Total Flavones Isolated from *Lycium Barbarum L.* Inhibit the Proliferation, Migration and Invasion of GlioblastomaU-87MG Cells by Decreasing Formyl Peptide Receptor 1 Expression

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Abstract

Background: To study the role and mechanisms of total flavones from *Lycium barbarum L.* (TFL) and FPR1 in the growth of glioblastoma U-87MG cells.

Main Methods: CCK-8, wound-healing and Transwell were used for investigating proliferation, motility and invasion of U87 cells after treating with total flavones. RT-qPCR and Western blot were used to study the effect of total flavones on proliferating cell nuclear antigen (PCNA), matrix metalloproteinase2 (MMP2) and FPR1. The short hairpin RNA and FPR agonist fMLP were used to delineate the role of FPR1.

Results: TFL was successfully isolated, and its concentration was determined to be 6.205 mg/l. TFL inhibited the proliferation, migration and invasion of U-87MG cells in a time and dose-dependent manner compared to controls. Decreasing FPR1 expression using short hairpin RNA significantly inhibited the migration and invasion of U-87MG cells. Notably, increased expression of FPR1 and treatment with FPR-agonist peptides such as N-formylmethionyl-leucyl-phenylalanine induced the migration and invasion of U-87MG cells, which was significantly decreased when the cells were treated with TFL.

Conclusion: TFL inhibits the proliferation, migration and invasion of human glioblastoma U-87MG cells through decreasing the expression of FPR1. These findings provide valuable evidence for the development of antitumor drugs.

Introduction

Glioma is the most common malignant brain tumor, which accounts for 80% of all malignant primary tumors of the central nervous system [1]. Glioblastoma, also known as glioblastoma multiforme (GBM), is the highest-grade subtype of glioma (grade IV), and is characterized by multilocus lesions, diffuse growth, and high invasiveness and recurrence rate. Although advances have been made in the treatment of GBM, the efficacy of the current treatment (which includes surgical resection, radiotherapy and chemotherapy) is limited due to the high invasiveness and recurrence rate of this malignancy. The median survival time for patients with GBM is about 1 years and the 5-year survival rate is 5.8% [2, 3]. Therefore, it is important to identify novel drugs to effectively inhibit the growth of GBM.

The antitumor effects of active ingredients extracted from plants have attracted great attention in recent years. Flavonoids, which are major active ingredients of medicinal plants, serve antitumor roles in numerous malignant tumors. Several studies have shown that flavonoid intake is associated with a decreased risk of breast cancer [4–6]. Dietary flavonoids such as luteolin and quercetin inhibit the stem cell properties and metastatic potential of prostate cancer Du145-III cells [7]. Silibinin, a natural flavonolignan, significantly inhibits the growth and progression of lung cancer. Flavonoids isolated from *Sinopodophylli Fructus* have bioactivity against human breast cancer cells [8]. Recently, several studies have indicated that flavonoids act as a type of antitumor compound in GBM, and are candidates for developing novel therapeutic drugs [9–12]. However, the biological function and molecular mechanism of flavonoids in the prevention and treatment of GBM are largely unknown.

Flavonoids are major compounds in *Lycium* plants. Although *Lycium* plants are extensively distributed in China's Ningxia, Inner Mongolia, Xinjiang, Gansu and Hebei provinces, Ningxia gouqi *(Lycium barbarum L.)* is the only flavonoid included in the Chinese Pharmacopoeia. It has been reported that flavonoids are mainly distributed in the fruits and leaves of *Lycium barbarum L.*, and the content of total flavonoids in cultivated *Lycium barbarum L.* is significantly higher than that in wild *Lycium barbarum L.* [13]. Recently, several studies have demonstrated that total flavonoids from *Lycium barbarum L.* (TFL) serve an antitumor role in hepatocellular carcinoma [14] and gastric cancer [15]. However, the effects and molecular mechanism of TFL on the proliferation, migration and invasion of GBM remain unclear.

Formyl peptide receptors (FPRs), including FPR1, FPR2 and FPR3, are located on the human chromosome 19q13.4, and are G-protein coupled receptors that can be activated by peptides, proteins and lipids [16]. Upon binding to ligands, FPR triggers intracellular signaling cascades that are involved in angiogenesis, cell proliferation and apoptosis. Recently, FPRs have been identified in several types of human cancer tissues and cells, including highly malignant glioma cells [17, 18], colorectal cancer [19], gastric cancer [20], lung cancer [21] and ovarian cancer [22], where they served important roles in tumor growth and angiogenesis. Although several studies have shown that FPRs were selectively expressed in highly malignant glioma cells, especially in U87 cells and contributed to the tumorigenicity of the human GBM cell line U-87MG *in vivo* [17, 23], the role of FPRs in human GBM remains unclear. Growing evidence is available for compounds that are novel and potent FPR1-specific antagonists, such as selected chromone/isoflavone derivatives and oligomer procyanidins, which are natural fractions isolated from grape seeds [24, 25].

It was previously demonstrated that FPR1, unlike FPR2 or FPR3, is mainly expressed on the surface of GBM U-87MG cells [26, 27]. However, the roles of TFL and FPR1 in the growth of GBM are largely unknown. The present study demonstrated that *Lycium barbarum L.* (TFL) inhibited the proliferation, migration and invasion of human GBM U-87MG cells through decreasing the expression of FPR1. Our findings indicate that TFL can inhibit the growth of GBM cells and provide valuable evidence for the development of antitumor drugs.

Materials And Methods

Extraction and identification of total flavonoids from fruits of L. barbarum

Extraction and separation of total flavonoids.

Fruits of cultivated L. *barbarum* (Wolfberry Research Institute, Ningxia Academy of Agriculture and Forestry Sciences, Yinchuan, China) were completely dried at 50℃ and fully grounded. The milled samples were refluxed with ether until the ether was colorless, and ultrasonically extracted twice with 80% ethanol at 60℃. Then, the mixture was filtered and concentrated. The extract was purified with an HPD600 macroporous adsorption resin column (Chemical Plant of Nankai University, Tianjin, China). Subsequently, the column was washed with 50% ethanol to elute flavonoids, and the solution was vacuum dried.

Identification and determination of total flavonoids.

The purified products were identified using reversed phase high-performance liquid chromatography (HPLC) [CBM-20A; Shimadzu (China) Co., Ltd, Beijing, China] according to a modified method [28, 29] (Fig. 1). The separation was performed on a Shim - pack VP-ODS 250 mm ×4. 6 mm (5 μm), acetonitrile/water/acetic acid (5:94.5:0.5) was used as the mobile phase A and acetonitrile/water/acetic acid (70:29.5:0.5) as the mobile phase B with gradient elution (0 ~ 14 min, 100% ~ 86% A, 14 ~ 22 min, 86% ~ 84% A, 22 ~ 40 min, 84% ~ 70%A and 40 ~ 55 min, 70% ~ 100% A). The flow rate was 0.5 ml/min, the detection wavelength was at 342 nm, the temperature was 30℃ and sample quantity was 5 μl. The chemical structures of the purified products were similar to that of rutin (National Institutes for Food and Drug Control, Beijing, China), suggesting that they were mainly flavonoids. Therefore, these compounds were named 'total flavonoids of *L. barbarum*' (TFL). The concentration of TFL was determined using an ultraviolet-visible (UV-vis) spectrophotometer [VIS-7220N; Beijing Beifen-Ruili Analytical Instrument (Group) Co., Ltd., Beijing, China] at 510 nm and the aluminum salt method [30], and calculated using rutin as the calibration standard. The reference solution of 0.2 mg/ml was prepared by precision weighing rutin 5.0 mg, dissolved in 80% ethanol, fixed to 25 ml. Taking the reference solution of 0, 0.2, 0.5, 1.0, 2.0, 3.0, 4.0 ml were separately added 5% sodium nitrite (w/v) 0.4 ml, 10% aluminum nitrate (w/v) 0.4mL, and 4% sodium hydroxide (w/v) 4 ml, adding water to a final volume of 10 ml and mixing, storing 15min. The absorbance at 510 nm was measured by spectrophotometer zeroed with liquid of no rutin. The regression equation was calculated by GraphPad Prism 6. After the sample was diluted, the absorbance was measured by the same method, and the concentration was calculated by the equation.

