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**Abstract**

A drimane type sesquiterpenoids, sulphureuine B was isolated from the edible mushroom *Laetiporus sulphureus* and its antiproliferative properties were investigated using U-87MG glioma cells. It was observed that sulphureuine B-induced apoptosis in U-87MG cells and the mechanisms involved are endoplasmic reticulum stress, mitochondrial and death receptor mediated pathways. Endoplasmic reticulum stress was identified from the results of enormous cytoplasmic vacuolation, CHOP elevation and caspase-12 cleavage. Further, we found that treatment of sulphureuine B-induced PERK, IRE1α, and ATF6α activations. In addition, sulphureuine B-induced Bcl-2 down-regulation, cleavage of PARP, and caspase-8 activation were also affected. All these experimental results clearly revealed that sulphureuine B-induced apoptosis mediated through endoplasmic reticulum stress, mitochondrial, and death receptor signaling pathways.

**Introduction**

Malignant gliomas are the major life-threatening primary brain tumor with the average survival of less than a year due to the ineffectiveness of radiation and other medical care (Legler et al., 1999). Glioblastomas existing as a spread over to wide area with invasion into typical brain but customarily repeated or developed after radiation as focal point (Garden et al., 1991), indicating that only a small proportion of tumor cells are accountable for regrowth. Even though there occurs the availability of treatment such as combination of surgery, radiation, chemotherapeutics for malignant glioblastomas, still these are considered as locally protruding tumors suffered with poor prediction. A survey from European Organization for Research and Treatment of Cancer (Stupp et al., 2005) displayed that the total viability was increased two more months with associated adjuvant radiation therapy with temozolomide chased by associated temozolomide when compared with the radiation treatment alone. Glioblastomas are very harmful neoplasms which can manifest clear angiogenesis with increased vascular endothelial growth factor expressions which further assist blood vessel production using precursors of endothelium (Plate et al., 1992, Plate et al., 1994). Also it is proved that in the glioblastoma medical care, either surgical treatment followed by radiation treatment or the surgery followed by chemotherapy yields a mean survival of less than 12 months (Fine et al., 1993). Earlier immunotherapy treatments for glioma tumor have concentrated on submissive and non-specific strategies and resulted uncertain benefits (Zeltzer et al., 1999).

Sesquiterpenoids are compounds possessing 15-carbons and can be obtained biosynthetically from 5-carbon isoprene units or can be industrially synthesized using feed stocks of monoterprenoids. These compounds attracted industrial attention due to their beneficial to the characteristic flavors and smell of spices, flowers,
and herbs (Bauer et al., 1997). Bioactive metabolites are found to be rich in mushroom sources. For example, lanostanoid triterpenes were isolated from *Laetiporus sulphureus* by Francisco and his group studied apoptosis induction in human myeloid leukemia cells, HL-60 (Francisco et al., 2004). *L. sulphureus*, is an edible mushroom and it belongs to *Polyporaceae* family. It is extensively spread in North America, Asia and Russia (Petrović et al., 2013). *L. sulphureus* is a member of *Aphyllophorales* fungi class and is very often distributed on conifers and hardwoods. It has been used in Asian folk medicine for long time (Zjawiony, 2004). Mainly, the *L. sulphureus* higher fungus is familiar for its enormous chemical components which includes triterpenes, sesquiterpenoids, alkaloids, and also well known for its biodiversity (Shiono et al., 2005).

Rapior et al narrated that the *L. sulphureus* has more or less pleasant odor (Rapior et al., 2000). The wide availability of fungi and their easy attainment make them more popular among the natural products community evaluation program (Berdy, 2005). The concentration towards *L. sulphureus* was brought into attention due to the eburicoic acid biosynthesis in 1960’s and there obtained 30% of *L. sulphureus* dry weight from this triterpenoid acid (Goad et al., 1966). Recent studies showed that natural products acquired biological activity and it contains enzymes which are specific to catalyze their biosynthesis (Li and Siehr, 1980; Alquini et al., 2004; Kobayashi and Kim, 2003). Recent reports also showed that sesquiterpenoids possessed antitumor activities (Weber et al., 2004; Tipton et al., 2003; El Ashry et al., 2003) but their molecular mechanism towards the actions remain unclear.

