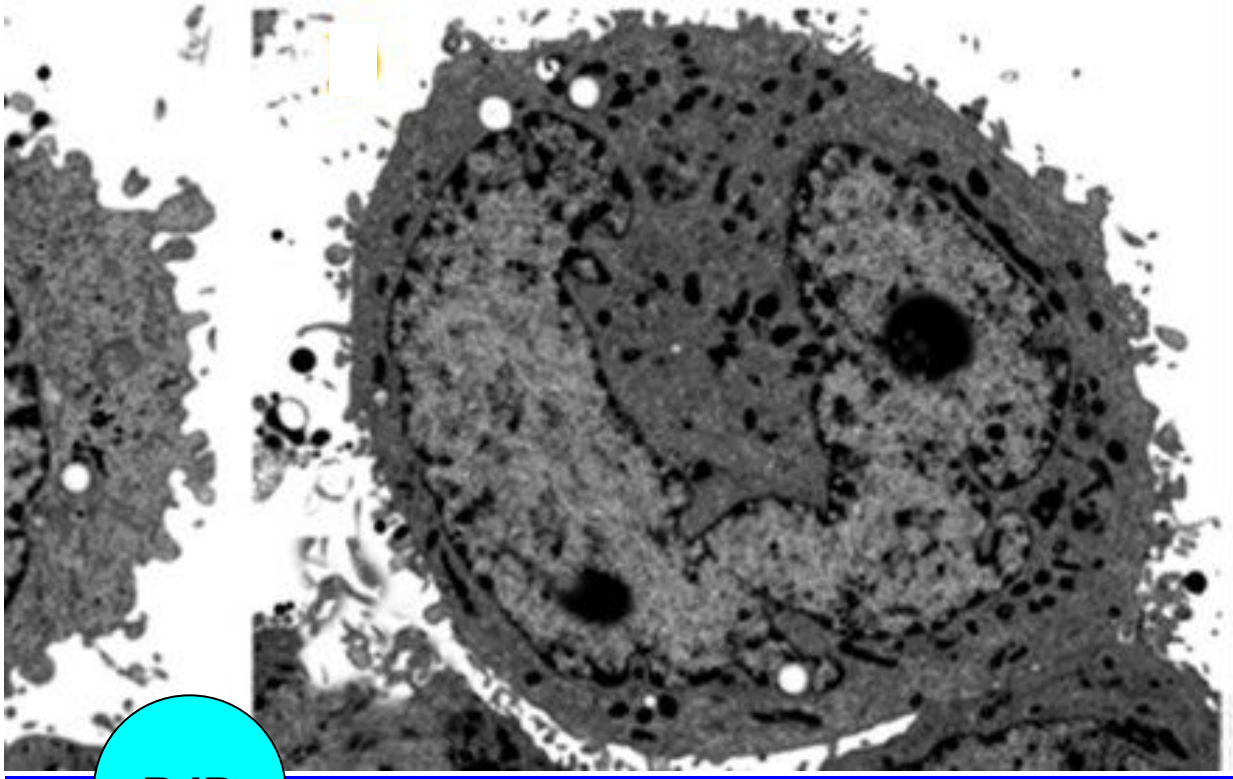

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Caryophyllene oxide exhibits anti-cancer effects in MG-63 human osteosarcoma cells via the inhibition of cell migration, generation of reactive oxygen species and induction of apoptosis

Zheng Pan[1], Shuan-Ke Wang[1], Xiao-Li Cheng[2], Xia-Wei Tian[1] and Jing Wang[1]

[1]The Orthopaedics Key Laboratory of Gansu Province, Second Hospital of Lanzhou University, Lanzhou University, China; [2]The Department of Experimental Teaching Center, Gansu University of Chinese Medicine, Lanzhou, China.

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Abstract

The main objective of the present study was to evaluate the antitumor and apoptotic effects of caryophyllene oxide in MG-63 human osteosarcoma cells. Cell viability of these cells was evaluated by MTT assay while as *in vitro* wound healing assay was used to study the effect of caryophyllene oxide on cell migration. Fluorescence microscopy and transmission electron microscopy were used to study the changes in cell morphology once the cells undergo apoptosis. Caryophyllene oxide significantly led to cytotoxicity in MG-63 cells showing dose-dependent as well as time-dependent effects. Caryophyllene oxide led to an inhibition of wound closure significantly. At caryophyllene oxide doses of 20, 80 and 120 µM, the percentage of cell migration was shown to be 94.2, 67.1 and 14.8% respectively. With an increase in the caryophyllene oxide dose, the extent of apoptosis also increased characterized by cellular shrinkage, membrane blebbing, chromatin condensation and apoptotic body formation.

