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Research Article
Inhibition of Proliferation, Invasion and Migration in U-251 MG Glioblastoma Cell Line by Gedunin

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Abstract

Background and Objective: Glioma is the most commonly detected primary brain tumor found in adults and has very high rate of morbidity and mortality. The present study was performed to investigate the effect of gedunin on viability, migration and invasion of human glioblastoma multiforme, U-251 MG cell line. **Materials and Methods:** Invasion assay was performed using 24-well cell culture invasion chamber (Corning Inc., Tewksbury, MA, USA) coated with 8.0 µm Matrigel™ (Becton Dickinson, Bedford, MA, USA). For real-time polymerase chain reaction U-251 MG cells were distributed on to the six-well tissue culture plates and treated with gedunin for 24 h. Qiagen RNeasy Mini Kit was used for the isolation of total RNA from the cells. **Results:** The results revealed that exposure of U-251 MG cells to gedunin reduced the viability of U-251 MG cells to 28% compared to 98% in control group. Analysis of cell migration and invasion showed a significant inhibition by gedunin at a concentration of 20 µM after 24 h. Gedunin treatment suppressed the expression of focal adhesion kinase (FAK), metalloproteinases (MMP)-7, MMP-9 and uPA which play a vital role in inducing migration of carcinoma cells. The inhibition was significant ($p<0.005$) at all the tested concentrations except 5 µM after 24 h in U-251 MG cells. In addition the real-time PCR analysis revealed a marked reduction in the expression of MMP-9, FAK and Rho kinases-ROCK-1 (ROCK-1) mRNA by gedunin treatment. **Conclusion:** Thus, gedunin treatment caused inhibition of proliferation, migration and invasion in U-251 MG cells through inhibition of MMP-9, FAK and ROCK-1. Therefore, gedunin is a potent agent for the treatment of tumor growth.

Key words: Gedunin, glioma, chemotherapy, metastasis, metalloproteinase, focal adhesion kinase

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Glioma is the most commonly detected primary brain tumor found in adults and has very high rate of morbidity and mortality[1]. It is found to be the most malignant and aggressive tumor with high rate of recurrence and mean survival period of around than 2 years[2,3]. Inspite of the advances in the field of chemotherapy and radiation therapy, the prognosis and therapy of glioma patients remains poor. It has been observed that in glioma patients the common cause of mortality is not the presence of glioma at its primary location but the tumor metastasis[4]. Thus, the inhibition of tumor metastasis to distant as well as adjacent organs is considered to be the promising strategy for the treatment of glioma. Taking this into the consideration studies are being devised to screen the molecules for their role in the inhibition of carcinoma cell proliferation, migration and invasion.

Gedunin is an isolate from the phytochemical analysis of the Indian neem tree (*Azadirachta indica*). This tetranortriterpenoid has been reported to exhibit a range of biological activities including, insecticidal, antimalarial and anticancer[5-7]. Studies have revealed through connectivity map that gedunin exhibits anticancer activity[8]. Analysis of the mechanism revealed that gedunin treatment imparts anti-proliferative effect through alteration in the expression of heat shock protein 90 kDa (Hsp90)[9,10]. The present study was aimed to investigate the effect gedunin on inhibition of cell proliferation, migration and invasive potential of U-251 MG glioma cells. It was observed that gedunin treatment inhibited proliferation, invasion and migratory potential of U-251 MG cells significantly.

MATERIALS AND METHODS

The study was carried out in 2015 and 2016.

Chemicals and reagents: The following reagents were purchased from the indicated suppliers. Matrigel invasion chambers were obtained from BD Biosciences (San Jose, CA, USA). Gedunin, dimethyl sulfoxide (DMSO), propidium iodide (PI), RNase, Tris-HCl, Triton X-100 and trypan blue were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The RPMI-1640, fetal bovine serum (FBS), glutamine, penicillin-streptomycin and trypsin-EDTA were obtained from Gibco-BRL (Grand Island, NY, USA).