The protein folding and its activity were regulated by multifunctional organ called endoplasmic reticulum. This endoplasmic reticulum appeared as a most important site of regulating the cell homeostasis and especially in controlling unfolded protein which is established to play a crucial role in cancer and other contagious diseases. The insufficient glucose contribution may affect the secretory protein pathway glycosylation as well as the production of ATP’s. These two factors resulted in the stockpile of unfolded proteins in endoplasmic reticulum and lead to cause endoplasmic reticulum stress (ER stress). The functions of modification in the post translation, accumulation of newly synthesized proteins, folding, and calcium storage regulation was operated and maintained by endoplasmic reticulum (Shoemaker et al., 2005). These are extremely important functions for the survival of cells such that their disorder can lead to cell damage, ER stress and finally apoptosis. Various pathways are activated by ER stress to eradicate the cell damage and these pathways are known as unfolded protein response (UPR). This UPR signal possesses dual role of weakening the damage related with ER stress or upon cell death induction through apoptosis (Ellgaard and Helenius, 2003; Wang et al., 2014). ER stress gained more attention towards molecular pathway and this can be modified to produce cytotoxicity in glioma and that can be a feasible aim to introduce new chemotherapeutic drugs to treat glioma cancer (Chakrabarti et al., 2011). However, there is no report mentioning about the mechanism of sulphureuine B-induced cell death in glioma cancer cells and this study was carried out to investigate the antiproliferative action of sulphureuine B and its mechanism of cell death in U-87 MG glioma cell lines. The comprehensive mechanisms, if explained, can help as to how the sulphureuine B can serve as a powerful drug candidate for future cancer treatment.

**Materials and Methods**

**Extraction and isolation of sulphureuine B**

Sulphureuine B (Figure 1) was extracted from the fruiting elements of *L. sulphureus*. Sulphureuine B was further isolated and purified according to the literature method (He et al., 2015). *L. sulphureus* fruiting bodies were collected at Fenyi, Jiangxi Province, China. Culture medium contains glucose (5%), pork peptone (0.15%), yeast (0.5%), KH₂PO₄(0.05%), and MgSO₄ (0.05 %). The initial pH was adjusted to 6.0, and then carried out fermentation using Erlenmeyer flask for one week till the mycelium biomass reached to the maximum. Later it was transferred to a fermentation tank (20 L) at 24°C, 250 rpm for 20 days, and ventilation was set to 1.0 air volume/culture volume/minute. The culture broth (20 L) was concentrated under vacuum and extracted with ethylacetate. The organic layer was evaporated in vacuum with crude yield of 5.8 g, which was separated by sephadex LH-20 (methanol) to get its fractions. Later it was subjected to a silica gel column and eluted with petroleum ether : acetone (1:1) to give subfractions (200 mg). It was then further purified by sephadex LH-20 (methanol) and silica gel column (petroleum ether : acetone, from 30:1 to 10:1) yield sulphureuine (2.5 mg) as colorless oil. ESI-MS m/z 272 [M]+; HR-EIMS m/z 272.1984 [M]+ (calcd for C₁₃H₂₅O₄, 272.1988). The structure of sulphureuine B was identified by the physicochemical methods and spectral data with 98% purity.

**Materials**

The MTT reagent 3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide), DMSO, Hoechst 33258,
rhodamine 123 and all the solvents and reagents used for biological experiments were obtained from Sigma-Aldrich Company (USA). The antibodies against Bax, Bcl-2, caspases 3, 8, 9 and 12, ATF-6α, FADD, fas, β-actin, Thr 981 (p-PERK), PARP, Bcl-XL, BID, CHOP, GRP 78, Ser 51 (p-eIF 2α), IRE 1α, XBP 1sand HRP conjugated antibodies were obtained from Santa Cruz Biotechnology (USA). Z-VAD/FMK and ECL were purchased from Cell signaling technology (Cell Signaling Technology, USA).

