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Anticancer Activity of New Synthetic 4-HYDROXYPROPIOPHENONEon MCF-7 Breast Cancer Cell Lines

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ABSTRACT

Breast cancer is the serious health concern in India causing the highest mortality rate in females, which occurs due to uncontrolled cell division and can be metastasize to other parts of the human body.Natural products are important leads in drug discovery. The search for effective plant-derived anticancer agents or their synthetic analogues has continued to be of interest to biologists and chemists for a long time. We investigated the, cytotoxicity and anticancer activity of new synthetic 4-Hydroxypropiophenone (4-HPPP) was tested against breast cancer cell line MCF-7. Cytotoxicity was examined using MTT assay. The ability to induce apoptosis and changes in mitochondrial membrane potential,levels of reactive oxygen species and comet Assay.Based on the results, we determined the effective dose of 4-HPPP as 100µM/ml for 24 hr. Cytotoxicity effects of 4-HPPP was confirmed by treatment of MCF-7cell with IC50 concentration of 4-HPPP resulted in sequences of events marked by the enhance the apoptosis accompanied by loss of cell viability, modulation of reactive oxygen species and cell cycle arrest through the induction of G0/G1 phase arrest on MCF-7cells. These findings show that the synthetic 4-HPPP, displays potential to be further explored in the development of new anticancer agents.

Keywords: 4-HPPP, Apoptosis, Mitochondria, MCF-7 cell, Reactive oxygen species and comet Assay

INTRODUCTION

Cancer is a multi-step disease incorporating physical, environmental, metabolic, chemical and genetic factors (Rahman*et al*., 2011). Breast cancer is one of the most recurring worldwide diagnosed and deadliest cancers next to lung cancer with a high number of mortality rates among females (Benson and Jatoi, 2012). At global level, it accounted for more than 1.6 million new cases in 2010. The incidence or prevalence rate of the breast cancer in India is expected to be more than 90,000 in the coming years and over 50,000 women die each year (Parveen Kumara *et al*., 2013). The anticancer drugs previously used for breast cancer cells exhibited relatively high toxicity not only to the tumour cells, but also to the normal cells of the body part in which the cancer had developed. The limited success of clinical therapies including radiation, chemotherapy, immunomodulation and surgery in treating cancer, as evident by the high morbidity and mortality rates, which also can produce severe side effects such as bone marrow depression, lecukopenia, anaemia, alopecia, and hyperuricaemia, teratogenicity, carcinogenicity, and also its reduced spermatogensis in men, amenoria in women because of this indication there is an imperative need of new cancer management. Throughout history, plants have always been an excellent source of pharmaceutical agents used in traditional medicine. More recently, a big number of important drugs have been obtained from plants, either directly, by extracting an active component or, more often, by structural modifications of natural compounds or by the synthesis of their analogues with improved pharmacological properties. Many of the compounds used for cancer chemotherapy, such as the Vinca alkaloids, paclitaxel, camtothecin and etoposide, were originally derived from plants (Cragg and Newman, 2005), and plants continue to be viewed as major sources for the development of new anticancer drugs. Anethole, also known as isoestragole occurs in nature as both *cis*and *trans*forms, wherein *trans*-isomer being more abundant (Zondek and Bergmann, 1938). Anethole is a major component of several essential oils, including anise seed oil (80-90%), star anise oil (90%) and sweet fennel oil (80%). The total global production of *trans*-anethole is approximately 0.75 million metric tone per annum (Svadkovskaya*et al*., 1970). Increasing demand for anethole led to development of new synthetic routes other than isolation from essential oils. Some derivatives of trans-anethole [1-methoxy-4-(1-propenyl)-benzene] were synthesized by introducing hydroxyl groups in the double bond of the propenyl moiety (Freire*et al*., 2005). 4-HPPP is a phenolic compound derived from spices possesses potent anticarcinogenic activities (Buu-Hot NGPH et al., 1952). It possesses a wide variety of biological activities, especially as antioxidants **(**Hemalatha .S and Kiresee Saghana P.R, 2015**).**To our knowledge there are no scientific reports available on the literature for in vitro inhibition of growth and induction of apoptosis in human breast cancer cell line MCF-7 by 4-HPPP. However, in the present study, we evaluated the effect of 4-HPPP on inhibition of growth induction and apoptosis in MCF-7 cancer cells. In vitro anticancer evaluation is universal model for evaluation of cancer activity. In vitro methods have an edge over come in vivo methods since they are less time consuming, more cost effective, large number of compounds can be tested with small amount of sample, easier to manage. Because of these advantages, the in vitro methods have been preferred to assess the anticancer activity (Narayanan *et al*., 1998). To our knowledge there are no scientific reports available on the literature for in vitro inhibition of growth and induction of apoptosis in human

