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ORIGINAL ARTICLE

β-caryophyllene, a Natural Bicyclic Sesquiterpene, Induces Apoptosis by Inhibiting Inflammation-Associated Proliferation in MOLT-4 Leukemia Cells

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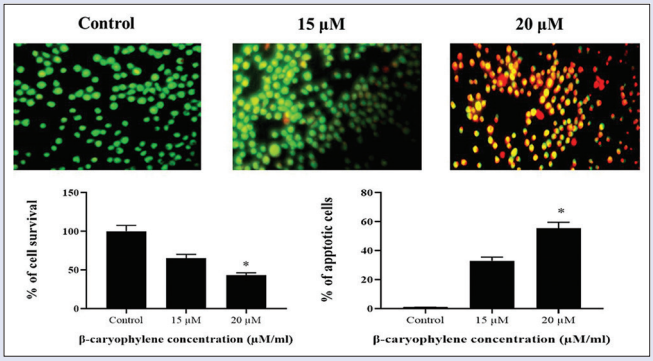
ABSTRACT

Background: Leukemia is known to be a common type of cancer mostly affecting children. The standard therapeutic treatment available for leukemia has many drawbacks with serious side effects. Therefore, plant-based chemotherapeutic agents that show less/no toxic side effects might be an efficient way to treat leukemia. Therefore, in this study, we aimed to explore the potential of β-caryophyllene, obtained from various plants sources, and found that it persuades oxidative stress-associated apoptosis during the repression of inflammation and proliferation in MOLT-4 leukemia cancer cells. **Materials and Methods:** In this study, MOLT-4 cells were incubated with β-caryophyllene (15 and 20 μM) for 24 h and found that β-caryophyllene increased the level of cytotoxicity and reactive oxygen species (ROS) and decreased the level of antioxidants, mitochondrial membrane potential, and apoptotic reaction in MOLT-4 cells. Cell proliferation and apoptosis are important cellular events, and inhibition of cell proliferation along with the generation of proapoptotic marker has been considered as a novel task for treatment of cancer. **Results:** According to our results, β-caryophyllene induced apoptosis by downregulating the expression of Bcl-2 family of proteins and upregulating the expression of caspases involved in BAX-associated apoptosis in MOLT-4 cells. It also downregulated the expression of biomarkers involved in proliferation (proliferating cell nuclear antigen and cyclin-D1) and inflammation (tumor necrosis factor-α, interleukin-6, nuclear factor-kappa B, and cyclooxygenase-2). **Conclusion:** In summary, β-caryophyllene potentially induced apoptosis by generating ROS and by inhibiting inflammation and proliferative genes in MOLT-4 leukemia cells.

Key words: Apoptosis, inflammation, leukemia, proliferation, β-caryophyllene

SUMMARY

- Leukemia is known to be one of the most common types of cancers mostly affecting children
- β-Caryophyllene potentially induced apoptosis by generating reactive oxygen species and by inhibiting inflammation and proliferative genes in MOLT-4 cells.



Abbreviations used: ROS: Reactive oxygen species; PBS: Phosphate-buffered saline; CAT: Catalase; SOD: Superoxide dismutase.

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INTRODUCTION

Leukemia is a common type of cancer mostly affecting children under the age of 14 years.[1] There are 900,000 new cases of hematological malignancies recorded each year such as myelomas, leukemias, and lymphomas, which accounts to about 6.9% of all the tumorigenic diseases and 6.5% of the deaths.[2] Specifically, pediatric leukemia has an extremely poor prognosis and high mortality rate in low-resource countries.[3] At present, leukemia is treated with standard chemotherapeutic agents and radiation therapy, which show severe toxicity and side effects.[4]

The extreme production of reactive oxygen species (ROS) results in oxidative stress and lack of antioxidants.[5] The imbalance in the redox homeostasis has been shown to be a specific mechanism in cancer-related treatment by inducing apoptosis.[6] Apoptosis can be induced by environmental and chemical stimuli. It is regulated by pro-apoptotic

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mediators present in the mitochondria and anti-apoptotic factors such as Bcl-2.[7] Moreover, proliferating cell nuclear antigen (PCNA) and cyclin-D1 are tumor cell proliferative markers that regulate the cell cycle.[8] In addition, the oxidative stress-depended inflammatory indicators of nuclear factor-kappa B (NF-κB), cyclooxygenase (COX)-2, tumor necrosis factor (TNF)-α, and interleukin (IL)-6 are participate in the enhancement of tumor cell proliferation which leads to severe tumorigenesis.[9] Downregulating the expression of Bcl-2, PCNA, and cyclin-D1 is being considered as an effective strategy to treat the cancer cells.

Despite the plenty of chemotherapeutic agents available in the market, the majority of them cause toxic side effects in patients with leukemia. Therefore, it is important to develop more conservative therapeutic methods to treat patients with leukemia. Among the numerous natural substances that are used to cancer,[10] β-caryophyllene is one of the natural bicyclic sesquiterpenes that is widely present various spices and plants.[11] It is present in the essential oils of basil (*Ocimum* spp.), cinnamon (*Cinnamomum* spp.), cannabis (*Cannabis sativa* L.), cloves (*Syzygium aromaticum*), lavender (*Lavandula angustifolia*), and oregano (*Origanum vulgare* L.).[12] It demonstrates various biological properties such as anti-inflammatory, anticarcinogenic, antioxidative, and antimicrobial.[11,13] Previously, a combined treatment with β-caryophyllene and aromadendrene oxide-2 and phytol was used to synergistically treat precancerous cells and cancerous cells of the skin.[13] Moreover, β-caryophyllene oxide inhibits mitogen-activated protein kinase- and PI3K/AKT-associated mammalian target of rapamycin signaling pathways in cancer cells.[14] In this study, we explored the effect of β-caryophyllene against the cell proliferation of MOLT-4 leukemia cells.