Cell culture: The U-251 MG cell line was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The cells were maintained in 75 cm^2 tissue culture flasks with RPMI-1640 medium supplemented with 10% FBS, 1% penicillin-streptomycin (100 Units mL^{-1} penicillin and 100 µg mL^{-1} streptomycin) and 1% glutamine and grown at 37°C under a humidified 5% CO_2 and 95% air at one atmosphere. Sub-confluent cells were passaged with a solution containing 0.25% trypsin and 0.02% EDTA.

Cell viability assay: The U-251 MG cells at a density of 2.5×10^5 cells per well were distributed onto 96-well plate (Marathon) and allowed to adhere overnight. The medium was removed and the cells were treated with new medium containing various concentrations of gedunin. The plates were incubated for 48 h at 37°C followed by addition of MTT (1 mg mL^{-1}) to each well and incubation was continued for 1 h more. Dimethyl sulphoxide (150 µL) was added to each well of the plate to dissolve the formazan crystals formed. The absorbance for each well of the plate was recorded spectro-fluorometrically at 570 nm using a Dynatech MR 700 microplate reader.

Invasion assays: On to the 24-well cell culture invasion chamber (Corning Inc., Tewksbury, MA, USA) coated with 8.0 µm Matrigel™ (Becton Dickinson, Bedford, MA, USA) were seeded U-251 MG cells. The cells were treated initially with gedunin for 48 h in 200 µL medium devoid of serum. The cells were put in to the upper chambers at a density of 2.5×10^5 cells and into the lower chamber was placed normal growth medium. The incubation for 24 h was followed by removal of cells from the upper chamber. In the lower chamber cells were fixed in 90% ethanol followed by crystal violet (Sigma-Aldrich) staining. The cells were then counted using the Olympus-CX31 microscope (Olympus Corp., Tokyo, Japan).

Migration assay: The U-251 MG cells in serum free DMEM medium containing charcoal-stripped FBS were left for 24 h. The lower chamber of the Transwell filter coated with type IV collagen contained DMEM medium. The cells treated with various concentrations of gedunin were distributed at a density of 3×10^5 cells on to the filter for 48 h. The cells were stained using hematoxylin and eosin (H and E) followed by the examination under a microscope.

Western blot analysis: The cells were incubated with gedunin and lyzed in 200 µL lysis buffer (40 mmol L^{-1} Tris-HCl, 150 mmol L^{-1} KCl, 1 mmol L^{-1} EDTA, 1% Triton X-100, 100 mmol L^{-1} NaVO3 and 1 mmol L^{-1} PMSF, pH 7.5). The nuclear lysates were harvested using NucBuster™ Protein Extraction kit (Novagen®, Merck KGaA, Darmstadt, Germany) according to the manual protocol. The proteins were then

resolved on 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore Corp., Billerica, MA, USA). The membranes were incubated with non-fat milk at 37°C for 1 h followed by incubation with primary antibodies at 4°C overnight. After incubation, membranes were rinsed in PBS and incubated with horseradish peroxidase-conjugated goat anti-mouse secondary antibodies for 1 h. The bands were visualized using enhanced Chemiluminescence kit (ECL Plus, GE Healthcare Europe GmbH, Freiburg, Germany).

Real-time polymerase chain reaction (PCR): Th eU-251 MG cells at a density 2×10^5 cells per well were distributed on to the six-well tissue culture plates and treated with gedunin for 24 h. Qiagen RNeasy Mini Kit was used for the isolation of total RNA from the cells. The high capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used for the transcription of RNA samples at 40°C according to the manufacturer's instructions. The cDNA was then reverse-transcribed for quantitative PCR at50°C for2 min, at 95°C for 10 min and 40 cycles at 95°C for 5 min, at 60°C for 1 min. The assays were performed using Applied Biosystems 7300 Real-Time PCR system 3 times.

Statistical analysis: Statistical analysis of the data obtained was performed using Student's t-test. The experiments were carried out independently in triplicates. The statistically significant differences were considered at $p<0.05$.

RESULTS

Inhibition of U-251 MG cell viability by gedunin: The results from MTT assay revealed that gedunin exhibits a concentration and time dependent inhibitory effect on the viability of U-251 MG cells. The reduction in cell viability was dominant at 20 µM concentration after 24 h of gedunin treatment. At this concentration gedunin reduced the viability of U-251 MG cells to 28% compared to 98% in the control group (Fig. 1).

