Antioxidant studies of hispolon and its derivatives.

Vara Prasad Saka
Vignan Pharmacy College
Chitra Vellapandian
SRM College of Pharmacy
Damodharan Narayanaswamy (damodhan@srmist.edu.in)
SRM College of Pharmacy https://orcid.org/0000-0003-4246-5351

Short Report

Keywords: Electromagnetic radiation, Hispolon, Inonotus hispidus, Mobile Phone, Reactive Oxygen Species, Apoptosis

Posted Date: September 16th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1644043/v2

Abstract

Hispolon, a polyphenolic yellow pigment isolated from Chinese mushrooms which was synthesized by researchers and its derivatives such as monomethyl ether, pyrazole and monomethyl ether pyrazole were evaluated for their chemical stability in cell culture medium, antioxidant effect in Chinese hamster ovarian (CHO) radioprotection in spleen lymphocytes. The results indicate that pyrazole derivatives were more chemically stable in cell culture medium. Further, pretreatment with hispolon and its derivatives in CHO cells showed significant inhibition in the radiation induced Reactive oxygen species (ROS) by pyrazole derivative of hispolon. Similarly, the treatment in the splenic lymphocytes showed no significant expression of antioxidant genes such as γGCL and HO-1, and also no significant radioprotection in the markers like fragmentation of DNA, and pre G1 gated cells. This may be because of the primary nature of lymphocytes with limited life span. In MMP assay hispolon pyrazole and monomethyl ether showed a significant rise indicates that these compounds are protecting through inhibition of radiation induced mitochondrial dysfunction. In conclusion, in line with the previous studies, the pyrazole derivatives of hispolon and hispolon monomethyl ether shows antioxidant and radioprotection.

Introduction

Reactive oxygen species (ROS) are oxygen containing highly reactive free radicals or the molecular species. ROS are naturally generated in every cell to perform important signaling functions. (Azzam et al., 2012; Murphy et al., 2011). The intracellular ROS level is controlled by a number of enzymatic and non-enzymatic antioxidants. An antioxidant is a substance that is present at low concentrations and significantly delays or prevents oxidation of the oxidizable substrate. The exposure of cells to external stimuli like ionizing radiation, leads to excessive generation of ROS through radiolysis of cellular water. Under these conditions, the intracellular defense machinery may not be enough to control the ROS level thereby facilitating damage to biological macromolecules such as nucleic acids, proteins and lipids (Azzam et al., 2012)(Einor et al., 2016). Therefore exogenously supplied antioxidants from natural origin may be effective in protecting cells from radiation-induced oxidative stress.

Hispolon (6-(3,4-dihydroxy-phenyl)-4-hydroxy-hexa-3,5-dien- 2-one; $C_{12}H_{12}O_4$), a yellow pigment and a natural polyphenol, isolated from the *Phellinus igniarius* and *Phellinus linteus*, is structurally similar to curcumin, but lacks one aryl moiety (Balaji et al., 2015; Hyang et al., 2015; Toopmuang et al., 2014; Wu et al., 2014). It exhibits protective effect against acute liver damage (Huang et al., 2012), inflammation (Chang et al., 2011), metastasis (Huang et al., 2010), proliferation of cancer cells (Huang et al., 2011), generation of ROS (Ali et al., 1996), estrogenesis in breast cancer (Hyang et al., 2015), (Wang et al., 2014). It has also been reported for anticancer potential against various cancer cells, including melanoma (Chen et al., 2014), leukemia (Chen et al., 2013), hepatocarcinoma (Huang et al., 2011), bladder cancer (Lu et al., 2009), and gastric cancer cells (Chen et al., 2008) through inhibition of cell growth, induction of cell cycle arrest and suppression of metastasis. All these reports have prompted several investigators to synthesize hispolon derivatives (Balaji et al., 2015). On similar lines our group has previously shown that hispolon and its derivatives hispolon pyrazole, hispolon monomethyl ether, and hispolon monomethyl ether

pyrazole showed potential anti-genotoxic effect in cells against radiation exposure. Here in present investigation, these hispolon derivatives were evaluated for chemical stability, antioxidant activity and to protect spleen lymphocytes from radiation induced cell death. The chemicals structures of the four hispolon derivatives used in present study are shown in figure 1.

