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Original Article

Effect of granatin B on the glioma cancer by inducing apoptosis

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Abstract: Glioblastoma is a highly malignant cancer of glioma cells. Present study investigates the anti proliferative activity of granatin B on glioma cell by inducing apoptosis. In this study Glioma cell (U87) was used on which anti proliferative activity of granatin B (0, 20, 40 & 80 μM) assessed by 3-(4, 5-dimethylthylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay. Thereafter Apoptosis of glioma cell was assessed by apoptosis detection kit suing flow cytometer, DAPI staining and by estimating the activity of caspase 3 & 9 using caspase 3 & 9 kit. Expression of MMP9 protein was determined through gelatin zymography. Possible mechanism of apoptosis induction was proved by estimating the effect of granatin B with MMP9 agonist on cell proliferation, caspase 3 activity & MMP9 expression on glioblastoma cell. Result of the study suggested that granatin B significantly decreases the cell proliferation of glioma cell compared to 0 μM treated group. It was also observed that treatment with granatin B significantly induces apoptosis and increases the activity of caspase 3 & 9 protein compared to 0 μM treated group. Expression of MMP9 protein was also decreases with granatin B treatment of glima cell. MMP9 agonist significantly reverses the effect of granatin B on cell proliferation, caspase 3 and expression of MMP9 protein in glima cell. Present study concludes the anticancer activity of granatin B on glioblastoma cell by inducing apoptosis.

Keywords: Granatin B, glioblastoma, apoptosis, caspase 3 & 9

Introduction

Cancer of glioma cell is the commonest type of brain tumor having high death rate in adult which commonly known as glioblastoma [1]. It is an aggressive type of cancer which includes characteristic symptoms such as headache, changes in mood and behavior, and other symptoms resembles to the strokes. Currently available therapy for the management of glioblastoma includes adjuent chemotherapy and radiotherapy after surgical resection. Still it was observed that life expectancy is not more than 5 years after the diagnosis of glioblastoma [2]. Moreover, the median survival of patient diagnosed with glioblastoma is just for 14 months [3]. Thus, an extensive research is going on for the development of effective therapy to control glioblasmota progression.

Reported literature suggested that *Punica granatum* having antimicrobial, antioxidant, anti inflammatory and cancer [4-6]. Granatin B is naturally isolated ellagitannin form *Punica granatum* [7]. Granatin B is a carbonic anhydrase inhibitor as per the previous reports [8]. Thus on the basis these medicinal properties this study evaluates the anticancer activity of granatin B on glioma cells.

Material and methods

Cell and culture media

Glioblastoma (U87) cell lines were procured from Shanghai Institutes for Biological Sciences, Shanghai, China. In a 5% CO_2 humidified incubator at 37°C cells lines were incubated. DMEM medium was used with (10% v/v) fetal bovine serum as a culture medium for the present study. Medium used in this study was replaced at a specific interval of time and cell were trypsinized routenly till it grows up to 80-90% of confluency. Granatin B was procured from Chromadex Inc. (Santa Ana, CA).

Estimation of cell viability by MTT assay

MTT assay was used to asses cellular viability. In which cell (5×103 cells/well) were placed at

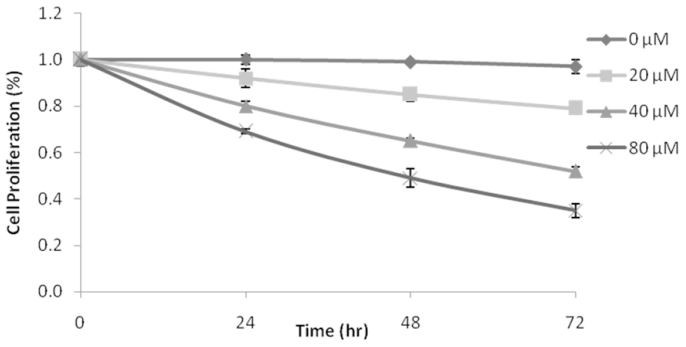

Figure 1. Effect of granatin B on the proliferation of glioma cells by MTT assay. **P<0.01 Vs granatin B 0 μM (n=3).

