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ORIGINAL ARTICLE

Moringa oleifera Leaf Extract Exerts Antiproliferative Effects and Induces Mitochondria Mediated Apoptosis within Rat Glioblastoma (C6) Cells

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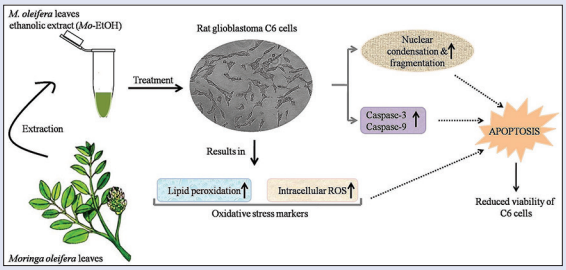
ABSTRACT

Background: Glioblastoma multiforme is a dreaded manifestation of brain tumors resulting in substantial mortality among affected individuals globally. *Moringa oleifera (Mo)* is well known from earlier times for its medicinal use in conventional medication for different ailments such as cancer. **Objective:** The present study aims to evaluate the antiproliferative efficacy of ethanolic *Mo* leaf extract (*Mo*Et-OH) in mouse-derived glioblastoma C6 cells. **Materials and Methods:** *Mo*Et-OH was prepared, and C6 cells were subjected to *Mo*Et-OH treatment at a dosage of 100, 200, and 400 µg/ml and incubated for 24 h. **Results:** Post-incubation, C6 cells exhibited a significant ($P < 0.05$) decline in their viability at 100 µg/ml, which further increased proportionally with increase in *Mo*Et-OH concentration ($P < 0.01$; $P < 0.001$). *Mo*Et-OH significantly enhanced the lipid peroxidation as assessed by measuring the increased levels of malondialdehyde at 100 µg/ml ($P < 0.05$), 200 µg/ml ($P < 0.01$), and 400 µg/ml ($P < 0.001$). *Mo*Et-OH-mediated evaluation of glutathione levels also exhibited similar trends. Moreover, reactive oxygen species estimation revealed a substantial increase in oxidative stress posttreatment with *Mo*Et-OH within C6 cells, even in a dose-dependent manner. *Mo*Et-OH also instigated apoptosis with glioblastoma cells through enhanced nuclear condensation and fragmentation as qualitatively evaluated through Hoechst 33342 staining. The apoptosis within C6 cells post-*Mo*Et-OH treatment was linked with enhanced expressional levels of caspase-9 and caspase-3 proportional to the *Mo*Et-OH concentration. **Conclusion:** Thus, our preliminary study elucidated that *Mo*Et-OH treatment results in antiproliferation within C6 cells by enhancing oxidative stress and instigating apoptosis by initiating nuclear fragmentation.

Key words: Apoptosis, C6 cells, *Moringa oleifera,* oxidative stress

SUMMARY

- *Mo*Et-OH extracts act as an herbal anticancer agent by decreasing the viability in C6 glioblastoma cells.
- *Mo*Et-OH augments ROS levels and nuclear condensation.
- *Mo*Et-OH treatment instigated apoptosis within C6 cells.
- Therefore, *Mo*Et-OH extracts may represent a beneficial therapeutic tool for use as part of a therapy for the treatment of debilitating glioblastoma multiforme.



Abbreviations used: *Mo*Et-OH: Ethanolic Mo leaf extract; GBM: Glioblastoma multiforme; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; C6 cells: rat derived glioblastoma cell line; ROS: reactive oxygen species

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INTRODUCTION

Glioblastoma multiforme (GBM) represents an incapacitating and heterogeneous malignancy of brain. Meager success rates of surgical intervention along with resistance toward radio- and chemotherapeutics delineate the seriousness of exploring novel, potent therapeutical candidates against this dreaded disease.[1] GBM further belongs to Grade IV as per the tumor grading nomenclature of the WHO. Moreover, it is documented as being the most aggressively malignant tumor with the characteristic of reproducing in a short period.[2] The disease's clinical symptoms include recurrent headaches, seizures (relating to the tumor site/s), cognitive decline, and focal neural deficit. Due to its localization, GBM is diagnosed through sophisticated instruments, namely magnetic resonance imaging and computed tomography.[3] As it is well documented that GBM is highly malignant, therefore, securing complete surgical removal of the tumor is still challenging in this era of technological advancements. Indeed, it is reported that patients who underwent surgical resection and also continued temozolomide administration survived only for about an average of 12–15 months.[4]