Cell culture

Human GBM U-87MG cells (American Type Culture Collection, Manassas, VA, USA,) which were unknown origin and which has been authenticated by STR profiling, were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) (both from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin and 100 μg/ml streptomycin, and incubated at 37°C in a humidified incubator containing 5% CO_2.

Determination of half maximal inhibitory concentration (IC_{50}) value of TFL

To assess the effect of the concentration of TFL on the growth of U-87MG cells, a Cell Counting Kit-8 (CCK-8; BestBio, Shanghai, China) assay was performed. In brief, U-87MG cells were seeded at a density of $5x10^3$ cells/well in 96-well plates and incubated for 24 h. Then, the cells were treated with 20, 40, 80, 120 or 160 mg/l TFL, and incubated for additional 24, 48 or 72 h. At various time points, 10 μl CCK-8 solution was added to each well and incubated for additional 2 h at 37°C. The absorbance was subsequently measured at a wavelength of 450 nm using the Multiskan MK3 plate reader (Thermo Fisher Scientific, Inc.). The IC_{50} value was calculated using probit regression analysis.

Cell proliferation assay

Cell proliferation activity was detected using the CCK-8 assay, similarly to the method of IC_{50} determination described above. The concentration of TFL used in the cell proliferation assay was 20, 40 and 80 mg/l. Normal U-87MG cells at different time points were used as control. The inhibition rate was calculated as 1- [optical density (OD) treated - OD blank]/(OD control - OD blank).

Wound healing assay

A wound healing assay was performed to analyze the role of TFL in the migratory activity of U-87MG cells. Briefly, cells were seeded at a density of $3x10^5$ cells/well in 6-well plates. When the cells reached 70-80% confluence, they were scratched from the surface of the plates using 200-µl pipette tips. Then, each well was washed three times, and fresh culture medium or culture medium containing 20, 40 or 80 mg/l TFL was added. At different time points, wells in each group were photographed using an Olympus IX71 microscope (Olympus Corporation, Tokyo, Japan).

Transwell assay

A transwell assay was performed to assess the effects of TFL on the migratory and invasive activities of U-87MG cells. For assessing their migratory activity, cells were seeded at a density of $3x10^3$ cells/well in the upper side of transwell chambers (BD Biosciences, Franklin Lakes, NJ, USA) containing serum-free DMEM with various concentrations of TFL. The lower chambers were filled with fresh medium containing 15% FBS. After 24 h of incubation, the cells on the upper surface were removed, while the cells on the lower surface were fixed in 4% formaldehyde and stained with 0.2 % crystal violet (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Subsequently, the migratory cells were photographed in 6 randomly selected fields under a light microscope (Olympus IX71; Olympus Corporation), and the number of migratory cells was counted and analyzed with GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA).

For assessing the invasive activity, the upper chambers were coated with 1 mg/ml DMEM-diluted Matrigel (BD Biosciences) prior to cell seeding. The remaining procedure was the same as that described above for the cell migration assay.

Establishment of an U-87MG cell line stably transfected with FPR1-short hairpin RNA (shRNA)

The U-87MG cell line was stably transfected with PDS019_Pl/FPR1-shRNA/GFP lentiviral recombinant plasmids (Novo Bio-Tech Corporation, Shanghai, China). U-87MG cells stably transfected with empty PDS019_Pl/shRNA/GFP plasmids served as controls. The transfected cells were selected and maintained in medium containing 10 µg/ml blasticidin (InvivoGen, San Diego, CA, USA). The sequences of the shRNAs targeting FPR1 are shown in Table I.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted using TRIzol reagent (Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer's protocol. Then, 1 μg total RNA was reverse transcribed to single-stranded complementary DNA with FastQuant RT Kit (TransGen Biotech Co., Ltd., Beijing, China) following the manufacturer's protocol. The expression levels of target genes were quantified using the SYBR Select Master Mix Kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) and the StepOnePlus Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) under the following conditions: 95°C for 4 min followed by 40 cycles consisting of 95°C for 20 sec, 60°C for 20 sec and 72°C for 20 sec. The relative expression levels of target genes were calculated using the $2^{-\Delta\Delta Cq}$ method, and GAPDH was used for normalization. The corresponding primer sequences are shown in Table 1.

Table 1 **Oligonucleotide sequences**

Name	Sequence (5′ to 3′)
PCNA	ATGTCGATAAAGAGGAGGAGG
	CTGAGTGTCACCGTTGAAGAG
FPR1	ACATTGCCAGTTATCATTCG
	AACGGCCACATTTATCCT
MMP2	GCTTCCAGGGCACATCCTA
	CTTCTGAGTTCCCACCAACAGT
GAPDH	AAATCCCATCACCATCTTC
	ATGACCCTTTTGGCTCCC
FPR1-shRNA	CACCGCTATCTCTTCCTGGATATCACGAATGATATCCAGGAAGAGATAGC
	AAAAGCTATCTCTTCCTGGATATCATTCGTGATATCCAGGAAGAGATAGC

Western blot analysis

Total protein was extracted from cells using radioimmunoprecipitation assay buffer containing a protease inhibitor cocktail (both from Beyotime Institute of Biotechnology, Haimen, China), and the protein concentration was calculated using the BCA Protein Assay Kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). Next, equal amounts of protein were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). After blocking in 5% non-fat dry milk in PBS-Tween 20 for 4 h, the membranes were incubated with rabbit polyclonal to matrix metalloproteinase 2 (MMP2) (Abcam ab37150, Cambridge, UK) (1:1500) or rabbit polyclonal to FPR1 (Abcam ab113531, Cambridge, UK) (1:500) overnight at 4°C. Subsequently, the membranes were washed in TBS-Tween 20 and incubated with horseradish peroxidase-conjugated goat anti-rabbit/mouse secondary antibodies (Abcam ab6721/ ab6789, Cambridge, UK) (1:1000~3000) at room temperature for 1 h. The protein bands were visualized using luminol (Thermo Fisher Scientific, Inc.) and detected using

an enhanced chemiluminescence detection system. Densitometry analyses of protein bands were performed using Image J software (version 1.46) (Softonic international Ed. Media TIC, Barcelona, Spain; https://imagej.en.softonic.com/) and all results were expressed as a relative ratio to GAPDH (Proteintech 60004-1-Ig, Chicago, USA) (1:1000)/Tubulin (Santa Cruz sc-8035, CA, USA) (1:500).

Statistical analysis

All data are expressed as the mean ± standard deviation from ≥3 independent experiments. GraphPad Prism 6 (GraphPad Software, Inc.) was used for statistical analysis. Statistical evaluation of the data was performed by one-way analysis of variance when ≥2 groups were compared with a single control, while Student's *t*-test was used for comparison of differences between 2 groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Concentration determination of TFL

Total flavonoids in fruits of cultivated *Lycium barbarum L.* were ultrasonically extracted with 80% ethanol and purified using an HPD600 macroporous adsorption resin column. Then, a reversed phase HPLC method was used for the determination of total flavonoids in *Lycium barbarum L.* extract. Rutin was used as the calibration standard, since its chemical structure is markedly similar to that of the purified products. The concentration of total flavonoids in *Lycium barbarum L.* extract was 6.205 mg/l, as detected by the aluminum salt method and calculated using regression analysis (equation, y = 1.2862x-0.0275; $R^2 = 0.9967$) (Fig. 2).