96 well plate and treated with granatin B at a different doses for the period of the duration of 3 days. MTT assay was performed as described by Xiao et al. MTT (50 μl) was added to the well plate and keep it for incubation for 4 hr. at 37°C. Thereafter from the reaction mixture supernatant was washed out and subsequently dimethylsulfoxide (200 μl) was added to all the well plate at room temperature. The absorbance was estimated at 570 nm wavelength [9].

Estimation of apoptosis

Apoptosis of cell was assessed by apoptosis assay in which U87 cancerous cells (1×106 cell/well) placed for 1 day in 6 well plates. Cell plates were treated with granatin B at different concentrations such as 20, 40 & 80 μM for the period of the duration of 2 days. Apoptosis of cells were estimated as per the instruction given by manufacturer (BD biosciences) by using apoptosis detection kit. In all the well plate Annexin V-FITC of 5 μl was added and kept it for 10 min at 37°C for incubation and thereafter propidium iodide (PI) at a volume of 5 μl was added to it. Flowcytometer was used for the estimation of cell apoptosis [10].

DAPI staining assay

Fluorescence microscope (Olympus Corp., Japan) was used for the determination of apoptotic cell in which U87 cancerous cells (1×106 cell/well) placed for 1 day in 6 well plates. Cell plates were treated with granatin B at different concentrations such as 20, 40 & 80 μM for the period of the duration of 2 days. All the cells were stained by using DAPI stain (1 μg/ml) [9].

Caspase-3 and caspase-9 activation assay

U87 cancerous cells (1×106 cell/well) placed for 1 day in 6 well plates. Cell plates were treated with granatin B at different concentrations such as 20, 40 & 80 μM for the period of the duration of 2 days. Activity of caspase 3 & 9 was estimated by as per the instructions of caspase 3 & 9 kit (Tiangen Biotech). All the glioma cells were incubated for 30 min by keeping it with cell lysis buffer. BCA protein reagent was used for the estimation of concentration of protein. Estimation of caspase 3 & 9 was achived using microplate spectrophotometer (Bio Rad lab., CA, USA) at a wavelength of 405 nm [9].

Real time PCR

U87 cancerous cells (1×106 cell/well) placed for 1 day in 6 well plates. Cell plates were treated with granatin B at different concentrations such as 20, 40 & 80 μM for the period of the duration of 2 days. RT PCR was used by assessment of expression of MMP9 of the cell. Extraction of total RNA was achieved from cell and thereafter expression of MMP 9 protein was estimated through a sequence detection system ABI 7300 HT (Applied Biosystems, Foster, USA).

Estimation of MMP 9 protein expression by zymography

U87 cancerous cells (1×106 cell/well) placed for 1 day in 6 well plates. Cell plates were treated with granatin B at different concentrations such as 20, 40 & 80 μM for the period of the duration of 2 days. Supernatant of culture media (20 μl) was added at an equal amount with buffer (sodium dodecylsulfate). Gel electrophoresis was used for this study in which after electrophoresis get was incubated at 37°C for the duration of 12 hr. thereafter gel was stained with Coomassie brilliant blue R-250.

Result

Evaluation of cell proliferation

Effect of granatin B on the glioma cells proliferation by MTT assay as shown in **Figure 1**. It