breast cancer cell line MCF-7 by 4-HPPP. However, in the present study, we evaluated the effect of 4-HPPP on inhibition of growth induction and apoptosis in MCF-7 cancer cells.

2. MATERIALS AND METHODS

Cell culture and drug treatment

Human breast carcinoma cell line (MCF-7) used in our study was obtained from National Centre for Cell Science (NCCS), Pune, India. Cells were cultured in DMEM with high glucose supplemented with 10% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin, in 5% CO2 incubator at 37°C. Cells were treated with different concentrations of 4-HPPP (1000, 500, 250, 125, 62.5, 31.25,…….. and 0.97µM/ml) and the cytotoxicity was observed by MTT (3-(4,5-dimethyl-2- thiaozolyl)-2,5-diphenyl-2H tetrazolium bromide) assay. IC50 values were calculated and the effective dose was used for further study.

Determination of effective dose of sinapic acid (MTT assay)

In order to determine optimal cell number required for the assay, serial dilutions of MCF-7 (2,000 cells/100 µl) were taken into a 96 well plate. Then the cells were pretreated with different concentration of drug (1000, 500. 250, 125, 62.5, 31.25,…….. and 0.97µM). Then the cells were incubated at 5% CO2 and 95% O2 environment at 37 ºC for 24 h. MTT (0.5 mg/ml) was added to the incubated cells and then further incubated for another 4 h. After that medium containing MTT was gently replaced by 200 µl DMSO to dissolve formazan crystals and the absorbance was measured in a microplate reader at 540 nm.

Determination of Reactive Oxygen Species

2’7′Dichlorodihydrofluorescein diacetate (DCFH-DA) is the cell permeable fluorescent and chemiluminescent probe used to direct measurement of the intracellular ROS levels. DCFH-DA is a nonpolar dye, converted into the polar derivative DCFH by cellular esterases that are nonfluorescent but switched to highly fluorescent DCF when oxidized by intracellular ROS and other peroxides (Hafer*et al.*, 2008). An aliquot of the isolated cells (8x106 cells/ml) were made up to a final volume of 2 ml in PBS (pH 7.4). Then, 1 ml cell suspension were taken, to which 10 µl DCFH-DA (10 µM) was added and incubated at 37 °C for 30 min. Then cells were incubated for 30 min in 6 well plates with 10 µM/ml of DCFH-DA in PBS. Finally, cells were

washed thrice with PBS and the fluorescence intensity was recorded using spectrofluorometer and the images were captured using fluorescence microscope (460 nm).

Mitochondrial membrane potential (ΔΨm)

Rhodamine 123 (Rh-123) is a lipophilic cationic dye, highly specific for mitochondria. Rh 123 uptake into mitochondria is driven by mitochondrial membrane potential that allows the determination of cell population with active integrated mitochondrial functions. Loss of ΔΨm leads to depolarization of mitochondrial membranes leading to collapse of mitochondrial functions ensurimg cell death. ΔΨm measurement was carried out essentially as described by Scaduto and Grotyohann, (1999). To the treated and control cells, 1µl of rhodamine-123 (5 m Mol) was added and kept in the incubator for 30 min. The cells were then washed with PBS and observed with a fluorescence microscope (450-490 nm). Polarized mitochondria emit orange-red fluorescence and depolarized mitochondria emit green fluorescence.

