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Regular paper

Anticancer activity of Germacrone terpenoid in human osteosarcoma cells is mediated *via* autophagy induction, cell cycle disruption, downregulating the cell cycle regulatory protein expressions and cell migration inhibition

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Germacrone a sesquiterpene is a potential pharmacological agent with important medicinal applications. It is a potential anticancer agent and has been reported for anticancer activity against hepatoma cells and breast cancer cells, additionally, it has also shown anti-inflammatory, antioxidant, and antifungal activity. Therefore, this study was designed to testify anticancer activity of germacrone terpenoid in human osteosarcoma cells along with studying its effects of autophagy induction, cell cycle disruption, downregulating the cell cycle regulatory protein expressions and cell migration inhibition. Cell proliferation rate was examined by MTT assay and phase contrast inverted microscopy was performed for morphological analysis. Further, flowcytometry was implemented to examine different cell cycle phases. Transwell assay was executed for the monitoring of cell migratory tendency of osteosarcoma cells. Finally, the levels of pro-autophagic and cell cycle allied proteins were checked by Western blot analysis. MTT assay results designated potential inhibition of osteosarcoma cell viability by germacrone drug in a dose and time-reliant manner. Further, phase contrast inverted microscopy depicted significant morphological changes in osteosarcoma cells after germacrone exposure, which were indicative of autophagic cell death. Next, transmission electron microscopy evaluated the formation of autophagic vesicles which are the trademark for autophagy. The autophagy allied protein expressions were observed through Western blotting indicating enhanced levels of pro-autophagic proteins (Becalin-1, LC3-I and –II). Hence, it may be depicted that the anti-proliferation effects of germacrone may be of autophagy inducing potential. Next, flowcytometric analysis revealed the cell cycle inhibitory effects of germacrone in osteosarcoma cells and the results indicated cell cycle arrest at S-phase. Cell cycle allied protein levels indicated declination in their expressions after germacrone exposure. Finally, transwell assay specified inhibitory effects on cell migration of osteosarcoma cells by germacrone in a dose-reliant manner. In conclusion, the results of the present investigation specified that germacrone drug is a potential anticancer agent against osteosarcoma cells. The anticancer effects were found to be mediated *via* autophagy induction, cell cycle disruption, downregulating the cell cycle regulatory protein expressions, and cell migration inhibition.

Key words: osteosarcoma, germacrone, autophagy, cell cycle

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Abbreviations: BCA, Bicinchoninic acid; FBS, Fetal Bovine Serum; ROS, reactive oxygen species; PBS, Phosphate-buffered saline

INTRODUCTION

In Asian countries including China and India, Zingiberaceae – a curcuma species, are among commonly used herbal medicines (Bamba *et al.*, 2011). Rhizoma curcuma is an important component of traditional Chinese medicine prescribed for the treatment of liver disorders and inflammation (Rodrigo *et al.*, 2010). It has been recorded with *in-vitro* anticancer activity against breast cancer through suppression of metastasis, angiogenesis, and cell proliferation (Chen *et al.*, 2021). Different types of phlegmonosis have been cured by the application of R. curcuma extracts (mainly volatile soil product) (Jiang *et al.*, 2010). It has revealed several pharmacological actions including antioxidant, anti-inflammation, as well as in the treatment of hepatitis (Radulovic *et al.*, 2010; Rozenblat *et al.*, 2008). Germacrone – a sesquiterpene, is present in high concentration in the volatile soil product of R. curcuma extract. Germacrone is a biologically as well as pharmacologically active compound and is used to treat several human malignancies. It exhibits antifungal, anti-inflammatory, depressant, vasodilator, antitussive, choleretic, hepato-protective, and anti-cancer potential (Cho *et al.*, 2009; Xiao *et al.*, 2002). Studies have shown protective effect of germacrone on liver injury in mice through tumor necrosis factor-α and lipopolysaccharide/D-galactosamine (Matsuda *et al.*, 1998; Morikawa *et al.*, 2002). Further, it has been reported with apoptosis inducing potency in Be17402 and HepG2 hepatoma cell lines with alterations in BAX and BCL-2 protein expressions (Zhang *et al.*, 2020). The levels of p53 were also upregulated along with enhancing ROS (reactive oxygen species). Germacrone induced apoptosis in breast cancer cells via stimulation of Caspase-dependent mitochondria-mediated apoptosis and cell cycle arrest (Liu *et al.*, 2013; Zhong *et al.*, 2011). Osteosarcoma is a lethal bone malignancy effecting a huge population worldwide (Tebbi & Gaeta, 1988; Misaghi *et al.*, 2018) Its frequency of happenings is increasing yearly at an alarming rate. Cisplatin, cyclophosphamide, and methotrexate are widely used chemotherapeutics against osteosarcoma (Ritter & Bielack, 2010; Benassi *et al.*, 1999). Poor diagnosis and lower survival rates are the major hurdles in osteosarcoma management. However, the accumulated drug resistance and lethal side effects (which causes damage to the normal tissues) increases the lethality of osteosarcoma management (Riddell *et al.*, 2016; Moding *et al.*, 2013).