Tfl Inhibits The Proliferation Of U-87mg Cells

To explore the role of TFL in regulating the proliferation of U-87MG cells, a CCK-8 assay was performed. U-87MG cells were treated with 20, 40, 80, 120 and 160 mg/l TFL for 48 or 72 h. Then, the proliferative activity of U-87MG cells was detected by CCK-8 assay and analyzed using GraphPad 6 software. The results revealed that the IC_{50} of TFL was 77 mg/l at 48 h and 75 mg/l at 72 h. According to the value of IC_{50}, various concentrations of TFL (20, 40 and 80 mg/l) were used to assess the effect of TFL on the proliferation of U-87MG cells at 24, 48 and 72 h by CCK-8 assay. The results revealed that TFL significantly inhibited the proliferation of U-87MG cells in a time and dose-dependent manner ($P< 0.05$) (Fig. 3A). In addition, the messenger RNA (mRNA) expression of proliferating cell nuclear antigen (PCNA), a biomarker of cell proliferation, significantly decreased in a dose-dependent manner (Fig. 3B).

Tfl Inhibits The Migration And Invasion Of U-87mg Cells

The roles of TFL in the migration and invasion of U-87MG cells were investigated using wound healing and transwell assays. U-87MG cells were treated with 20, 40 and 80 mg/l TFL for 24 or 48 h. The results revealed that the migratory (Fig. 4A and B) and invasive (Fig. 4C) activities of U-87MG cells significantly decreased upon treatment with TFL, compared with those of the control group. To further investigate the role of TFL in the migration and invasion of U-87MG cells, the expression of MMP2, which serves an important role in the invasion and metastasis of tumor cells, was examined using RT-qPCR and western blot analysis. The results revealed that the relative mRNA (Fig. 5A) and protein (Fig. 5B) expression of MMP2 in U-87MG cells was markedly decreased following treatment with TFL in a dose-dependent manner.

Decreased FPR1 expression inhibits the migration and invasion of U-87MG cells

It has been reported that FPR can influence the expression of MMP2 [31], and our group previously identified that FPR1 is mainly expressed on the surface of U-87MG cells. Thus, it was speculated that FPR1 may be involved in the migration and invasion of U-87MG cells. To evaluate the role of FPR1 in the migration and invasion of U-87MG cells, an FPR1-shRNA-stably transfected U-87MG cell line was established, which was confirmed by RT-qPCR and western blot assay (Fig. 6A and B: Lane1-3). The results revealed that the migration and invasion capacities of U-87MG cells were significantly decreased in FPR1-shRNA-transfected U-87MG cells (Fig. 6C and D) ($P < 0.05$).

Note

The initial experiment was to test the relationship between FPR1 and ANXA1, but according to the specific experimental content, subsequent experiments were adjusted. Based on the authenticity of the experimental results, Only the experimental result related to FPR1(Lane 1–3 and 6 in Fig. 6B) were selected in this paper. Other results (Lane 4 and 5 in Fig. 6B) were omitted.

Tfl Decreases The Expression Of Fpr1

Based on the results that TFL inhibited the migration and invasion of U-87MG cells by inhibiting the expression of MMP2, it appeared that FPR1 is involved in the migration and invasion of U-87MG cells, and influenced the expression of MMP2. It was therefore speculated that the inhibitory effects of TFL on the migration and invasion of U-87MG cells may be associated with the expression of FPR1. To explore the role of TFL in the expression of FPR1, an RT-qPCR assay was performed. U-87MG cells were treated with 20, 40 and 80 mg/l TFL for 48 h. Then, the expression of FPR1 was detected by RT-qPCR assay. The results indicated that the expression of FPR1 significantly decreased upon treatment with TFL in a dose-dependent manner (Fig. 7) compared with the control group ($P < 0.05$).

TFL inhibits the N-formylmethionyl-leucyl-phenylalanine (fMLP)-induced migration and invasion of U-87MG cells

To confirm whether the inhibitory effects of TFL on the migration and invasion of U-87MG cells were mediated by decreased FPR1 expression, fMLP, an FPR-agonist peptide, was used to activate the expression of FPR1. A total of 100 nM fMLP was added to FPR1-shRNA-stably transfected U-87MG cells, and the relative protein expression levels of FPR1 were obviously increased compared with untreated cells (Fig. 6B: Lane 3 and 6). After 24 h of incubation with 100 nM fMLP and 80 mg/l TFL, the migration and invasion abilities of U-87MG cells were evaluated using a transwell assay. U-87MG cells, FPR1-shRNA-stably transfected U-87MG cells and cells treated with 100 nM fMLP were used as the control. The results indicated that, after 24 h of incubation with 100 nM fMLP, the migration and invasion of U-87MG cells increased significantly, and the promoting effect of fMLP was significantly inhibited when TFL was simultaneously added (Fig. 8A and B). This phenomenon was also confirmed on FPR1-shRNA-transfected cells. The mRNA and protein expression of MMP2 was further investigated. The results revealed that the expression of MMP2 was significantly increased following treatment with the FPR-agonist peptide fMLP, which was inhibited by TFL (Fig. 8C). Similar results were observed in FPR1-shRNA-transfected U-87MG cells (Fig. 8D).

Discussion

The antitumor effects of active ingredients extracted from Chinese herbal medicinal plants have attracted increasing attention in recent years. With the in-depth study of the anticancer effect of Chinese herbal medicines and the aim of research on anticancer drugs gradually shifting from cytotoxic drugs to tumor cell-specific signal targeting drugs, natural flavonoids have become the focus of research due to their low toxicity and high antitumor activity [32]. Studies on natural flavonoids are expected to provide useful evidence for the screening and structural modification of novel and more effective multi-target antitumor drugs. Furthermore, flavones from natural sources have small molecular weights and can therefore be rapidly absorbed by the human body and pass through the blood-brain barrier [33]. Recently, several studies have demonstrated that natural flavonoids serve an important role in the angiogenesis, proliferation, invasion, metastasis and apoptosis of different types of tumor cells [34–36]. Although various studies have demonstrated that flavonoids serve an antitumor role in GBM, the effects and molecular mechanism TFL on the proliferation, migration and invasion of GBM are largely unknown.

In the present study, total flavonoids present in the fruits of cultivated *Lycium barbarum L.* were extracted and identified. The chemical structure of the purified products was remarkably similar to that of rutin, and the content of TFL was determined to be 6.205 mg/l with an UV-vis spectrophotometer at 510 nm and the aluminum salt method, using rutin as the calibration standard. Then, the role of TFL in the proliferation, migration and invasion of GBM U-87MG cells were explored. The IC_{50} value of TFL was observed to be 77 mg/l at 48 h and 75 mg/l at 72 h, and TFL significantly inhibited the proliferation of U-87MG cells in a time and dose-dependent manner, which was further confirmed by decreased mRNA expression of PCNA, a common biomarker of cell proliferation [37]. It has been demonstrated that the poor prognosis and high recurrence rate of GBM are largely due to its high invasiveness [38]. Flavonoids could inhibit the expression of MMP2 and MMP9 [39, 40], which serve important roles in the invasion and metastasis of

tumor cells. The present results revealed that TFL inhibited the migration and invasion of U-87MG cells, and decreased the mRNA and protein expression of MMP2 in a dose-dependent manner, which indicates that TFL serves an antitumor role in GBM U-87MG cells.