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Moreover the selective permeability of blood-brain barrier towards drugs, the intrinsic resistance of tumor cells towards apoptosis along with the dependency of affected individuals on others represents few of several challenges that demand attention towards systemic therapies against GBM. Glioblastoma cells derived from rodents (C6) share several histopathological characteristics with human GBM. Furthermore, nuclear polymorphism with concomitant high mitotic index also renders C6 pragmatic for evaluation of novel therapeutical drugs.[5] The increased exploration and use of traditional medicine can be employed explicitly as an alternate source of cancer patients' treatment, to reduce its global burden substantially.[6,7] *Moringa oleifera* (*Mo*) is an endemic tree of India belonging to family Moringaceae, which is also well distributed in Saudi Arabia and Iran and is grown primarily for its medicinal and industrial attributes.[8,9] *Mo* is locally named as drumstick or the "tree of life."[9,10] Exhaustive literature supports the notion that *Mo* has intrinsic medicinal characteristics, but comparatively, its leaves are documented for higher presence of vitamins (A and C), potassium, iron, and calcium.[11,12] In addition, *Mo* leaves of possess rich quantities of bioactive constituents, namely carotenoids, flavonoids, and alkaloids supplemented with enhanced levels of cystine, tryptophan, methionine, and lysine.[13] *Mo* is reported for its use as a traditional medicine in severe disease conditions, including diabetes, hepatic disorders, and cardiovascular disorders.[14-16]

Mo leaf extract is implicated in disrupting cancer cells' proliferation and is reported to augment the amount of glutathione (GSH)-S-transferase within Swiss mice and further instigated apoptosis within cervical cancer cells.[9,17] The authors believe that to date, there is absence of any literature focusing on exploring the anticancerous efficacy of *Mo* leaf ethanolic extracts on glioblastoma cells. Thus, this contemporary study tries to survey the antiproliferative attributes of ethanolic leaf extract of *Mo* against rat glioblastoma C6 cells. The study hypothesized that ethanolic leaf extract of *Mo* would alleviate the survival of glioblastoma cells derived from rodents through instigating apoptosis plausibly via the intrinsic mitochondrial pathway.

MATERIALS AND METHODS

Materials

Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), and antibiotic-antimycotic solution were procured from Gibco, Thermo Fischer Scientific, USA. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and N-Acetyl-L-cysteine (NAC) were supplied by HiMedia, India. Hoechst 33342, caspase-3 and caspase-9 inhibitors, Z-DEVD-FMK and Z-LEHD-FMK, respectively, and 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) were subsequently purchased from Sigma, USA. GSH-Glo™ glutathione assay kit and caspase-3 and caspase-9 kits used were from Promega, USA, and BioVision, USA, respectively.

Plant collection and preparation of *Mo* ethanolic leaf extract (*Mo*Et-OH)

Mo leaves were obtained from botanical garden in Mysore, Bengaluru, India, in the month of September 2020. The plant part was further authenticated by Prof. J. Suresh, JSS College of Pharmacy, Mysore, Bengaluru, India, and submitted in the Department of Pharmacology with reference no: MO-2020/01 for future reference purposes. Leaves were shade dried. *Mo*Et-OH was prepared using the standard protocol. Briefly, 500 mg of *Mo* leaves was soaked allowed to stand (5 days) in 70% ethanol (1500 ml), usually accompanied by random gentle stirring. After that, the solution's insoluble components were manually removed, and postremoval, the resulting mixture was percolated twice using Whatman No. 1 filter papers. It was eventually transferred within the beaker whose empty weight was recorded. The percolated solution was entirely extracted using a soxhlet. Eventually, the *Mo*Et-OH was concentrated to 10 g through a hot plate with a temperature adjusted to 50°C. The *Mo*Et-OH was then aliquoted and kept at 4°C until subsequent use.

