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Abstract

The aim of this work was to identify the anticancer activity of Caryophyllene oxide (CPO) against MCF7 cell lines. CPO as phytochemical terpenoids is a chemoprevention derivative enhances breast cancer growth inhibition and proliferation. The cytotoxicity of CPO against breast cancer cells (MCF-7) was concentration dependent and the inhibitory concentration **(IC_{50})** of CPO was 24 µM/ ml. Also, toxicity of CPO was accompanied with the arrest of cell cycle significantly at the phase (G2/M) in compare with control. Also, the MCF-7 cells apoptotic profile showed a significant elevated **Early** and **Late** apoptosis and necrosis as well compared with control. In the meantime, DNA fragmentation detected via conduction of comet assay indicate elevated the percent of tail moment, tail DNA, and tail length versus control DNA fragmentation profile. In conclusion, Caryophyllene oxide is one of the most promising anticancer agents for breast cancer in worldwide.

1. Introduction

Cancer poses a major risk as a health problem in the United States and many other parts of the world. Recently, in the United States one in 4 deaths is due to cancer.[1] Cancer incidence will increase by 58%, from 84,703 cases in 2015 to 133,937 cases in 2035 and also cancer mortality will increase by 67% during this period, from 52,282 to 87,430 deaths according to the World Health Organization (WHO).[2] Natural chemoprevention is a promising approach in the prevention of cancer.

Terpenoids are organic chemicals that are naturally produced by many plants, including cannabis plants. An effective anticancer drugs which were discovered from natural plants plays an important role in chemotherapy.[3] caryophyllene oxide (CPO) is a sesquiterpene isolated from essential oils of medicinal plants such as clove (Eugenia caryophyllata), black pepper (Piper nigrum L.) , oregano (Origanumvulgare L.), guava (Psidiumguajava) and cinnamon (Cinnamomum spp.) ,and act as a strong anticancer agent also can stop the proliferation of a wide variety of tumor cells, consisting of breast ,lung ,liver, and prostate adenocarcinoma as reported in several studies.[4,5]

In vitro study, several studies have elucidated that Caryophyllene oxide(CPO) can exert its anticancer effects by inhibiting the activity of JNK, ERK and p38 MAPK in tumor cells; and can also inhibit constitutive activation of signaling cascade as(PI3K/AKT/mTOR/S6K1).[6] CPO is also known to have important effects on several diseases including cancer. Preventive effects of cancer related to CPO on metabolic pathways via induction of apoptosis and modulation of cellular signal transduction, Antiproliferation , transformation and promotion of cell cycle arrest, as well as tumor invasion and/or metastasis, inhibition of angiogenesis have been demonstrated in many vitro.[7] CPO showed cytotoxic effect on various cancer cell lines, such as HeLa , HepG2 , AGS , SNU- 1 , SNU- 16 and A549. The mechanism underlying the effect of CPO on breast cancer cells (MCF7) has not been fully explained yet. Therefore, aim of the work to identify the antitumor activity of CPO against MCF7 cell lines.

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Material and Methods

All reagents have been purchased from Sigma-Aldrich Company Ltd. in (United Kingdom, Gillingham). Caryophyllene oxide (CPO) was prepared as a working solution as 10µM in dimethyl sulfoxide (DMSO), supplied from ICI-UK.

Cell lines:

Breast cell lines (MCF7) were obtained from VACSERA-Egypt specially the cell culture department. MCF7 cells were imported from the American type Culture Collection (ATCC) in the form of frozen ampoule with the reference number HTB-22. MCF7 cells were cultured in 75-cm^3 tissue culture flasks [Griener-Germany] using the reagent called (RPMI-1640) with 10% (FBS) fetal bovine serum as a growth medium. Cells were grown in 5% CO_2 in air-humidified incubator at 37°C(Jouan-France). The medium was replaced every 3 days, and cells were passaged when they were ~80% confluent. Trypsin (0.25%) and Fetal Bovine serum (FBS) and RPMI-1640 were kindly supplied.