Introduction

Osteosarcoma remains one of the most common malignant cancer of the bones in children as well as the adults. This is a highly malignant bone tumor with its incidences more in males than in females. The tumor mostly occurs in the metaphyseal area of long bones involving the growth plate, with majority of the cases occurring in the distal femur and proximal febia (Longhi et al., 2006; Cotterill et al., 2004). Around 25% of the osteosarcoma patients have already advanced to metastatic stage at diagnosis and lungs are the most common region where the tumor migrates (Wesolowski and Budd, 2010).

One of the main problems remains the poor prognosis of osteosarcoma coupled with the growing drug resistance to chemotherapeutics and metastasis to the lungs (Link and Meyers, 2002). Treatment of osteosarcoma involves both surgery and cytotoxic chemotherapy or their combination. Presently, cytotoxic chemotherapy plays a key role in the management of osteosarcoma therapy which has enhanced cure rates from 20 to 75% (Eilber et al., 1987; Link et al., 1991).

The advantage of primary neo-adjuvant chemotherapy is better than surgery alone, and this is due to the recurrent presence of subclinical micrometastatic disease (Ayerza et al., 2010). However, chemotherapy is associated with lot of serious, life-threatening toxicities leading to disadvantages of chemotherapy. This paves the way for the design and development of alternate chemotherapeutic drugs especially from natural products which are cheap, relatively non-toxic and easily available. Natural products have avital role as anti-cancer agents, and several of the cytotoxic drugs

used clinically today are derived from plants. The vinca alkaloids, isolated from the plant *Vinca rosea*, and the taxanes, from the bark of the Western yew *Taxus brevifolia* are the examples of cytotoxic compounds that are commonly used in cancer treatment (Cragg et al., 2009; Gordaliza, 2007).

The objective of the present study was to demonstrate the antitumor effects of caryophyllene oxide in MG-63 human osteosarcoma cells along with examining its effect on apoptosis induction, cancer cell migration and generation of reactive oxygen species (ROS).

Materials and Methods

Chemicals and reagents

Caryophyllene oxide (purity >98%; as determined by high-performance liquid chromatography),3-(4,5-dimethyl-2-thiazolyl)‑2,5-diphenyl-2H‑tetrazolium bromide (MTT) were obtained from Sigma-Aldrich Chemical Co. (USA). 2,7‑Dichlorodihydro-fluorescein diacetate (DCFH‑DA) and Hoechst 33342 were purchased from Wuhan Boster Biological Technology Ltd. (China). Dulbecco's Modified Eagle's Medium and RPMI-1640 medium were purchased from HyClone (USA). Fetal bovine serum, penicillin, and streptomycin were purchased from Tianjin Hao Yang Biological Manufacture Co., Ltd. (China).

Cell line and culture conditions

MG-63 human osteosarcoma cell line was obtained from American type culture collections and maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and antibiotics (100 U/mL penicillin G and 100 µg/mL streptomycin) at 37°C in a humidified incubator containing 5% CO_2 and 95% air.

Cell proliferation assay using MTT (Video Clip)

MG-63 human osteosarcoma cells were seeded in a 96-well plates at 2 × 10^6 cells per well. After 24 hours, caryo-phyllene oxide dissolved in DMSO at various concen-trations (0, 20, 40, 80 and 120 µM) was added to the cells. After incubation times of 24, 48 and 72 hours, MTT solution was added followed by addition of DMSO to dissolve formazan crystals. The number of viable cells is proportional to the formation of formazan crystals which were dissolved in ethanol and the optical density was measured on a microplate reader (Bio-Tek, America) at a wavelength of 490 nm.