MATERIALS AND METHODS

Reagents and antibodies

β-Caryophyllene, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), acridine orange (AO), dichlorodihydrofluorescein diacetate (DCFH-DA), rhodamine-123, and Hoechst were purchased from Sigma chemicals, USA. Ethylenediaminetetraacetic acid, phosphate-buffered saline, Roswell Park Memorial Institute (RPMI) 1640 Medium penicillin/streptomycin, heat-inactivated fetal bovine serum (FBS), glutamine, and caspases 3, 8, and 9 assay kit were acquired from Invitrogen, China. The antibodies (BAX, cyclin-D1, Bcl-2, and PCNA) were obtained from Santa Cruz Biotechnology.

Cell culture

MOLT-4 cells were cultured in RPMI-1640 supplemented with 10% FBS and 1% penicillin/streptomycin in a humidified atmosphere at 37°C and 5% CO_2.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

MTT test was conducted based on a previously defined protocol.[15] Briefly, MOLT-4 were cultured in 96-well plates for 24 h. Then, different concentrations (5–35μM) of β-caryophyllene were added to MOLT-4 cells and incubated for 24 h at RT. After this, MTT reagent was added to each well and incubated for 4 h at 37°C. Next, the medium was discarded and dimethyl sulfoxide (100–200 μL) was added to dissolve the formazan crystals. The absorbance was read at 570 nm on an ELISA plate reader.

Dichlorodihydrofluorescein diacetate staining

Intracellular ROS was estimated by DCFH-DA, which can significantly reduce keen on the intracellular framework, have oxidized into fluorescent DCF by means of ROS.[16] The fluorescence intensity of the dye directly corresponds to the amount of ROS generated in the cells. The cells were grown in Petri dishes and β-caryophyllene (15 and 20 μM) was added. Then, the cells were incubated in a CO_2 incubator for 24 h. Subsequently, cells were kept for recolored using DCFH-DA for 10 min. Finally, the intensity of the fluorescence was read at excitation wavelength of 485 ± 13.5 nm and emission wavelength of 530 ± 12.5 nm.

Rhodamine-123 staining

The mitochondrial membrane potential (MMP) ($\Delta\psi_m$) was measured as per the previously described procedure using rhodamine-123.[17] MOLT-4 cells were incubated in Petri dish and β-caryophyllene (15 and 20 μM) was added to the cells (24 h). Then, rhodamine-123 was added to the cells and incubated for 30 min. Finally, the intensity of the fluorescent was estimated using spectrofluorometer (at 485/530 nm).

Apoptotic signature

Fluorescence microscopic assessment of apoptotic signature was examined by AO/ethidium bromide (EB) two-fold staining method that was used to know apoptosis via morphological examination.[17] Then, β-caryophyllene (15 and 20 μM) was added to the cells and incubated for 24 h. Next, dye solution was added and the cells were observed using an inverted fluorescent microscope. The same procedure was used in this study, and instead of AO/EB, propidium iodide (PI) could be used for microscopic examination.

Biochemical parameters

We estimated the activities of catalase (CAT), superoxide dismutase (SOD), reduced glutathione (GSH), and the level of lipid peroxides formed were quantified using standard biochemical tests.

Immunoblotting analysis

The expression of PCNA, Bax, cyclin-D1, and Bcl-2 was analyzed via immunoblotting.[18] The total protein was analyzed by the method of Lowry *et al.*[19] The extracted proteins were isolated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (8%–12%) technique. The protein bands were transferred onto polyvinylidene difluoride (PVDF) membranes, and the proteins were subsequently blocked for 1 h in cold temperature. Then, the membranes were rinsed with TBST. Next, the membranes were incubated with primary antibodies at various dilutions based on the manufacture's protocol and incubated overnight. Then, the membranes were washed again with TBST and incubated with horseradish peroxidase-conjugated secondary antibodies for 45 min. PVDF membranes were again rinsed with TBST, and the protein band expression has been developed by addition of enhanced chemiluminescence substrate.

Detection of caspases

Caspases 3, 8, and 9 were detected using caspase ELISA assay kit, and the experimental protocol was followed as per the manufacturer's instructions (Invitrogen). Briefly, β-caryophyllene (15 and 20 μM) was added to the cells and incubated for 24- and 48-h respective CO_2-based incubator. Then, the reagents were added in order and incubated for 2 h in dark environment. Then, the absorbance was measured on an ELISA plate reader at 400 or 405 nm.

Reverse transcription–polymerase chain reaction

The total RNA was extracted by Qiagen mini column kit and stored at −80°C until use. The mRNA expression of TNF-α, NF-κB, IL-6, and COX-2 was analyzed as per the method described by Balupillai *et al.*[20]

Statistical analysis

In this study, data were analyzed using SPSS version 17 software (SPSS Inc., Chicago, IL, USA). Unless otherwise mentioned, all data are presented as mean ± standard deviation. The difference in means of different groups was compared based on analysis of variance (ANOVA) and DMRT. The data were considered as statistically significant if $P < 0.05$.