Cytotoxic activity

Cancer cell line was maintained according to the manufacturer protocol and cell count was maintained and cell count was optimized as 2x105 / ml. Cells were dispensed to 96 well plates (TPP, Swiss). Cells were incubated till confluency at 37oC (Jouan, France). Growth medium was decanted from and TC plates. Test drug was 2fold serially diluted and treatment medium was applied for 24 hrs. Cytotoxic effect was detected using inverted microscope (Hund –Germany). Using sterile phosphate buffer saline (PBS), detached cells were washed. The remaining living cells were stained using MTT stain 0.5mg/ ml. Stained plates are incubated for 4 hrs at 37^{o}C. Developed MTT formazan crystals were dissolved using DMSO (ICI-UK). Developed color was read at 570 nm using ELISA reader (ELX-800 Biotek –USA). Cell viability was determined according to the following equation

$$\text{Viab \%} = \frac{\text{Mean OD of test dilution X100}}{\text{Mean OD of cell control}}$$

IC50 values were determined using Master plex 2010 soft ware

Flow cytometric analysis

Analysis of Flow cytometric was measured with the use of FACS Caliber Flow Cytometer (USA, CA, Sunnyvale, Becton Dickinson) with equipping a compact air-cooledlow- ion laser beam (488 nm) with power 15 mW argon. As described previously, Cell cycle analysis was performed [8]. Briefly, after sample preparation, the cells were incubated with concentration (200 µL) of solution consists of200 µg/ml RNase A (Invitrogen Biotechnology, Carlsbad, CA, USA) 20 µg/ml of PI (Sigma, St. Louis, MO, USA) and 0.1%Triton v/v in PBS. After incubation in dark for 30 min, flow cytometric analysis was performed, and samples were analyzed. Results are showed as a percentage of cells in G2/M, G0/G1 or S phases. [9]

Apoptosis detection

The apoptosis detection Kit dependent on observation of apoptotic cells in different phases induced post apoptosis initiation, treated cell lines transfer the membrane [PS] phosphatidylserine from inner face of plasma membrane to cell surface. PS can be easily detected by fluorescent on the cell surface which conjugated with Annexin V, a protein highly affinity for PS. The staining step takes only 10 minutes. Analyzed detection can be measured by fluorescence microscopy or by flow cytometry. When performing both Annexin V-FITC and Propidium Iodide [PI] staining, the kit can differentiate between apoptosis and necrosis.

Comet assay (Single cell gel electrophoresis):

As previously demonstrated, the evaluation of damage in DNA using the single-cell gel electrophoresis was performed in CPO treated MCF-7 .[10] Using Comet Assay II automatic digital analysis system, one hundred cells of each group were analyzed. Quantification of DNA damage in obtained these images were measured with the CASP software to directly obtain a percent of tail moment, DNA in tail and tail length.

Analysis of Statistical

The results are showed the mean ± SD. The significance in Statistical was calculated using the one-way analysis (ANOVA) and Student–Newman–Keulsposthoc tests for different comparisons. All analyses were measured with SPSS.