In vitro wound healing assay for cell migration

MG-63 human osteosarcoma cells were put in a sterile 12-well plates and horizontal lines were drawn on the base of the plate by keeping it upside down. Then 2 mL of cell culture containing media was transferred into each well. The plate was covered by the lid and then kept in CO_2 incubator for 48 hours at 37°C. Then the plate was taken out from the CO_2 incubator and a scratch in each well was made using a 50 µL micropipette tip. After that the cells in the plate were subjected to varying doses of caryophyllene oxide (0, 20, 80 and 120 µM) and then incubated for 48 hours, fixed and stained with 5.5% ethanol containing 1.5% crystal violet powder for 30 min. Then using a phase contrast microscope (Olympus, Tokyo, Japan), randomly selected fields were chosen and photographed. Image J software (1.46 version) was used to determine the length of the wounds.

Fluorescence microscopic study of apoptosis using Hoechst 33342

MG-63 human osteosarcoma cells were treated with various concentrations (0, 20, 80 and 120 µM) of caryophyllene oxide and then the cells were placed in a CO_2 incubator for 48 hours at 37°C. Following incubation, the cells were fixed with 3.5% formaldehyde for 20 min and then washed with PBS three times. The solution of Hoechst 33342 was added to the cells and after 15 min of staining, the cells were observed under a fluorescence microscope at 400x magnification (Nikon, Japan).

Cell morphological and apoptotic evaluation by Transmission Electron Microscopy (TEM)

MG-63 human osteosarcoma cells (2 x 10^6 cells/well) were seeded in three flasks. The cells were treated with increasing doses (0, 20, 80 and 120 µM) of caryophyllene oxide for 48 hours and the cells were then harvested and washed with PBS three times. After wards, 2.0% glutaraldehyde was added for microtome sectioning using ultramicrotome (JEOL Co: Japan). TEM analysis was performed using a TEM (JEM-4000; JEOL Co; Japan).

ROS analysis

MG-63 human osteosarcoma cells (2 x 10^6 cells/well) treated with the specified concentrations of caryophyllene oxide (0, 20, 80 and 120 µM) for 1 hour, were loaded with 10 µM fluorescent probe 2', 7'-dichlorodihydrofluorescein diacetate (Wuhan Boster Biological Technology Ltd. (China) at 37°C for 1 hour (Zhang et al., 2014). The fluorescence intensity was analyzed on FACS Calibur instrument (BD Biosciences, USA) system using Cell Quest™ Pro software.

Statistical analysis

All results were depicted as mean ± standard error (SE) from at least three independent experiments. The differences between groups were analyzed by one-way ANOVA, significance of difference was designated as $^a p<0.05$, $^b p<0.01$.

Results

Antitumor activity

Caryophyllene oxide induced significant, time-dependent as well as concentration-dependent cytotoxic effects in MG-63 osteosarcoma cells (Figure 1). In order to evaluate the potency of the compound, its IC_{50} values were measured at different time intervals which were found to be 43.2, 31.6 and 24.2 µM at 24, 48 and 72 hours time intervals respectively. The IC_{50} value at different time intervals reveal that cytotoxicity of this compound increases with the increase in the incubation time for which the cells are exposed.

Cell migration tendency

In the present study, the anti-migratory effects of caryophyllene oxide on the MG-63 human osteosarcoma were also demonstrated and the results are shown in Figure 2. Results indicate that caryophyllene oxide led to an inhibition of wound closure significantly and in a dose-dependent manner. At caryophyllene oxide doses of 20, 80 and 120 µM, the percentage of cell migration was shown to be 94.2, 67.1 and 14.8% respectively. Untreated control cells exhibited 100% cell migration. These results are promising because suppression of cancer cell migration can be an effective approach to treat this deadly disease. Many of the malignant cancers possess high migratory capabilities and hence high incidences of mortality.

Apoptotic evaluation by fluorescence microscopy

One of the best methods to evaluate whether a compound induces apoptosis or not is the fluorescence microscopy technique. Since cellular morphological changes during the process of apoptosis can be easily detected by using different staining agents in combination with fluorescence microscopy. The results of the current study reveal that with an increase in the caryophyllene oxide dose, the extent of apoptosis also increased characterized by cellular shrinkage, membrane blebbing, chromatin condensation and apoptotic body formation (Figure 3). The untreated control cells showed normal morphology with no signs of apoptosis.

Apoptotic evaluation by TEM

The fact that caryophyllene oxide does induce apoptotic effects in MG-63 human osteosarcoma cells was further confirmed by TEM. The results which are shown in Figure 4 indicates that as compared to the untreated control cells, caryophyllene oxide-treated cells showed significant damage to the nuclear membrane as well as mitochondria. The increase in the dose of caryophyllene oxide was accompanied with increasing number of vacuoles in the cytoplasm. TEM results also indicated that swelling of endoplasmic reticulum as well as mitochondria was detected.