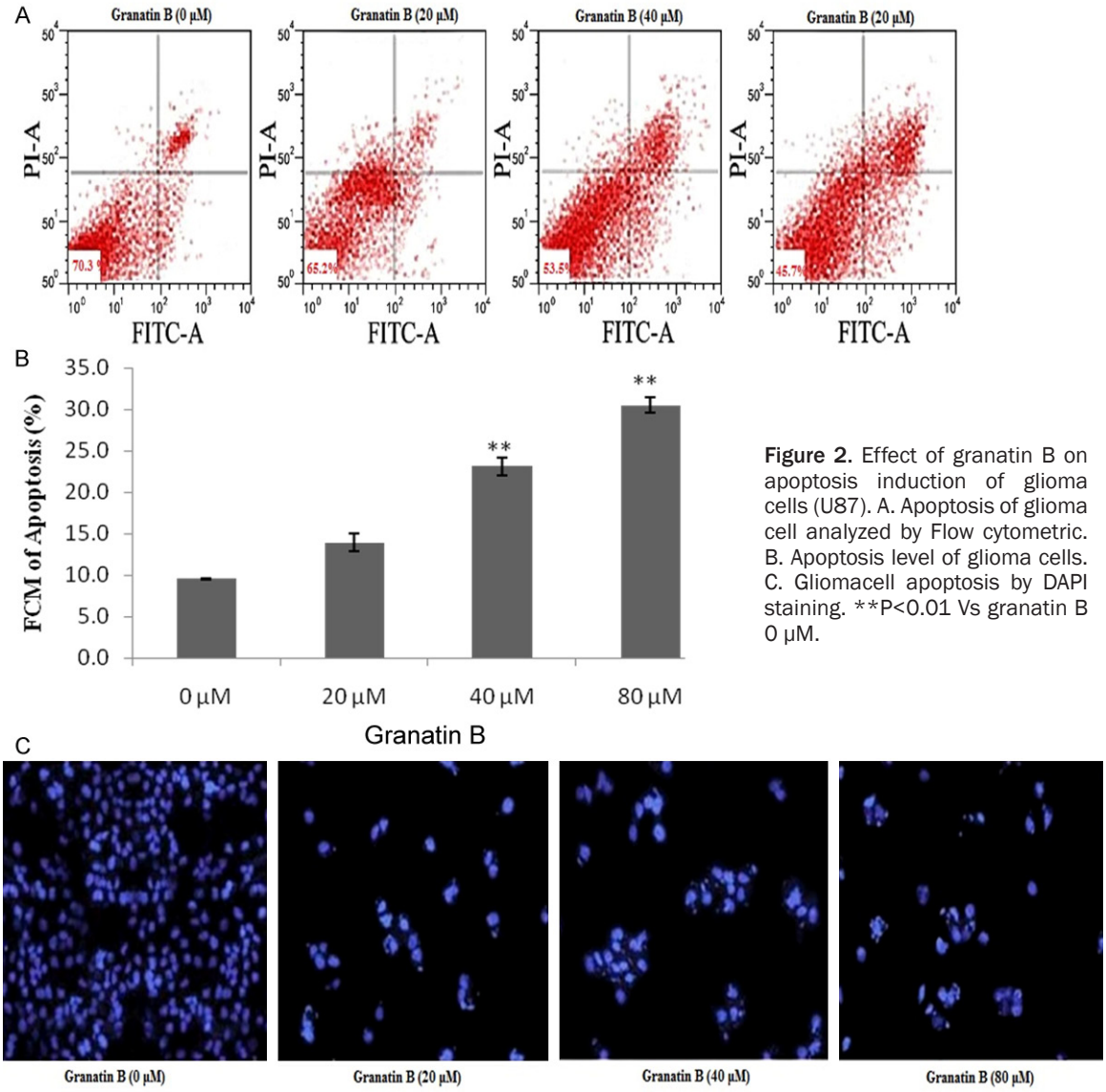

Figure 2. Effect of granatin B on apoptosis induction of glioma cells (U87). A. Apoptosis of glioma cell analyzed by Flow cytometric. B. Apoptosis level of glioma cells. C. Gliomacell apoptosis by DAPI staining. **P<0.01 Vs granatin B 0 μM.

was observed that granatin B at different dosage inhibits the glioma cells proliferation and this inhibition of proliferation was achieved in a dose and time dependent manner. There was significant decrease in the cell proliferation in granatin B (40 and 80 μM) after 2 and 3rd day of treatment compared to 0 μM treated group.

Figure 2 shows the effect of granatin B on apoptosis induction of glioma cancerous cells by Annexin V-FITC detection kit and through DAPI staining. It was observed that treatment with granatin B at 20, 40 & 80 μM significantly (P<0.01) induces the apoptosis of glioblastoma cells compared to 0 μM treated groups as shown in **Figure 2A** and **2B**. Moreover, DAPI staining reveal the effect granatin B on cell apoptosis, as it significantly increases the apoptosis in a dose dependent manner compare to 0 μM treated group as showin in **Figure 2C**.

