Methyl gallate isolated from *Spondias pinnata* exhibits anticancer activity against human glioblastoma by induction of apoptosis and sustained extracellular signal-regulated kinase 1/2 activation

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ABSTRACT

Background: *Spondias pinnata* has been reported for its efficient anticancer effects, but the studies were mostly focused on its extract. **Objective:** Since its bioactive compounds are largely unknown, this study was designed to characterize the lead components present in it and their anticancer activity against human glioblastoma cell line (U87). **Materials and Methods:** Major compounds from the ethyl acetate fraction were isolated by column chromatography and their anticancer potentials against U87 cells were evaluated. Furthermore, flow cytometric and immunoblotting analyses were performed to demonstrate the mechanism of apoptosis inducing activity of methyl gallate (MG) against U87 cell line. **Results:** Four major compounds were isolated from the ethyl acetate fraction. Amongst these, two compounds showed promising activities and with the help of different spectroscopic methods they were identified as gallic acid and MG. Flow cytometric studies revealed that MG-induced apoptosis in U87 cells dose-dependently; the same was confirmed by activation of caspases through cleavage of endogenous substrate poly (adenosine diphosphate-ribose) polymerase. MG treatment also induced the expression of p53 and B-cell lymphoma-2-associated X and cleavage of BH3 interacting-domain with a concomitant decrease in B-cell lymphoma-2 expression. Moreover, MG-induced sustained phosphorylation of extracellular signal-regulated kinase (ERK1/2) in U87 cells with no change in the phosphorylation of other mitogen-activated protein kinases (c-Jun N-terminal of stress-activated protein kinases, p38). **Conclusion:** MG is a potent antioxidant and it induces sustained ERK1/2 activation and apoptosis in human glioblastoma U87, and provide a rationale for evaluation of MG for other brain carcinoma cell lines for the advancement of glioblastoma therapy.

Key words: Anticancer, apoptosis, cytotoxicity, methyl gallate, *Spondias pinnata*, U87

INTRODUCTION

Cancer is a universal public health crisis because of its high occurrence and mortality rate. Regardless of recent advances in the development of new diagnostic and therapeutic tools, it remains of some urgency to identify new and better therapeutic agents and regimes for cancer. Bioactive natural products provide structures that will become major sources of novel agents with pharmacological promise.[1]

Glioma is the most common and highly malignant type of primary brain tumor in humans[2] which compared to other cancers, is relatively rare but their high malignancy rate make them responsible for a very high mortality rate. Despite invasive surgery, radiotherapy and chemotherapy, malignant brain tumors remain a therapeutic challenge,[3] due to its characteristic resistance to apoptosis.[4] Doxorubicin, a well-known chemotherapeutic drug used in treating gliomas has some serious side effects that are responsible for congestive heart failure.[5] Thus, major attention is being given to search for better and safer antioxidants of natural origin, which may raise the efficiency of cancer treatment.

Spondias pinnata (Linn. f.) Kurz. (Family - Anacardiaceae) is a deciduous tree widely distributed in South-East Asian

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countries. The plant is cultivated in various parts of India as the fruits are eaten as a vegetable when green and as a fruit when ripe and local people make chutney, jam and pickle. Fruits are very nutritious and rich in vitamin A, minerals and iron content.[6] Ethnomedically, *S. pinnata* is used for its antibacterial activity[7] and treatment of dysentery.[8] It is also reported that the bark of *S. pinnata* acts as a very good antioxidant and free radical scavenger[9] as well as a potent iron chelator.[10] Our recent findings suggest that the bark of *S. pinnata* also possesses promising anticancer activity.[11] The gum exudate of the species has been reported to contain acidic polysaccharides.[12] Oleanolic acid has been isolated from the fruit of this species, and its antimicrobial properties have been established previously.[13] These findings further culminate our interest in isolating and characterizing the active principles from *S. pinnata* and investigated for their various pharmacological potentials like anticancer activity.