Materials And Methods

Chemicals:

Hispolon derivatives were procured from Natsol pvt ltd, vizag. Propidium iodide (PI), diethyl pyrocarbonate (DEPC), agarose, sodium dodecyl sulphate (SDS), dimethyl sulfoxide (DMSO), triton X-100, SYBR Green-II dye, cell culture medium (RPMI), fetal calf serum (FCS), penicillin, streptomycin, EDTA, trizol reagent, cDNA synthesis kit, 10× SYBR green polymerase chain reaction (PCR) mix and RNAase were purchased from Sigma Chemical Company (St. Louis, MO, USA). Dichloro fuorescein diacetate (DCF-DA), 5, 5 ’, 6, 6’-tetrachloro-1, 1’, 3, 3’-tetraethyl benzimidazolo carbocyanine iodide (JC-1) were obtained from Molecular probes, USA. The gene-specific primers for RT-PCR were custom synthesized from the local agents. All other chemicals with maximum available purity were purchased from reputed local manufacturers/suppliers.

Assessment of stability of hispolon compounds in cell culture medium:

To assess the stability, hispolon and its derivatives dissolved in DMSO was added to achieve final concentration of 10 µM and 0.1 % DMSO in RPMI medium containing 10% fetal calf serum and incubated at 37°C for a time period of 4.5 h and observed for absorbance at 360 nm for hispolon and hispolon monomethyl ether; 316 nm for hispolon pyrazole and hispolon monomethyl ether pyrazole for every 30 min. and was plotted as function of time to see the % stability (Ying-Jan Wang , et al, 1997).

Animal maintenance:

Six- to eight-week-old inbred BALB/c male mice, weighing approximately 20–25 g, reared in the animal house of the Bhabha Atomic Research Centre were used. The guidelines issued by the Institutional Animal Ethics Committee of the Bhabha Atomic Research Centre, Government of India, regarding the maintenance and dissection of small animals were strictly followed (Khan et al., 2011).

Cell culture and treatment condition:

CHO cell line (obtained from NCCS, Pune, India) was grown in DMEM medium supplemented with 10% FCS, 100 IU/ml penicillin, and 100 µg/ml streptomycin. Monolayer cultures in tissue culture flasks (Nunc, Roskilde, Denmark) were incubated in 95% air, 5% CO_2 humidified atmosphere at 37°C. The culture medium was changed every 48 h. Stock solution of hispolon and its derivatives was prepared in DMSO and added to the culture medium at the desired concentrations. The maximum concentration of DMSO was 0.1% (Khan et al., 2011).

Preparation of splenic lymphocytes and treatment:

Spleens were aseptically removed and placed in sterile dishes containing RPMI medium. Single-cell suspensions were prepared by gently teasing the spleen on a sterile cell strainer placed in the dish. Splenocytes were centrifuged and red blood cells were lysed by brief hypotonic shock. In all in vitro experiments, cells were pretreated with hispolon and its derivatives (10 μM) for 2 h in RPMI medium before irradiation. DMSO was used as vehicle control in vitro (Khan et al., 2011; Raghuraman et al., 2017).

Irradiation protocol:

CHO cells and lymphocytes were treated with hispolon and its derivatives (10 μM) and incubated at 37°C for 2 h and then subjected to irradiation at a dose rate of 1 Gy min-1 and a source to sample distance of 60 cm. After being irradiated, 10% serum was added to the culture medium and the cells were cultured for desired time points before being processed for various assays (Khan et al., 2011; Raghuraman et al., 2017).

Measurement of ROS:

To monitor the level of ROS, cells (1 × 104) were treated with hispolon and its derivatives (10 μM) for 2 h, washed with PBS, and loaded with 5 μM DCHF-DA for 30 min at 37°C. The levels of intracellular ROS and superoxide anion were represented by the fluorescence intensity of 2′,7-dichlorofluorescein (DCF) after excitation at 488 and emission at 530 nm on a multimode plate reader (Synergy H1, BioTek, USA) (Kunwar et al., 2012; Raghuraman et al., 2017).

Mitochondrial membrane potential (MMP) assay:

For this study, lymphocytes (1 × 105) were labelled with JC-1 (10 μg ml-1, final concentration) for 20 min at 37ºC in the dark and analyzed for fluorescence emissions at 535 and 610 nm after excitation at 485 and 565 nm, respectively, using a multimode microplate reader. The ratio of fluorescence intensities at 610 nm and 535 nm was represented as the MMP (Verma et al., 2016).

Estimation of cell death in splenic lymphocytes by PI staining:

PI staining was done as reported earlier (Khan et al., 2011). In brief, lymphocytes (1 × 106) were stained with a solution containing 50 μg ml-1 PI, 0.1% sodium citrate and 0.1% Triton X-100 and kept overnight at 4ºC in the dark. The labelled cells were acquired using a flow cytometer (Partec, Germany) and characterized for cell cycle phases using FlowJo® software. The pre-G1 phase population represented the apoptotic cells.

