using a HyperSep C18 column (Thermo Scientific, 320 Rolling Ridge Drive, Bellefonte, PA, USA) and passed through a 0.22-m filter membrane prior to HPLC analysis. Thermo Fisher Scientific's UltiMate3000 HPLC system was used with an Accucore C18 column (4.6 mm150 mm, 2.6 µm), acetonitrile as mobile phase A, 0.5% acetic acid water as mobile phase B, gradient elution, flow rate of 1.5 mL/min, detection wavelength of 285 nm, column temperature of 30 °C, and injection volume of 10 µL.

2.6 Statistical investigation

For data processing and analysis, GraphPad Prism 7.0 (Graph Pad Software, La Jolla, USA) and SPSS statistical software programs (SPSS Institute Inc., Chicago, IL, USA) were used. The data is presented as mean ± STDE, and the significant difference between groups was determined using one-way ANOVA and Duncan's multiple range tests.

3 Results

3.1 In vitro antioxidant activity

Geniposide, LC-NBRC101979, and geniposide + LC-NBRC101979 solutions show DPPH and ABTS free radical scavenging actions, with geniposide + LC-NBRC101979 having a larger impact than geniposide and LC-NBRC101979 (Figure 2).

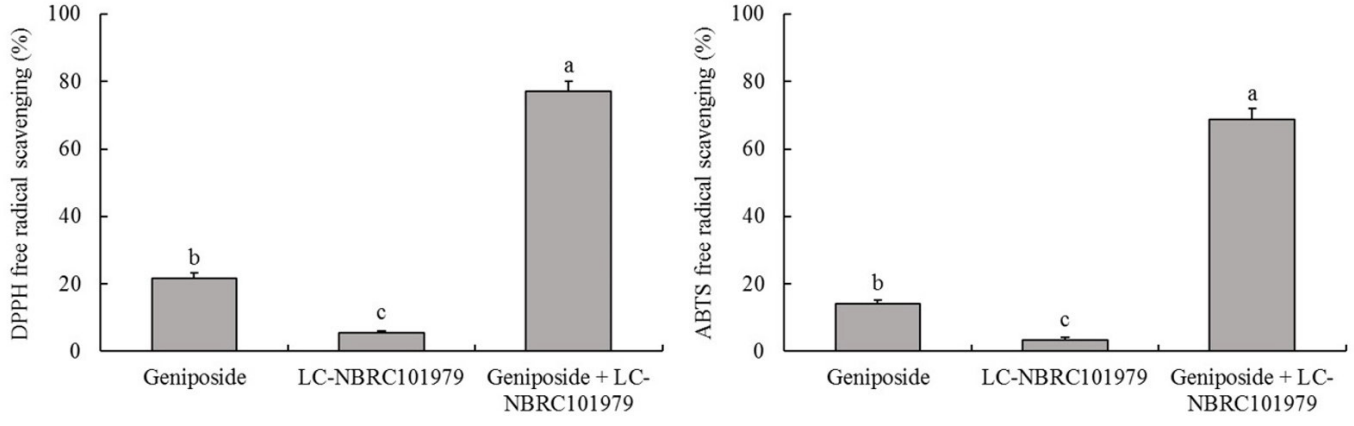

Figure 2. Scavenging ability of experimental subjects to DPPH and ABTS free radicals. a-c represents statistical results, and different letters represent significant differences between corresponding groups. Geniposide: 100 µg/mL geniposide solution treatment; LC-NBRC101979: 10^6 CFU/mL LC-NBRC101979 solution treatment; Geniposide + LC-NBRC101979: 100 µg/mL geniposide and 10^6 CFU/mL LC-NBRC101979 solution treatment.

3.2 Cytotoxicity evaluation

The concentrations of Geniposide, LC-NBRC101979, and geniposide + LC-NBRC101979 solutions were substantially positively linked with the reduction of U87 cell growth during a 24-hour treatment period with these substances (Figure 3). The three solutions mentioned above had no discernible impact on the growth of HBE cells, nevertheless.

3.3 Apoptosis-related gene expression alterations

After 24 hours of treatment with geniposide, LC-NBRC101979, and geniposide + LC-NBRC101979, the effects of geniposide, LC-NBRC101979, and geniposide + LC-NBRC101979 on glioma cells were evaluated at the gene level, and the cells' mRNA levels were quantified (Figure 4). The expression of apoptosis-related genes caspase-3, COX-2, p53, c-myc, and p21 was up-regulated after geniposide, LC-NBRC101979, and geniposide + LC-NBRC101979. Bcl-2 and NF-κB expression, on the other hand, were down-regulated. The induced level of geniposide + LC-NBRC101979 was more noticeable, as was the apoptosis.