MATERIALS AND METHODS

Chemicals
Cell proliferation reagent WST-1 and Annexin-V-FLUOS kit were procured from Roche diagnostics, Mannheim, Germany. US-origin fetal bovine serum (FBS) was purchased from HyClone Laboratories Inc., Utah, USA. All the other chemicals and solvents are of minimum analytical or molecular biology grade and procured locally. Anti-B-cell lymphoma (Bcl-2) (NT), anti-caspase-9 and anti-p53 antibodies were purchased from AnaSpec, Inc., USA. Anti-poly (adenosine diphosphate-ribose) polymerase (PARP), anti-caspase-3, anti-caspase-8, anti-Bcl-2-associated X (Bax), anti-BH3 interacting-domain (Bid), anti-pERK-1/2, anti-pJNK, anti-p38 and anti-beta-actin antibodies were purchased from OriGene Technologies Inc., Rockville, USA. Alkaline phosphatase conjugated anti-Rabbit secondary antibody was purchased from RockLand Immunochemicals Inc., Gilbertsville, USA.

Extraction and isolation
Seventy percent methanolic extract of the *S. pinnata* bark was prepared according to the previous method.[9] The lyophilized extract (1.1 kg) was re-extracted with 2 × 10 L of n-hexane, chloroform, ethyl acetate sequentially. All the fractions were evaporated under reduced pressure and the crude ethyl acetate fraction (20 g) was subjected to silica gel column chromatography eluting with dichloromethane (DCM) followed by DCM-methanol ([100:0], [50:1], [100:3], [20:1]). The elutes were monitored by thin-layer chromatography and grouped into four fractions namely SPE1, SPE2, SPE3 and SPE4 which upon evaporation yielded 2.4 g, 80 mg, 1.8 g and 180 mg powdered mass respectively. The purity of SPE1, SPE2, SPE3 and SPE4 was determined by reverse phase ZIC®-HILIC column with a gradient mobile system of acetonitrile and 5 mM ammonium acetate in water.

In vitro anticancer study
Cell lines and culture
Human glioblastoma (U87) were purchased from the National Centre for Cell Science, India and maintained in the laboratory. U87 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) FBS, 100 U/ml penicillin G, 50 µg/ml gentamycin sulfate, 100 µg/ml streptomycin and 2.5 µg/ml amphotericin B. The cell line was maintained at 37°C in a humidified atmosphere containing 5% CO_2 in CO_2 incubator.

WST-1 cytotoxicity assay
Cell viability was quantified using the WST-1 cell proliferation reagent, Roche diagnostics, according to the previously described method.[11] In brief, $1 × 10^4$ cells were treated with compounds ranging from 1 to 30 µg/ml for 48 h in 96-well culture plate. After treatment, 10 µl of WST-1 cell proliferation reagent was added to each well followed by 2 h of incubation at 37°C. Cell viability was quantified by measuring absorbance at 460 nm using a microplate enzyme-linked immunosorbent assay reader MULTISKAN EX (Thermo Electron Corporation, USA).

Structure elucidation
The chemical structures of the most bio active compounds were elucidated by molecular mass using EIMS (JEOL JMS-700, Germany), Fourier transform-infrared spectroscopy spectra recorded in KBr (Perkin Elmer, USA) and different nuclear magnetic resonance (NMR) experiments including ^1H, ^{13}C, ^{13}C DEPT-135, ^1H-COSY, HSQC and HMBC with a Bruker-500 MHz NMR Spectrometer (Germany).

Cell cycle analysis
Cell cycle analysis was performed by flow cytometry using the method previously described.[11] U87 cells ($1 × 10^5$) were treated with methyl gallate (MG) ranging from 10 to 100 µM for 24 h. After treatment, nuclear DNA of cells was then stained with propidium iodide (PI) and cell phase distribution was determined on FACSVerse (Becton Dickinson) equipped with 488 nm (blue), 405 nm (violet) and 640 nm (red) solid state laser light using FACSuite software version 1.0.3.2942 (Becton Dickinson). A total 10,000 events were acquired, and data analysis was done using the same software. A histogram of DNA content (x-axis, red fluorescence) versus count (y-axis) was plotted.