Caryophyllene oxide-induced ROS generation

The effect of caryophyllene oxide on the ROS generation in MG-63 human osteosarcoma cells was studied using flow cytometry using 2'-7'-dichloro-

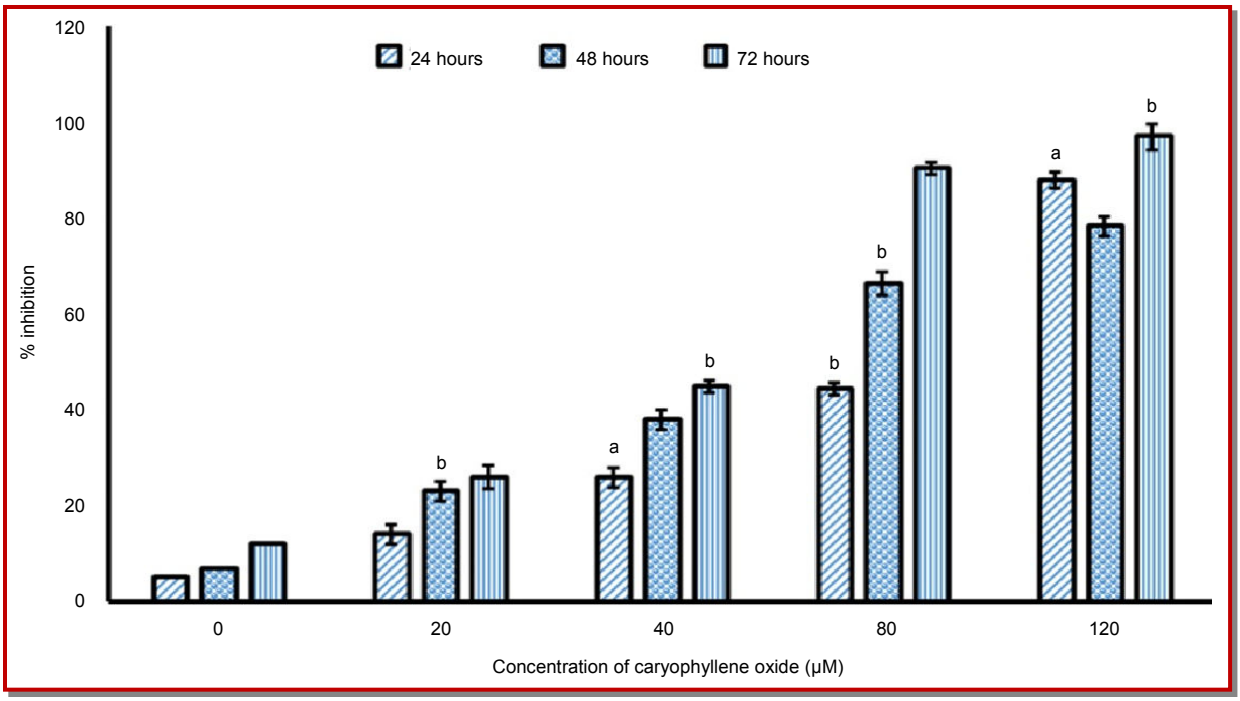

Figure 1: Cytotoxic effect of caryophyllene oxide in human osteosarcoma cells (MG-63) at different doses. Data are shown as the mean ± SD of three independent experiments. [a]p<0.05, [b]p<0.01, vs 0 µM (control)