Cell lines and culture

U-87MG glioma cells were obtained from American Type Culture Collection (USA). U-87MG cells were cultured using Dulbecco’s modified Eagle’s medium (GIBCO, USA) supplemented with fetal calf-serum (10%), streptomycin (100 µg/mL), penicillin (100 IU/mL) and L-glutamine (0.02%) and maintained at 37°C with 5% CO₂ in a humidified atmosphere.

Cytotoxicity

The MTT assay was carried out to study inhibitory action of sulphureuine B on U-87 MG and its cell viability. The cell lines at a density of 5 x 10⁴ cells/well were seeded on 96 well microplates and kept for incubation for about 24 hours. After one day incubation, these cells were treated with various drug concentrations and their cell growth was measured. Cell viability was expressed as absorbance ratio of treatment to control and their absorbance was recorded at 540 nm using V-730 UV-visible spectrophotometer (Ascaso, USA).

Hoechst and rhodamine staining

The cells were stained with Hoechst 33258 and/or rhodamine 123 dye. For the Hoechst dye staining, cells were resuspended at 106 cells/mL and stained with the dye at a concentration of 5 mg/mL for 90 min at 37°C. After the incubation, cells were kept in serum media at 48°C to prohibit leakage of Hoechst dye from the cells. For the rhodamine 123 staining, 106 cells/mL were exposed to 0.1 mg/mL of rhodamine 123 dye for 20 min at 37°C in the dark. The cells were then washed and resuspended with the serum-enriched media and kept on ice. For combined staining, the same method was used as described for the Hoechst staining, but rhodamine 123 was added during the last 20 min of the incubation. Propidium iodide (2 mg/mL) was added to the final suspension before the flow cytometric sorting to exclude dead cells from the flow cytometric profile. The fluorescence microscopy was used to determine the cell morphology (Leica Microsystems, Germany).

Dual staining annexin-V/PI assay

The apoptosis participation in sulphureuine B-induced cell death was investigated using dual staining annexin-V/propidium iodide assay. Cells (1 x 10⁴) were washed and resuspended with PBS. Apoptotic cells were identified by double supravital staining with recombinant FITC (fluorescein isothiocyanate)-conjugated annexin-V and propidium iodide, using the annexin-V-fluos staining kit according to the manufacturer’s instructions. Flow cytometric analysis was performed immediately after supravital staining. Data acquisition and analysis were performed in a Becton Dickinson FACSCalibur flow cytometer using CellQuest software. Before sulphureuine B treatment with U-87MG cells, 2 µM of Z-VAD-FMK pan caspase inhibitor was also added.

Mitochondrial membrane potential measurement

In this experiment, rhodamine 123, a membrane permeable fluorescent cationic dye was used to determine the measurement of mitochondrial membrane potential. In brief, cells were treated with sulphureuine B and incubated with rhodamine 123 for about 30 min at 37°C in dark. It is then harvested and resuspended in PBS (200 µL) and subsequently measured using attune NxT acoustic focusing cytometer (Life Technologies, New York, USA).

Western blot

The U-87MG cells were first harvested and washed twice using PBS in cold. Later it was lysed in whole cell lysis buffer which are protease inhibitors supplied for 1 hour at 4°C and centrifuged. After centrifugation, Pierce BCA protein assay kit (Life Technologies, New York, USA) was used to measure the concentration of proteins. The equal content of total protein was separated using 12% SDS PAGE and then transferred onto the PVDF membrane (Life Technologies, New York, USA). At room temperature, skimmed milk of 5% was used to block the membranes for about 1 hour and then incubated with primary antibodies overnight at 4°C and HRP-conjugated secondary antibody for 2 hours at room temperature and then finally visualized by ECL substrates.

Transmission electron microscope (TEM) studies

This study was carried out as reported in the previous literature methods (Cheng et al., 2009). U-87MG cells were treated with 50 µM sulphureuine B for the indicated time periods. The collected cells were fixed with NaCl / Pi containing 3% glutaraldehyde, postfixed with NaCl / Pi containing 1% OsO₄. The samples were dehydrated in graded alcohol, embedded and sectioned. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined using a JEM-1200 transmission electron microscope.

si-RNA transfection

In this experiment, cells were first transfected with si-CHOP/si-NC at a final concentration of 25 nM using lipofectamine reagent according to the manufacturer’s guidelines. The transfected cells were used for subse-
sequent experiments 24 hours later. si-NC (si RNA’s) act as a negative control. After 24 hours, the transfected cells were utilized for further experiments.