DNA ladder assay:

Lymphocytes (1 × 106) were lysed and their DNA content was analyzed on an agarose gel as described earlier (Khan et al., 2011).

Real-time polymerase chain reaction (RT-PCR) :

In brief, total RNA was isolated from lymphocytes (5 × 106) 24 h post irradiation using a TRIzol reagent according to the manufacturer's instructions. Approximately, 4 µg of the total RNA was used for cDNA synthesis by reverse transcription (cDNA synthesis kit, Thermo Scientific, USA), and real-time PCR was carried out using the template (cDNA), SYBR green master mix (Roche Applied Science, Germany) and gene-specific primers in a Rotor-Gene Q (QIAGEN, Germany) according to the protocol standardized earlier. The threshold cycle (CT) values estimated from the above runs for the target genes were normalized against a housekeeping gene, b-actin, according to the method described earlier (Verma et al., 2016). The primers (forward and reverse) used for cDNA amplification are given in Table 1.

Table 1: List of gene specific primers

Name of gene Primer	sequence	Gen Bank accession no.
βActin	GGCTGTATTCCCCTCCATCG CCAGTTGGTAACAATGCCATGT	NM_007393
γGCL	GGGGTGACGAGGTGGAGTA GTTGGGGTTTGTCCTCTCCC	NM_010295
HO-1	AAGCCGAGAATGCTGAGTTCA GCCGTGTAGATATGGTACAAGGA	NM_010442

Statistical analysis:

All the experiments were carried out in duplicates. The results are presented as means ± SEM, n = 2 from an independent experiment. The data were analyzed by paired T test using Origin (version 9) software to confirm the variability of the data. The P values < 0.05 were considered as statistically significant.

Results

Assessment of stability of hispolon compounds in cell culture medium:

The stability of hispolon compounds (10 µM) in presence of cell culture medium was monitored for a period of 4.5 h with an interval of 30 min using UV-Visible spectrophotometer. The percent stability calculated from the absorbance values normalizing with respect to the absorbance at 0 min time point as a function of time is shown in figure 1. The results clearly indicate time dependent degradation of hispolon compounds in presence of cell culture medium. From the results it was found that hispolon

pyrazole and hispolon monomethyl ether pyrazole are more stable when compared to hispolon and hispolon monomethyl ether.

Antioxidant effect of hispolon and its derivatives in CHO cells:

The results indicate that cells treated with hispolon and its derivatives alone did not show any significant change in the ROS level compared to control. The exposure of radiation at 2 Gy led to a significant increase in the ROS generation compared to the control. Pre-treatment with hispolon for 2 h did not show any significant change in the ROS level compared to radiation control. Similar treatments with hispolon pyrazole, hispolon monomethyl ether and hispolon monomethyl ether pyrazole showed lowering of the ROS level compared to radiation control. Among these three compounds, hispolon pyrazole was the most effective in reducing radiation induced ROS level.

Antioxidant effect of hispolon and its derivatives in spleen lymphocytes:

The antioxidant effect of hispolon and its derivatives were estimated by the amplification of anti-oxidant genes such as *γGCL* and HO-1. There is no significant expression of antioxidant genes when treated with the hispolon and its derivatives. In line with the above results and from the previous observations of the host lab, it was shown that the hispolon and its derivatives are radioprotective but not by inhibiting ROS generated by means of radiation.

Radioprotective effect of effect of hispolon and its derivatives in spleen lymphocytes:

The ability of hispolon and its derivatives to protect the radiation induced cell death in spleen lymphocytes was evaluated by the PI assay at 24 h post irradiation Fig. 4 C and D show representative gated histograms of the PI stained cells and the quantification of pre-G1 cells, respectively. Furthermore, the radioprotective effect was validated by evaluating the other markers of apoptosis, such as the MMP and fragmentation of DNA and the results of these studies were presented in fig 4 A and B respectively. The groups treated with hispolon and its derivatives alone shows marked protection from DNA fragmentation except hispolon monomethyl ether (fig B.4) and also in MMP. Similar treatment with hispolon and its derivatives prior to the irradiation, hispolon pyrazole and hispolon monomethyl ether pyrazole shows marked increase in the MMP but could not protect fragmentation of DNA. These results also can be correlated with the results from the PI stained cells where quantification of pre G1 cells shows that the radiation significant increase in the pre G1 peak when compared to the control and also there was no significant protection of cells when pretreated with hispolon and its derivatives. Remarkably hispolon monomethyl ethers was toxic to both non irradiated cells as well as non-irradiated cells.