Apoptosis studies with AO/EB staining method

The ethidium bromide/acridine orange stain (EBr/AO stain) is a viability stain that detects apoptotic cells. Ethidium bromide is a dye that is only able to pass through the membrane of a dead or dying cell. Acridine orange is a membrane permeable dye that stains all the cells. Each dye that is taken up by a cell fluoresces AO makes a cell green, and EB makes a cell red (Baskic*et al*, 2002). Apoptotic studies were performed with a staining method utilizing AO and EBr according to the method of Lakshmi *et al*, (2008). The MCF-7 cells were grown in 6 well plates for 24h, and then treated with IC50 concentration of 4-HPPP were incubated in CO2 incubator for 48h, then the cells were trypsinized and stained with 1:1 ratio of AO/EBr. Stained cells were immediately washed again with PBS and viewed under a fluorescence microscope using blue filter with a magnification of both 20X and 40X

Evaluation of DNA damage by single cell gel electrophoresis (comet assay)

Single cell gel electrophoresis (SCGE), or comet assay, used to estimate DNA damage at the single cell level by the method of Singhet al., 1988. Frosted slides were prepared by pouring 3-5 ml of 1% normal agarose over frosted glass slides (Gold Coin Microslides, Blue Label Scientifics). It was allowed to dry at room temperature and placed in hot-air oven at 70-80 °C for 30 min.

Freshly suspended cells (50 µl) were mixed with 200 µl in 0.8% low-melting point agarose (LMPA) was cast on to frosted microscopic slides, kept for 10 min in ice box to solidify. The cells were then lysed by immersing the slides in the lysis buffer for 1 h at 4 °C. After lysis, slides were placed in a horizontal electrophoresis tank. The unit was filled with alkaline electrophoresis buffer to a level of 0.25 cm above the slides. The cells were exposed to the alkaline electrophoresis buffer for 30 min to allow DNA unwinding. Electrophoresis was conducted in a cold condition for 20 min at 25 V or 300 mA. After electrophoresis, the slides were placed horizontally and neutralised with Tris-HCl buffer. Finally, 50 µl of ethidium bromide was added to each slide and analysed using a fluorescence microscope. To prevent additional DNA damage, all steps were conducted under dim light or in the dark, with 40x magnification in a fluorescence microscope connected to a computer-based image analysis system. Images were captured and analyzed by image analysis software, CASP (Koncaet *al*., 2003).

Statistical analysis

Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) by using statistical package of social science (SPSS) version 12.0 for windows. The values are mean ± S.D. for six samples in each group. *p* values <0.05 were considered as level of significance.

RESULTS

Determination of IC50 concentration using MTT assay

The cytotoxic effect of 4-HPPP was examined on cultured MCF-7 cancer cells by exposing the cells to (1000, 500. 250, 125, 62.5, 31.25,…….. and 0.97µM) concentrations for 24 hr. The graph was plotted as percentage of inhibition on (X-axis) against the concentration of 4-HPPP (Y-axis). The IC50 value was determined by the concentration of the drug which reduces the absorbance to half that of the control and the cell viability was depicted in Figure. 1. 4-HPPP can effectively inhibit the viability of MCF-7 cell lines in a dose-dependent manner. The IC50 values of 4-HPPP were 100 µg/ml of 4-HPPP posses 50% cell vaiability for 24 hr. obviously; 4-HPPP at a concentration of 100µM/ml for 24hr shows higher cytotoxic activity. These results suggest that 4-HPPP can effectively inhibit the proliferation of the MCF-7 cells.

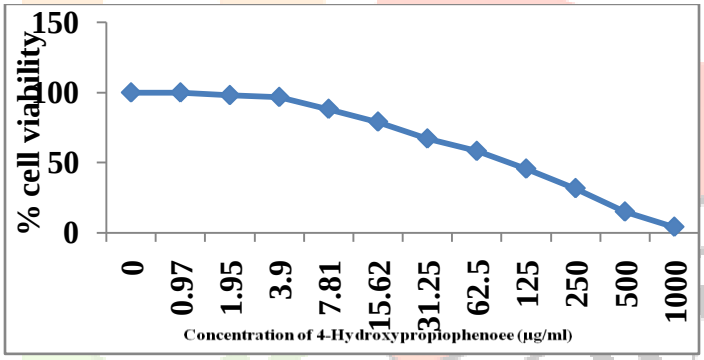

Figure 1. MTT Assay on 4-HPPP**treated MCF-7 cells.** MCF-7cells were exposed to various concentrations of 4-HPPP(1000, 500. 250, 125, 62.5, 31.25,…….. and 0.97µM). The cytotoxicity activity was measured by MTT assay and IC50 values were calculated 100µM/ml for 24hr

Effect of 4-HPPP on MCF-7 cell lines in ROS levels by fluorescence microscope

The effect of 4-HPPP on ROS production in MCF-7 by using DCFH-DA in flurometrically. Figure 2 shows the fluorescence intensity of DCFH-DA in MCF-7 cells after exposure to 4-HPPP IC50 concentrations for 24hr, and the bar diagram of the fluorescence intensity of ROS production in control, DMSO and 4-HPPP treated MCF-7 cells. The intracellular ROS production was significantly higher in 4-HPPP treated cells, when compared to the control and DMSO alone treated cells.