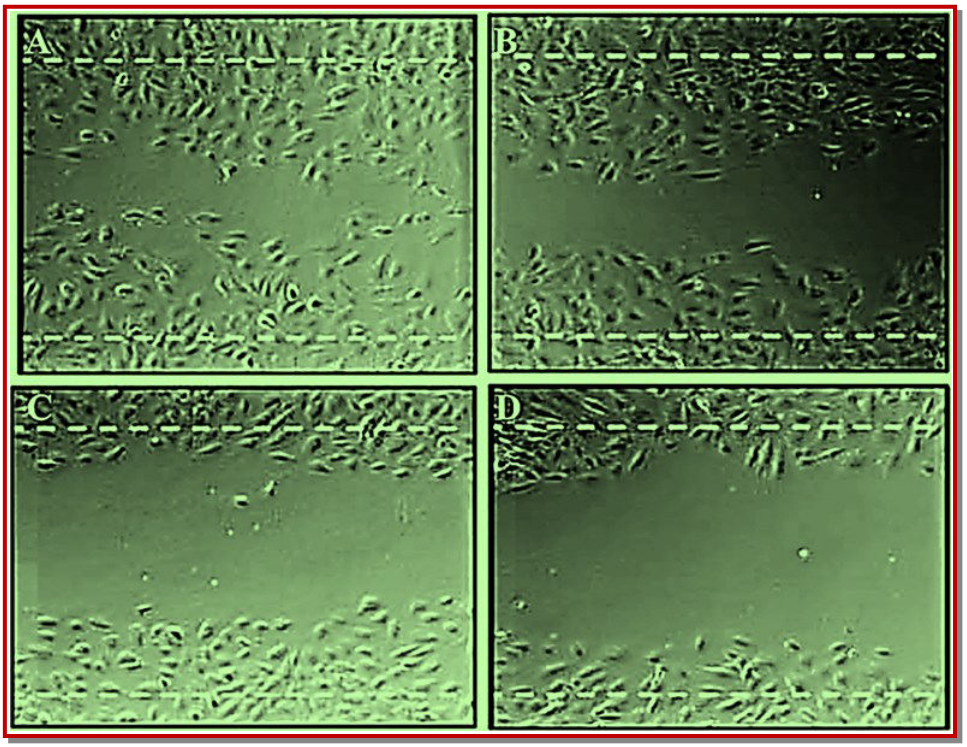

Figure 2: Inhibition of cancer cell migration in MG-63 human osteosarcoma cells induced by caryophyllene oxide. Phase contrast microscope was used for capturing images after the cells were treated with 0 (A), 20 (B), 80 (C) and 120 (D) µM dose of caryophyllene oxide for 48 hours

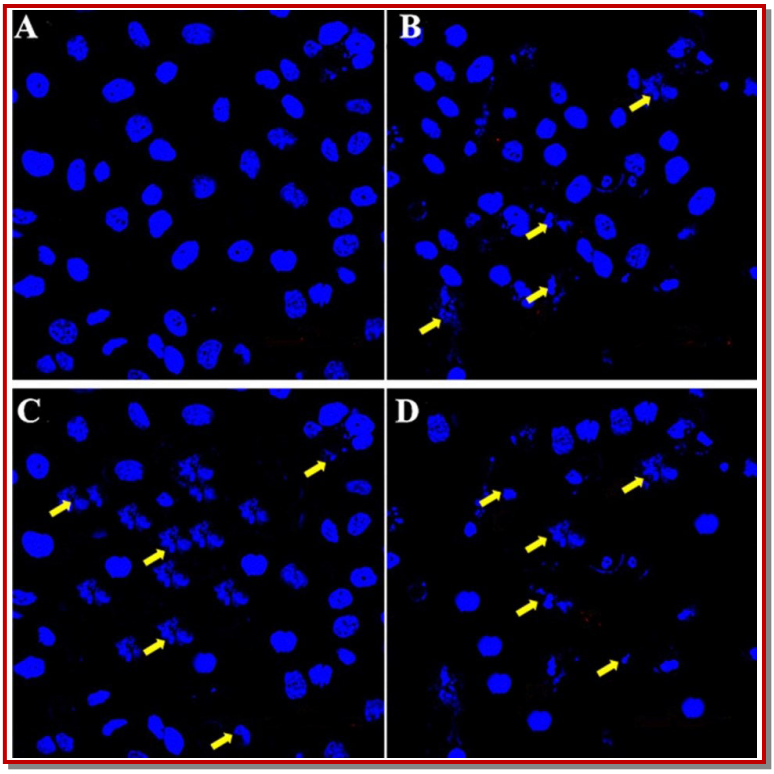

Figure 3: Effect of caryophyllene oxide on the apoptosis induction in MG-63 human osteosarcoma cells. The cells were treated with 0 (A), 20 (B), 80 (C) and 120 (D) µM dose of caryophyllene oxide, stained with Hoechst 33342 and then observed under fluorescence microscope at 400x magnification