3.4 Oxidative damage of normal cells

Keep track of how geniposide, LC-NBRC101979, and geniposide combined with LC-NBRC101979 affect several redox status markers in cells that have been exposed to H_2O_2. Table 2 presents the outcomes. MDA, LDH, and NO levels among oxidative damage indicators were greater in the H_2O_2-induced injury model group than in the healthy control group. The levels of CAT, SOD, GSH-Px, and T-AOC, on the other hand, were lower than those in the healthy control group. In the geniposide, LC-NBRC101979, and geniposide + LC-NBRC101979 groups, MDA, LDH, and NO levels were lower than those of the model group, whereas CAT, SOD, GSH-Px, and T-AOC levels were higher. Different alterations were more pronounced in the geniposide + LC-NBRC101979 therapy group.

3.5 Effect of LC-NBRC101979 on geniposide transformation

The examination of the transformation of geniposide by LC-NBRC101979 utilizing HPLC technology is displayed in Figure 5. The outcomes of the experiment demonstrate that LC-NBRC101979 can convert the majority of geniposide into geniposide with improved biological activity and stability.

4 Discussion

The effect of active substances on cancer cells was first observed whether they could promote apoptosis of cancer cells, rather than lead to cell death by toxic effects. Active substances that can only act on cancer cells without affecting normal cells can be called tumor suppressants. This study shows that geniposide and *Lactobacillus casei* can both be considered as tumor suppressants, and under the joint action of *Lactobacillus casei* and geniposide, the effect of inhibiting the proliferation of cancer cells is very strong.

Inflammation is the complicated biological response of the body to irritation, damage, or infection (Mantovani, 2010). Numerous studies have demonstrated that inflammation increases the formation and progression of cancers (Kumar et al., 2016; Li et al., 2005). Although acute inflammation might be beneficial, persistent inflammation can increase tumor growth. When there is inflammation in the body, the prognosis of cancer patients is bad (Deng et al., 2016). Tumor necrosis factor (TNF) is a key mediator of tumor-associated inflammation and one of the most critical molecules for activating the NF-κB signaling pathway, which inhibits cancer cell death (Wang et al., 2016; Wang et al., 2022).

Apoptosis is the autonomous and orderly death of cells regulated by genes in order to preserve the internal environment's integrity. It entails the activation, expression, and control of a number of genes involved in the process of removing damaged or malignant cells. Apoptosis is a process in which several genes are tightly controlled. These genes, including as the Bcl-2 family, the

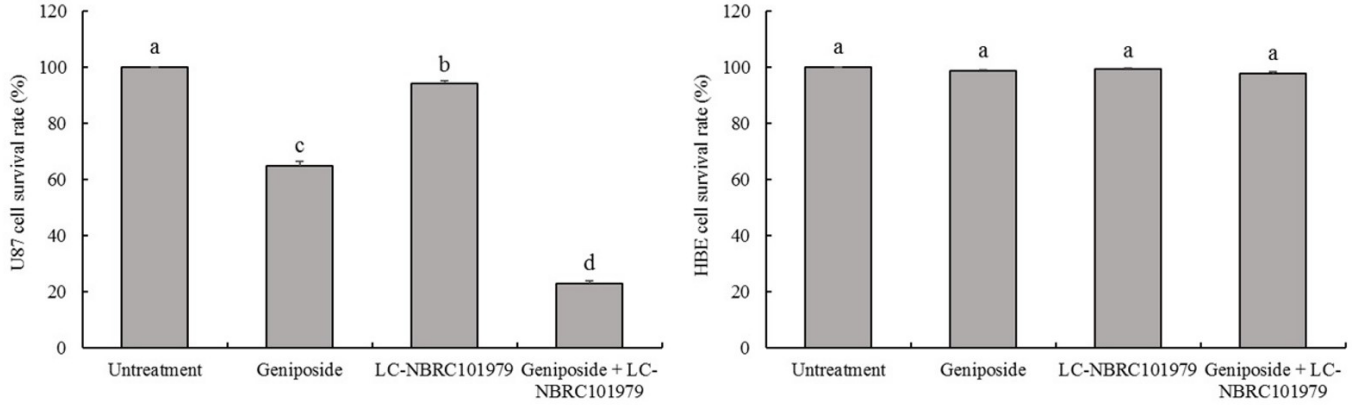

Figure 3. Toxic effect of experimental object on U87 human glioma cells and HBE normal human bronchial epithelial cells. a-d represents statistical results, and different letters represent significant differences between corresponding groups. Untreatment: U87 cells without sample treatment; Geniposide: U87 cells treated with 100 μg/mL geniposide solution; LC-NBRC101979: U87 cells treated with 10^6 CFU/mL LC-NBRC101979 solution; Geniposide + LC-NBRC101979: U87 cells treated with 100 μg/mL geniposide and 10^6 CFU/mL LC-NBRC101979 solution.