It is known that chemokines and their receptors serve important roles in the development and metastasis of tumors. FPR1, a member of the G-protein coupled receptor family, is mainly expressed by cells of myeloid origin, where it mediates the innate immune response to bacterial formylated peptides. High expression of FPR1 has been detected in various cancer types, such as colorectal cancer [19], gastric cancer [41], neuroblastoma [42], breast cancer [43] and GBM [23, 27]. However, the role of FPR1 in human GBM remains unclear. In a previous study, it was observed that the expression of MMP2 and MMP9, which are involved in the migration and invasion of tumor cells, decreased in FPR-siRNA xenografts compared with that of U-87MG cell xenografts, and isoflavone (a kind of flavonoid) could be used as a specific FPR1 antagonist [24]. It was previously demonstrated that FPR1 is mainly expressed on the surface of GBM U-87MG cells and could inhibit the migration and invasion of U-87MG cells as well as the expression of MMP2 in U-87MG cells. Therefore, the present study speculated that the inhibitory effects of TFL on the migration and invasion of U-87MG cells may be associated with the expression of FPR1. It was observed that the migratory and invasive abilities of U-87MG cells were significantly inhibited when the expression of FPR1 was decreased using specific shRNA, and the expression of FPR1 was significantly decreased upon treatment with TFL in a dose-dependent manner. These results indicated that TFL inhibited the migration and invasion of U-87MG cells by decreasing the expression of FPR1.

It was previously demonstrated that FPR, when activated by agonists, could regulate the proliferation and migration of tumor cells [18, 23](17,23). To further confirm whether the inhibitory effects of TFL on the migration and invasion of U-87MG cells were mediated by decreased FPR1 expression, fMLP, which is the first-identified FPR-agonist peptide with high affinity for FPR1 [44], was used to activate the expression of FPR1. The migration and invasion of U-87MG cells, as well as the expression of MMP2, increased significantly following treatment with the FPR-agonist peptide fMLP, and the promoting effect of fMLP could be significantly inhibited when TFL was simultaneously added. This phenomenon was also confirmed in FPR1-shRNA-transfected cells. A previous study demonstrated that the phosphatidylinositol-3-kinase/Akt/mammalian target of rapamycin signaling pathway serves an important role in GBM cell proliferation and migration. However, the molecular mechanisms of TFL in FPR1 expression regulation are largely unknow. Therefore, further studies should focus on the signaling pathway that is involved in the regulation of FPR1 expression in GBM [45]. In another study of ours, the results showed that TFL inhibited the migration of breast cancer through regulating the activity of PI3K/Akt/mTOR pathway. It was suggested that the antitumor role of TFL in glioblastoma cells may be related to the PI3K/Akt/mTOR pathway.

There are still some limitations in this paper. Firstly, it should be noted that this study was carried out only in U87-MG cells due to its higher expression of FPR, and was not verified in other glioblastoma cell lines. Secondly, in this study, we just want to know whether the TFL has effects on the proliferation, migration and invasion of glioblastoma U87 cells or not, and the components of TFL compounds is not yet clear.

Therefore, there was no comparison with conventional chemotherapeutic drugs. Thirdly, there was no results for *in vivo* experiment. The nude mice subcutaneous transplantation experiment has been done, but the tumorigenicity of animals was not stable. The best way is to do orthotopic transplantation, but it is difficult for us due to the technical reasons. In our further studies, the active monomer components will be isolated from TFL, and the antitumor mechanism of them will be further explored.

In summary, the present results demonstrated that TFL inhibits the proliferation, migration and invasion of GBM U-87MG cells by decreasing the expression of FPR1. These data suggest that TFL exerts antitumor activities in GBM cells and that FPR1 can be used as a target gene for the treatment of GBM. Further studies should focus on the identification and exploration of the antitumor activities of flavonoid monomers, and the regulatory mechanism of FPR1 in the genesis and progression of GBM.

Abbreviations

GBM glioblastoma;

MMP2 matrix metalloproteinase 2;

TFL total flavonoids from *Lycium barbarum L.*

FPR1 formyl peptide receptor 1;

shRNA short hairpin RNA;

ANXA1 Annexin 1; BOC2, FPR antagonist;

fMLP N-formylmethionyl-leucyl- phenylalanine .

Declarations

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Ethics statement

Our study did not require an ethical board approval because it did not contain human or animal trails.

Author contributions

HY and YC designed and supervised the experiments. QW, YL, BP and YZ carried out the experiments. HY, YL and QW analyzed and interpreted the experimental results. YL and QW contributed with materials and analysis tools. HY and YC wrote the manuscript. HY and YC reviewed and edited the manuscript. All authors read and approved the manuscript.

Consent for publication

Not applicable

Competing interests

The authors have declared that there is no conflict of interest.

Data availability

The authors declare that all data generated or analysed during this study are included in this published article.

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Figures

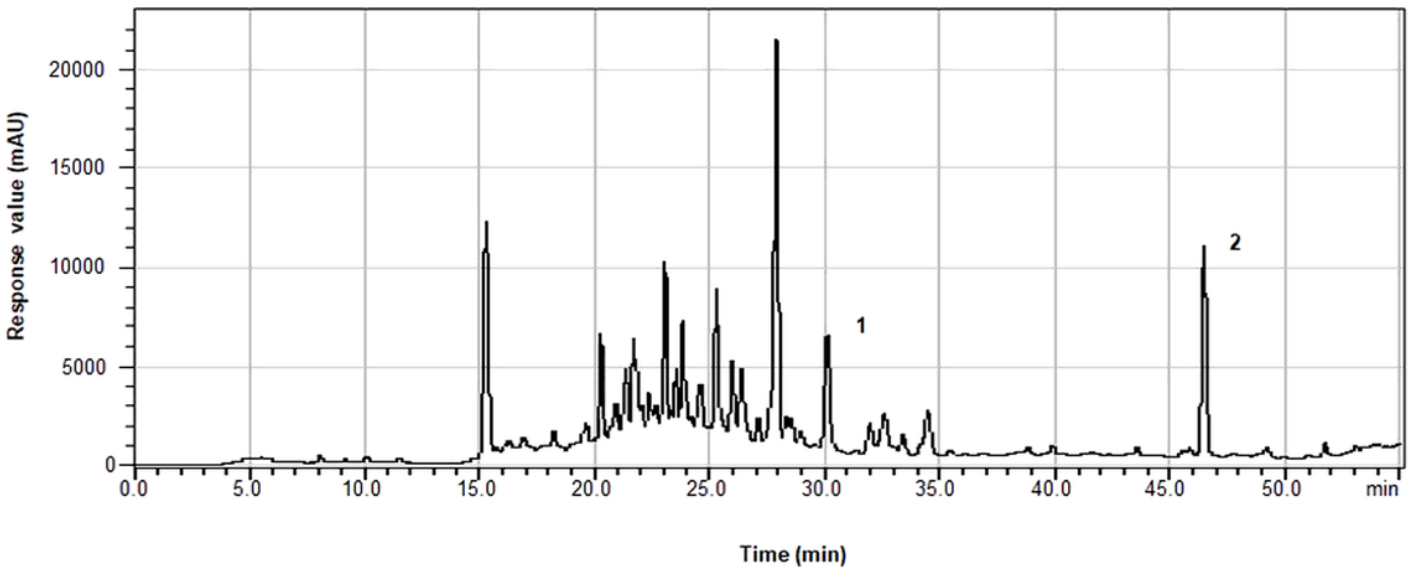

Figure 1

Chromatogram of fruits of cultivated L. barbarum (Wolfberry Research Institute, Ningxia Academy of Agriculture and Forestry Sciences. Yinchuan, China). Among the chromatographic peaks, peaks 1 and 2 are Chlorogenic acid and rutin, respectively.