Discussion

Hispolon, a yellow pigment, is identified in mushrooms and were evaluated and reported for various biological activities. It was also reported to have cytotoxic nature in various cancer cells by inducing apoptosis through mitochondrial dysfunction as a result of ROS progression and also identified to be

protective in normal tissues by inhibiting ROS (Chen et al., 2008, 2013; Huang et al., 2011; Lu et al., 2009, 2013). This prompted us to hypothesize that these agents can be explored to protect cells from ROS induced by radiation, which is a prerequisite for developing any radio protector as well as therapeutic antioxidant. In order to address this issue, we employed CHO cells of epithelial origin, being a model cellular system for radiation related research (Raghuraman et al., 2017). All the four hispolon compounds used in the present study screened for the antioxidant activity against radiation induced ROS using this cell line. The results clearly indicate that the replacement of hydroxyl group and keto group with methoxy and pyrazole moieties protects the cells from ROS generated because of radiation. This effect is expected because of the chemical stability imparted as a result of the substitution of hydroxyl and keto groups with methoxy and pyrazole in hispolon. This phenomenon may be because of the hydroxyl radicals and keto groups are more prone to auto-oxidation which results in production of phenoxyl radicals as seen in case of curcumin (K. INDIRA PRIYADARSINI, et al, 2003).

Since lymphocytes are the most sensitive cellular system against radiation toxicities, the anti-oxidant and radioprotective activity of hispolon and its derivatives was evaluated in these cells also (Khan et al., 2011). The antioxidant activity of hispolon and its derivatives were thought to be cytoprotective responses through Nrf-2 mediated by inducing the transcription of genes involved in antioxidant defense mechanisms was investigated on the relative expression of direct Nrf-2 target genes like γ GCL and HO-1 (Raghuraman et al., 2017). All the four compounds of hispolon were not able to induce the expression of both the genes except the methyl pyrazole derivative increased the expression of HO-1 by 22 fold and the results suggest that all the compounds are not radioprotective through antioxidant mechanism. Furthermore it was observed that pretreatment of hispolon and its derivatives did not influence the cell death and DNA damage induced by radiation in spleen lymphocytes. It was evident by the marked increase in characteristic fragmentation of DNA, pre G1 peak and MMP. This may be expected due to that lymphocytes are primary cells with no cell cycle and limited life span which results in the apoptosis with the track of time. From the above results, it was concluded that the hispolon and its derivatives are radioprotective but not through ROS inhibition.

In conclusion, hispolon pyrazole and hispolon monomethyl ether pyrazole are chemically stable and showed anti-oxidant activity.