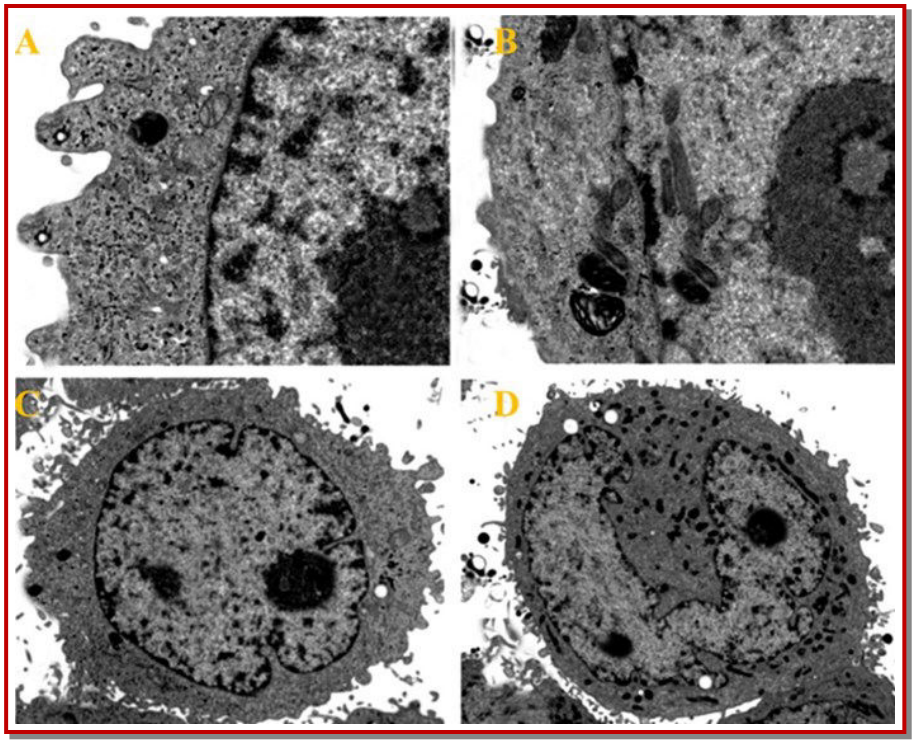

Figure 4: Study of the apoptotic cell death induced by caryophyllene oxide in MG-63 human osteosarcoma cells using transmission electron microscopy. The cells were treated with 0 (A), 20 (B), 80 (C) and 120 (D) µM dose of caryophyllene oxide and then observed under Transmission Electron Microscope at 10,000x magnification

fluorescin diacetate fluorescent dye.

Figure 5 reveals that caryophyllene oxide-treated MG-63 human osteosarcoma cells exhibited a significant increase in intracellular ROS production. It not only induced dose-dependent, but also a time-dependent increase in ROS. The production of ROS, which damages DNA, proteins and lipids, has been associated with a number of human diseases, such as atherosclerosis and cancer.

Discussion

The primary objective of the current research work was to evaluate the anti-cancer activity of caryophyllene oxide against MG-63 human osteosarcoma cells along with demonstrating its effects on cell migration, ROS generation as well as apoptosis induction. Cell viability experiments which were carried out using MTT assay indicated that caryophyllene oxide is a potent cytotoxic agent inducing time-dependent as well as dose-dependent cell cytotoxicity in these cells. *In vitro* wound healing assay showed that caryophyllene oxide led to a suppression of wound closure considerably. Caryophyllene oxide also led to apoptosis induction characterized by cell shrinkage, membrane blebbing, chromatin condensation and formation of apoptotic bodies. TEM examination revealed that caryophyllene oxide led to significant damage to nuclear membrane as well as to the mitochondria. ROS experiments using flow cytometry and 2'-7'-dichlorofluorescin diacetate fluorescent dye exhibited that caryophyllene oxide led to a significant time-dependent as well as concentration-dependent build-up of ROS in MG-63 human osteosarcoma cells, after the cells were treated with 0, 20, 80 and 120 µM dose of caryophyllene oxide. It has been reported earlier that caryophyllene oxide exhibits cytotoxic activity against a range of cancer cells including HepG2 human leukemia cancer cells, AGS human lung cancer cells, SNU-1 human gastric cancer cell and SNU-16 human stomach cancer cells (Jun et al., 2011).