Statistical analysis
Statistical analyses were performed using Student’s t-test. All the represented data were performed in at least three independent measurements. All the data are expressed as mean ± SD and p<0.05 is considered as statistically significant.

Results
Anti-proliferation and apoptosis inducement against U-87MG cells

MTT assay was used to determine the cell viability by treating U-87MG cells with various concentrations of sulphureuine B (5, 10, 25, 50, and 100 µM) for 24, 36 and 48 hours. Time-dependent and dose-dependent responses were identified from MTT results with an IC50 value of 16 µM at 24 hours (Figure 2).

To further investigate the reducing cell survival induced by sulphureuine B, we examined morphological changes in the cells using phase contrast microscopy method. Membrane swelling and apoptosis morphological changes were identified while treating the sulphureuine B when compared to that of control group (Figure 3; upper row). These observations were further tested with Hoechst staining.

The regular distribution of low dense fluorescence was observed in the control whereas condensed, bright fluorescence and fragmentation was observed in the case of sulphureuine B treated cells (Figure 3; middle row). Sulphureuine B-treated U-87MG cells showed the apoptotic characteristics such as condensation, vacuolation of cytoplasm and nuclear fragmentation (Figure 3; lower row). All these observations support for the apoptosis inducement of sulphureuine B in U-87MG cells.

Participation of caspase activation in apoptosis

Figure 4a showed the PARP cleavage was induced by sulphureuine B, indicating the activation of caspase which is considered as notable characteristics for apoptosis. In addition, we further studied the caspase activation was induced by sulphureuine B and we used western blot analysis to detect the caspases-3, -8 and -9.

Results depicted in Figure 4A clearly disclosed the procaspase-3, -8 and -9 decrease and significant elevation in the cleaved caspase-3, caspase-8 and caspase-9, suggesting the caspase activation. To find out whether the cell death induced by sulphureuine B was caspase dependent, we pre-treated U-87MG cells with 5 µM pan caspase inhibitor, Z-VAD-FMK before treatment with sulphureuine B. Results indicated that treatment of Z-VAD-FMK suppressed sulphureuine B induced cell death. We observed the cell death induced by

![Figure 2: Inhibitory effect of sulphureuine B against U-87MG cells with various concentrations of 5, 10, 25, 50, and 100 µM for 24, 36 and 48 hours](image)

![Figure 3: U-87MG cells treated with 20 µM of sulphureuine B using phase contrast microscopy (upper row); Hoechst staining results by the treatment of U-87MG cells with 20 µM of sulphureuine B (middle row); TEM ultrastructure results upon treating cells alone or with 20 µM sulphureuine B (lower row)](image)
Figure 4: A) Western blot analysis observed upon treating U-87MG cells with 20 µM sulphureuine B for 0, 6, 12 and 24 hours using β-actin as control; B) MTT results upon treatment of U-87MG cells with 20 µM sulphureuine B with or without Z-VAD-FMK (5 µM) and compared with control of sulphureuine B alone; C) Flow cytometry results upon treatment of U-87MG cells with 20 µM sulphureuine B with or without Z-VAD-FMK (5 µM) and compared with control of sulphureuine B alone.
sulphureuine B was reduced from the level of 45.3 ± 0.3% to 24.6 ± 2.8% when it underwent with Z-VAD-FMK co-incubation (Figure 4B). Further, annexin-V/PI flow cytometry results revealed that treatment of Z-VAD-FMK reduced that apoptosis cell population upon sulphureuine B induction. It was also identified that the late apoptosis ratio was decreased from the level of 26.8 ± 1.0% to 9.7 ± 0.6% upon z-VAD-FMK co-treatment (Figure 4C). These results clearly indicated the partial involvement of caspase activation in the sulphureuine B induced apoptosis.