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Figures

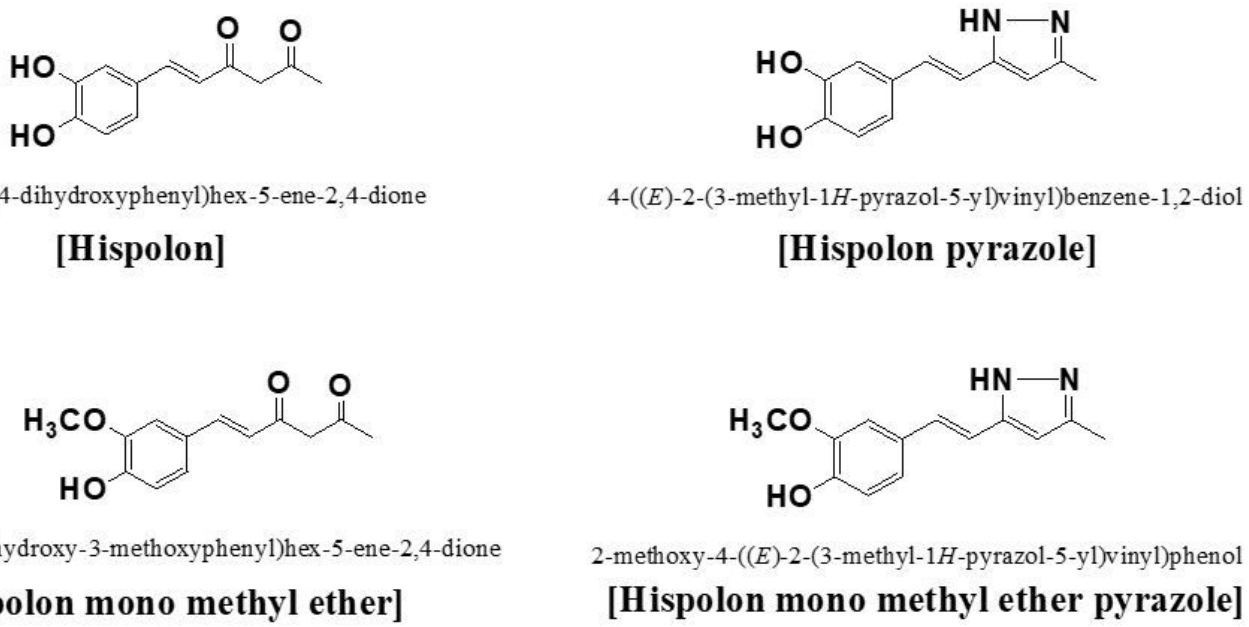

Figure 1

Chemical structures of hispolon and its derivatives

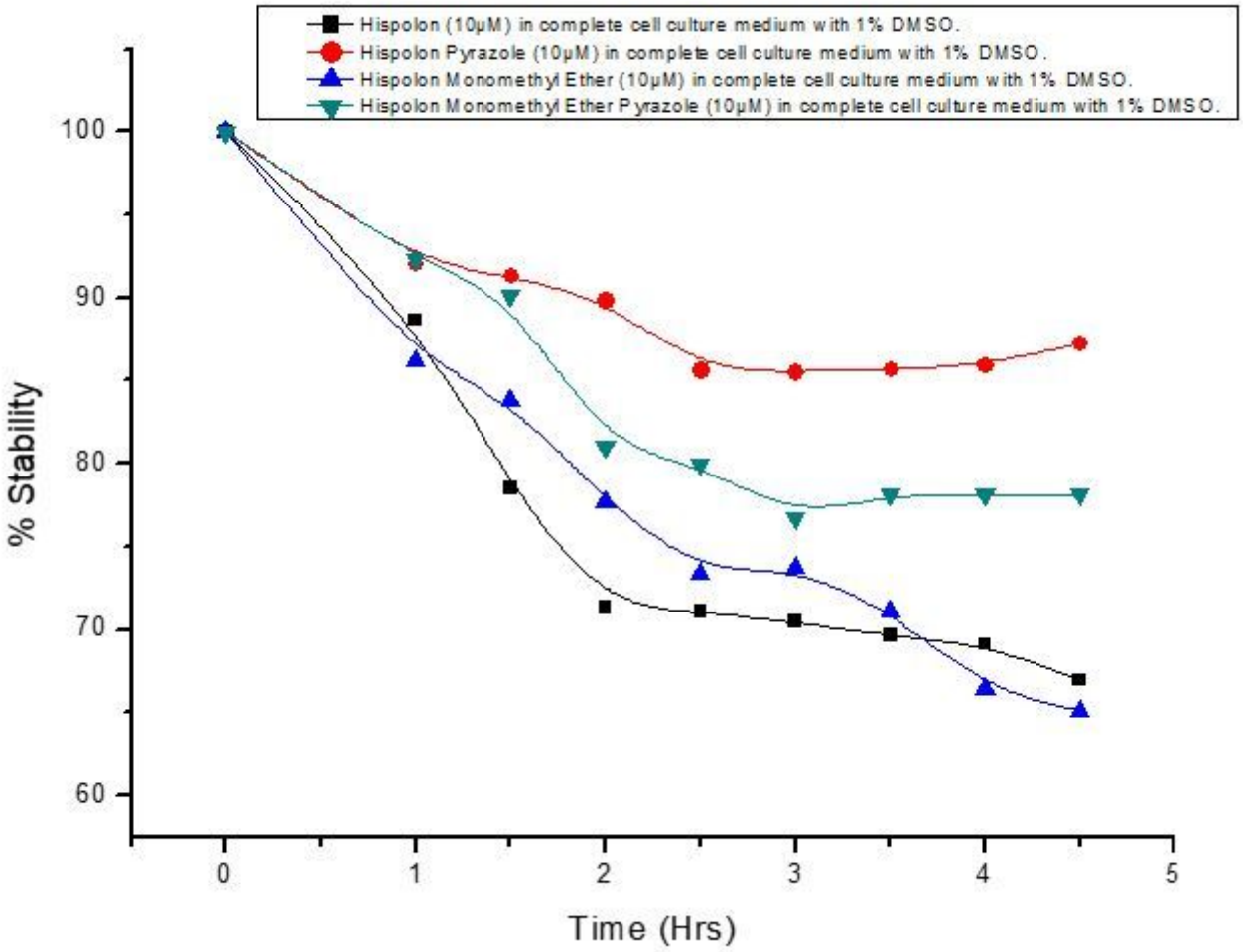

Figure 2

Assessment of stability of hispolon compounds (10 μM) in complete cell culture medium incubated at 37°C for a period of 4.5 H using UV-Visible spectrophotometer at 360 nm for hispolon and hispolon monomethyl ether and 316 nm for hispolon pyrazole and hispolon monomethyl ether pyrazole.