Inhibition of U-251 MG cell migration by gedunin: The effect of gedunin on migration potential of U-251 MG cells was investigated using Transwell migration assay. The results revealed that gedunin treatment inhibited the migration of U-251 MG cells significantly compared to the control after 48 h (Fig. 2). The concentration of gedunin at which migration potential of U-251 MG cells was reduced significantly ($p<0.002$) was 15 µM.

Inhibition of U-251 MG cell invasion by gedunin: The U-251 MG cells after treatment with gedunin were analyzed for invasive potential. It was observed that compared to the control group gedunin treated cells showed significantly lower tendency of invasion following 48 h of the treatment (Fig. 3). The reduction in invasive tendency of U-251 MG cells by gedunin treatment showed concentration dependent effect and was significantly higher at 15 µM concentration.

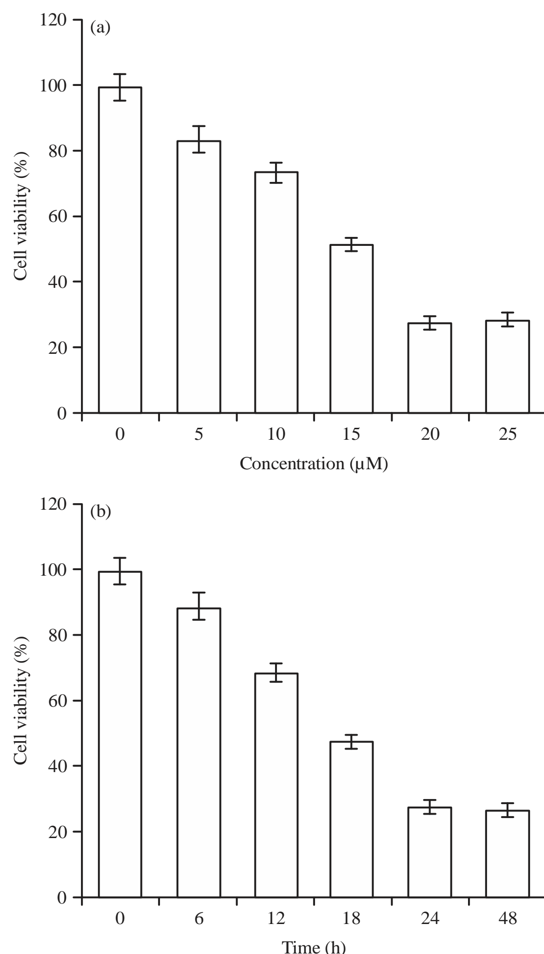

Fig. 1(a-b): Inhibition of U-251 MG cell viability by gedunin treatment. The cells were incubated with the indicated concentrations of gedunin followed by viability analysis using MTT assay
The bars represent the mean of SD

Effect of gedunin on the expression of levels of FAK, MMP-7, MMP-9 and uPA proteins: To investigate the factors involved in gedunin induced inhibition of invasive tendency, the expression of FAK, MMP-7, MMP-9 and uPA proteins were analyzed. It was observed that exposure of U-251 MG cells to 20 µM concentration of gedunin caused a marked reduction in the expression of FAK, MMP-7, MMP-9 and uPA proteins (Fig. 4).