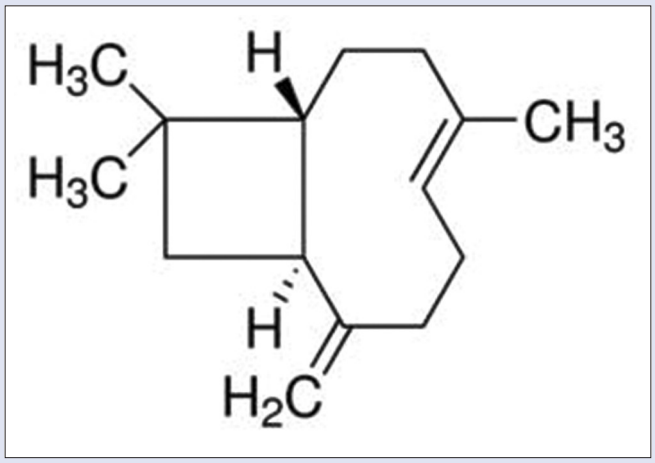

Figure 1: The chemical structure of β-caryophyllene

RESULTS

Cytotoxicity of β-caryophyllene against MOLT-4 cells

Figure 1 shows the structure of β-caryophyllene. Cytotoxicity of β-caryophyllene against the MOLT-4 cells was analyzed by MTT assay. Figure 2 shows that β-caryophyllene significantly decreased the viability of the cells at different concentrations. The half maximal inhibitory concentration of β-caryophyllene was found to be 15 μM. Based on this information, we chose 15 and 20 μM for further experiments.

β-Caryophyllene produces reactive oxygen species in MOLT-4 cells

The production of ROS in MOLT-4 cells was visualized using DCFH-DA staining method. Figure 3 shows the level of ROS production in MOLT-4 cells after incubation with β-caryophyllene. The two tested concentrations of β-caryophyllene (15 and 20 μM) induced a high degree of ROS production in MOLT-4 cells (the top green-hued fluorescence). Among the two tested concentrations, 20 μM β-caryophyllene demonstrated higher production of ROS than that of 15 μM.

β-Caryophyllene alters mitochondrial membrane potential ($\Delta\psi_m$) in MOLT-4 cells

Premature apoptotic phase was activated through the changes in the MMP ($\Delta\psi_m$), which was evaluated using lipophilic dye rhodamine-123. Compared to the cells treated with β-caryophyllene, the normal control cells produced high intensity of green fluorescence [Figure 4]. Furthermore, among the two concentrations of 15 and 20 μM β-β-caryophyllene, 20 μM reduced green fluorescence intensity in MOLT-4 cells.

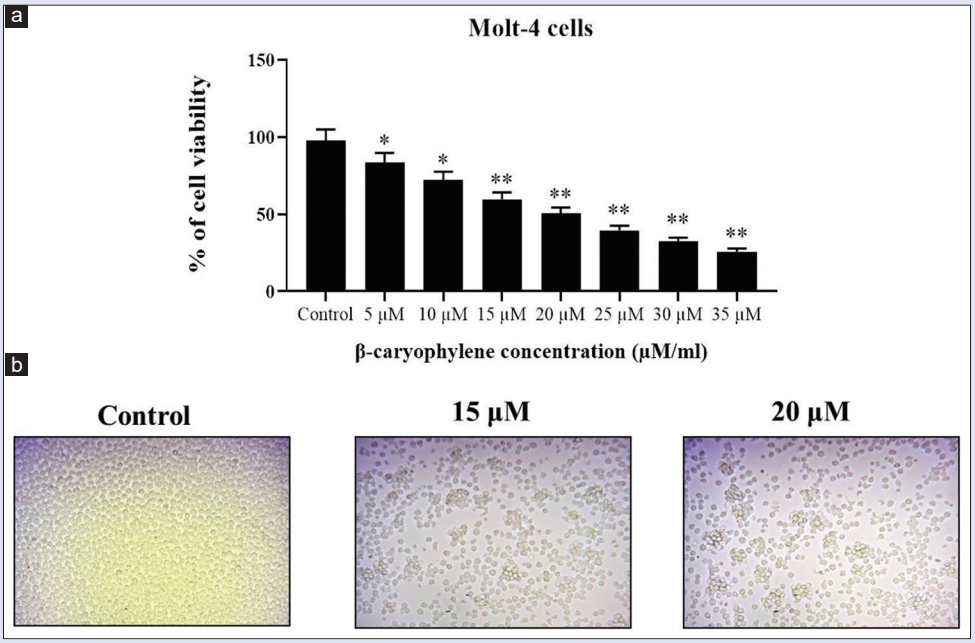

Figure 2: (a) The cytotoxicity effect of β-caryophyllene on leukemia cancer cell line MOLT-4 measured by MTT assay. (b) Microscopic images show β-caryophyllene against cytotoxic effect of MOLT-4 cells. The statistical analysis was carried out using one-way analysis of variance. Values are represented mean ± standard deviation of three experiments. $P < 0.05$ was significantly different from the control sample