Annexin V staining

This assay was performed using Annexin-V-FLUOS Staining kit, Roche diagnostics. U87 cells (1×10^5) were treated with MG ranging from 10 to 100 μM for 24 h. After treatment cells were labeled with PI and FITC according to the protocol of the kit manufacturer. The distribution of apoptotic cells was identified by flow cytometer on FACSVerse (Becton Dickinson) equipped with 488 nm (blue), 405 nm (violet) and 640 nm (red) solid state laser light using FACSuite software version 1.0.3.2942 (Becton Dickinson). A total 10,000 events were counted. Cells that were Annexin V (−) and PI (−) were considered as viable cells. Cells that were Annexin V (+) and PI (−) were considered as early stage apoptotic cells. Cells that were Annexin V (+) and PI (+) were considered as late apoptotic or necrotic cells.

Western blot analysis

1×10^6 U87 cells were treated with MG (50 μM) for various time intervals (3–36 h). After treatment, cells were lysed with triple detergent cell lysis buffer (50 mM tris-Cl, 150 mM NaCl, 0.02% sodium azide, 0.1% sodium dodecyl sulfate (SDS), 1% triton X-100, 0.5% sodium deoxycholate, 1 μg/ml aprotinin, 100 μg/ml phenyl methyl-sulfonyl fluoride, pH 8) and the lysates were then centrifuged at 13,800 $\times g$ for 20 min at 4°C. Protein concentration was measured by Folin-Lowry method. Proteins (40 μg) in the cell lysates were resolved on 12% SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane and the membranes were then blocked with 5% nonfat dry milk in tris-buffered saline (TBS). After overnight blocking, the membranes are incubated for 3 h with corresponding antibodies. Postwash with TBS-T (0.01% of Tween-20 in TBS) membranes were incubated with alkaline phosphatase conjugated anti-Rabbit immunoglobulin G antibody at room temperature in the dark for 4 h, followed by washing. The blots were then developed with the 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate, and the images were taken by the imaging system EC3 Chemi HR (UVP, USA). The blots were then analyzed for band densities using ImageJ 1.45s software (National Institute of Health, USA).

Statistical analysis

All spectrophotometric data was reported as the mean ± standard deviation of 6 measurements. The statistical analysis was performed by KyPlot version 2.0 beta 15 (32 bit) (KyensLab Incorporated, Tokyo, Japan). The inhibitory concentration 50 (IC_{50}) values were calculated by the formula, $Y = 100 \times A1/(X + A1)$ where $A1 = IC_{50}$, $Y =$ response ($Y = 100\%$ when $X = 0$), $X = IC_{50}$. The IC_{50} values were compared by paired *t*-test. $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

For the past 30 years, the emphasis in natural product research has been in the field of anticancer drugs.[13,14] The traditional medicinal use of *S. pinnata* was previously supported with the finding of antioxidant, hepatoprotective and anticancer properties of this plant. So, the current study focuses on the bioactivity guided isolation and characterization of the active principles from *S. pinnata* bark and since our preceding studies showed effective antioxidant and anticancer activities with the ethyl acetate fraction of 70% methanolic extract of *S. pinnata* bark, this fraction was subjected to different isolation methods and four major compounds were isolated (SPE1, SPE2, SPE3 and SPE4) and used for further anticancer investigations.