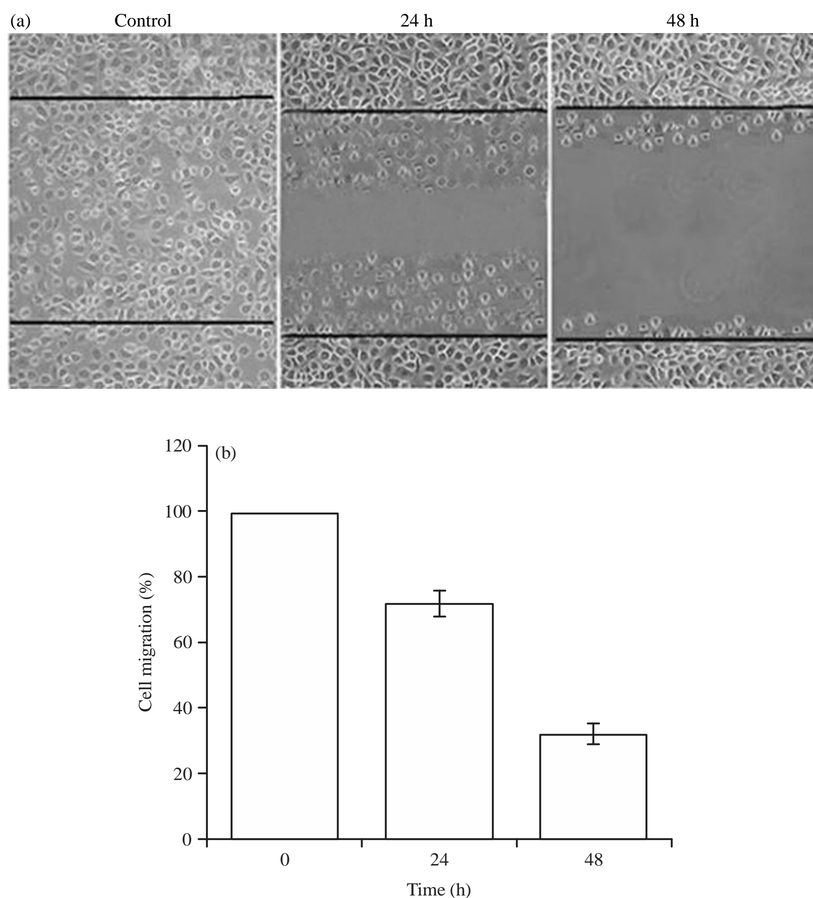

Fig. 2(a-b): Inhibition of U-251 MG cell migration by gedunin, (a) Effect of gedunin on cell migration by transwell assay examined using light microscope and (b) Migration potential of the cells was also quantified

Data is the Mean±SD of 3 independent experiments

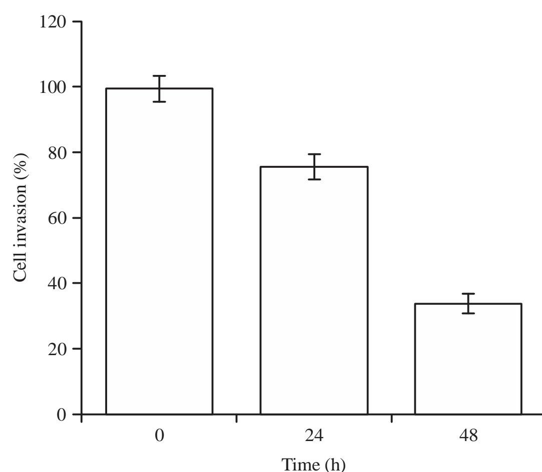

Fig. 3: Inhibition of invasive potential of U-251 MG cells by gedunin treatment. BGM U-251 MG cells were incubated with the indicated doses of gedunin for 48 h. The cells after gedunin treatment were subjected to matrigel assay and examined under light microscope. The quantification of the invasive potential of U-251 MG cells was carried out using qRT-PCR

The expressed data is the Mean±SD of 3 experiments performed independently

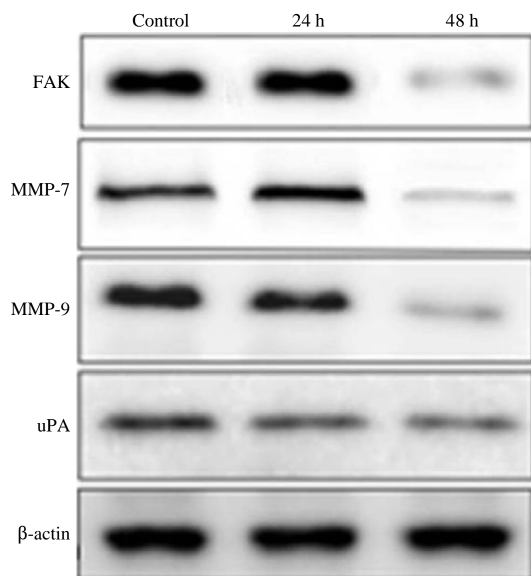

Fig. 4: Inhibition of invasive protein expression by gedunin treatment in U-251 MG cells. Effect of gedunin on the expression of FAK, MMP-7, MMP-9 and uPA proteins was analyzed by western blot assay