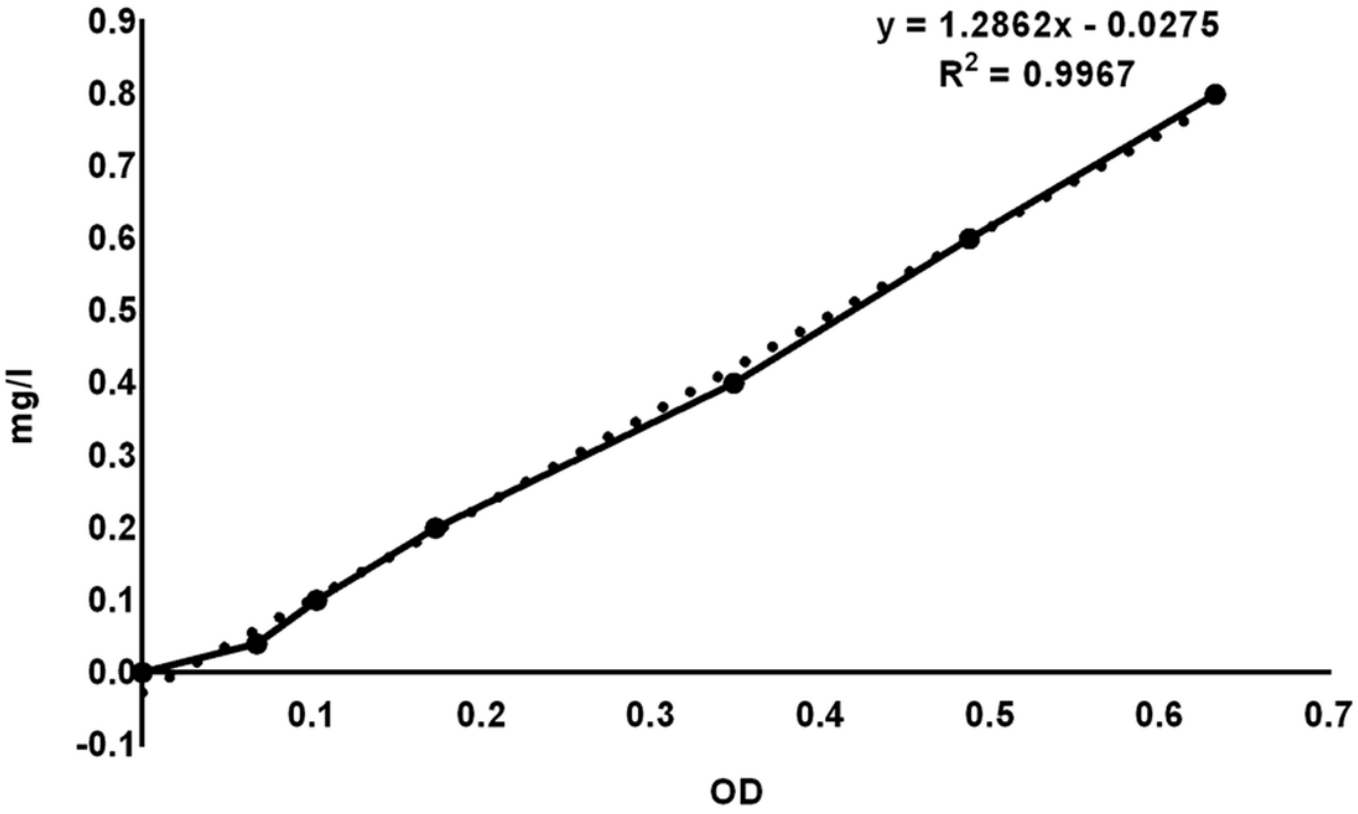

Figure 2

Calculated results using regression analysis. The concentration of total flavonoids in Lycium barbarum L. was detected by aluminum salt method and calculated using a regression-equation method with rutin as the calibration standard. The concentration of TFL (6.205 mg/l) was determined using an ultraviolet-visible spectrophotometer.

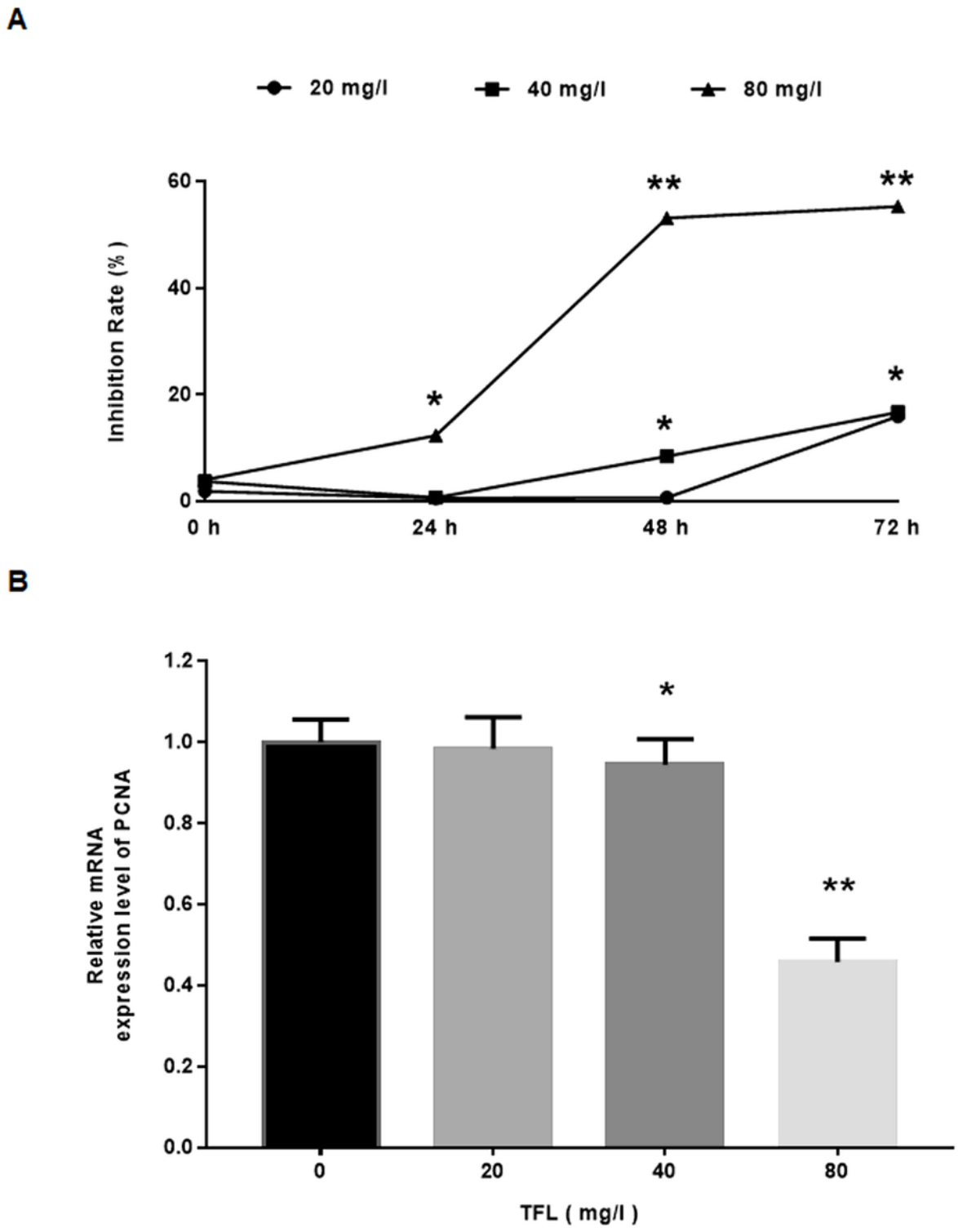

Figure 3

TFL inhibits the proliferation of GBM U-87MG cells. GBM U-87MG cells were treated with 0, 20, 40 and 80 mg/l TFL. Then, the effects of TFL on the proliferation of GBM U-87MG cells were evaluated using (A) a Cell Counting Kit-8 assay and (B) reverse transcription-quantitative polymerase chain reaction, which detected the messenger RNA expression of proliferating cell nuclear antigen (PCNA) at 48 h after TFL treatment. Data were represented as the mean ± standard deviation and were analyzed using GraphPad Prism 6 software. *P<0.05, **P<0.01. GBM, glioblastoma; TFL, total flavonoids from Lycium barbarum L.