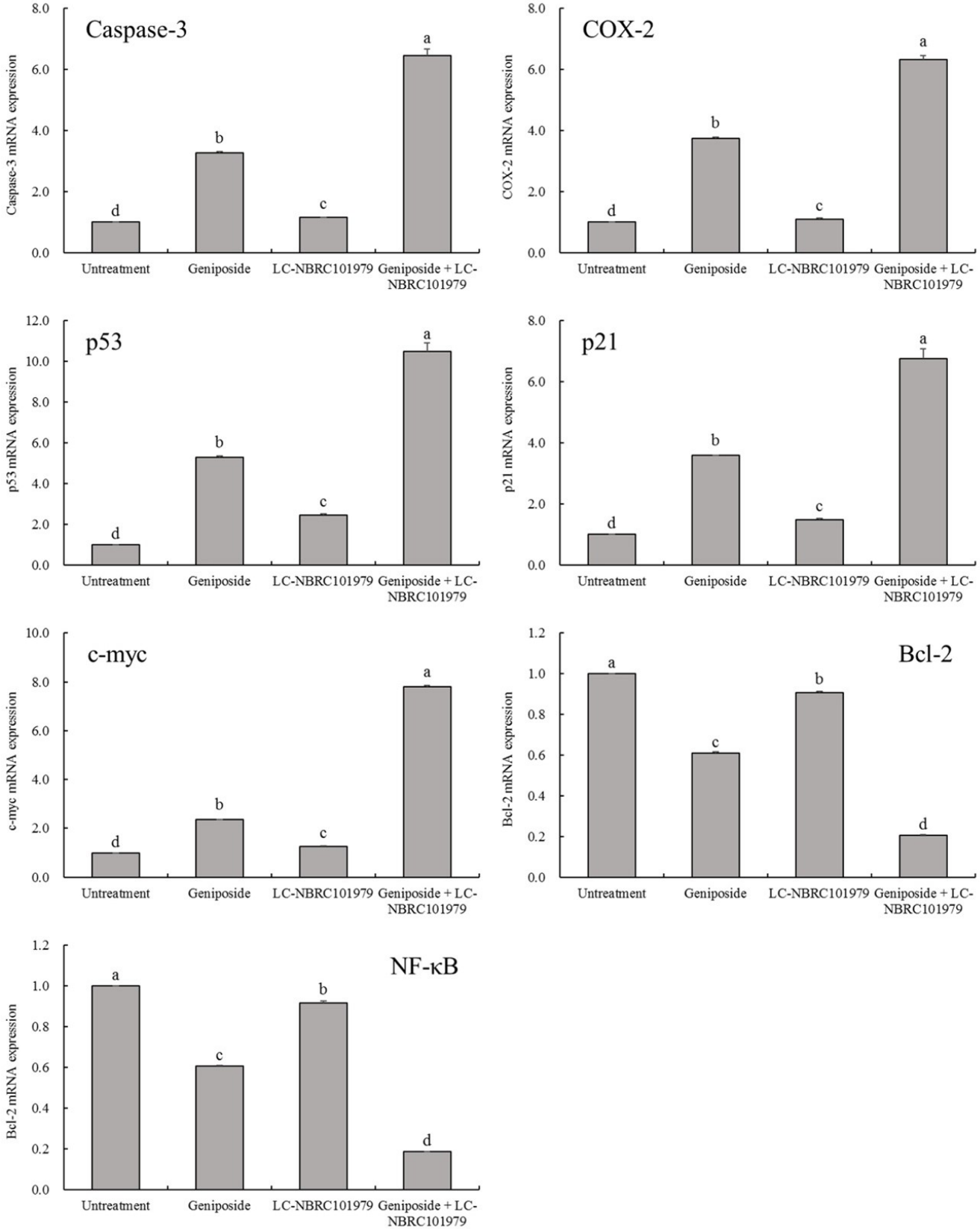

Figure 4. Effects of experimental objects on mRNA expression of U87 human glioma cells. a-d represents statistical results, and different letters represent significant differences between corresponding groups. Untreatment: U87 cells without sample treatment; Geniposide: U87 cells treated with 100 μg/mL geniposide solution; LC-NBRC101979: U87 cells treated with 10^6 CFU/mL LC-NBRC101979 solution; Geniposide + LC-NBRC101979: U87 cells treated with 100 μg/mL geniposide and 10^6 CFU/mL LC-NBRC101979 solution.