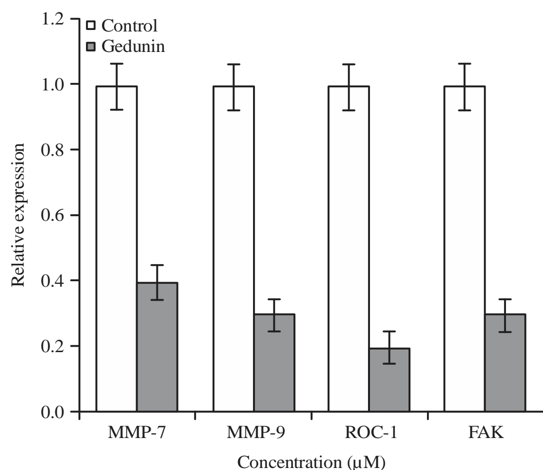

Fig. 5: Inhibition of the expression of mRNA corresponding to genes involved in cell migration. The cells were incubated with gedunin followed by real-time PCR to determine the mRNA expression

The expressed data is the Mean±SD of 3 experiments performed independently

Effect of gedunin on the expression of MMP-9, ROCK-1 and FAK mRNA: The effect of gedunin on the mRNA corresponding to MMP-9, ROCK-1 and FAK genes was also analyzed. It was observed that the cells exposed to 20 µM concentration of gedunin showed significant ($p<0.005$) reduction the expression of MMP-9, ROCK-1 and FAK mRNA (Fig. 5).

DISCUSSION

In the study it was observed that gedunin treatment reduced the viability and inhibited the invasive and migratory tendency of U-251 MG cells significantly. Uncontrolled division is the characteristic and most dangerous feature of the carcinoma cells. Therefore, the treatment strategies which inhibit the proliferation of cancer cells is considered to be a promising therapeutic method for cancer treatment. The present study revealed that exposure of the glioma cells to gedunin caused a marked reduction in the rate of cell proliferation. It is reported that invasion of carcinoma tissues is associated with degeneration of the basement membranes and extracellular matrix (ECM)[11]. The process of membrane degeneration is mediated by the metalloproteinase (MMPs) enzymes which play a dominant role in invasion of the carcinoma tissues[12,13]. The carcinoma tissues express higher level of MMPs which clearly indicates their role in the promotion of invasive and migratory potential[14-18]. The results from the present study demonstrated that gedunin caused a marked reduction in the invasive and migratory tendency of U-251 MG cells. Analysis of the factors involved in migration and invasion of the carcinoma cells demonstrated that gedunin exhibited inhibitory effect on migration and invasion through gene level suppression. Gedunin treatment caused inhibition of MMP-9, Rho kinases-ROCK-1 (ROCK-1) and focal adhesion kinase (FAK) mRNA expression. These factors have been shown to play a vital role in the process of cancer cell migration and[19-21]. The results from the present study revealed that gedunin treatment in glioma cells inhibited the expression of MMP-9, Rho kinases - ROCK-1 (ROCK-1) and focal adhesion kinase (FAK) mRNA expression. These findings showed that gedunin exhibited inhibitory effect on the migratory and invasive potential of glioma cells through suppression of MMP-9, Rho kinases-ROCK-1 (ROCK-1) and focal adhesion kinase (FAK) mRNA expression. The expression of uPA is found to be higher in the carcinoma cells and it induces degradation of the basement membranes[22,23]. The results from the present study showed that exposure of glioma cells to gedunin significantly reduced the expression of uPA.

CONCLUSION

Thus gedunin treatment exhibits inhibitory effect on the proliferation, migration and invasive potential of the glioma cells. Therefore, gedunin can be of the therapeutic importance for the treatment of glioma.

SIGNIFICANCE STATEMENT

This study demonstrates the effect of gedunin on viability, migration and invasion of human glioblastoma multiforme, U-251 MG cells. Gedunin treatment caused inhibition of proliferation, migration and invasion of U-251 MG cells through inhibition of matrix metalloproteinase-9 (MMP-9), FAK and ROCK-1. Therefore, gedunin is a potent agent for the treatment of tumor growth.

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