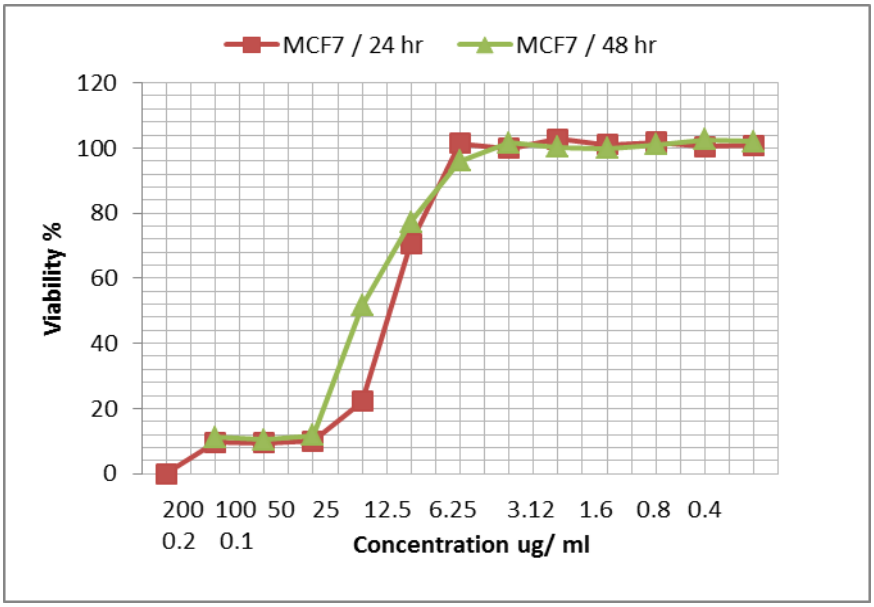
Results

Cytotoxic activity

The cytotoxic effects of CPO on MCF7 cell line‘s viability was measured by using MTT assay. The Data obtained illustrated the percentage of mean viability of CPO treated cells was dependent on concentration. IC_{50} value of CPO was 24.1uM/ml. In the

meantime, Flow cytometric analysis demonstrated that the DNA accumulation (Figure 2a). Cell cycle phase distribution analysis showed a significant inhibitory effect of CPO on cell cycle phases compared with the untreated group (Figure 2b). The CPO treated group showed the arrest of cell cycle at G1 phase with increasing the number of apoptotic cells at sub-G1 phase significantly compared with untreated group.

Fig. (1): Evaluation of viability % of MCF7 cells post treatment with different concentrations of caryophylleneoxide for 24&48 hours.



2.Cell cycle analysis

The use of flow cytometry was demonstrated whether induced inhibition in the cell cycle progression in MCF7 cell line due to CPO (Figure 2a). Cell cycle phase distribution analysis showed a significant inhibitory the effect of CPO on the cell cycle phases of the MCF7 obtained from treated group compared with the untreated group (Figure 2b). The CPO treated group showed the arrest in the cell cycle at G2/M phase by a significant way ($p<0.001$) compared with its value in cell control group (Figure 2b)

Figure (2, a) Effect of Caryophyllene oxide on the cell cycle progress and apoptosis with MCF7 cells. Data are expressed as percentage of the cell cycle in the sub-G1, G0/G1, S, G2/M phases of the cell cycle. The CPO induced the arrest of cell cycle in G2/M phase. Values are mean ± SD, * Significant different ($p<0.001$) from the MCF7 control group**