Methods

Cell culture maintenance

Rat glioblastoma (C6) cells were obtained from National Center for Cell Sciences, India. The cells were cultivated throughout using DMEM media supplemented with FBS (10% v/v) and antibiotic-antimycotic solution (1% v/v), respectively, in controlled atmosphere at 37°C constituted by CO_2 (5%). The culture flasks were monitored routinely and passaged twice a week after attaining >90% confluency. Before seeding, the count of C6 cells was determined as per the requirements during experiment individually using a hemocytometer along with trypan blue. C6 cells without any treatment served as control.

Assessment of MoEt-OH-mediated toxicity on glioblastoma cells

*Mo*Et-OH-mediated pernicious influences (if any) on rat glioblastoma cells were quantified through MTT assay with slight modifications described earlier.[18] Briefly, 10^4 C6 cells/well were allocated to a 96-well plate and left undisturbed overnight under standard conditions. Subsequently, the wells were supplemented with media consisting of different *Mo*Et-OH concentrations (100, 200, and 400 µg/ml) and further incubated for 24 h. Thereafter, media from every well was decanted, replaced with MTT dye (5 mg/ml; 10 µl), and the plate was left undisturbed under standard conditions for an additional 4 h. Post-incubation, C6 cells (*Mo*Et-OH treated and control) were supplemented with dimethyl sulfoxide (100 µl/well) to solubilize the crystalline formazan, and were left in a darkened area (30 min; room temperature) before recording the absorbance-based readings of treated and control cells at 570 nm using a spectrophotometer (Bio-Rad, USA). The cellular viability among different treated samples was quantified in percent (%) in correlation with the control. It was quantified as $A_t \times 100/A_c$ where A_t = absorbance of dosed groups and A_c = absorbance of control cells.

Estimation of malondialdehyde levels post-MoEt-OH treatment

Malondialdehyde (MDA) serves to be an essential end product of lipid peroxidation which indicates degree of oxidative stress. Therefore, the presence of MDA was quantified in C6 cells posttreatment with varying concentration (100, 200, and 400 µg/ml) of *Mo*Et-OH using the thiobarbituric acid (TBA) reacting substance approach following the protocol described previously.[7,19] Concisely, *Mo*Et-OH (varying concentrations as stated) was added to wells of different formulated groups that were maintained at ambient conditions (24 h). Thereafter, they were mechanically lysed, bypassing the suspension mixed with H_3PO_4 (0.2%; 100 µL) using a needle (25G; repeatedly for at least 25 times). The suspension from different groups was relocated to fresh tubes having additional 200 µl of 2%, 400 µl of 7% H_3PO_4, and 400 µl TBA/butylated hydroxytoluene solution. All samples were heated to 100°C (15 min) with their pH preadjusted to 1.5. The samples were subsequently cooled, added with 1.5 ml of butanol. After that, each reaction mixture was vortexed to separate different phases. Eight hundred microliters was abstracted from the segregated upper phase of butanol and centrifuged (16,000 rpm; 5 min) at 37°C. Finally, sample from different groups (100 µl) was placed in every single well, and their O.D. was recorded at 532 nm (600 nm reference wavelength) through a spectrophotometer (Bio-Rad, USA). Each group's mean absorbance was estimated by dividing the absorbance of respective groups by the absorption coefficient (ε) 153/mM and expressed in µM units.

Quantitative evaluation of glutathione in MoEt-OH-treated C6 cells

GSH levels among different treatment groups were quantified by utilizing a luminescent GSH-Glo™ assay kit using the manufacturer's instruction. Briefly, 10^4 C6 cells were transferred to each well of a 96-well plate, treated with varying *Mo*Et-OH concentrations (as stated above) 24 h under the aforesaid culture environment. Post-incubation, media from treated and control cells (100 µl) was transferred to wells of an opaque 96-well plate. Detection reagent tagged with luciferin was reconstituted and supplemented in respective wells (50 µl), and the plate was incubated at 37°C for about 15 min. After that, the luminescence of different treated groups and control was recorded through Modulus™ luminometer from Turner Biosystems, USA. Standard controls during the assay were prepared (0-100 micromolar) from the stock solution (5 mM) as indicated in the user manual provided and the results were expressed in relative light units (RLU).