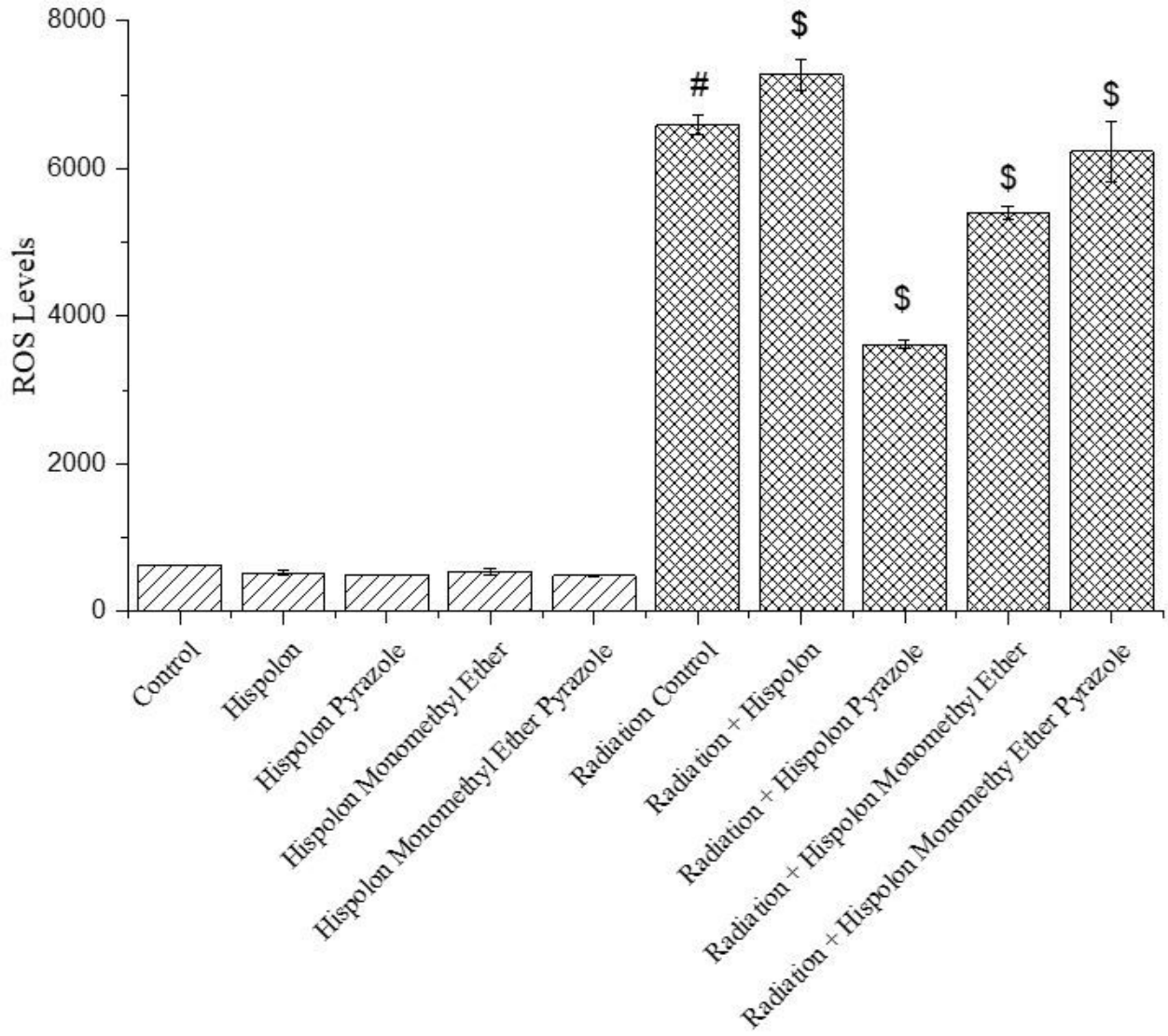

Figure 3

Effect of hispolon and its derivatives on radiation induced ROS levels in CHO cells; Intracellular ROS level estimated 30 min after irradiation using the DCF-DA probe (λex – 488 nm). The fluorescence intensities of oxidized DCF under different treatments are representative of the ROS levels. #p<0.05 when compared to control; $p<0.05 when compared to radiation control.