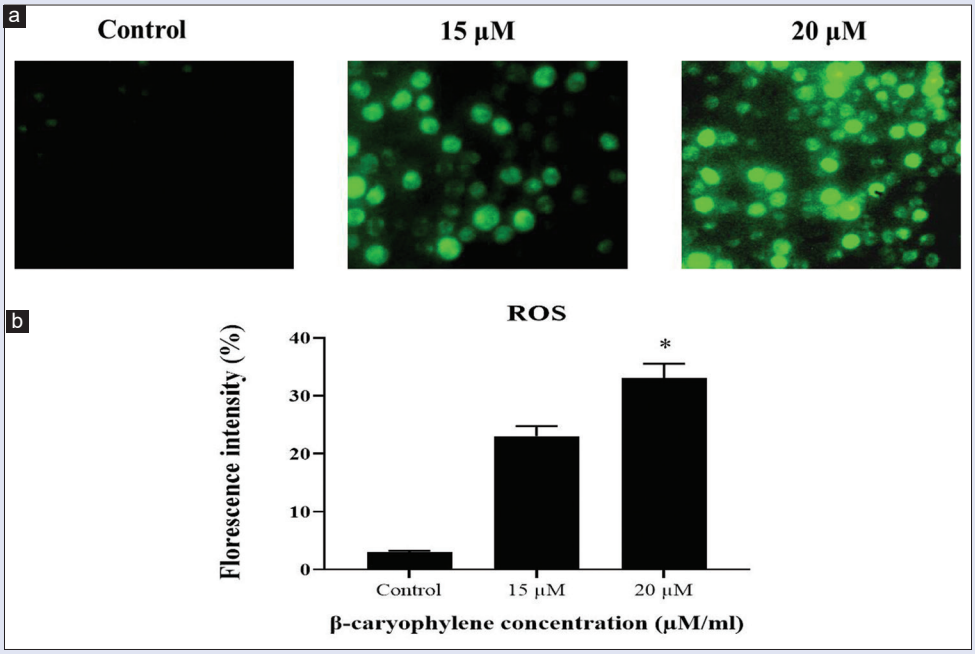

Figure 3: The effect of β-caryophyllene reactive oxygen species generation in MOLT-4 cell lines was evaluated using dichlorodihydrofluorescein diacetate staining. (a) Fluorescence microscopic for β-caryophyllene on intracellular reactive oxygen species generation. (b) Percentage of reactive oxygen species generation was detected by spectrofluorometer. The statistical analysis was carried out using one-way analysis of variance. Values are represented mean ± standard deviation of three experiments. *$P < 0.05$ was significantly different from the control sample

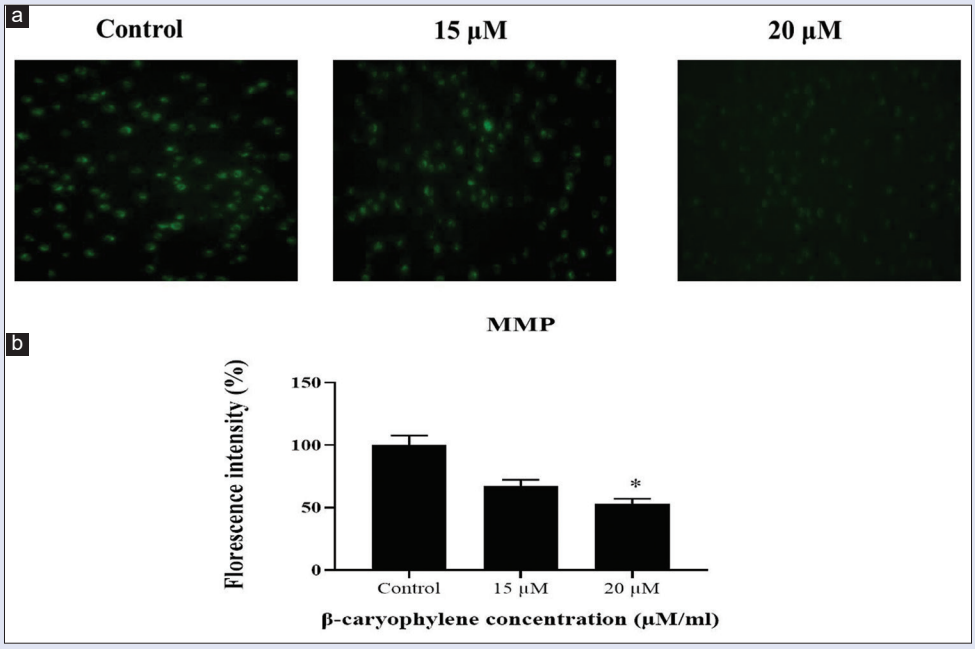

Figure 4: The effect of β-caryophyllene mitochondrial membrane potential alteration in MOLT-4 cell lines was evaluated using rhodamine-123 staining. (a) Fluorescence microscopic for β-caryophyllene on mitochondrial membrane potential alteration. (b) Percentage of fluorescence intensity for mitochondrial membrane potential was detected by spectrofluorometer. The statistical analysis was carried out using one-way analysis of variance. Values are represented mean ± standard deviation of three experiments. *$P < 0.05$ was significantly different from the control sample

β-Caryophyllene induced apoptosis in MOLT-4 cells

The apoptotic activity of β-caryophyllene in MOLT-4 cells was evaluated by the AO/EB staining method. The microscopic staining of AO/EB analysis exhibited the hallmark attribute of apoptotic cells after treatment with β-caryophyllene [Figure 5]. The red-colored EB fluorescence dye revealed dead apoptotic cells with the condensed nuclei, whereas the AO (green) was taken up by the live cells alone. Among the two tested concentrations, the cells treated with 20 µM β-caryophyllene