Therefore, this study was undertaken to investigate anticancer activity of germacrone terpenoid in human osteosarcoma cells, along with investigating the autophagy inducing, cell cycle disruptive, downregulating the cell cycle regulatory protein expressions and cell migration inhibition effects of germacrone.

MATERIALS AND METHODS

Cell viability detection

MTT cell viability assay was performed to assess the cell viability potency of osteosarcoma cells (Saos-2). In brief, cells were seeded at a density of 3×10^3 cells/well in a 96-well plate by incubating at 37°C in CO_2 (5%) humidified incubator. Afterwards, cells were left untouched overnight for settlement. Settled cells were then treated with germacrone molecule at changing doses viz 0, 5, 25, 50 and 150 µM for 24 h and 48 h. Thereafter, MTT stock solution of 10 ml with a concentration of 5 mg/ml were supplied to each well and incubated in dark for 2h at 37°C. Finally, for optical density determination, absorbance was measured at 492 nm using the Automated Microplate Reader (Sunrise, Tecan, Switzerland).

Phase contrast inverted microscopy

Inverted microscopy was executed to monitor cellular morphology of germacrone molecule treated osteosarcoma Saos-2 cells. Cells were seeded into 6-well plates (cultural plates) and treated with changing germacrone drug doses viz 0, 5, 50, and 150 µM. Treated cells were incubated for 24 h followed by monitoring of morphological modifications with a phase contrast inverted microscope (Leica DMI 3000B, Germany) at 200× of magnification.

Cell cycle phase analysis

Saos-2 osteosarcoma cells were seeded with a concentration of 10^5 cells and incubated with changing doses of germacrone drug (0, 5, 50, and 150 µM). Afterwards, treated Saos-2 cells were washed two times in PBS. Further, cells were centrifuged and collected for fixation at –20°C in 70% ethanol (ice-cold) overnight. Staining of the cells was accomplished with PI solution (100 µl) in a dark room for about 30 min. Ultimately, the cells were analyzed for different cell cycle phases.

Transwell assay

Saos-2 osteosarcoma cells were transfected with germacrone at altering concentrations viz 0, 5, 50, and 150 µM. These cells were then assembled and transferred to the upper chambers of transwell well with RMPI-1640 medium. Lower chambers were filled with medium (600 µl) containing 10% FBS (Corning Incorporated, Corning, NY, USA). After 12 h of incubation membranes were subjected to fixation in alcohol for 10 min at 4°C. Migrated cells were subjected to staining with crystal violet at room temperature for 5min. unmigrated cells were cleansed with a cotton swab. Finally, different sections were photographed at 200X magnification under an inverted light microscope (TS100; Nikon Corporation, Tokyo, Japan).

Western blotting analysis

Soas-2 osteosarcoma cells were cultured up to 80% confluence and subjected to germacrone exposure with variant doses viz 0, 5, 50, and 150 µM. Afterwards, cells were lysed with a cell lysis buffer. BCA assay was executed to quantify protein content within each lysate. 45 µg of protein from each lysate was resolved by SDS-PAGE followed by electrophoretically transference to nitrocellulose membranes. Blocking of the membranes was performed with Tris-Buffered-saline with Tween (TBST) containing non-fat dry milk (5%) overnight at 4°C. Blocking was followed by appropriate primary antibody treatment (antibodies against Becalin-1, LC3-I, LC3-II, Cyclin-A, Cyclin-B1 and Cyclin-D1). Followed by TBST washing and secondary antibody treatment at room temperature for 2 h. Finally, proteins are thoroughly washed with TBST, and signal was detected through ECL (enhanced chemiluminescence) assay kit (Thermo Scientific, Rockford, USA).

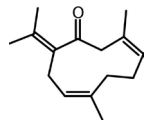

Figure 1. Chemical structure of germacrone molecule

Statistical analysis

Unpaired two-tailed Student's *t*-test was implemented for statistical analysis and employed to assess the statistical significance among the germacrone treated and control groups. $P<0.05$ was considered as statistically significant.