Evaluation of MoEt-OH reactive oxygen species within C6 cells

The potency of *Mo*Et-OH in instigating reactive oxygen species (ROS) posttreatment within C6 cells was examined quantitatively as described.[20] Concisely, 1.5×10^4 C6 cells were transferred in black 96-well plate, and were incubated overnight under standard conditions. Post-incubation, the media of different groups including control was reinstated with different *Mo*Et-OH concentrations (100, 200, and 400 µg/ml), and the plate was maintained at standard culture condition for 12 h. Post-incubation, media of respective groups was decanted, and cells were the subjected to 10 µM DCFH-DA for another 30 min in dark at 37°C. DCF-DA-mediated intensity of fluorescence was recorded at excitation/emission wavelength: 485/528 nm using a Synergy H1 hybrid multi-mode microplate reader (BioTek, USA). Alterations in levels of ROS among different groups were described as DCF-DA-mediated fluorescence intensity percentage in juxtaposition with the control.

NAC-mediated inhibitory effects on reactive oxygen species on C6 cells

To establish the effectiveness of *Mo*Et-OH in inducing oxidative stress within glioblastoma C6 cells, N-acetyl-L-cysteine (NAC), a compelling ROS suppressor, was also utilized during this exploratory investigation. 1.5×10^4 C6 cells were placed in a black 96-well plate and subjected to NAC treatment (10 mM) for 2 h under standard conditions. Subsequently, the media was reinstated with media consisting of different *Mo*Et-OH concentrations (100, 200, and 400 µg/ml). Post-incubation, the wells were washed gently using 1X phosphate-buffered saline (PBS) and evaluated for DCF-DA-mediated fluorescence using a microplate reader as mention in the preceding section. In addition, to evaluate the effects of *Mo*Et-OH-instigated intracellular ROS on apoptosis within treated C6 cells, To assess the toxic effects of *Mo*Et-OH on NAC pretreated C6 cells, MTT assay was performed as stated earlie.

Determination of morphological aberrations within nucleus post-MoEt-OH treatment

Aberrations of nuclear morphology within apoptotic C6 cells were visualized using Hoechst 33342 dye as previously described.[21] C6 cells at a density of 5×10^3 cells/well were seeded in a 6-well plate and were allowed to adhere overnight under standard conditions, as stated earlier. C6 cells after that were subjected to various concentrations of *Mo*Et-OH (100, 200, and 400 µg/ml) and incubated under the standard conditions for 24 h. Subsequently, the media was decanted from each well. The wells were gently washed using 1X PBS, treated with 5 µg/ml of fluorescent Hoechst 33342 dye, followed by an additional incubation for 10 min. Eventually, a blue fluorescent channel (Excitation/Emission: 390/446 nm) of the FLoid imaging station (Thermo Fischer Scientific, USA) was employed to visualize and record changes within fluorescent nuclei of different formulated groups and control.

Evaluation of caspase-3 and caspase-9 activities post-MoEt-OH treatment within C6 cells

Activity of specific caspases was evaluated within rat glioblastoma C6 cells using colorimetric-based kits as per the manufacturer's procedure. Primarily, 3×10^6 C6 cells were given *Mo*Et-OH treatment at stated concentrations as per the process and incubation time discussed. Subsequently, the cells belonging to different treatment groups and control were lysed using chilled 50 µl lysis buffer with additional incubation of 10 min on ice. The resulting cell suspension was centrifuged (10,000 × *g*; 4°C for 1 min), followed by collection of the supernatant, and was stored on ice till further proceedings. Subsequently, the supernatant (50 µl) was mixed with 10 mM Dithiothreitol (DTT) (50 µl; reaction buffer). Reaction substrate (50 µl; 4 mM DVD-PNA) was thereupon added to wells, and incubated additionally for 10 min. Post-incubation, the absorbance of different treated groups was documented at 405 nm through a microplate reader (Bio-Rad, USA). Percent (%) change within selected caspase activity was further evaluated by comparing the absorbance of different groups.

Evaluation of caspase-3 and caspase-9 activities in inhibitor pretreated glioblastoma cells

*Mo*Et-OH-instigated cytotoxicity within C6 cells was further delineated through caspase-3 (Z-DEVD-FMK) and caspase-9 (Z-LEHD-FMK) suppressors as per protocol. Briefly, 10^4 C6 cells/well were seeded and postadherence; these were subjected to Z-DEVD-FMK and Z-LEHD-FMK (50 µM; 2 h). Thereafter, pretreated cells were again subjected to Mo*Et*-OH concentrations (100, 200, and 400 µg/ml) and were incubated further for 24 h. Eventually, the viability of C6 cells was assessed through MTT dye, as stated in subsection "Assessment of *Mo*Et-OH-mediated toxicity on glioblastoma cells."