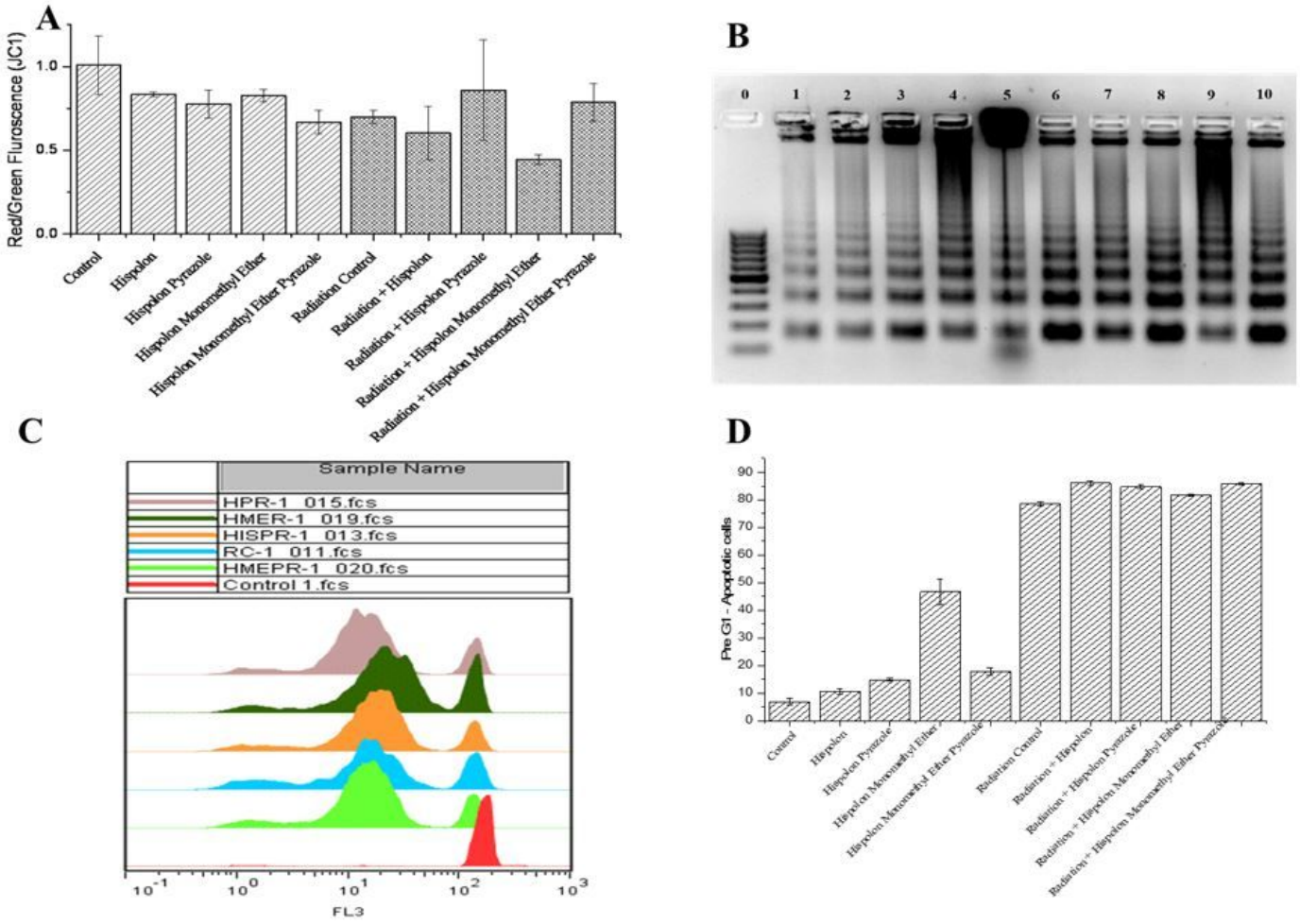

Figure 4

Radio protective effect of effect of hispolon and its derivatives in spleen lymphocytes; A Red/Green florescence intensity ratio of the splenic lymphocytes; B DNA Fragmentation in splenic lymphocytes; 0-control, 1-DMSO control, 2-Hispolon Control, 3-Hispolon Pyrazole Control, 4-Hispolon Monomethyl Ether Control, 5- Hispolon Monomethyl Ether Pyrazole Control, 6-Radiation Control (RC), 7-Radation and Hispolon (HISPR), 8- Radiation and Hispolon Pyrazole (HPR), 9- Radiation and Hispolon Monomethyl Ether (HMER), 10- Radiation and Hispolon Monomethyl Ether Pyrazole (HMEPR); C Pre G1 gated population showing apoptosis in radiation groups