RESULTS

Effects of germacrone on osteosarcoma cellular viability

Effects on the cellular viability of osteosarcoma cells by germacrone (Fig. 1) drug were analyzed by MTT cell viability assay. The results showed that the cellular viability was potentially inhibited with time along with dose-dependent approach. The cellular proliferation rate after 24h of exposure with germacrone (0–150 µM) was recorded as nearly 100%, 80%, 70%, 35%, and 20%. The proliferation suppression rate enhanced after 48 h of drug exposure and was observed to be nearly 100%,

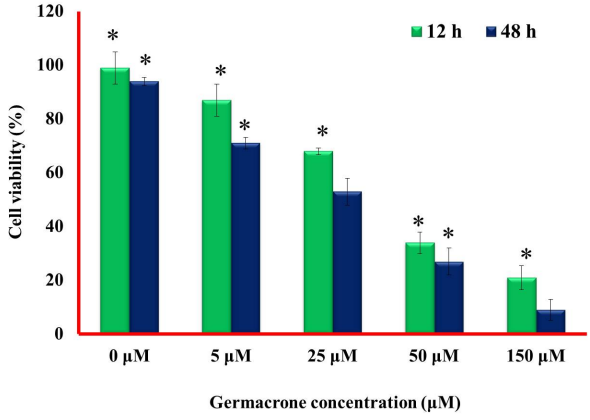

Figure 2. MTT assay results representing dose as well as time-reliant cell viability inhibition of osteosarcoma Soas-2 cells. Individual experiments were executed in triplicates. $P<0.05$ was considered as statistically significant and data was presented as ±S.E.M.

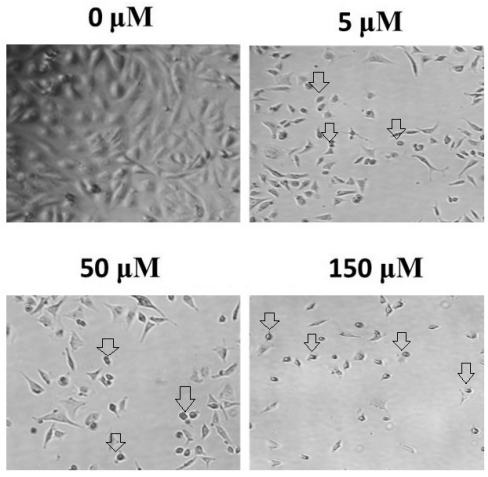

Figure 3. Phase contrast inverted microscopy results representing cellular morphology of germacrone treated Soas-2 cells.
Individual experiments were executed in triplicates.

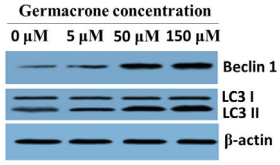

Figure 4. Western blotting assay representing levels of pro-autophagic proteins after germacrone exposure of Soas-2 cells.
Individual experiments were executed in triplicates. $P<0.05$ was considered as statistically significant and data was presented as ±S.E.M.

70%, 50%, 30% and 10% (drug concentrations ranging from 0–150 µM) (Fig. 2).

Morphological study of osteosarcoma cells after germacrone exposure

Germacrone exposure induced significant morphological changes in osteosarcoma Soas-2 cells. Morphology was monitored by phase contrast inverted microscope at varying drug concentrations (0–150 µM). Results showed normal cells with regular membranes, organelles, and nucleus. After drug exposure condensed nucleus, irregular organelles, disrupted membrane and vesicle like structures were observed (Fig. 3). These modifications increased with increasing doses of germacrone.

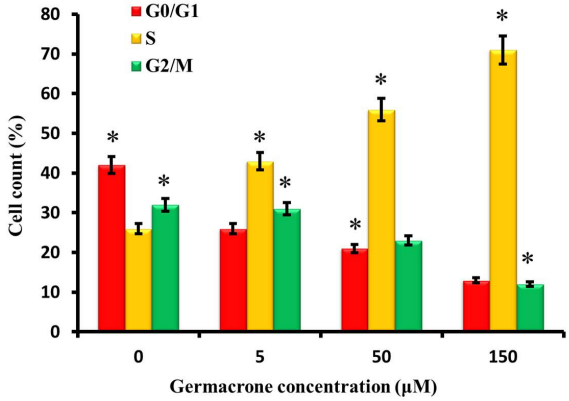

Figure 5. Flow cytometry analysis of cell cycle phases indicating S-phase cell cycle arrest.
Individual experiments were executed in triplicates. $P<0.05$ was considered as statistically significant and data was presented as ±S.E.M.

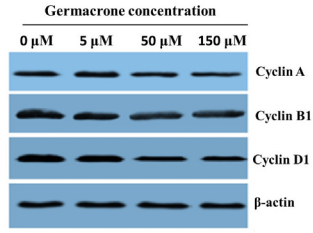

Figure 6. Western blotting assay representing levels of cell cycle allied proteins after germacrone exposure of Soas-2 cells.
Individual experiments were executed in triplicates. $P<0.05$ was considered as statistically significant and data was presented as ±S.E.M.