**Apoptosis in intrinsic and extrinsic pathways**

Caspase-9 and -8 activations indicated that sulphureuine B induced apoptosis may be proceeded through mitochondrial-mediated intrinsic pathway and death receptor mediated extrinsic pathway. The mitochondrial membrane potential was damaged by the ratio of Bcl-2/Bax protein down regulation. Hence, we performed rhodamine 123 staining to determine the mitochondrial membranes integrity of the cells by using flow cytometry analysis and the fluorescence microscopy. Results showed that treatment of sulphureuine B leads to MMP decrease in a time dependent manner as given in Figure 5A and 5B. Western blot analysis detected many proteins such as Bax, Bcl-2, Bcl-xL of Bcl-2 family and fas, FADD of death receptor related proteins. The results indicated that Bax expression was significantly increased whereas Bcl-2 and Bcl-xL protein levels are found to be decreased (Figure 5C). Also, Fas & FADD protein expression levels are also increased. In addition, we found that treatment of sulphureuines B induced the Bid cleavage, which is a caspase 8 downstream protein (Figure 5D). These resulted proved that sulphhu-reuine B stimulated apoptosis is mediated through both extrinsic and intrinsic pathways.

**Endoplasmic reticulum stress and unfolded protein response**

Enormous cytoplasmic vacuolation was identified in U-87MG cells upon treatment with sulphureuine B for about 24 hours (Figure 6A). A normal phenotype was observed with the control group cells. On comparison with control group, sulphureuine B treated cells showed the apoptotic characteristics such as cytoplasmic vacuolation, condensation and nuclear fragmentation. Prolonged treatment with sulphureuine B leads to the formation of hypervacuolation (Figure 6B). Further, the cytoplasmic vacuolation induction by sulphureuine B was not due to autophagy due to the non-inducement of autophagosome formation by sulphureuine B.

It is possible that sulphureuine B induced endoplasmic reticulum changes in morphology may have parallel effect with ER stress and its malfunction. We determined whether the sulphureuine B induces ER stress via the detection of caspase-12, GRP78, and CHOP expression levels. As shown in Figure 6C, results showed that upon treatment with sulphureuine B, the caspase-12 activation was observed along with the up-regulation of GRP78 and CHOP expression levels in time dependent response. ER stress can cause to happen UPR via the activation of PERK, ATF6, and IRE1 signaling proteins and hence we studied the sulphureuine B effects on 3 pathway signaling of UPR using immune-blotting experiment. The results showed that upon treatment with sulphureuine B, auto-phosphorylation of PERK was identified using Thr981 and also eIF2α phosphorylation was also identified for 6 hours but its corresponding phosphorylated protein level was slightly reduced after 6 hours, which is due to eIF2α dephosphorylation caused by apoptosis elevation. Upon sulphureuine B treatment, ATFTα was found to be readily decreased from 6 hours to 24 hours, evidencing the protein cleavage into the activated form. UPR signal activation was observed from the IRE1α up-regulation and XBP1 activation (Figure 6D). Mitochondrial and death receptor signaling pathways are regulated by the pro apoptotic CHOP protein molecule. In order to examine its involvement in the intrinsic and extrinsic pathways, we studied the caspase-8 and bcl-2 expressions using siRNA as control and CHOP siRNA transfection studies. Results indicated that there is a decrease in CHOP by using the particular CHOP siRNA. Also, CHOP expression decrease further reduced the activation of caspase-8 and regained the expression of Bcl-2. CHOP inhibition also blocked PARP cleavage induced by sulphureuine B (Figure 6E). All these results clearly revealed the significant importance of CHOP in sulphureuine B-induced apoptosis.