Table 2. Oxidation damage indexes in U87 cells.

Group	LDH ($U/10^4$)	GSH-Px (U/mg prot)	CAT ($U/10^4$)	MDA ($nmol/10^4$)	SOD ($U/10^{4)}$	T-AOC ($U/10^4$)	NO ($\mu mol/10^4$)
Untreatment	13.18 ± 0.45^a	0.27 ± 0.02^d	0.29 ± 0.07^d	2.15 ± 0.02^a	2.04 ± 0.34^d	3.20 ± 0.26^d	11.90 ± 0.40^a
Geniposide	8.33 ± 0.22^c	1.10 ± 0.03^b	1.33 ± 0.07^b	1.41 ± 0.02^c	6.29 ± 0.31^b	10.68 ± 0.44^b	7.31 ± 0.19^c
LC-NBRC101979	12.06 ± 0.43^b	0.62 ± 0.05^c	0.57 ± 0.09^c	1.97 ± 0.03^b	3.43 ± 0.16^c	5.60 ± 0.57^c	10.61 ± 0.38^b
Geniposide + LC-NBRC101979	3.10 ± 0.06^d	1.83 ± 0.22^a	2.19 ± 0.10^a	0.67 ± 0.02^d	10.64 ± 0.35^a	17.69 ± 0.37^a	2.69 ± 0.31^d

a-d represents statistical results, and different letters represent significant differences between corresponding groups. Untreatment: U87 cells without sample treatment; Geniposide: U87 cells treated with 100 μg/mL geniposide solution; LC-NBRC101979: U87 cells treated with 10^6 CFU/mL LC-NBRC101979 solution; Geniposide + LC-NBRC101979: U87 cells treated with 100 μg/mL geniposide and 10^6 CFU/mL LC-NBRC101979 solution.