Statistical analysis

The data herewith represent the mean ± standard error of the mean of each experiment executed thrice in triplicates. The comparison between means of different groups and control was ascertained through one-way ANOVA, and subsequently by Dunnett's *post hoc* test. The difference among treatment groups was contemplated to be significant at * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ (*,**,*** represented the level of significance.) through GraphPad Prism (version 5.0) (CA, USA) software.

RESULTS

*Mo*Et-OH extract inhibits proliferation in C6 glioblastoma cells

MTT assay was carried out during our investigations to evaluate cytotoxic effect of *Mo*Et-OH extract on glioblastoma C6 cell proliferation. Treatment with various doses of *Mo*Et-OH extract (100, 200, and 400 µg/ml) post 24 h of incubation elucidated a substantial decrease in live glioblastoma C6 cells, which accounted for 74.23% ±2.94% (100 µg/ml; $P < 0.05$), 35.67% ±1.89% (200 µg/ml; $P < 0.01$), and 30.96% ±1.96% (400 µg/ml; $P < 0.001$) in comparison with control [Figure 1a] in a dose-reliant manner.

*Mo*Et-OH extract-induced reactive oxygen species in glioblastoma C6 cells

ROS is a well-known instigator of oxidative stress within biological systems. Among several mechanisms modulated by ROS, lipid peroxidation was assayed by quantifying the MDA amount, as shown in

Figure 1b. A substantial increase in MDA concentration was found to be 0.5 ± 0.047 μM ($P < 0.05$), 0.124 ± 0.07 μM ($P < 0.01$), and 0.90 ± 0.124 μM ($P < 0.001$) in *Mo*Et-OH-treated C6 cells at concentration of 100, 200, and 400 μg/ml, respectively, as compared to the untreated cells where MDA concentration was 0.016 ± 0.009 μM. Furthermore, GSH levels were found be significantly decreased to $3.166 \times 10^6 \pm 0.072$ ($P < 0.05$), $3.0 \times 10^6 \pm 0.094$ ($P < 0.01$), and $2.533 \times 10^6 \pm 0.054$ RLU ($P < 0.001$) in the *Mo*Et-OH-treated cells in juxtaposition with control [Figure 1c].

*Mo*Et-OH extract enhanced the reactive oxygen species generation in C6 glioblastoma cells

The quantitative estimation of ROS generation was accomplished in *Mo*Et-OH extract-treated C6 cells. As observed, the intracellular level of ROS was enhanced by 25.04% ±5.08% ($P < 0.05$) when correlated with control following treatment with 100 μg/ml of *Mo*Et-OH. Indeed, ROS generation was further increased to 68.68% ±5.62% ($P < 0.01$) and 188.08% ±3.87% ($P < 0.001$) in C6 cells at 200 μg/ml and 400 μg/ml *Mo*Et-OH concentrations, respectively [Figure 2a]. Therefore, these findings pointed out that *Mo*Et-OH enhanced the ROS generation in C6 cells which increased proportionately with the increase in *Mo*Et-OH concentration. Furthermore, to confirm that treatment *Mo*Et-OH extract mediated the ROS generation in glioblastoma C6 cells, quantitative assessment of ROS level in glioblastoma cells was further ascertained using NAC, a well-known ROS inhibitor, followed by *Mo*Et-OH treatment. The results indicated the fact that pretreatment with NAC (10 mM) completely ameliorated the enhanced ROS within the glioblastoma cells ($P < 0.05$), which confirms that *Mo*Et-OH extract treatment increases the production ROS in C6 cells [Figure 2b].