**Discussion**

Terpenoids are distributed word wide and it belongs to wide class of secondary metabolites of lipophilic characteristics and derived from iso-pentyl pyrophosphate (Harborne and Toma’s-Barbera’n 1991; Harrewijn et al., 2001). The enormous variety of terpenoids exists in plants, and they can produce main constituents of essential oil from the extracts of plants. An early report demonstrated that *L. sulphureus* extracts contains large amount of hydrocarbons say about approximately 80% and 43.1% of acetone & dichloromethane and methanol extracts, respectively. Among them, nonane, n-decane and 4-methyl nonane were found to be in more percentages. Wu et al identified 40 main volatile components and quantified (Wu et al., 2005). It is proved that sesquiterpenoids such as α and β tumerones, curlone, are the turmeric oil constituents possessing antioxidative and mosquitocidal properties (Jayaprakasha et al., 2002; Roth et al., 1998). Production of E2 and NO prostaglandin was also inhibited utilizing these components (Hong et al., 2002; Lee et al., 2002). In recent years, natural product substrates provides an effective sources for anti-cancer treatment (Pan et al.,
Sulphureuine B was isolated from the natural compound of *L. sulphureus*. Current study deals with inhibitory effects of sulphureuine B towards the glioma U-87MG cells for the first time as a dose and dependent responses. The sulphureuine B induced apoptosis was also elaborately studied. The mechanism involved in its action was also described as via ER stress, mitochondrial, and death-receptor pathways. The ER stress in cancer cells involvement is found to be in enormous amount in the anticancer therapies and the reports demonstrated that autophagy and apoptosis in glioma cancer cells was induced by ER stress (Shen et al., 2014). Also, reports showed that apoptosis observed in glioma cells was normally concentrated on ROS generation, cell cycle arrest and the mitochondrial mediated pathway (Chang et al., 2004, Sun et al., 2009, Lv et al., 2013). From our results, we observed that eIF2α & PERK phosphorylation, IRE1α up-regulation, ATF6α cleavage, caspase 12 cleavage, CHOP & GRP78 expression and XBP1s expression was induced by sulphureuine B. All these results evidenced for the sulphureuine B induced apoptosis mediated through endoplasmic reticulum stress.

Earlier reports predicted that mitochondrial and death-receptor pathways are integrated with endoplasmic reticulum induced apoptosis (Zhu et al., 2011). From the results obtained in our studies, we identified Bcl-2/Bax ratio & Bcl-xl suppression, caspases-8 & 9 activation, Bid cleavage, mitochondrial potential membrane loss, Fas & FADD proteins upstream which are the major

Figure 5: A) Rhodamine 123 assay results upon treating U-87MG cells with 20 µM sulphureuine B for 24 hours; B) Mitochondrial membrane potential results upon treating U-87MG cells with 20 µM sulphureuine B for about 6, 12 and 24 hours stained by Rhodamine 123; C) Western blot results upon treating U-87MG cells with 20 µM sulphureuine B to detect Bax/Bcl-2 ratio and Bcl-xl levels using β-actin as control; D) Immunoblotting results upon treating U-87MG cells with 20 µM sulphureuine B to detect Fas and FADD using β-actin as control.
Figure 6: A) Cytoplasmic vacuolation results upon treating U-87MG cells with 20 µM sulphureuine B for 24 hours; B) TEM ultrastructure results upon treating U-87MG cells with 20 µM sulphureuine B for 0, 6, 12 and 24 hours; C) Western blot results upon treating U-87MG cells with 20 µM sulphureuine B to detect GRP78, CHOP, and caspase-12 for 0, 6, 12 and 24 hours; D) Immunoblotting results upon treating U-87MG cells with 20 µM sulphureuine B to detect p-PERK, ATF6α, pEIF2α, IRE1α and XBP1s, Fas & FADD using β-actin as control; E) si-RNA transfection results upon transfecting U-87MG cells with si-RNA as negative control or with CHOP si-RNA and lysed after treated with 20 µM sulphureuine B to detect the CHOP, caspase 8, PARP, Bcl-2 expression levels using western blot analysis. β-actin was used as control.
characteristic features for the function of mitochondrial and death-receptor pathways. Si-RNA transfection results came out with CHOP expressions. Further, it inhibits PARP cleavage and activates caspase-8. These results disclosed the vital role of CHOP in endoplasmic reticulum stress stimulated apoptosis on sulphureuine-B treated U-87MG cells.

**Conclusion**

Sulphureuine B obtained from *L. sulphureus* can function as a favorable antiproliferative agent which are potent to inhibit the U-87MG glioma cells operating via endoplasmic reticulum stress, mitochondrial and death receptor signaling pathways. Our findings supports for utilizing sulphureuine B as a powerful anticancer drug for the treatment of malignant glioblastoma.

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**Conflict of Interest**

Authors declare no conflict of interest

**References**