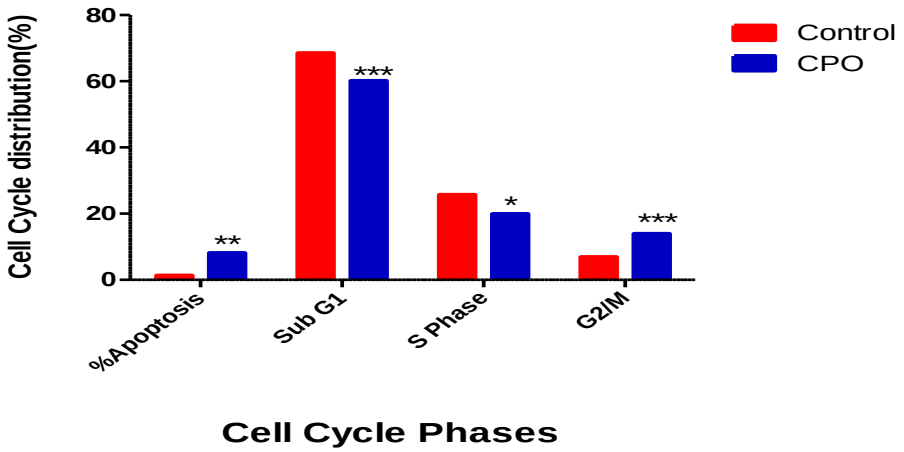
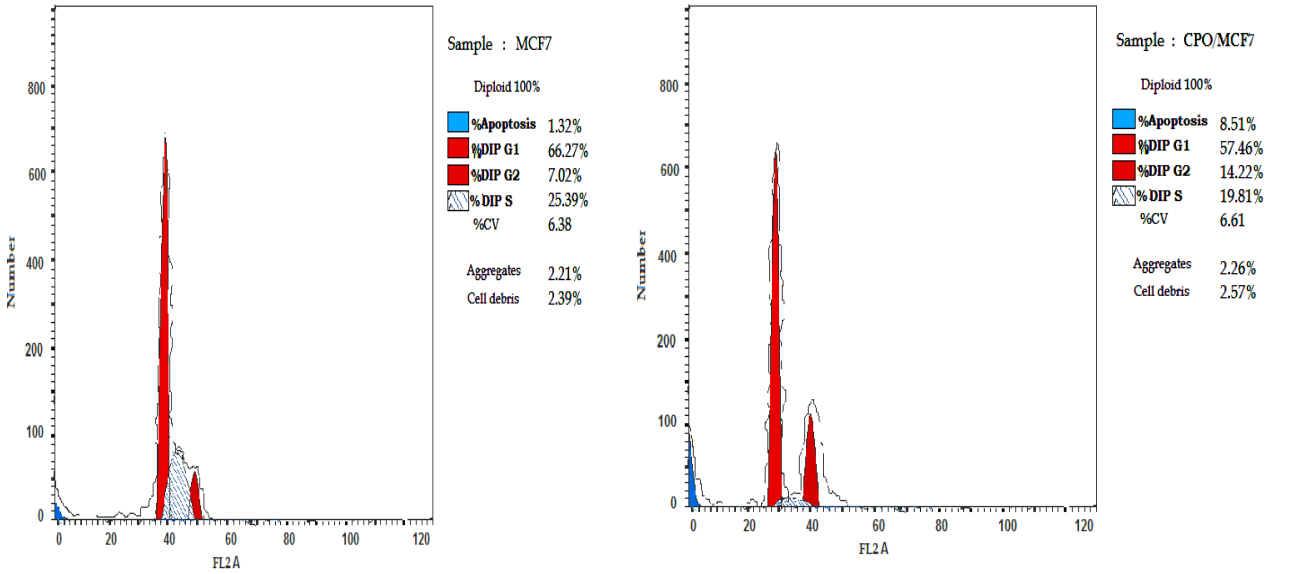


Figure (2, b): Flow cytometric histogram of cell cycle phases in MCF7 cells.



Annexin V-FITC Apoptosis

There was a significant in the apoptotic profile of treated cells [P<0.01] increased late and early apoptosis compared with untreated cell control. Also, the necrotic potential was significant [P<0.05] elevated than in case of cell control as well [Figure3-a].

Figure (3-a): Effect of caryophyllene oxide on phases of MCF7 apoptosis. The values are demonstrated the percentage of the cell phases in the viable, early, late apoptosis and necrotic stages compared with from the MCF7 control group.