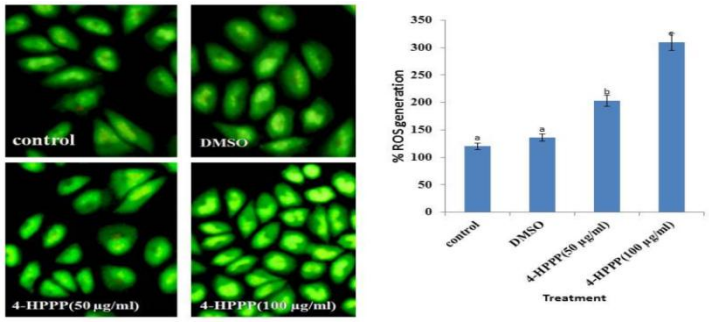

Figure 2. Effect of 4-HPPP on ROS generation observed by Spectrofluorometric readings of DCF fluorescence in MCF-7 cells. Values are given as means ± SD of six experiments in each group. Values not sharing a common superscript differ significantly at $P<0.05$ (DMRT).

Effect of 4-HPPP on MCF-7 cell lines in mitochondrial membrane potential

Mitochondrial membrane potential changes, as measured by Rh-123 fluorescence in different treatment groups, are shown in 3. Mitochondrial membrane potential was reduced significantly in the 4-HPPP treated group compared to control and DMSO. Fluorescence microscope image, show accumulation of Rh-123 dye in the control and the accumulation was decreased in 4-HPPP treated groups as the membrane potential decreased. 4-HPPP treated cells showed more decrease in MMP compared to control and DMSO.

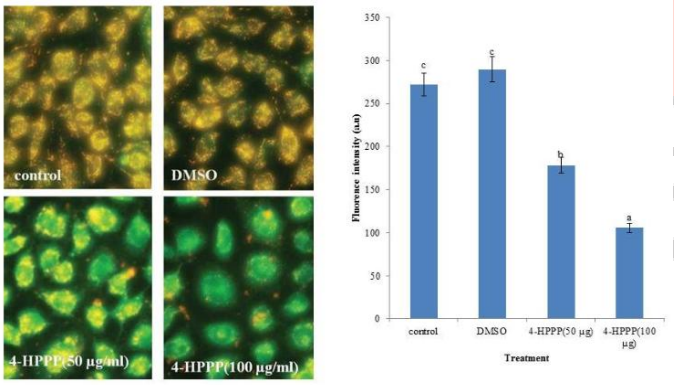

Figure 3. Fluorescent microscopy images of control cells and cells treated with different concentration of 4-HPPP for 2 h at 37◦C. Yellow-orange fluorescence is present in the cell areas with high mitochondrial membrane potential, while green fluorescence in the cell areas with low mitochondrial membrane potential. The stained cells were visualized under an inverted fluorescence microscope.

Effect of 4-HPPP on MCF-7 cell lines in Apoptosis assay with AO/EB staining method

AO/EB staining was done on MCF-7 cells treated with IC50 concentration of 4-HPPP for 24 hr were shown in figure 4. acridine orange (AO) and ethidium bromide (EtBr) to differentiate cells that are apoptotic and/or viable. 4-HPPP treated cells showed condensed nuclei, membrane blubbing and apoptotic bodies (EtBr stained cells). In contrast, the control cells (AO stained cells) also showed apoptosis. 4-HPPP treated cells showed increase percentage of apoptotic cells when compared to control and DMSO.