The cytotoxic activity of the four isolated compounds against U87 cells is shown in Figure 1. Results suggest that SPE3 and SPE4 showed significant toxicity against U87 cells dose-dependently with an IC_{50} value 59.28 ± 3.92 μg/ml and 8.44 ± 0.61 μg/ml respectively while SPE1, SPE2 showed negligible toxicity in any of the examined concentrations with a very high IC_{50} value of 841.54 ± 89.32 μg/ml and 280.42 ± 34.89 μg/ml respectively. As a whole, we can say that SPE3 and SPE4 are the lead compounds as an anticancer agent in ethyl acetate fraction of *S. pinnata* bark. These two purified compounds are then subjected for their structural elucidation using various spectroscopic methods which indicated SPE3 to be gallic acid [Figure 2a] and SPE4 as MG [Figure 2b]. The spectroscopic data are shown as follows:
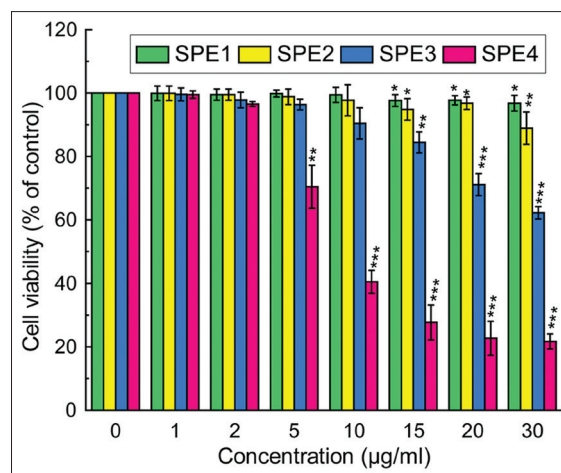
Figure 1: Effect of the isolated compounds (SPE1, SPE2, SPE3, SPE4) on cell proliferation and viability of U87 cells. Cells were treated with increasing concentrations of compounds for 48 h; cell proliferation and viability was determined with WST-1 cell proliferation reagent. Results were expressed as cell viability (% of control). Each value represents mean ± standard deviation (*n* = 6) (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ vs. 0 µg/ml)

SPE3: The white 99.04% pure compound; melting point: 220–223°C; IR: 3402, 1693, 1617; EIMS (m/z): 170 [M]+, 153, 125, 85/cm; ^{1}H NMR (500 MHz, DMSO d6): δ 6.71 (2H, s, Ar-H-2, H-6), 8.64 (s, Ar-4, 1-OH), 8.99 (s, Ar-3, 5, 2-OH), 12.02 (s, -COOH); ^{13}C NMR (500 MHz, DMSO d6): δ 109.17 (C-2, C-6), 120.88 (C-1), 138.44 (C-4), 145.85 (C-3, C-5), 167.93 (C-7).

SPE4: The white solid 99.94% pure compound; melting point: 195–200°C; IR: 3435, 1698, 1618, 1374/cm; EIMS (m/z): 184 [M]+, 153, 126, 115, 91; ^{1}H NMR (500 MHz, DMSO d6): δ 3.73 (3H, s, -OCH$_3$), 6.93 (2H, s, Ar-H-2, H-6), 8.93 (s, 1-OH), 9.26 (s, 2-OH). ^{13}C NMR (500 MHz, DMSO d6): δ 52.05 (C-8), 108.96 (C-2, C-6), 119.75 (C-1), 138.87 (C-4), 146.04 (C-3, C-5), 166.79 (C-7).

Gallic acid is a phenolic compound and previously reported effective against many types of the malignancies[15] including various gliomas especially U87.[16] MG is a derivative of
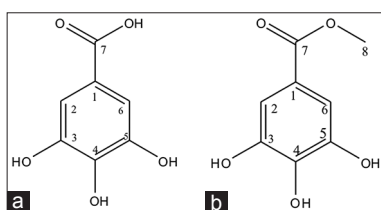

Figure 2: Chemical structure of (a) Gallic acid and (b) Methyl gallate isolated from *Spondias pinnata* bark