*Mo*Et-OH mediates nuclear condensation in glioblastoma C6 cells

Hoechst 33342 staining was accomplished to qualitatively analyze that the *Mo*Et-OH extract-induced cytotoxicity in C6 cells was due to apoptosis induction. Treatment with various doses of *Mo*Et-OH extract (100, 200, and 400 μg/ml) for 24 h induced significant

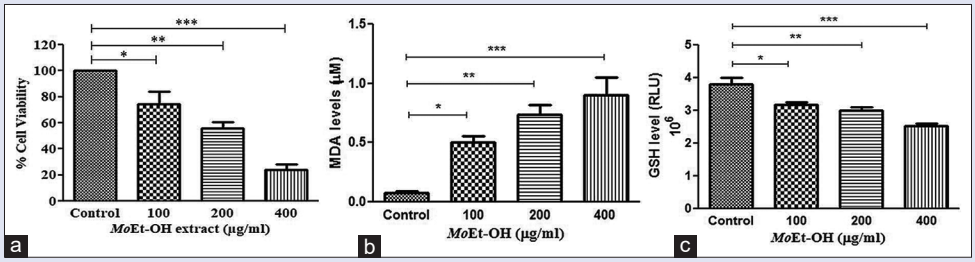

Figure 1: (a) Cell viability percentage of C6 cells subjected to varying concentrations of *Mo*Et-OH and induction of oxidative stress as assessed by (b) malondialdehyde and (c) glutathione levels. Data reported are mean ± standard error of the mean of each experiment executed in triplicate in triplicate. Statistical significance between control and treated groups was analyzed using one-way ANOVA and Dunnett's *post hoc* test where significance was illustrated when *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ through (GraphPad Prism, version 5)

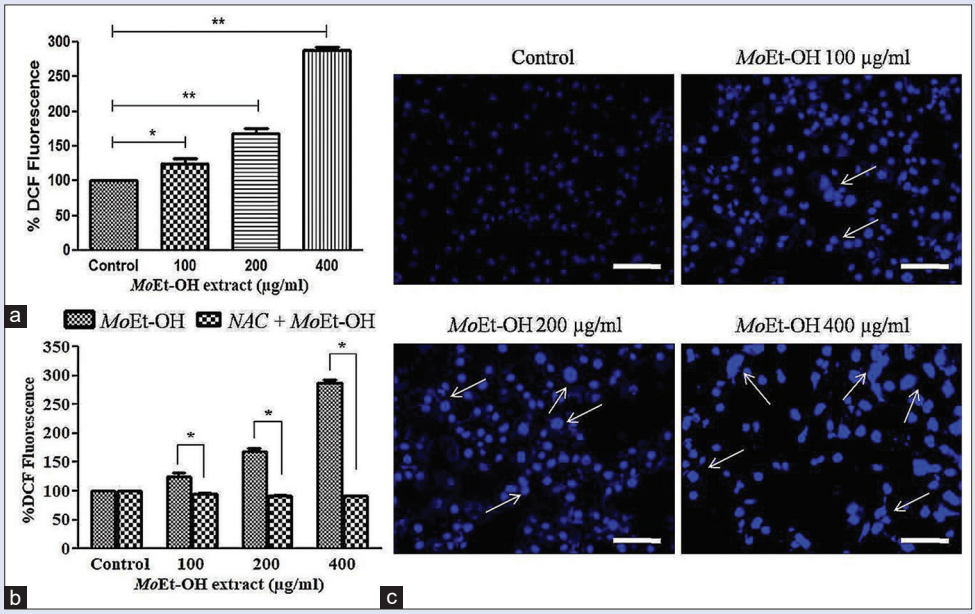

Figure 2: (a) Quantitative estimation of *Mo*Et-OH-mediated DCF-DA fluorescence intensity (%) within C6 cells, (b) level of reactive oxygen species within C6 cells pretreated with NAC and subsequently treated with *Mo*Et-OH (100–400 μg/ml), and (c) fluorescent photomicrograph of C6 cells treated with 100, 200, and 400 μg/ml of *Mo*Et-OH for 24 h followed by subsequent staining with Hoechst 33342 (scale bar = 100 μm). Data reported are mean ± standard error of the mean of each experiment executed in triplicate in triplicate. Statistical significance between control and treated groups was analyzed using one-way ANOVA and Dunnett's *post hoc* test where significance was illustrated when *$P < 0.05$ and **$P < 0.01$ through (GraphPad Prism, version 5)

modifications within the nuclear morphology of C6 cells, as presented in Figure 2c. As observed from the fluorescent micrographs, treatment of *Mo*Et-OH extract induces nuclear fragmentation and condensation in C6 cells. The augmentation within nuclear fragmentation and condensation was also proportionally dependent on *Mo*Et-OH concentration. However, the control cells exhibited unaltered morphology.