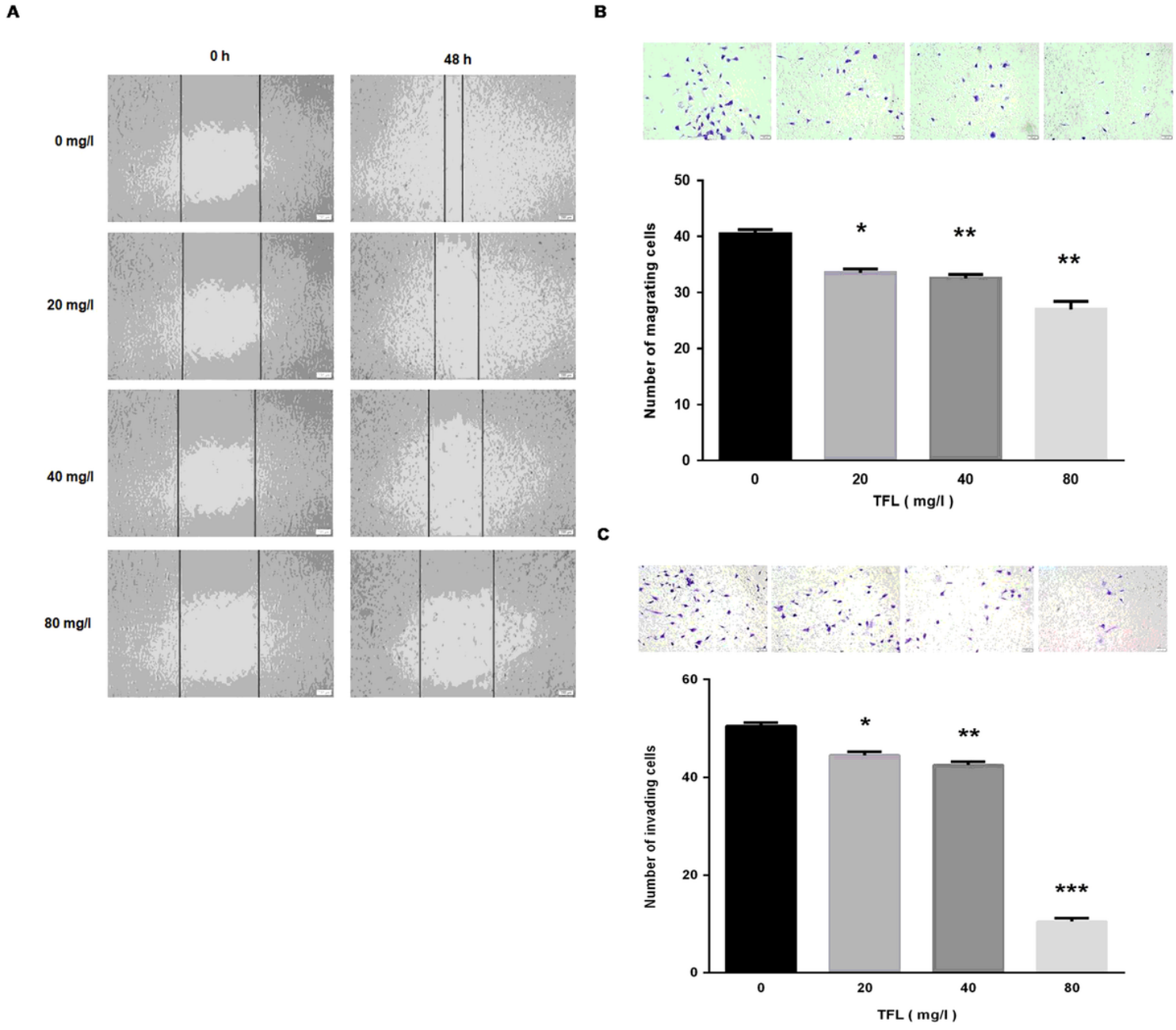

Figure 4

TFL inhibits the migration and invasion of GBM U-87MG cells. GBM U-87MG cells were treated with 0, 20, 40 and 80 mg/l TFL. Then, the migratory ability of GBM U-87MG cells was detected using (A) a wound healing assay at 0 and 48 h after TFL treatment, while the (B) migratory and (C) invasive activities of GBM U-87MG cells were detected using a transwell assay at 24 h after TFL treatment. Cells were stained

with 0.1% crystal violet and photographed under a bright field (magnification, x100). The number of cells in each group was calculated and analyzed by GraphPad Prism 6 software, and compared with that in the control group. Data are represented as the mean ± standard deviation. *P<0.05, **P<0.01, ***P<0.001. GBM, glioblastoma; TFL, total flavonoids from Lycium barbarum L.