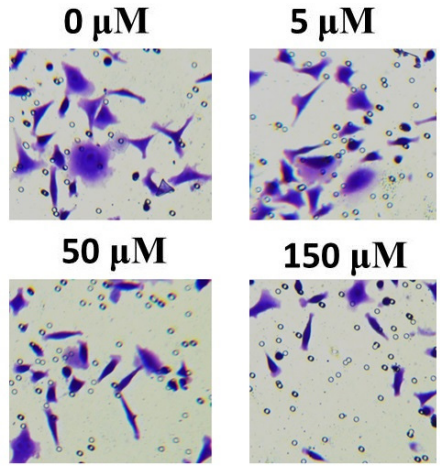

Figure 7. Transwell migration assay results representing suppressive effects of germacrone on Soas-2 cells.
Individual experiments were executed in triplicates.

Germacrone induced autophagy in osteosarcoma cells

Western blotting analysis was implemented to detect the induction of autophagy in osteosarcoma cells. The results showed dose-reliant increase in the levels of pro-autophagic proteins. The levels of Becalin-1 and LC3-II increased and that of LC3-I remained constant indicative of autophagy (Fig. 4).

Cell cycle analysis

Flow cytometric analysis was executed to explore effect of germacrone on different cell cycle phases. Results represented that the number S-phase cells increased in a dose-dependent manner compared to the other phases. The number of S-phase cells increased to 25%, 45%, 55% and 70% at germacrone doses of 0, 5, 50, and 150 µM (Fig. 5). These results indicated that germacrone arrested cell cycle in osteosarcoma at S-phase of cell cycle. Western blotting analysis was performed to monitor cell cycle allied proteins. Cyclin-A, Cyclin-B1, and Cyclin-D1 expressions were found to decrease with increasing drug doses, indicating cell cycle arrest (Fig. 6).

Germacrone inhibited cellular migration of osteosarcoma cells

Cell migration was examined via transwell assay after germacrone drug exposure. Results indicated that cell

migration of Soas-2 cells reduced significantly with increasing germacrone drug doses (Fig. 7). Thus, results suggested that germacrone is a potential cell migration inhibitor in osteosarcoma Soas-2 cells.

DISCUSSION

Autophagy is termed as type-II PCD (programmed cell death), and it is conserved in all eukaryotes as a cell survival pathway. Autophagy brings about selective disintegration of the components present in a cell including protein aggregates, long-lived proteins, intracellular pathogens, and cytoplasmic cell organelles. This disintegration results in energy generation and recycling of nutrients (Levine & Klionsky, 2004). Basal autophagic levels are needed for the maintenance of cellular homeostasis. Stress conditions that result in upregulation of autophagy are extracellular and intracellular stresses. Extracellular stress includes hypoxia, infections and nutritional deprivation, and intracellular stress includes high bioenergetics requirements, accumulation of impaired cell organelles and proteins. It allows mammals to maintain different pathophysiological and physiological processes like immunity, microorganism elimination, development, differentiation, and antiaging mechanisms. In lower eukaryotes, autophagy helps overcome starvation. Thus, bringing about autophagy in cancerous cells serves as a therapeutic strategy to overcome cancer. Herein, the current study was designed to uncover anticancer activity of germacrone terpenoid in human osteosarcoma cells. It was testified for its mediation *via* autophagy induction, cell cycle disruption, downregulating the cell cycle regulatory protein expressions, and cell migration inhibition. Cellular viability was estimated by MTT assay and results suggested that germacrone inhibited cell viability of Soas-2 osteosarcoma cells in a dose as well as time-dependent manner. Further, phase contrast inverted microscopy was performed to assess morphology of germacrone treated Soas-2 osteosarcoma cells. It revealed that cellular morphology changed significantly with increasing drug doses and those changes were indicative of autophagic cell death induction. Moreover, autophagy was also examined through estimation of allied protein levels with a Western blot assay. It revealed enhanced activity of pro-autophagy proteins. Next, flow cytometric analysis revealed that S-phase cells increased as compared to the others with increased drug doses. This indicated a cell cycle arrest at S-phase. It was further checked by Western blot assay which indicated dose-dependent declination of Cyclin-A, Cyclin-B1, and Cyclin-D1 levels. Finally, cell migration ability of Soas-2 osteosarcoma cells was testified by transwell assay. Results indicated dose-reliant potential suppression in cell migration after germacrone treatment.

CONCLUSION

In conclusion, results of the current study revealed that germacrone drug is a potent anticancer agent against human osteosarcoma. The anticancer effects were mediated *via* autophagy induction, cell cycle disruption, downregulating the cell cycle regulatory protein expressions, and cell migration inhibition. Hence, germacrone can be considered for osteosarcoma chemotherapy but further *in-vitro* and *in-vivo* studies are recommended.

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