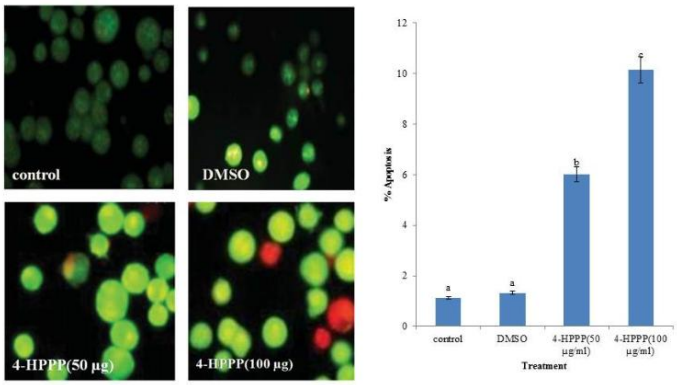

Figure 4. Cellular morphological changes were observed under a fluorescence microscope using AO/EtBr staining (20x). 4-HPPP treatment increased frequency of apoptotic cells in a dose dependent manner. Green colored nuclei indicate live cells. A condensed or fragmented nucleus with green or orange-red color indicates apoptosis and even nucleated red color indicates necrosis. Different dose of drug induced apoptosis but not necrosis in cancer cells.

Effect of 4-HPPP on MCF-7 cell lines in Commet Assay

The comet assay has been of particular advantage in assessing DNA damage. It shows the levels of comet attributes in control, DMSO and/or 4-HPPP treated MCF-7 cells. 4-HPPP (50 and 100 µg/ml) treatment significantly increases the levels of DNA damage in a concentration dependant manner. Fluorescence microphotographs show diminished comet tail in control and DMSO treated whereas 4-HPPP treated cells showed distinct comet formation.

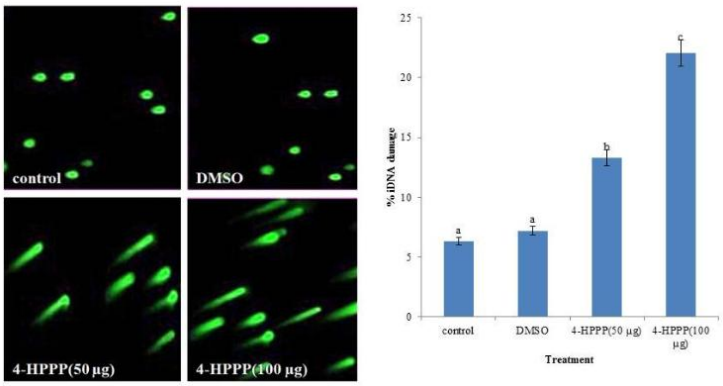

Figure 5.Effect of drug on DNA damage MCF-7cells. Values are given as means ± SD in each group. Values not sharing a common superscript differ significantly at *P*< 0.05. Fluorescence microscopic images of oxidative DNA damage (comet assay)

DISCUSSION

The results of cell line study suggest that 4-HPPP induces apoptosis in cancer cells by analysis of apoptotic factors. Nature is a phenomenal repository of an amazing array of structurally and functionally diverse organic compounds which are useful as drugs, fine chemicals, fragrances, flavors and biologically active dietary constituents (Treben,1986;Harborne and Dey, 1989; Albert, 1996; Clardy and Walsh, 2004). 4-HPPP is synthesized phenylpropanoid compound of trans-anethole which is major components of the essential oil of

fennel (*Foeniculumvulgare miller*), a member of the parsley plant family (Tanira*et al.*, 1996). The apoptosis inducing effect of 4-HPPP was confirmed by ROS, MMP and double AO/EtBr staining assays and comet assay. Our results revealed that 4-HPPP is more potent to inhibit the cell proliferation and induce apoptotic cell death in MCF-7human breast carcinoma cell line. In the present study, cytotoxic potential of 4-HPPP on MCF-7 cell lines using the MTT assay. The results of the MTT assay conclude that, the 4-HPPP caused cytotoxicity in a dose and time dependent manner, the IC_{50} values obtained after 24 hr treatment of different concentrations of 4-HPPP (100 µg /ml for 24 hr).