Evaluation of activity of caspase 3 & 9

Activity of caspase 3 & 9 were estimated by using caspase 3 & 9 assay. It was observed that the activity of caspase 3 & 9 significantly (P<0.05, P<0.01) induced in the granatin B at a dose of 20, 40 & 80 μM treated group compared to 0 μM treated group. Activity of caspase 3 & 9 found to be increases in a dose dependent manner as shown in **Figure 3A** and **3B**.

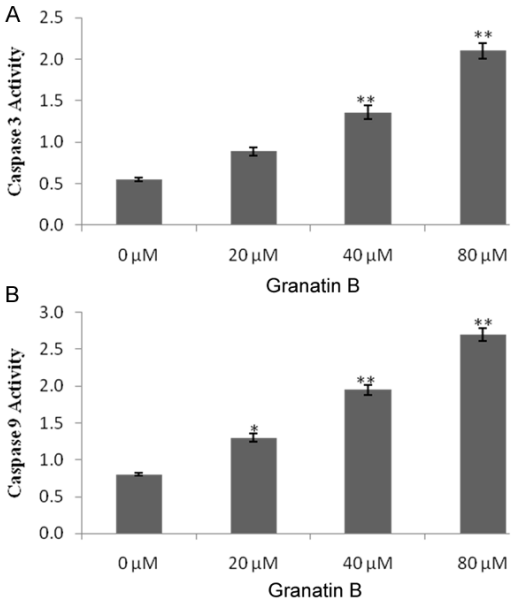

Figure 3. Effect of granatin B on activation of caspase 3 & 9 in glioma cancerous cells. A. Activity of caspase 3. B. Activity of caspase 9. *P<0.05, **P<0.01 Vs granatin B 0 μM.

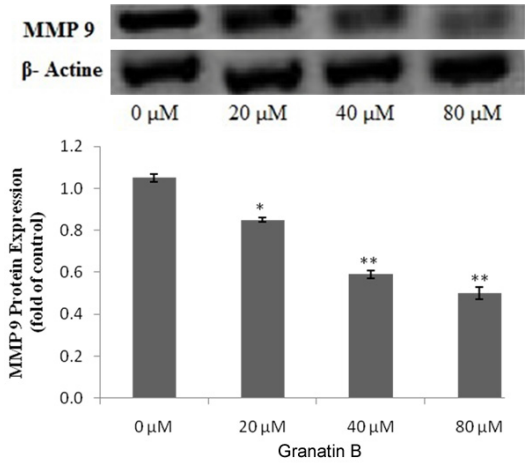

Figure 4. Effect of *Granatin B* on protein expression of MMP9 in glioma cancerous cell. *P<0.05, **P<0.01 Vs granatin B 0 μM.