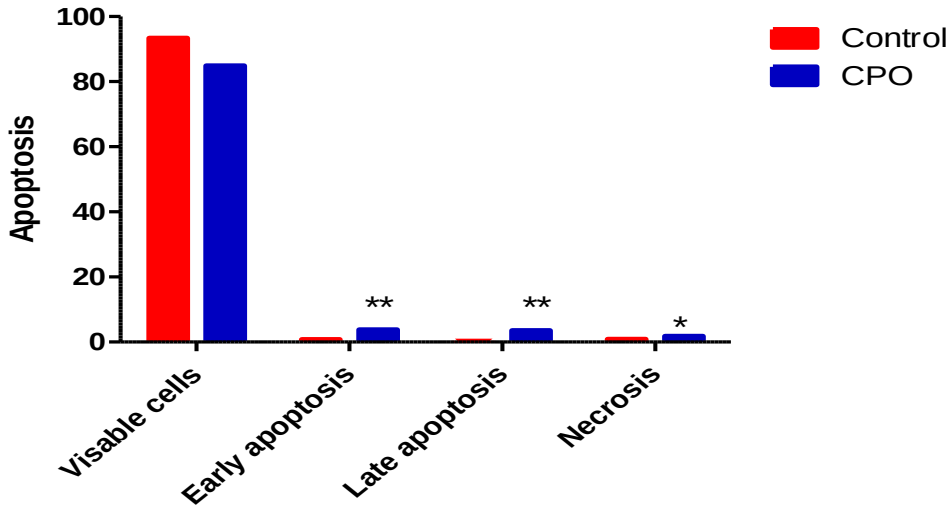
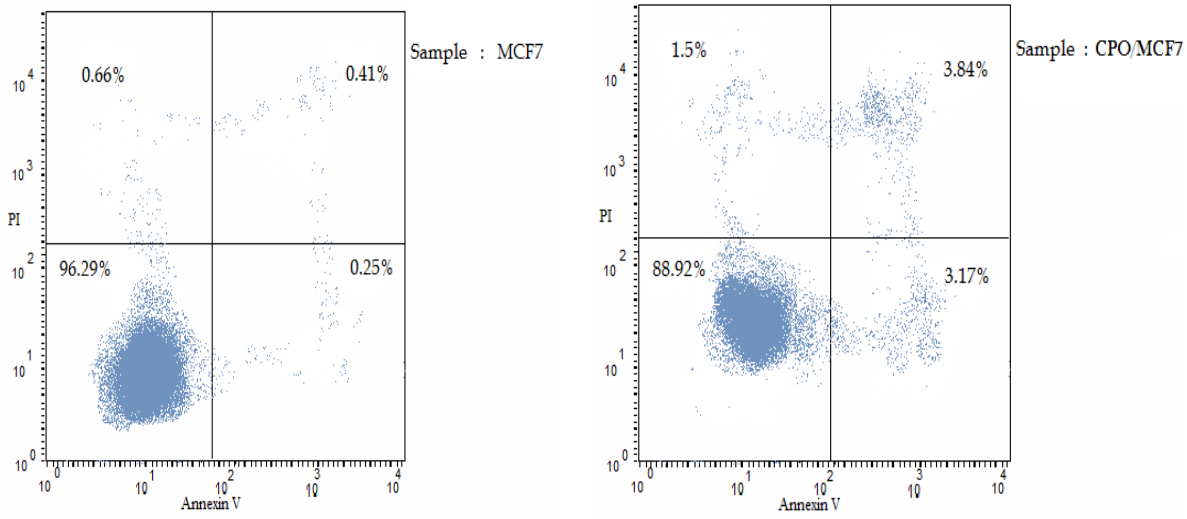


Figure (3, b): Percentage of apoptosis for control cells and PCO treated MCF7 cells using Annexin V- FITC



Regarding the comet assay data, it was found a significant in tail length, content of DNA in tail area, tail moment in compare with the DNA in tail region, tail length and tail moment of control untreated cells [Figure .4]

Figure (4-a): CPO effect of on the percentage DNA damage with MCF7 in the three groups by using the comet assay which detects the DNA fragmentation. The three Comet parameters include the Percentage of tail moment, tail length and DNA in tail. Microscopic images of representative comets for the different groups are shown. Values are mean ± SD ***P < 0.001, *P < 0.05, **P < 0.01vs control.