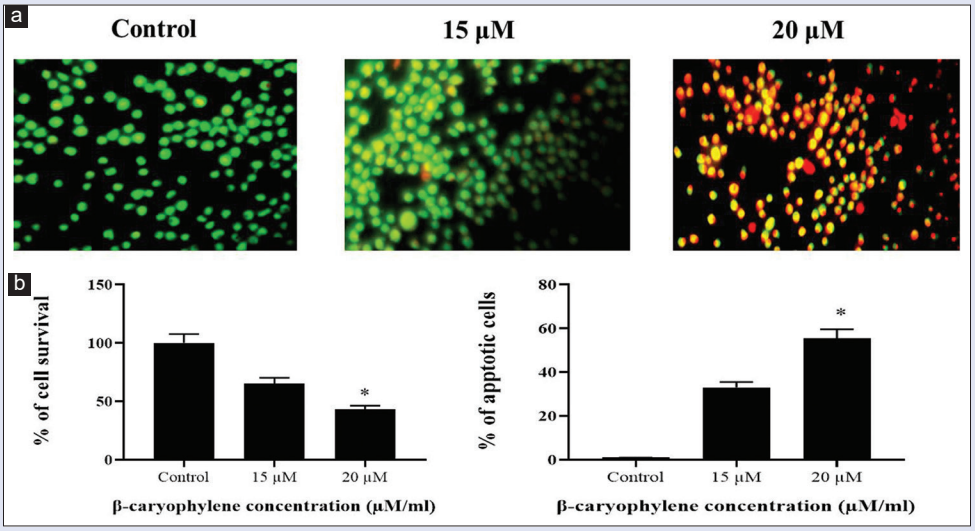

Figure 5: β-caryophyllene on apoptotic morphological changes was analyzed by dual staining (acridine orange/ethidium bromide). (a) Microscopical images shows that control cells shows acridine orange-stained cells and β-caryophyllene-treated cells showed ethidium bromide-stained cells. (b) Bar diagram shows that the percentage of apoptotic cells was calculated. The statistical analysis was carried out using one-way analysis of variance. Values are represented mean ± standard deviation of three experiments. **P < 0.05 was significantly different from the control sample*

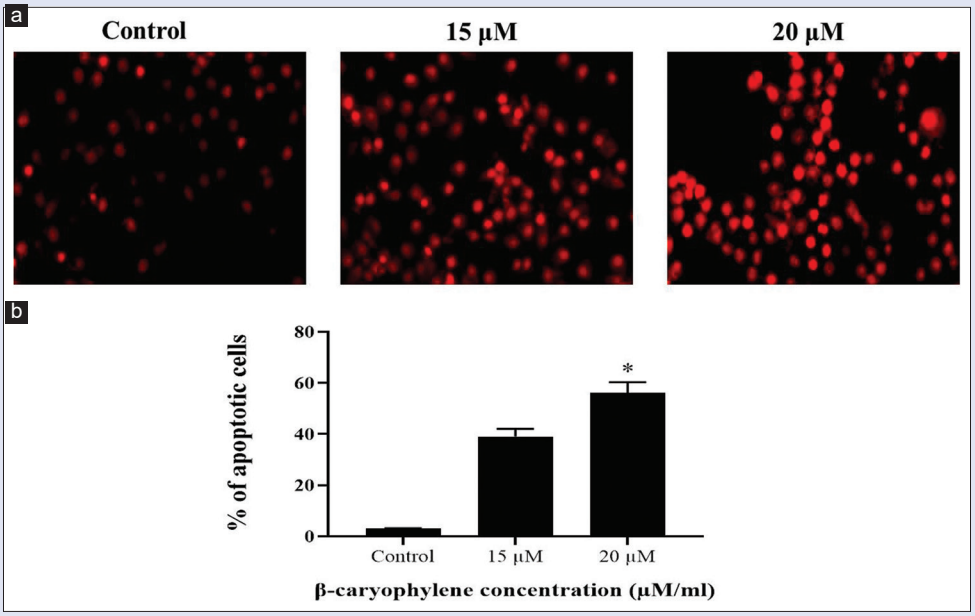

Figure 6: β-caryophyllene on nuclear fragmentation changes was analyzed by propidium iodide staining. (a) Microscopical images shows that control cells shows cells no fragmentation and β-caryophyllene treated cells showed increased. (b) Bar diagram shows that the percentage of apoptotic cells was calculated. The statistical analysis was carried out using one-way analysis of variance. Values are represented mean ± standard deviation of three experiments. **P < 0.05 was significantly different from the control sample*

demonstrated orange color, which indicated the early stage of apoptosis, and red-colored fragmented nuclei, which represented late apoptosis. For further confirmation, we performed PI staining [Figure 6], which revealed that cells treated with 20 µM β-caryophyllene effectively demonstrated DNA damage in MOLT-4 cells.

Effect of β-caryophyllene on antioxidant levels and lipid peroxidation status in MOLT-4 cells

Figure 7 shows the effect of β-caryophyllene in MOLT-4 cells. Both the tested concentrations (15 and 20 µM) of β-caryophyllene demonstrated high levels of Thiobarbituric acid reactive substances (TBARS) formation resulting in the depletion of antioxidant markers such as SOD, CAT, and GSH. However, the control cells showed increased levels of antioxidants and decreased levels of TBARS in MOLT-4 cells.

Effect of β-caryophyllene on the expression of apoptotic and proliferative proteins in MOLT-4 cells

The expression range of Bcl-2 and Bax is acting inversely in the occupation of apoptosis [Figures 8 and 9]. Bax induces apoptosis, whereas Bcl-2 blocks it. The levels of Bcl-2, PCNA, and cyclin-D1

