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Haberlea rhodopensis alcohol extract normalizes stress-responsive transcription of the human TP53 gene

Neli Dimitrova[1§], Dessislava Staneva[2§], Borislav Popov[1], Albena Alexandrova[3], Milena Georgieva[2], George Miloshev[2*]

[1]Department of Molecular Biology, Immunology and Medical Genetics, Faculty of Medicine, Trakia University, Stara Zagora, Bulgaria
[2]Laboratory of Molecular Genetics, Epigenetics and Longevity, Institute of Molecular Biology "RoumenTsanev", Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria
[3]Laboratory of Free Radical Processes, Institute of Neurobiology, Bulgarian Academy of Sciences, 23 Acad. G. Bonchev str, 1113 Sofia, Bulgaria
[§]These authors contributed equally

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TP53 gene expression

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ABSTRACT

The Orpheus flower *Haberlea rhodopensis* (Friv.) of the family *Gesneriaceae* can go into anabiosis for long periods in an almost entirely desiccated state. It is an endemic relict from the Balkan Peninsula. Alcohol extracts from *H. rhodopensis* contain many biologically active substances with potent antioxidant, antigenotoxic, radioprotective, revitalizing and antiaging capabilities. However, regulating the gene networks responsible for these activities is vastly unknown. This study explores the cellular mechanisms underlying the protective effect of *H. rhodopensis* extracts (HRE). HeLa cells (human cervix epithelial carcinoma, HeLa ATCC® CCL-2™) were used as a model. We examined the changes in catalase activity and TP53 mRNA level shortly after oxidative (H_2O_2) and ionizing radiation (IR) induced stress with and without pre-incubation with HRE extracts. The dynamics in the activity of catalase, a main cellular antioxidant enzyme, and the expression of the stress-responsive gene TP53 were investigated by UV spectrophotometric assay and RT-qPCR, respectively. Under the applied stress conditions, H_2O_2 treatment and gamma radiation, catalase activity increased. This was a sign of induced ROS generation. In the first hours after treatment, the two stressors led to opposite changes in the levels of TP53 gene expression, which were alleviated by pre-incubation with HRE in a concentration-dependent manner. The broad biological activities of the studied extract, taking into account our results, show that ability of HRE to reduce the effect of stress is achieved through complex molecular mechanisms

* Corresponding author
E-mail: karamolbiol@gmail.com; gmlab@chromatinepigenetics.com (Prof. George Miloshev)

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aimed at preserving cellular homeostasis. Mechanisms include the normalization of antioxidant enzyme activity such as catalase and the activity of TP53, one of the genes responsive to stress, by up or down-regulation.

1 Introduction

In the family *Gesneriaceae*, the Orpheus flower, *H. rhodopensis* (Friv.), is a well-known but endangered endemic remnant from the Balkans. It grows in various rocky regions of the Rhodope and Balkan mountains, northern Greece, and the Bulgarian Sredna Gora. *H. rhodopensis* belongs to a group of highly resurrecting plants because of its prominent feature to survive long-lasting anabiosis in an almost fully desiccated state and to recover quickly in the availability of water (Gechev et al. 2013). This ability is provided through the continuous high-level expression of desiccation stress-responsive proteins, even in non-stress conditions (Gechev et al. 2013). The potential of resurrection plants to attenuate the oxidation caused by free radicals and to protect membranes from desiccation was also discovered. Resurrection plants also accumulated significant levels of carbohydrates and phenolic compounds, both of which are crucial for the survival of plants under harsh environments (Muller et al. 1997; Berkov et al. 2011).