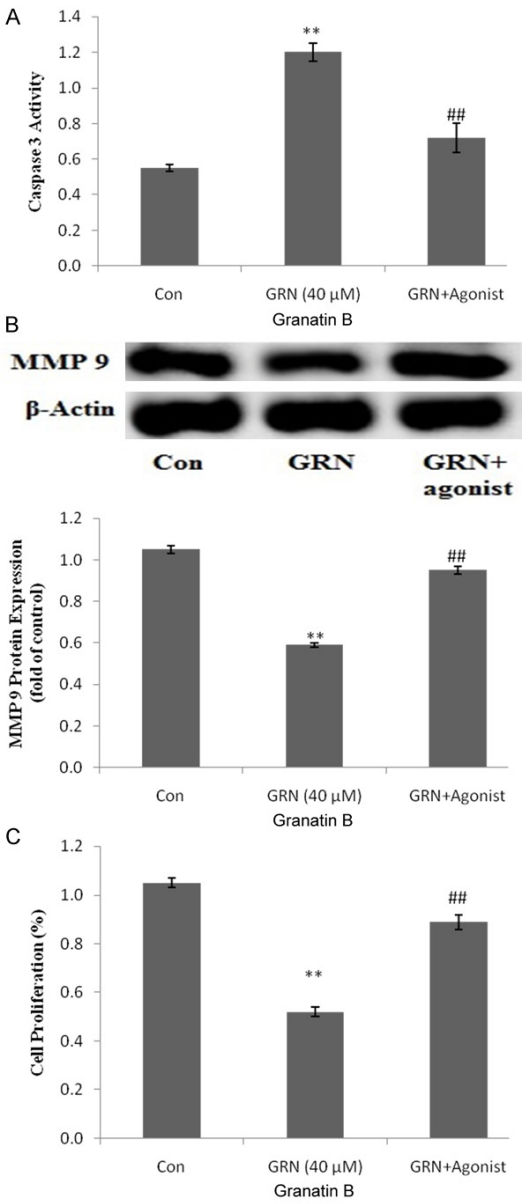

Figure 5. Effect of MMP 9 agonist on the activity of *Granatin B* in glioma cancerous cell. A. Caspase activity 3. B. Protein expression of MMP9. C. Cell proliferation. **P<0.01 Vs Control, #P<0.01 Vs GRN (40 μM).

Effect Of granatin B on MMP 9 protein expression

Expression of MMP9 protein was estimated by zymographic analysis in the given study. Granatin B treated group (20, 40 & 80 μM) shows the significant decreased (P<0.05, P<0.01) in the protein expression of MMP9 compared to 0 μM as shown in **Figure 4**. It was observed that this decreased in the protein expressions of MMP9 in dose dependent manner.

Effect of granatin B and MMP 9 agonist on the protein expression of MMP 9

MMP 9 agonist reverses the effect of granatin B on caspase activity, MMP9 proteins expression & cell proliferation in glioma cells as shown in **Figure 5**. It was observed that caspase 3 activity significantly reversed in GRN+ agonist treated group compared GRN (40 μM) treated groups (**Figure 5A**). Protein expressions of MMP9 and cell proliferation were significantly

decreases (P<0.01) in GRN (40 μM) treated group compared to control group. Whereas GRN (40 μM) co administered with MMP9 agonist significantly reverse the decreased activity of Protein expressions of MMP9 and cell proliferation of glioma cells (**Figure 5B** and **5C**).

Discussion

Glioblastoma is an aggressive type of neuronal cancer and its uncontrolled proliferation limits clinical treatment to it [11]. This study investigates the anticancer activity of granatin B on glioblastoma cell lines. Granatin B has a strong carbonic anhydrase activity and the herbs contain this ellagitannin posses anti inflammatory and antioxidant activity [5, 6, 8]. Previously reported study suggested that activity of carbonic anhydrase inhibitors found to inhibit the glioblastoma cancer by decreasing apoptosis [12]. It was found that granatin B significantly decreases (P<0.01) the cell proliferation and induces the apoptosis of glioma cells. granatin B reduces the glioma cell proliferation by inducing apoptosis and thereby it posses anti cancer activity.

Caspase 3 & 9 is the member of cysteine-aspartic acid protease family which activates the process apoptosis [13]. It induces the intrinsic mitochondrial pathway and thereby induces the process of apoptosis [14]. In the given investigation granatin B significantly increases the activity of caspase 3 & 9.

MMP 2 and 9 are the types of metalloproteinase enzyme and it becomes a topic of interest for the research. Several studies suggested that MMP 2 & 9 inhibitor induces apoptosis of cancerous cells [15]. It was also observed that expression of MMP 9 protein significantly decreases with the granatin B treatment which was also reversed when co administered with MMP9 agonist. Hence it confirms that granatin B induces apoptosis by inhibiting expression of MMP 9 protein in glioma cell. Present study concludes the anticancer activity of granatin B in glioblastoma cell by inducing apoptosis. Study also suggested that it induces apoptosis by inhibiting the expression of MMP9 protein specifically.

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Disclosure of conflict of interest

None.

Authors' contribution

Shao-Ping Wang, and Yan-Jun Zhong design and perform the given research work. Mei Yang and Jian Xue contributed in the interpretation of result.

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