gallic acid. The anticancer properties of MG are not widely proven rather there are some reports regarding its antitumor[17] and proliferation inhibition activities.[18] However, its biological activity has not been extensively examined in glioma and other cancer cells. With the interest of revealing the underlying mechanism of MG-induced cytotoxicity against U87, further flow cytometric as well as immunoblotting studies were carried out. The effect of MG on cell cycle distribution of U87 cells resulted in an increase in accumulation of Sub-G1 phase that refers to the apoptotic cells [Figure 3]. The apoptosis was found to increase dose-dependently with nearly 40% cells in sub-G1 at a concentration of 100 µM. Furthermore, the dose-dependent induction of apoptosis in MG treated U87 cells was confirmed by Annexin-V staining [Figure 4]. The results showed an increase in early apoptotic cells with an increase in dose of MG. At zero dose of MG, 4.12% cells were found in early apoptotic phase while 4.46%, 5.63%, 28.12%, 43.63% and 50.08% cells were found in early apoptotic phase with an increase in doses 10 µM, 25 µM, 50 µM, 75 µM and 100 µM respectively suggesting that the MG treatment induced apoptosis but not necrosis in U87 cells.

Mitochondria play a pivotal role in the activation of caspase cascade and thereby signal transduction of apoptosis.[19] Effects of MG on the proteolytic activation of caspase-9 and caspase-3 were then examined. As shown in Figure 5a and b, MG treatment resulted in a significant increase in
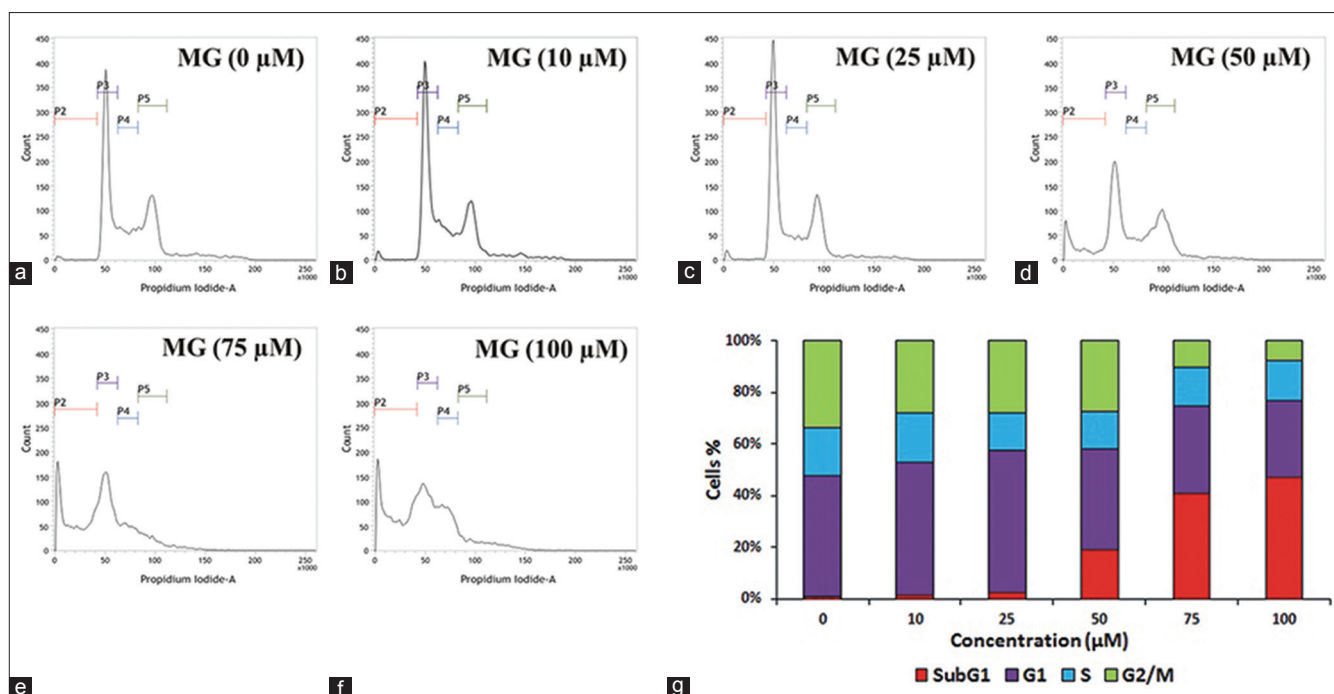
Figure 3: Cell cycle distribution of methyl gallate (MG) treated U87 cells. Sub-G1 (P2), G1 (P3), S (P4), and G2/M (P5) phases of U87 cells treated for 24 h with different concentrations: (a) 0 µM, (b) 10 µM, (c) 25 µM, (d) 50 µM, (e) 75 µM, (f) 100 µM of MG, (g) Graphical representation of % cell population in different phases
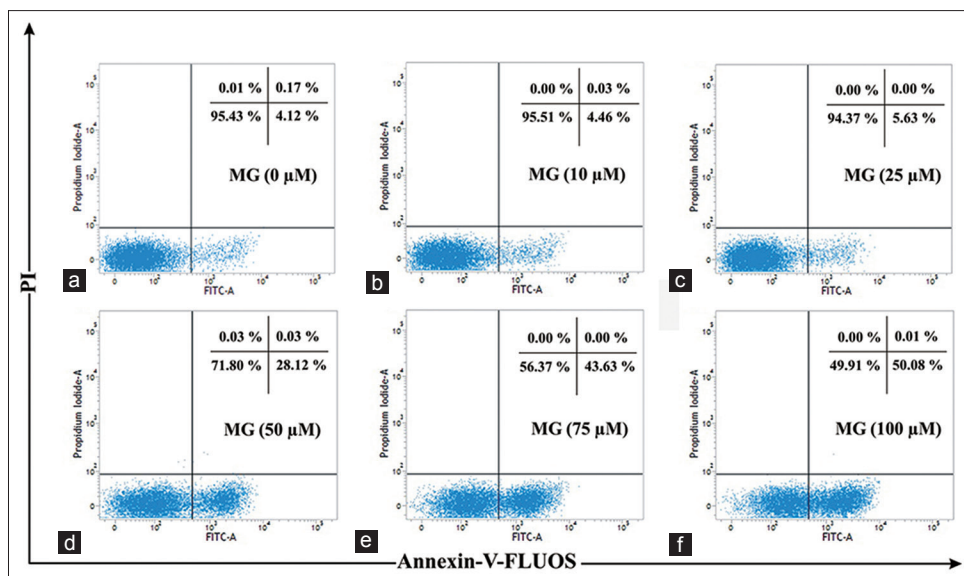
Figure 4: Dot plots of Annexin-V-FLUOS and propidium iodide staining of methyl gallate (MG) treated U87 cells. U87 cells were treated for 24 h with different concentrations: (a) 0 μM, (b) 10 μM, (c) 25 μM, (d) 50 μM, (e) 75 μM, (f) 100 μM of MG. Numbers in boxes represent % of total cells in respective boxes
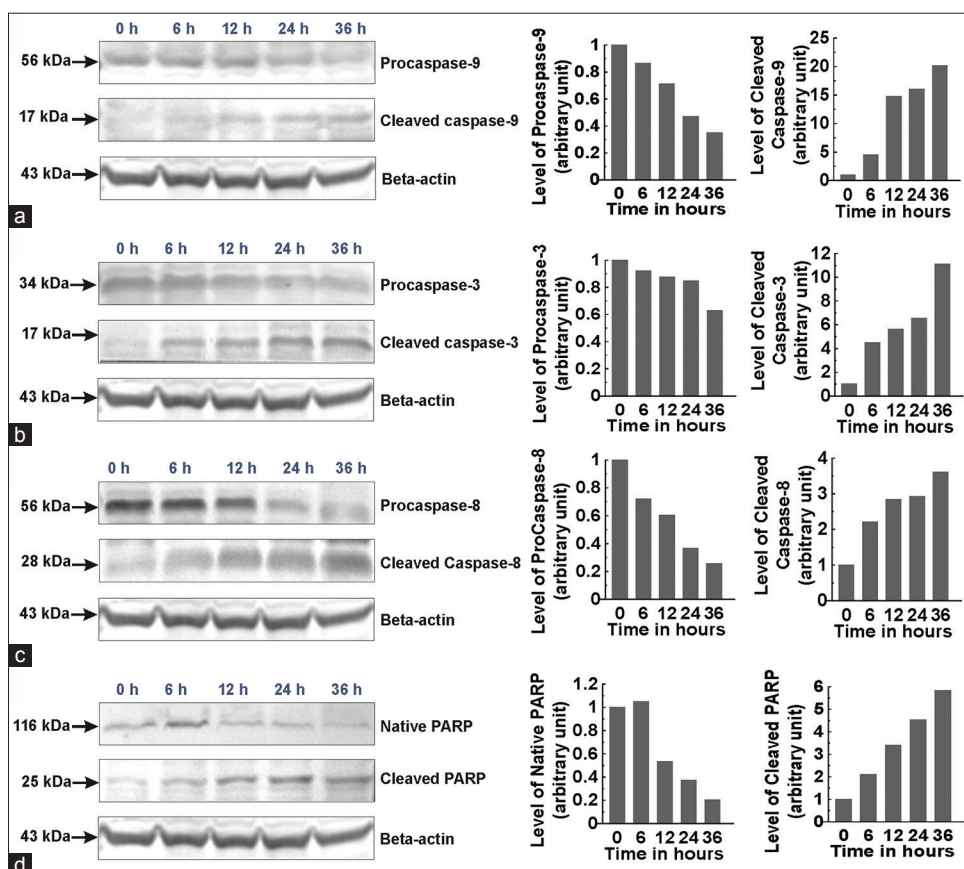
Figure 5: Western blot analysis of caspases and poly(adenosine diphosphate-ribose) polymerase (PARP) of U87 cells treated with 50 μM methyl gallate. Graphs adjoining the blots represent the expression levels of corresponding proteins for indicated time intervals: (a) Pro and cleaved caspase-9, (b) Pro and cleaved caspase-3, (c) Pro and cleaved caspase-8, (d) Native and cleaved PARP