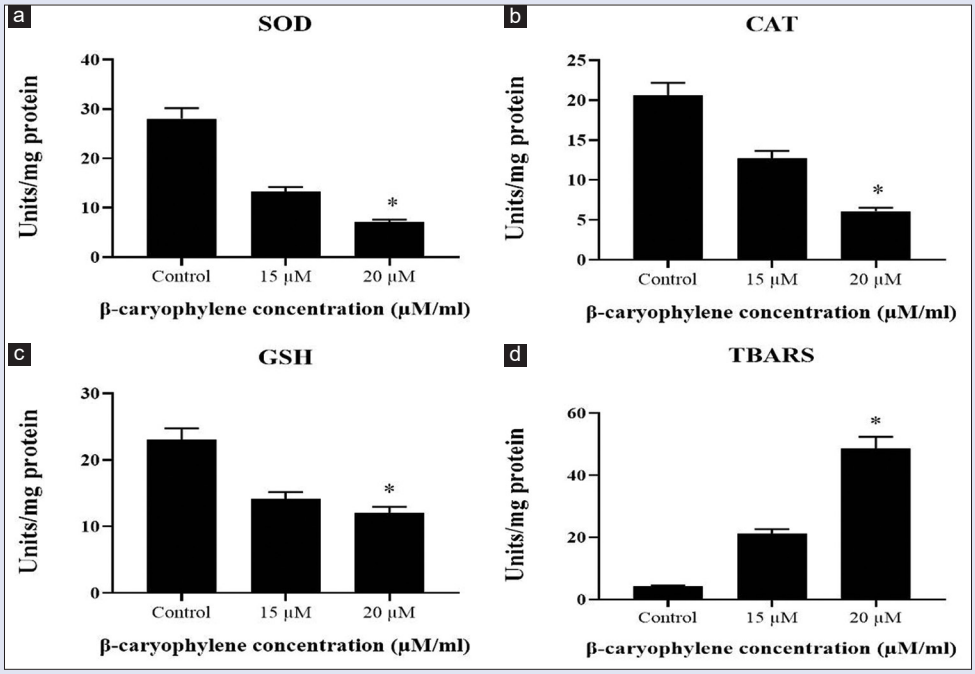

Figure 7: β-caryophyllene depletes antioxidants and induces lipid peroxidation in MOLT-4 cells. (a-c) Superoxide dismutase, catalase, and glutathione. (d) Lipid peroxidation status in MOLT-4 cells. Values are not sharing a common marking. (*) differ significantly at $P < 0.05$ (Duncan's multiple-range test)

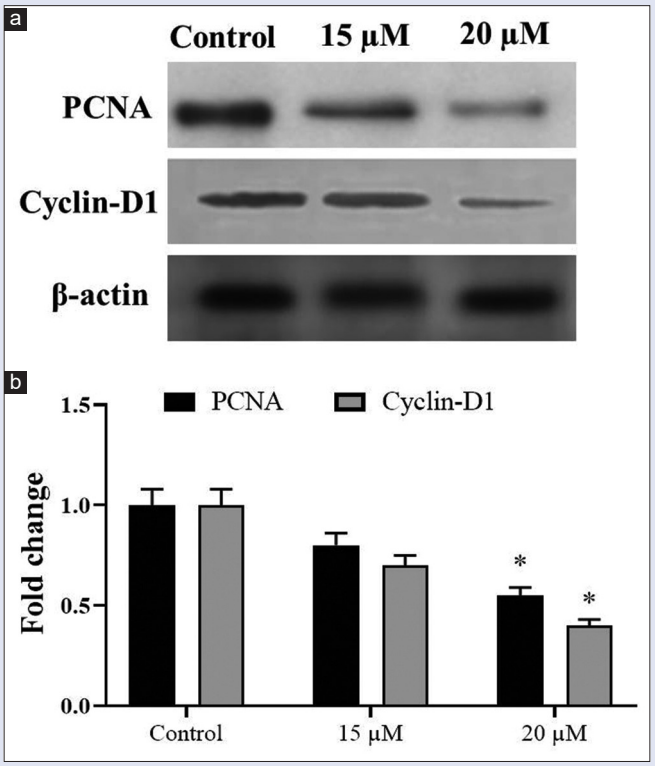

Figure 8: β-caryophyllene induces an apoptotic marker assessed by Western blotting. (a) Western blot markers for Bax and Bcl-2. (b) The interest of proteins was quantified by densitometry and normalized to respective β-actin loading control. *$P < 0.05$ was significantly different from the control sample

proteins were found be upregulated, whereas the level of Bax protein was found to be downregulated in control cells. In contrast to control cells, the effect of β-caryophyllene on MOLT-4 cells was found to be significant ($P < 0.05$). It increased the levels of Bax proteins and decreased the levels of Bcl-2, PCNA, and cyclin-D1 proteins. This shows that β-caryophyllene induced apoptosis and inhibited proliferation in MOLT-4 cells. Furthermore, β-caryophyllene decreased the enzyme activity of caspases 3, 8, and 9 in MOLT-4 cells [Figure 10]. In addition, β-caryophyllene treatment enhanced the enzyme activity of caspases 3, 8, and 9 in MOLT-4 cells.

Effect of β-caryophyllene on the expression of inflammatory markers in MOLT-4 cells

The expression of mRNA of inflammatory markers was analyzed by reverse transcription–polymerase chain reaction. Both the tested concentrations of β-caryophyllene (15 and 20 µM/mL) demonstrated significant ($P < 0.05$) downregulation in the mRNA expression levels of NF-κB, TNF-α, COX-2, and IL-6 when compared with the vehicle control [Figure 11].

DISCUSSION

β-Caryophyllene is a natural plant compound under the cannabinoid class and a part of bicyclic sesquiterpene.[21] However, the mechanism of action of sesquiterpenes in cancer treatment has not been studied much. β-Caryophyllene demonstrated excellent anticancer and antiproliferative activities; however, the mode of action and their optimum concentrations still remain unclear.[11] The previous docking report evidenced that the β-caryophyllene had superior binding affinity to NF-κB, PI3K, and AKT and potentially inhibited the expression range of inflammation and cell survival genes in cancer cell line. In general, plant-based agents induce DNA fragmentation and nuclear

damage by increasing oxidative stress in the cell.[22] A recent report of β-caryophyllene clearly demonstrated the method of toxicity in oral tumor by the initiation of ROS and activation of apoptotic pathway.[23] Similarly, in this study, β-caryophyllene demonstrated apoptotic activity by increasing the production of ROS in MOLT-4 cells. Dahham *et al.*[24] proposed that apoptosis was induced by β-caryophyllene after treatment of HCT cells. Usually in malignancy, the declined ROS causes decrease of free cytochrome-c in the mitochondria, which halt the apoptosis.[25] On the contrary, previous report of the β-caryophyllene established apoptosis by producing excess ROS and induced $\Delta\psi_m$ loss that released free cytochrome-c into the cytosol and induced the stimulation of caspase-8,-9-mediated caspase-3 in KB cells.[22] This agrees with our results that the significant loss of MMP in rhodamine-123 dye at 20 μM of β-β-caryophyllene treatment and ELISA results demonstrated the substantial activation ability of caspase-3-mediated 8 and 9, which explicated the occurrence of apoptosis through mitochondrial-mediated apoptotic pathway.