presented in histograms; D Quantification of pre G1 gated population showing apoptosis in sham controls and radiation groups.

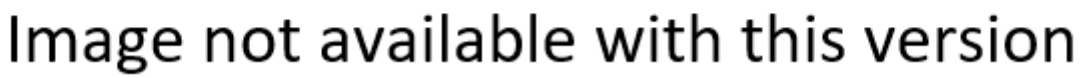

Figure 5

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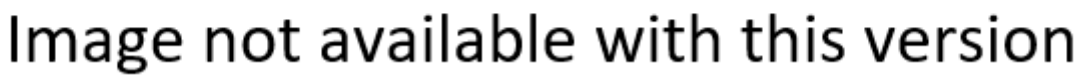

Figure 6

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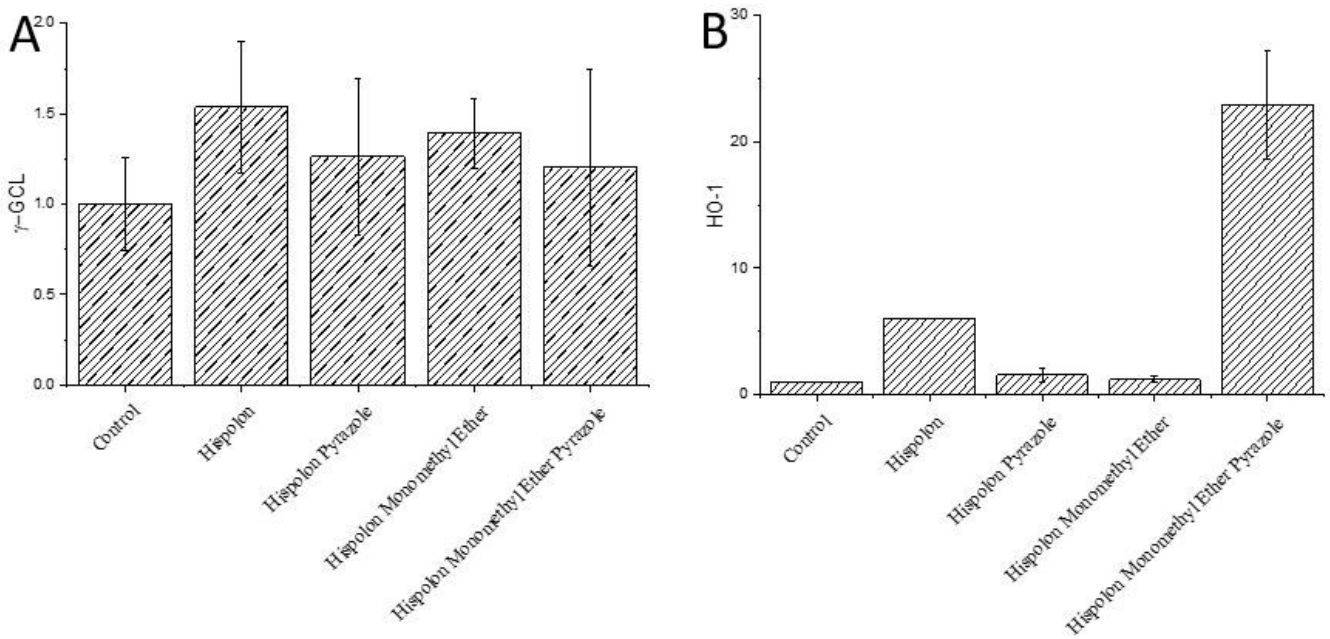

Figure 7

Effect of pretreatment of hispolon compounds on the mRNA expressions of γGCL and HO-1 by RT-PCR. The expressions in treatment groups were normalized against the sham control group and the relative expression changes have been plotted. Actin expression was used as the internal control for all the genes.