the active form of caspase-9 and caspase-3 in U87 cells. Furthermore, procaspse-8 level decreased, caspase-8 level elevated [Figure 5c]. The activation of caspases in MG treated

U87 cells was further confirmed by detecting the cleavage of PARP, an endogenous substrate of activated caspase-3 and a hallmark of apoptosis. As shown in Figure 5d, treatment

of U87 cells with MG resulted in the cleavage of PARP to a 25 kDa fragment. The observation of MG-mediated activation of caspase-9, caspase-3, subsequent cleavage of PARP in U87 cells, suggesting that mitochondrial-mediated caspase cascade pathway plays a very important role in MG-induced apoptosis in U87 cells.[20] Bcl-2 family proteins, including Bcl-2 and Bcl-2-related family members such as Bax, Bid, Bcl-extra-large and Bcl-2-associated death promoter, play an important role in the regulation of apoptosis. The balance between the expression levels of Bcl-2 (anti-apoptotic) and Bax (pro-apoptotic), is critical for cell survival and death as the increase in Bax/Bcl-2 ratio contributes to the release of cytochrome C from mitochondria and decides the susceptibility of cells to undergo apoptosis.[21] As shown in Figure 6a, MG treatment increased the level of Bax expression while reduced Bcl-2 expression in a time-dependent manner. A densitometric analysis of the bands revealed that MG increased the Bax/Bcl-2 ratio, which is responsible for MG-induced apoptosis in U87 cells. As cleaved caspase-8 level was elevated there was an appearance of the t-Bid [Figure 6b] after the treatment with MG. Previously it is reported that, caspase-8 links intrinsic pathway with extrinsic pathway by cleaving Bid into truncated-Bid and plays an important role in activation of both these pathways.[22] It is possible that p53 molecule plays a role in the alteration of the expression of Bax and Bcl-2. Our results showed that MG treatment induces the expression of p53 in U87 cells [Figure 7a]. This suggests that p53 is responsible for the upregulation of Bax and down regulation of Bcl-2 in MG treated U87 cells. The ability of wild type p53 to upregulate Bax and downregulate Bcl-2 and proceed to the apoptosis is demonstrated previously.[23]