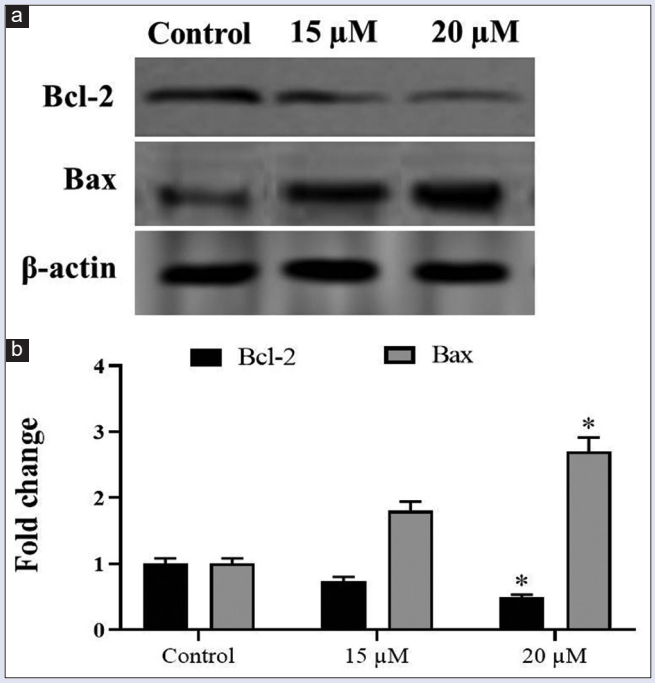

Figure 9: β-caryophyllene inhibits proliferative markers assessed by Western blotting. (a) Western blot markers for proliferating cell nuclear antigen and cyclin-D1. (b) The interest of proteins was quantified by densitometry and normalized to respective β-actin loading control. *P < 0.05 was significantly different from the control sample

Blebbing, shrinkage, condensation, and nuclear fragmentation are the characteristics of apoptosis.[26] In KB cells, the data of AO/EB staining methods confirmed that β-caryophyllene exhibited apoptosis in green, bright green, and orange colors, which denoted three stages of apoptosis event cell (alive, early, and late).[23,27] In AO/EB staining methods, AO emits green fluorescence represented by both live and dead cells, whereas EB emits red fluorescence and represent only dead cells. In this study, AO/EB staining showed early, late, and necrotic cell death and exhibited decrease in the percentage of cell survival and elevation in the percentage of apoptotic cells at 20 μM of β-caryophyllene against MOLT-4 cancer cells.

A previous *in vitro* study conducted on copaene, a sesquiterpene-type drug, showed reduced expression of antioxidant markers and decreased levels of TBARS in human lymphocyte cells.[28] In this study, β-caryophyllene (15 and 20 μM) showed high levels of TBARS and depleted levels of antioxidant markers such as SOD, CAT, and GSH than that of control cells.

PCNA is mostly present during all stages of the cell cycle which involves in the proliferation of tumor cells when it is over expressed. It is involved in the regulation of cell cycle.[29] In human glioblastoma U251 cells,

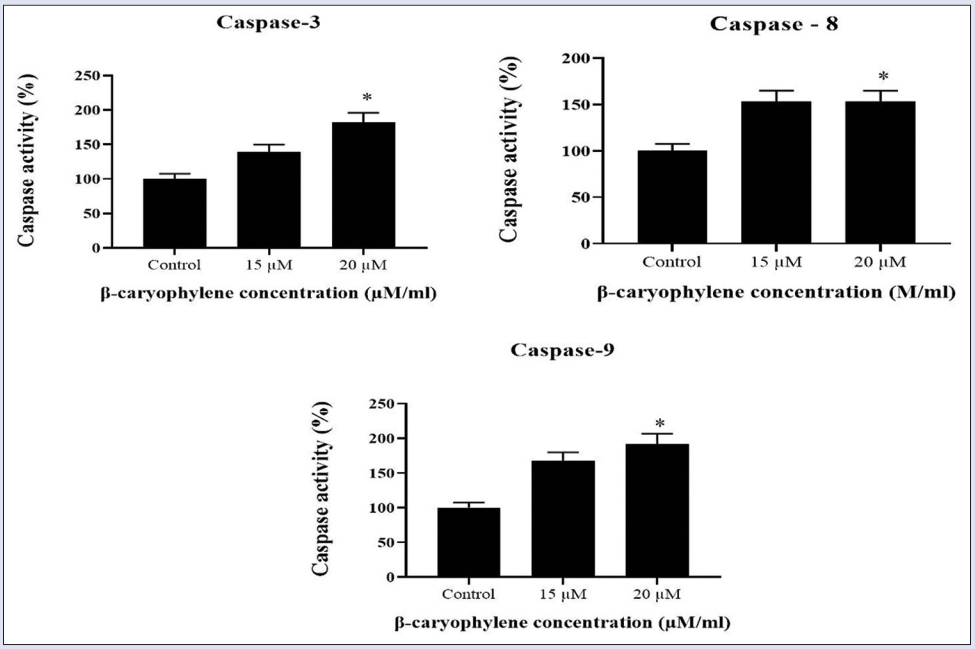

Figure 10: Colorimetric ELISA assay for caspase-8, -9, and -3 activity expression in β-caryophyllene-treated MOLT-4 cells. The statistical analysis was carried out using one-way analysis of variance. Values are represented mean ± standard deviation of three experiments. P < 0.05 was significantly different from the control sample. *P < 0.05 was significantly different from the control sample