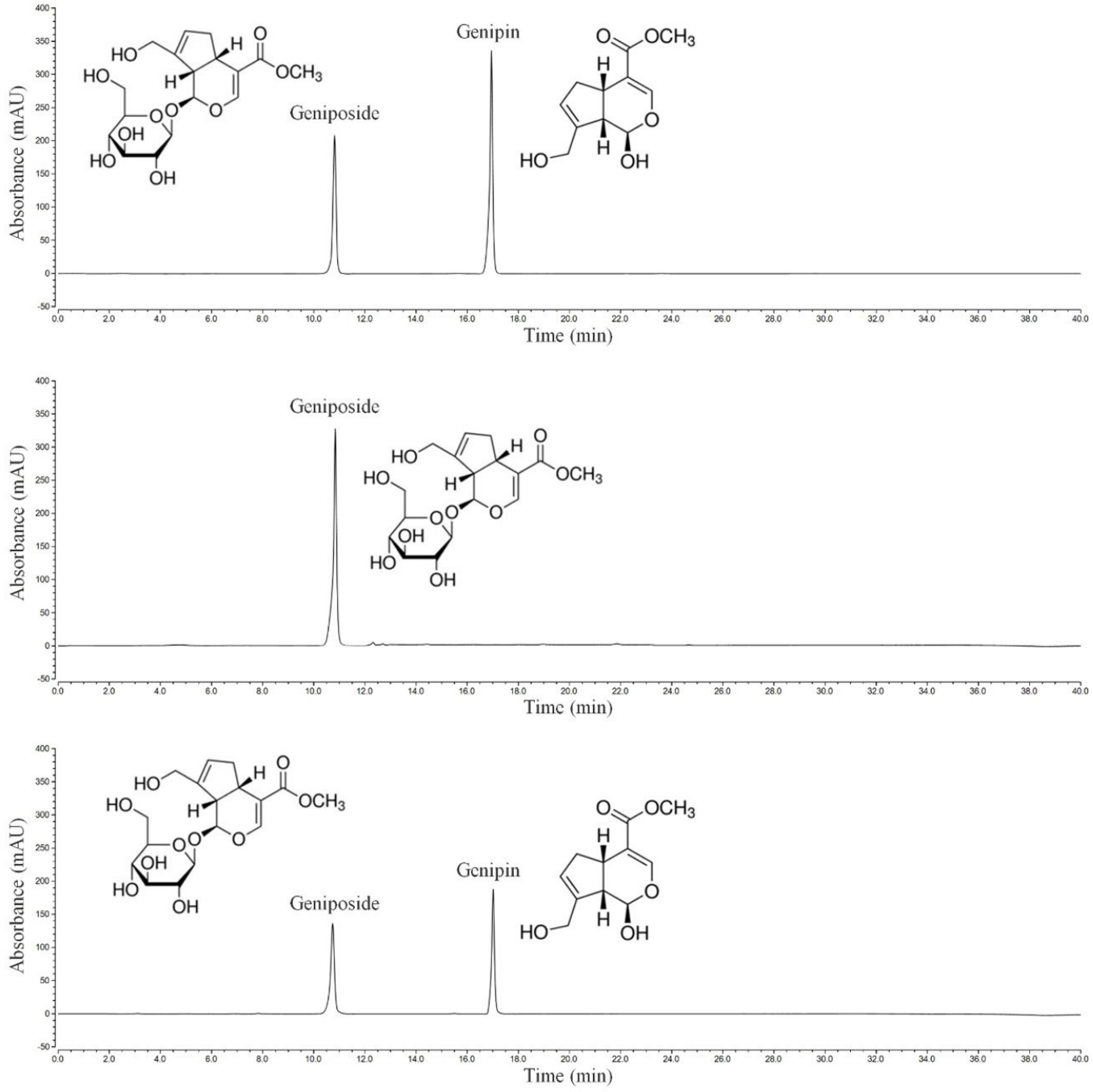

Figure 5. Transformation ability of *Lactobacillus casei* to geniposide (HPLC analysis).

caspase family, oncogenes like c-myc, and the tumor suppressor gene p53, are relatively conservative among species (Lee et al., 2005; Li et al., 2022). Most anticancer drugs cause apoptosis by activating corresponding apoptosis pathways, such as those of the Bcl-2 family proteins, which play an important role in cell death and are reflected by a rise in the Bax/Bcl-2 ratio.

The p53 gene is strongly associated with human malignancies and has the ability to control the cell cycle and prevent cell carcinogenesis (Cooper et al., 2004). More than half of cancer patients had p53 gene mutations and inactivation, according to research (Mansourian et al., 2020). The p53 protein causes apoptotic cell death when a cell is injured and cannot be repaired. The cell cycle process is governed by the cyclin/cyclin-dependent kinase (CDK) complex (Reyes et al., 2019). At the G1-S checkpoint of the cell cycle, once the DNA has been damaged and cannot be changed in the cell, high p53 protein expression results in high p21 expression, stopping the cell cycle from continuing (Wang et al., 2018). The experimental results showed that geniposide and *Lactobacillus casei* had the effect of regulating the above expression, and the effect of geniposide increased significantly after the action of *Lactobacillus casei*.

Oxidative stress affects the cell signal transduction system, making cancer cells lose normal contact inhibition and continue to proliferate, more and more (Azab & Mostafa, 2022). In addition, oxidative stress can also promote the formation of tumor neovascularization, thereby promoting the growth and metastasis of cancer. This study shows that geniposide and *Lactobacillus casei* can reduce the degree of oxidative stress damage to normal cells to a certain extent, but the effect is not strong. However, under the combined action of *Lactobacillus casei* and geniposide, this effect is greatly enhanced.

Lactobacillus casei can produce β-glycosidase, which β-glycosidase can convert geniposide into geniposide (Qian et al., 2018). The study showed that the stability and activity of geniposide were much better than geniposide. Through HPLC analysis, it was found that *Lactobacillus casei* could transform geniposide into geniposide, thus showing better anti-tumor effect in cell experiments.

5 Conclusion

This study found that a *Lactobacillus casei* NBRC101979 had a transformation effect on geniposide, and could promote the transformation of geniposide into geniposide, thus inhibiting U87 human glioma cells by regulating oxidative stress and p53 pathway. At the same time, the study showed that *Lactobacillus casei* NBRC101979 and geniposide mixture can produce stronger effects than geniposide alone. This study provides a new idea for further development and utilization of *Gardenia jasminoides*.

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