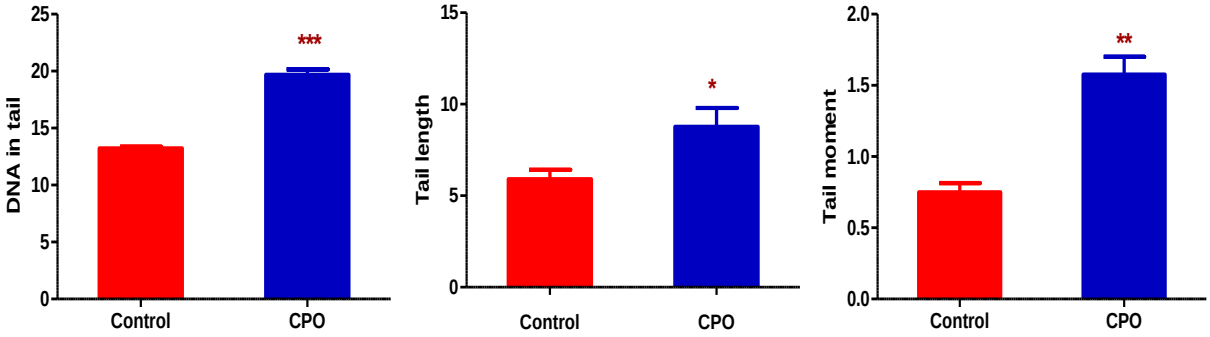
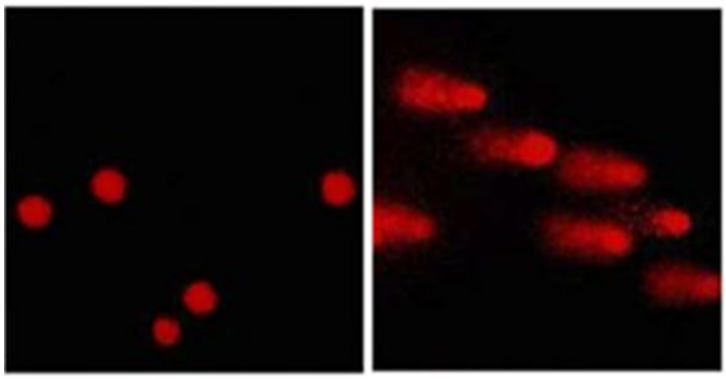


Figure (4-b):-Evaluation of DNA fragmentation using comet assay compared with untreated cell control.



A-Control **B-CPO treated cells**

Discussion

Nature chemoprevention has an effective role all over the world in the treatment and prevention of serious human diseases for thousands of years. More than 60% of recently used anticancer agents are derived in one way or another from natural sources.[15] The biological activities of CPO have been ascribed in many studies. Particularly, it seems to have an antioxidant capacity [11], anti-leishmaniasis activity [12] , Stimulate apoptosis in tumor cells [13] and the effect of cytotoxicity has been demonstrated against cancer cells .[14] In this study, the anticancer effect of CPO on MCF7 was demonstrated. Tests were carried out on proliferation of a panel of human cancer and normal cell lines on the CPO for its inhibitory effect.[7] In the present study the cytotoxic activity of CPO was proved and our results was in accordance with the report of [16] reporting that CPO was used against several cancer cell lines of human by using MTT assay. Compared with previous studies, the results of this study showed that CPO has an excellent activity against cancer cell lines of humans. It has been found that cytotoxicity of CPO is dependent on time and dose.[14]

The current study demonstrated that CPO was effective in inducing the growth's inhibition, cell cycle deregulation and apoptosis. The data revealed that cell cycle arrest in G0/G1 phase was significant, with increasing in the percentage of apoptotic MCF7 cells after CPO treatment. Therefore, CPO-induced the inhibition of MCF7 cell proliferation could be linked with arrest at G0/G1 phase in the cell cycle Progress. The finding indicates that CPO reduced cell proliferation, confirming the antitumor effects reported using essential oils breast cancer.

The bioactive CPO can prevent tumor development not only through scavenging free radicals but also by inducing pro-oxidative effects on biomolecules including DNA in the cell. These data were further investigated utilizing comet assay. The comet parameters indicated a significant effect of CPO via stimulation of MCF7 cell death signals, represented as a marked percentage increase in tail cells and tail length of treated group. This indicates a potential cytotoxic effect of CPO on genomic integrity of MCF7cells.

Conclusion:

It can be concluded that CPO has anticancer activity via inhibition of cell proliferation, Incite cell arrest/ DNA distribution at pre-G1 and G2/M phases. This arrest proved via significant DNA fragmentation marked via tail length, and DNA content in tail length and moment

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