Several other sesquiterpenoids and oxygenated sesquiterpenoids have been reported to exhibit anti-cancer activity against a range of solid tumor cell lines (Kubo et al., 1996; Wang et al., 2014). Withaferin A, resibufogenin and nobiletin exhibit similar effects (Chen et al., 2014; Niu et al., 2014; Zhang et al., 2014) .

Caryophyllene oxide has also been reported to inhibit cell growth in human prostate and breast cancer cells by inducing apoptosis and suppressing the PI3K/AKT/mTOR/S6K1 pathways (Park et al., 2011). To the best of our knowledge, the current research work is the first such report on caryophyllene oxide against MG-63 human osteosarcoma cancer cell line.

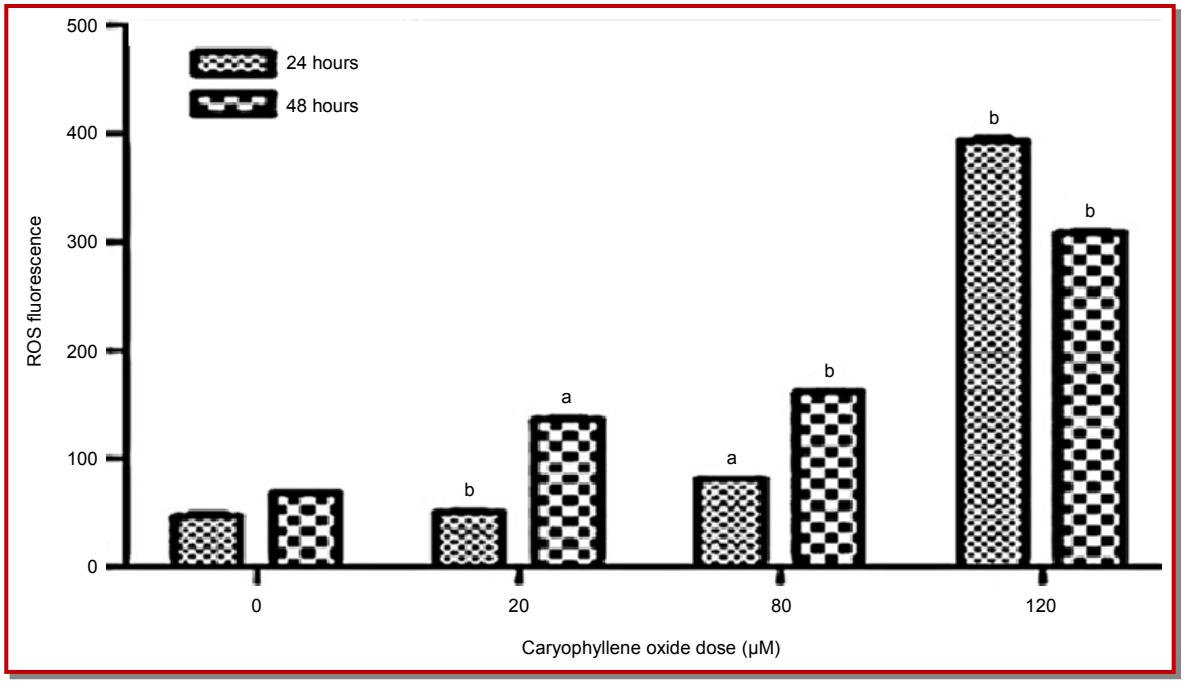

Figure 5: Reactive oxygen species (ROS) generation induced by different doses of caryophyllene oxide at two different time intervals in MG-63 human osteosarcoma cells. The cells were treated with 0, 20, 80 and 120 μM dose of caryophyllene oxide, stained with 20 μM 2, 7-dichlorodihydrofluorescein diacetate for 20 min and then analyzed by flow cytometry. Data are the mean ± SE of three experiments. [a]p<0.01 vs. control, [b]p<0.05 vs. control

Conclusion

Caryophyllene oxide exerts strong anti-cancer effects against MG-63 human osteosarcoma cells by inhibiting cancer cell migration tendency and inducing apoptosis characterized by cellular shrinkage, membrane blebbing, chromatin condensation and apoptotic body formation. Caryophyllene oxide also induced dose-dependent as well as time-dependent ROS production in these cells.

Conflict of Interest

All authors have completed the ICMJE uniform disclosure form and declare no support from any organization for the submitted work.

Acknowledgement

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Author Info
Shuan-Ke Wang (Principal contact)
e-mail: wangshuanke@hotmail.com

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