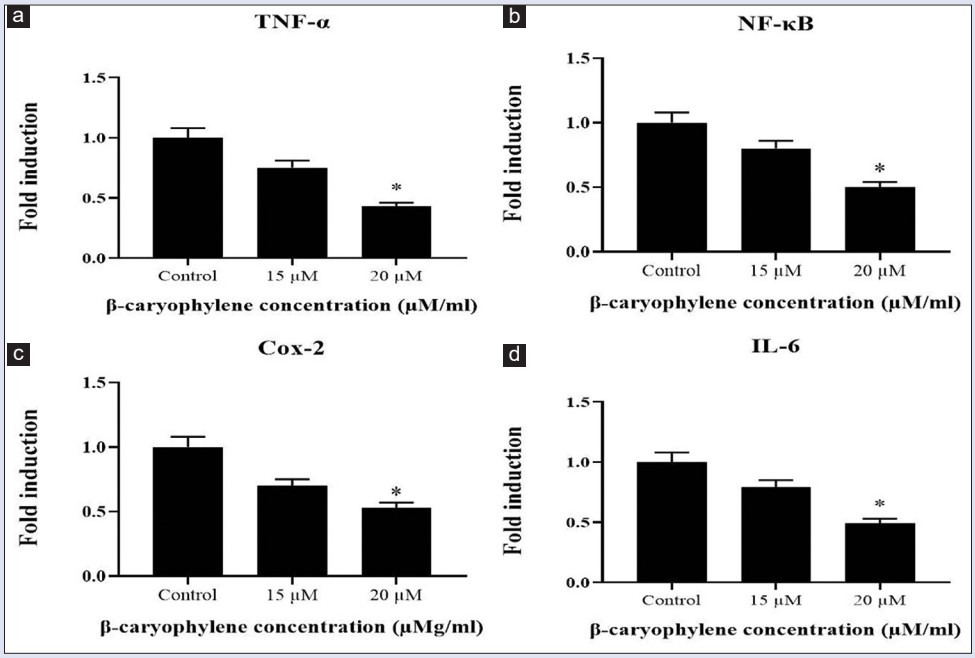

Figure 11: β-caryophyllene inhibits inflammatory markers assessed by reverse transcription–polymerase chain reaction. (a-d) mRNA expression of tumor necrosis factor-α, nuclear factor-kappa B, interleukin-6, and cyclooxygenase-2 was used and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. The statistical analysis was carried out using one-way analysis of variance. Values are represented mean ± standard deviation of three experiments. $P < 0.05$ was significantly different from the control sample. *$P < 0.05$ was significantly different from the control sample

sesquiterpenes were shown to express the antitumor activity by inhibiting the expression of PCNA and tubulin polymerization.[30,31]

Cyclin-D1 is known to be elevated in all types of malignancies and it is an essential regulator which controls the cells entry from G0/G1 to S phase.[32-35] The supporting and reported evidence of sesquiterpenes and β-caryophyllene completely reduced the manifestation of PCNA and cyclin-D1.[24,36,37] Similarly, this study showed reduced levels of PCNA and cyclin-D1 after incubating the cells with 20 µM β-caryophyllene, which suggests the antiproliferative effect of β-caryophyllene.

The upregulation of Bax and suppression of Bcl-2 modify the Bax/Bcl-2 ratio and allow membrane permeabilization in favor of apoptosis.[38] β-Caryophyllene strongly correlates and supports the previous evidence of β-caryophyllene, which downregulates the expression of Bcl-2 and upregulates the expression of Bax protein.[23]

The generation of cytokines in cancer is stimulated by the processes of inflammation and tumor initiation.[39] In this case, the increased production of transcription factor NF-κB, enhanced proliferation of cancer cells, and invasion in cancer cells. It controls the transcription of various inflammatory genes such as TNF-α, nitric oxide synthase (iNOS), COX-2, and IL-6, which prolongs the process of inflammation and cancer.[40,41] Therefore, these proteins are routinely detected in inflammation and in a variety of cancers.[42] β-Caryophyllene has been shown to be a strong inhibitor of inflammation[43] and a report of evodiamine showed significant blocking of NF-κB signaling that ultimately stimulates cell death in human melanoma cells.[44] In addition, the evidence of β-caryophyllene on human oral cancer KB cells significantly declined the levels of markers (TNF-α, iNOS, COX-2, and IL-6) that are involved in inflammation, which clearly indicated that the β-caryophyllene suppressed NF-κB signaling pathway activation.[23] This study coupled with previous studies shows that β-caryophyllene significantly reduced the transcription of NF-κB and also downregulated the expression of other inflammatory genes such as TNF-α, COX-2, and IL-6 in MOLT-4 cancer cells.

CONCLUSION

This study shows that β-caryophyllene induced cytotoxicity, increased ROS production, and increased apoptosis in MOLT-4 cells. In addition, it induced pro-apoptotic markers such as BAX, thereby inhibiting the enhancement of PCNA, cyclin-D1, and Bcl-2 expressions in MOLT-4 cells. In addition, β-caryophyllene suppressed the inflammatory biomarkers in MOLT-4 cells. In summary, β-caryophyllene inhibited the proliferation of leukemia via suppression of proliferation and inflammation.

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Conflicts of interest

There are no conflicts of interest.

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