Mitogen-activated protein kinases (MAPK) (extracellular signal-regulated kinase 1/2 [ERK 1/2], c-Jun N-terminal of stress-activated protein kinases 1/2 [JNK1/2] and the p38 protein kinases) participate in diverse cellular functions such as cell proliferation, cell differentiation, cell motility and cell death. Cellular stresses and stimuli induce cell apoptosis via sustained activation of the MAPK signaling pathways.[24] We thus examined the effect of MG treatment on activation of ERK1/2, JNK and p38 in U87 cells. The phosphorylation level of ERK1/2 was increased [Figure 7b] after MG treatment, which lasted 18 h. However, no significant changes in phosphorylation levels of JNK and p38 were observed [Figure 7c and d]. It was proved that the transient activation of ERK1/2 plays an essential role in cell proliferation, and that sustained ERK1/2 activation induces apoptosis.[25] These results suggested that sustained activation of the ERK is involved in MG-induced growth inhibition and apoptosis in U87 cells.

CONCLUSION

We can say that MG, isolated for the first time from *S. pinnata* bark, inhibited cell growth and induced cell apoptosis in glioma cells. The further western blotting studies revealed the underlying principle of MG-induced apoptosis in glioma cells that involve the activation of caspase-3 and caspase-9 and Bid truncation. We found that increased p53 level, Bax/Bcl-2 ratio and sustained ERK1/2 activation were also associated factors for MG-induced apoptosis [Figure 8]. The results provide a rationale that MG can be developed as a potential anticancer agent against human glioblastoma. Furthermore, extensive *in vitro* studies against different glioblastoma cell line and *in vivo* research with xenograft and preclinical models with MG will justify its use as potent drug, thus making it a promising candidate for management of brain cancer patients.

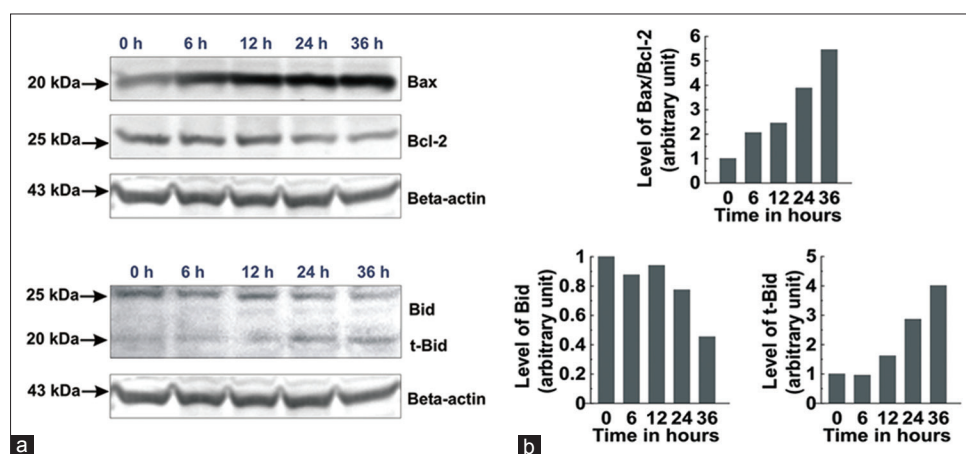**Figure 6:** Western blot analysis of B-cell lymphoma (Bcl-2) family proteins of U87 cells treated with 50 μM methyl gallate. Graphs adjoining the blots represent the expression levels of corresponding proteins for indicated time intervals: (a) Bcl-2-associated X and Bcl-2, (b) BH3 interacting-domain (Bid) and t-Bid

Figure 7: Western blot analysis of Mitogen-activated protein kinases and p53 of U87 cells treated with 50 μM methyl gallate. Graphs adjoining the blots represent the expression levels of corresponding proteins for indicated time intervals: (a) p53, (b) phosphorylated extracellular signal-regulated kinases-1/2, (c) pJNK, (d) p38

Figure 8: Proposed mechanism of methyl gallate-induced apoptosis in U87 cells

ACKNOWLEDGMENTS

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