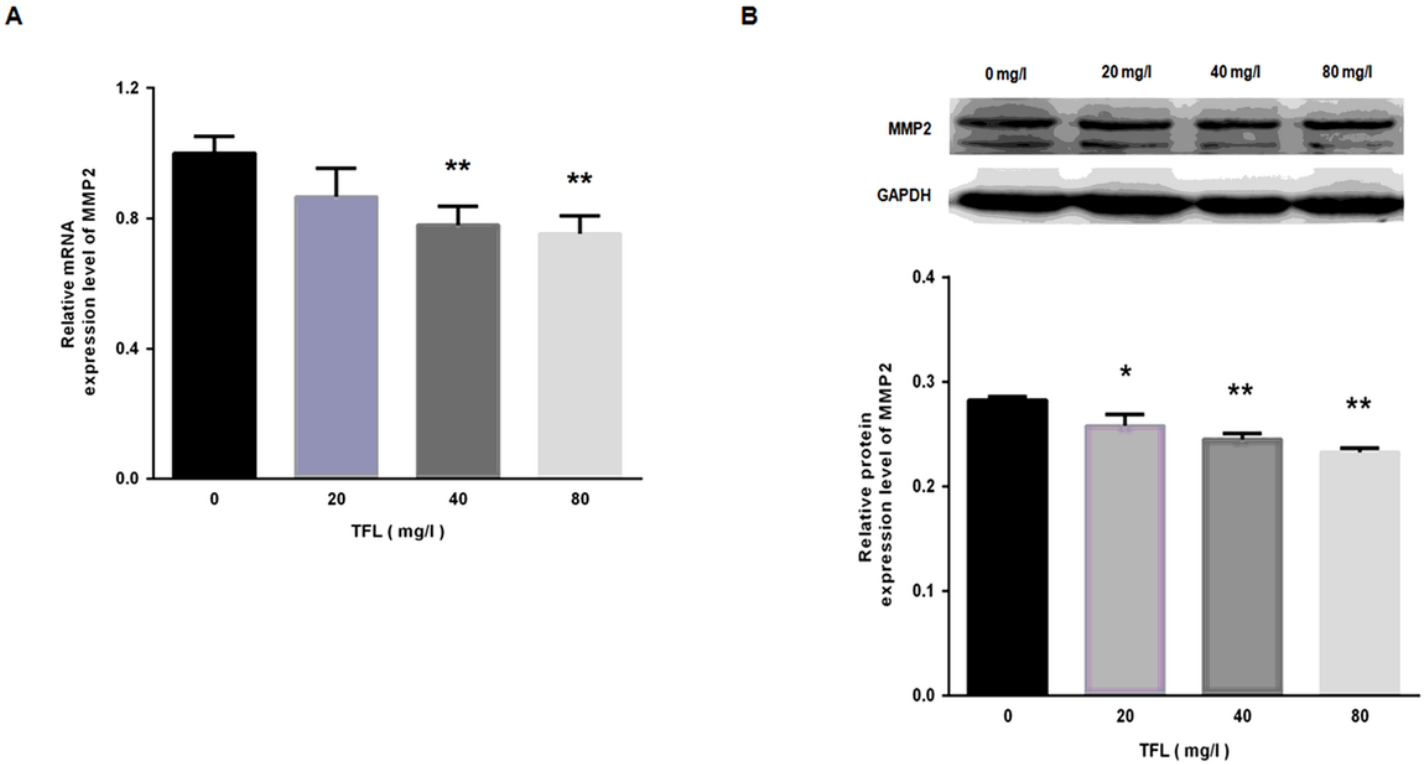

Figure 5

TFL inhibits the expression of MMP2 in GBM U-87MG cells. GBM U-87MG cells were treated with 0, 20, 40 and 80 mg/l TFL for 48 h. Then, the messenger RNA and protein expression of MMP2, a key migration and invasion-associated biomarker, were examined using (A) reverse transcription-quantitative polymerase chain reaction and (B) western blotting, respectively. The two bands for MMP2 in Fig. 5B were represented the inactive proform (72 kDa) and active form (66 kDa) of MMP-2 respectively. Data were represented as the mean ± standard deviation and were analyzed using GraphPad Prism 6 software. *P<0.05, **P<0.01. GBM, glioblastoma; MMP2, matrix metalloproteinase 2; TFL, total flavonoids from Lycium barbarum L.

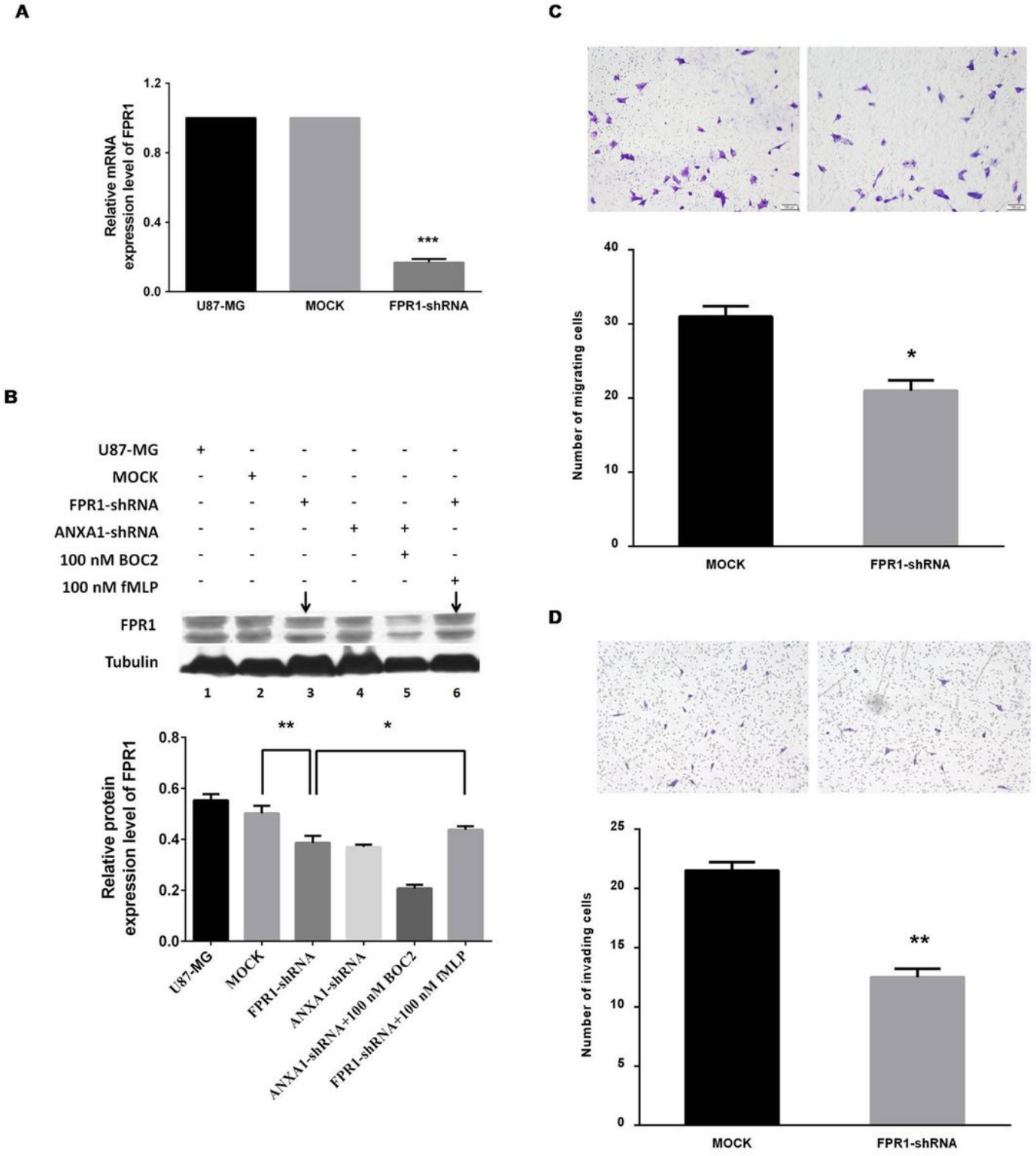

Figure 6

FPR1 regulates the migration and invasion of GBM U-87MG cells. GBM U-87MG cells were stably transfected with FPR1 shRNA or its control shRNA (mock). Then, the messenger RNA and protein expression of FPR1 were examined by (A) reverse transcription-quantitative polymerase chain reaction and (B) western blotting (Lane1-3). Following treatment of FPR1-shRNA-stably transfected U-87MG cells with 100 nM fMLP, an FPR-agonist peptide, the protein expression of FPR1 was detected by western

blotting (Lane 3 and 6 denoted by vertical black lines). To explore the role of FPR in the (C) migration and (D) invasion of GBM U-87MG cells, a transwell assay were performed. The number of cells in each group was calculated upon staining the cells with 0.1% crystal violet and photographing them under a bright field (magnification, x100). Data were represented as the mean ± standard deviation and were analyzed using GraphPad Prism 6 software. *P<0.05, **P<0.01, ***P<0.001. GBM, glioblastoma; FPR1, formyl peptide receptor 1; shRNA, short hairpin RNA; ANXA1, Annexin 1; BOC2, FPR antagonist; fMLP, N-formylmethionyl-leucyl-phenylalanine.