Assessment of caspase-3 and caspase-9 activities in *Mo*Et-OH extract-treated glioblastoma C6 cells

Caspases (cysteine proteases) are the critical element in apoptotic pathways. Therefore, we inspected the intracellular activity of caspase-3 and caspase-9 in *Mo*Et-OH extract-treated C6 cells. Our data substantiated a substantial augmentation in the activities of caspase-3 and caspase-9 in *Mo*Et-OH-treated cells when compared with control. As evident, in C6 cells, caspase-3 activity significantly increased by 57.33% ± 2.98%, 89.46% ± 4.86%, and 113.84% ± 5.97% in comparison to control, at the concentration of 100, 200, and 400 µg/ml of *Mo*Et-OH ($P < 0.05$), respectively [Figure 3a]. Furthermore, augmentation in caspase-9 activities was observed to be 35.54% ± 4.44%, 66.73% ± 2.99%, and 102.58 ± 2.61, respectively, in *Mo*Et-OH extract-treated C6 cells ($P < 0.05$). This activity, in turn, was found again to be proportionally dependent on *Mo*Et-OH concentration.

Attenuation of *Mo*Et-OH extract-induced apoptosis in C6 glioblastoma cells by caspase inhibitors

To establish the association between activated caspases and *Mo*Et-OH-instigated apoptosis, cellular viability of C6 cells pretreated (2 h) with Z-DEVD-FMK (50 µM; caspase-3 inhibitor) and Z-LEHD-FMK (50 µM; caspase-9 inhibitor) was estimated. As observed, pretreatment of caspase inhibitors led to a substantial decrease ($P < 0.05$) in *Mo*Et-OH-mediated cytotoxicity within C6 cells [Figure 3b and c]. Therefore, the authors concluded that caspase activation played an important role in *Mo*Et-OH-mediated apoptosis.

DISCUSSION

Mo is a common edible plant occurring in many Asian, Southeast Asian countries and contains various compounds with powerful health benefits including antioxidant and anticancer properties.[22] *Mo* exerts its anticancer potential by modulating numerous signaling pathways, which negatively modulates proliferation and progression of cancer cells.[7] Earlier reports have suggested anticancer efficacy of *Mo*, therefore, we speculated that it holds the potential of further refinement as an effective plausible therapeutic against glioblastoma. In this study, the authors tried to initially explore the effects of *Mo*Et-OH on C6 cells. Our preliminary results demonstrated that *Mo*Et-OH treatment substantially decreases the viability of C6 cells in a dose-reliant fashion, clearly establishing its anticancerous potency against rat glioblastoma cells.

Apoptosis is a crucial physiological process required for homeostatic maintenance and development in multicellular organisms by eliminating tumor cells which further inhibits metastasis of cancers. Impaired apoptosis is considered as a peculiar attribute of carcinogenesis. Induction of apoptosis represents an important therapeutic target for treating cancer.[23,24] The specific attributes of cells undergoing apoptosis include disrupted nuclear and cytoplasmic organization, condensation of chromatin, and the disintegration of the nucleus, resulting in apoptotic bodies' formation.[25] Hoechst 33342 staining results further indicated that *Mo*Et-OH was competent in instigating programmed cell death or apoptosis. The attribute of cell death was clearly evident through nuclear fragmentation and condensation during visualization of *Mo*Et-OH-treated C6 cells.