ROS plays a very important role in apoptosis induction under both physiological and pathological conditions. Accumulation of ROS coupled with an increase in oxidative stress has been implicated in the pathogenesis of several diseases including cancers (Thannickal and Fanburg, 2000). Increasing evidence suggests that compounds induce apoptosis associated with oxidative stress derived from ROS and mitochondrial dysfunction. ROS is critical for the metabolic and signal transduction pathways associated with cell growth and apoptosis (Ramesh*et al.*, 2009). However, excessive production of ROS leads to oxidative stress and cellular damage. Several anticancer agents including anthracyclines, cisplatin, bleomycin and irradiation currently used for cancer treatment have shown to cause increased intracellular ROS generation. Moreover, some studies have reported that anticancer agents from phenolic compounds such as emodin and quercetin induce apoptosis in human cancer cells through ROS dependent mechanism (Hockenbery*et al.*, 2002). The present study, showed the intracellular ROS levels were significantly increased in MCF-7 cells treated with IC_{50} concentration of 4-HPPP in a dose dependent manner.

Mitochondria have been shown to play a central role in the apoptotic process, because both the intrinsic pathway and the extrinsic pathway can converge at the mitochondrial level and trigger mitochondrial membrane permeability (Kuo*et al.*, 2010). Most of the conventional anticancer treatments are thought to induce cell death through indirect activation of the mitochondria dependent pathway of apoptosis, a pathway often found altered in drug-resistant cancer cells (Ma *et al.*, 2012).Demonstrated that *Artemisinin* and its derivatives shows ROS-mediated anti-cancer activity through the reduction of $\Delta\Psi m$ human prostate cancer cells (Reungpatthanaphong*et al.*, 2003) In this study, 4-HPPP on the intracellular ROS by increasing its level in the cell; it is possible that considerable increase in intracellular ROS level enhances the cell death by the treatment of 4-HPPP. This result is in agreement with a recent study of Ma *et al.*, (2012) that an active *Anthraquinone* derivative of emodin induces apoptotic cell death through the associated loss of $\Delta\Psi m$.

Apoptosis is an important homeostatic mechanism that balance cell division and cell death thus a maintaining the appropriate cell member in the body. Disturbances of apoptosis in cancer cells have been studied in detail and induction of apoptosis was one of the strategies for cancer drug development (Martin and Green, 1995; Hu and Kavanagh, 2003). In the present study apoptosis identification was done using analysis of morphological alterations, since it constitutes a standard procedure because of its simplicity, low cost and precision (Doonan and Cotter, 2008). The technique uses acridine orange dye, an intercalating agent, nucleic

acid specific which emits green fluorescence when linked to DNA. This dye penetrates in live cells or in cells beginning apoptosis. Normal cells have bright and homogeneous nucleus while apoptotic cells presents dense area of chromatin condensation (Mishell*et al.*, 1980*;* Wyllie, 1980). Apoptosis is regulated by a complex network of pro-apoptotic and anti-apoptotic proteins. The apoptotic signals can be initiated by external stimuli/ligands and by cellular stress caused by cytotoxic drugs, leading to altered mitochondrial permeability. As a consequence of alterations in mitochondrial permeability and pore transition, release of cytochrome-C into the cytoplasm occurs where it can bind and induce conformational change of apoptotic protease activating factor-1(APAF-1), resulting in the formation of the "apoptosome" complexes. These apoptosomes recruit and activate caspase-9 which in turn activates the effector caspases 3, 6, and 7.

More than 50% of neoplasms undergo aberrations in the apoptotic machinery which leads to abnormal cell proliferation (Reed, 2002). Accumulated evidences indicated that most of chemotherapeutic agents halt tumor cells proliferation *via* induction of apoptosis (Pommier*et al*, 2004). In the present study, 4-HPPP showed significant apoptotic effect compared with the normal cells and was similar to DMSO treated cells. These results are in accordance with other studies where the application of phenolic compounds resulted in an inhibition of cell viability, linked to induction of apoptosis (Corsi*et al*., 2002)

The induction of the DNA single strand breaks is often used to predict the drug sensitivity of tumor cells. The extent of DNA damage was greater in the 4-HPPP treated group compared to normal control. Impairment of ATP production and energy metabolism are the obvious mechanisms proposed to explain inhibition of DNA repair pathways (Dwarkanath*et al*., 2001). The reason for increased DNA damage in 4-HPPP treatment groups, compared to normal control, might be the increased generation of ROS by 4-HPPP compound.

Our results showed that the 4-HPPP may have potent cytotoxic effects on MCF-7 cancer cells, by inhibiting the growth of the cancer cells, through the apoptotic cell death. Thus, the present study suggests that 4-HPPP may be a promising anticancer therapeutic agent for breast cancer cell lines.

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