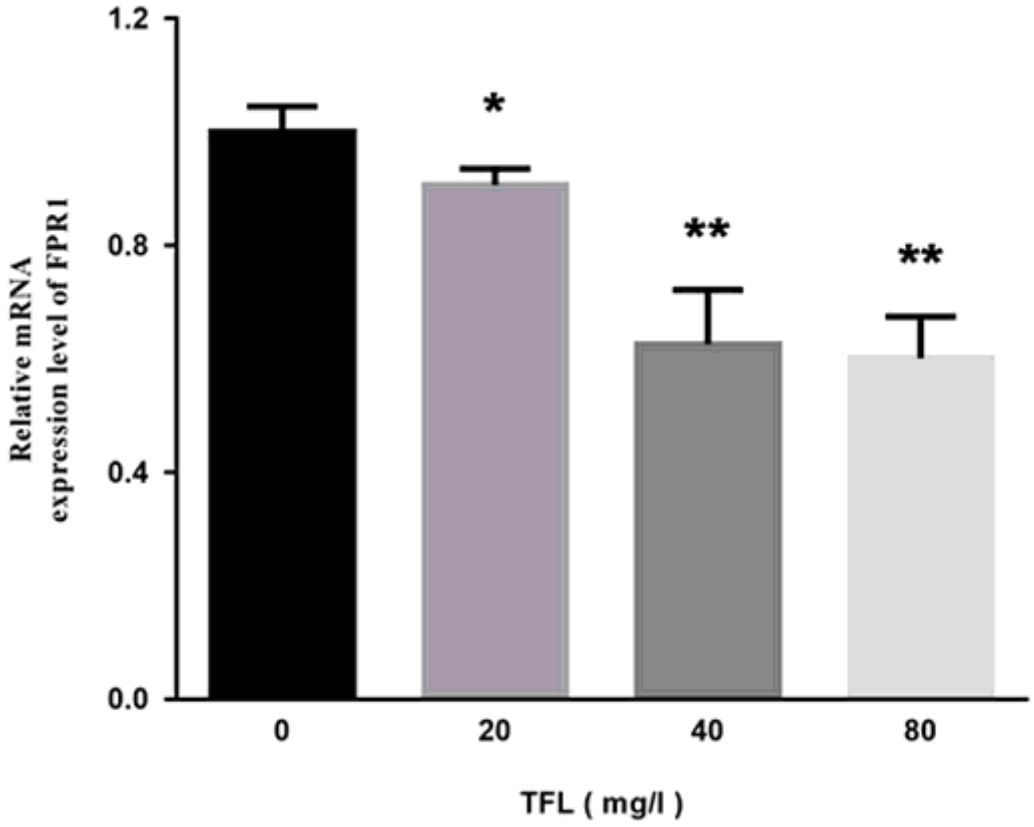

Figure 7

TFL inhibits FPR1 expression. The messenger RNA expression of FPR1 in glioblastoma U-87MG cells was detected using reverse transcription-quantitative polymerase chain reaction at 48 h after TFL treatment. Data were represented as the mean ± standard deviation and were analyzed using GraphPad Prism 6 software. *P<0.05, **P<0.01. TFL, total flavonoids from Lycium barbarum L.

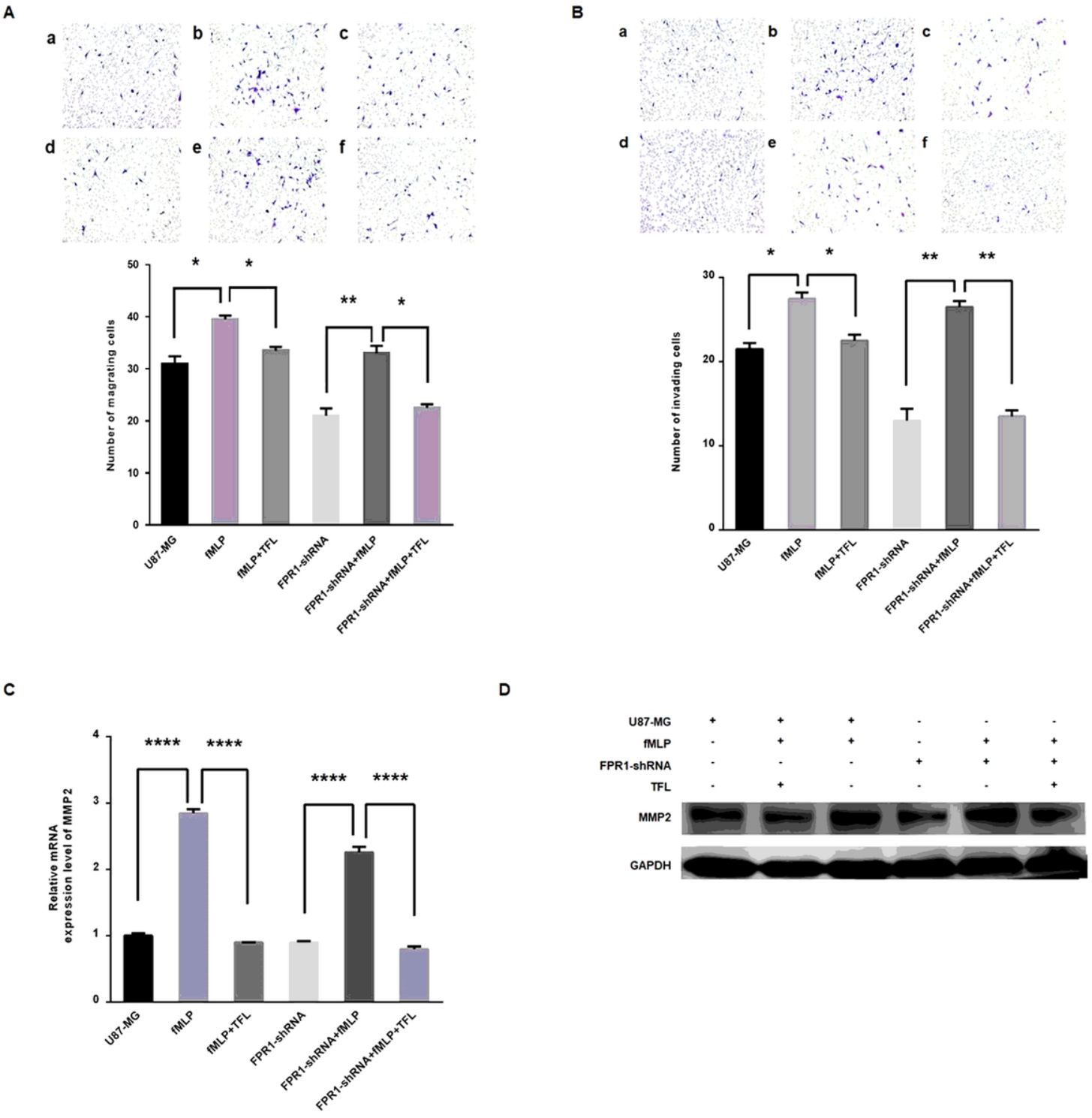

Figure 8

TFL inhibits fMLP-induced migration and invasion. U-87MG cells and FPR1-shRNA-stably transfected U-87MG cells were treated with 100 nM fMLP and 80 mg/l TFL, respectively. After 24 h of incubation, the abilities of (A) migration and (B) invasion of U-87MG cells were examined using a transwell assay. Cells were stained with 0.1% crystal violet and photographed under a bright field (magnification, x100). The number of cells in each group was calculated, analyzed using GraphPad Prism 6 software and compared with that in the control group. U-87MG cells and FPR1-shRNA-stably transfected U-87MG cells were

treated with 100 nM fMLP. After 24 h of incubation, the culture media were refreshed, and 80 mg/l TFL was supplemented to the culture for another 48 h of incubation. Then, the (C) messenger RNA and (D) protein expression of matrix metalloproteinase 2 in each group of U-87MG cells was detected using reverse transcription-quantitative polymerase chain reaction and western blotting. Data are expressed as the mean ± standard deviation, and are normalized to the expression of the reference gene or protein. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. FPR1, formyl peptide receptor 1; fMLP, N-formylmethionyl-leucyl-phenylalanine; shRNA, short hairpin RNA; TFL, total flavonoids from Lycium barbarum L.