Chronic inflammation characterized by enhanced ROS levels is documented as a well-known trigger for the onset of several diseases.[26] They damage the phospholipids present within the cell membrane resulting in lipid peroxidation.[27] This report further demonstrated that *Mo*Et-OH significantly enhanced the peroxidation of lipids. The lipid peroxidation would undoubtedly obstruct the cell membranes and their function. Furthermore, increased lipid peroxidation could disrupt the mitochondrial membrane resulting in disruption of phosphorylation which further causes electrons to escape the respiratory chain. Furthermore, oxidants also compromise the cellular viability by reacting with proteins and DNA components.[28] Hydrogen peroxide (H_2O_2) oxidizes cysteine in GSH which ultimately produces glutathione disulfide, thereby lowering the antioxidant efficacy of GSH. Our results suggested that GSH levels reduced substantially within *Mo*Et-OH-treated C6 cells with a concomitant escalation in lipid peroxidation. Enhanced ROS generation within *Mo*Et-OH pretreated C6 cells was also seen which could be correlated with the mitochondrial apoptotic pathway. However, pretreatment with NAC attenuated the ROS generation after *Mo*Et-OH treatment in C6 cells, which strengthens our observation that *Mo*Et-OH increased ROS generation within C6 cells. Furthermore, pretreatment of NAC negatively modulated substantially the levels of cytotoxicity induced by *Mo*Et-OH without NAC, making it evident that enhanced ROS was critical in regulating *Mo*Et-OH-mediated apoptosis.

Earlier findings have substantiated that caspases are cysteine proteases and considered a critical apoptotic component.[29] Among several other initiator proteases of apoptotic pathway regulated via mitochondria, caspae-9 exerts its effect at multiprotein activation platforms and caspase-3 is a crucial regulator of apoptosis. It mediates the breakdown of several

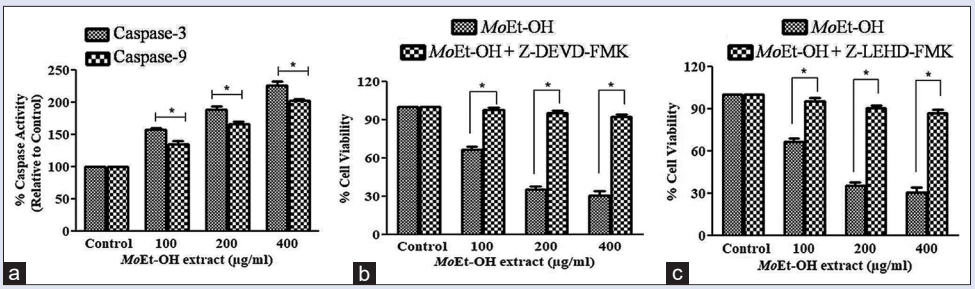

Figure 3: *Mo*Et-OH-instigated activation of (a) caspase-9 and caspase-3 posttreatment at varying concentrations of 100, 200, and 400 µg/ml in C6 cells and cell viability (%) of C6 cells pretreated with (b) caspase-9 and (c) caspase-3 inhibitors. Data reported are mean ± standard error of the mean of each experiment executed thrice in triplicate. Statistical significance between control and treated groups was analyzed using one-way ANOVA and Dunnett's *post hoc* test where significance was illustrated when *$P < 0.05$ through (GraphPad Prism, version 5)

cellular proteins.[30] Dose-dependent activation of caspases (9 and 3) suggested that treatment *Mo*Et-OH initiates mitochondrial-mediated apoptosis in glioblastoma C6 cells. Moreover, *Mo*Et-OH-induced cytotoxicity in C6 cells was considerably decreased by caspase inhibitors such as Z-LEHD-FMK, and Z-DEVD-FMK, suggesting a pivotal role of caspase-9 and caspase-3 activation during *Mo*Et-OH-induced apoptosis. Thus, it is oblivious to say that *Mo*Et-OH might instigate apoptosis within C6 cells via caspase-dependent pathways. In sum, these evidences suggested that the *Mo*Et-OH extracts could suppress the growth and instigate mitochondrial-mediated apoptosis in glioblastoma cells. Furthermore, our results add to the growing evidence supporting the promising role of *Mo* as an anticancer drug agent and open a new window for studying molecular mechanistic action of *Mo*Et-OH extracts on the role of predominant signaling pathways in the development and progression of cancer.

CONCLUSION

Our preliminary study showed that the *Mo*Et-OH extracts act as an herbal anticancerous agent by decreasing the viability in C6 glioblastoma cells with concomitant escalation of ROS levels and nuclear condensation which eventually resulted in apoptosis of C6 cells. Thus, *Mo*Et-OH extracts may represent a beneficial therapeutic tool for use as part of a therapy for the treatment of debilitating GBM. Although the results of this study appear to be encouraging, exhaustive studies in animal models of GBM are further warranted.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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