A phytochemical study indicated the glucosides myconoside, paucifloside and flavone 8-C glycosides as the compounds present in alcohol extract from *H. rhodopensis* (Ebrahimi et al. 2011). Several research groups reported that *H. rhodopensis* contains lipids, disaccharides and polysaccharides, tannins, flavonoids, and free phenolic acids, of which syringic acid is most prevalent (Stefanov et al. 1992; Muller et al. 1997; Radev et al. 2009; Berkov et al. 2011). Evidence shows that among the components with biological activity found in *H. rhodopensis*, the myconoside has potent antioxidant and hepatoprotective effects (Kondeva-Burdina et al. 2013). Together with the phenolic content, these compounds significantly contribute to the antioxidant properties of *H. rhodopensis* extracts (Mihaylova et al. 2011). In particular, ethanolic extracts of *H. rhodopensis* leaves have been reported to exhibit vital antimicrobial and antioxidant activities, reduce the clastogenic effect of γ-irradiation, and exert *in vivo* anticlastogenic and antimutagenic potential against the anticancer drug cyclophosphamide (Popov et al. 2010a; Ebrahimi et al. 2011; Popov et al. 2011). Methanolic extracts of *in vitro* propagated *H. rhodopensis* plants also showed antioxidant properties, modulated genotoxic and inflammatory stress and improved yeast *Saccharomyces cerevisiae*'s cell viability during chronological ageing (Hayrabedyan et al. 2013; Georgieva et al. 2015). Notably, it has been shown that *H. rhodopensis* possesses a more significant number of antioxidant genes than plant species whose genomes were sequenced at the time (Gechev et al. 2013).

The significant devastating effect of hydrogen peroxide and gamma radiation exposure is attributed to the reactive oxygen species (ROS) overgeneration and the subsequent oxidative destruction of DNA, lipids and proteins in the exposed biological samples (Giorgio et al. 2007; Valko et al. 2007; Gomes et al. 2018). The ionizing radiation (IR) can damage DNA directly (when electrons attack DNA) or indirectly by radiolysis of intracellular water, forming different types of ROS, e.g. hydroxyl ($^{\bullet}OH$) and superoxide ($^{\bullet}O_2^-$) radicals, hydrogen peroxide (H_2O_2), lipid hydroperoxides, etc. (Oneill and Fielden 1993). IR is arguably one of the primary sources of genotoxic stress, causing various DNA lesions like bulky adducts, single-strand and double-strand DNA breaks, and intra-strand cross-linking (Hickman and Samson 1999). In addition, enzymes of the intracellular antioxidant system, catalase (CAT), superoxide dismutase (SOD), mitochondrial Mn-SOD, glutathione peroxidase (GPx), aldehyde dehydrogenase (ALDH), etc., play a crucial role in removing ROS and protecting cells from oxidation-induced damage. Cells respond to genotoxic stress by initiating a multistep process involving cell cycle arrest and maintenance of this arrest during DNA repair. Therefore, numerous crucial genes involved in DNA damage repair and cell cycle regulation exhibit altered expression following radiation exposure. This leads to reprogramming many biochemical and cellular processes to synchronize the adequate response to radioactive stress. Numerous studies reported interlinks between ROS content, p53 expression and antioxidant enzymes, including CAT, at transcript, protein and activity levels (Bai and Cederbaum 2003; Hussain et al. 2004; Liu et al. 2008; O'Connor et al. 2008; Popowich et al. 2010; Kang et al. 2013; Park and Kwak 2022). The transcriptional level of the TP53 gene was significant for cell cycle control, DNA repair mechanisms, and redox enzyme regulation (O'Connor et al. 2008; Kang et al. 2013; Stevenson 2016).

In the present study, changes in the CAT enzyme activity and the transcription of the TP53 gene in HeLa cells pre-incubated with *H. rhodopensis* extracts (HRE) and then treated with hydrogen peroxide or exposed to gamma ionizing radiation (γ-IR) have been studied to evaluate the molecular effects of HRE in a genotoxic stress context.

2 Materials and methods

2.1 Collection of *H. rhodopensis* plants and ethanol extracts preparation

After obtaining official permission from the Ministry of Environment and Water (MoEW), *H. rhodopensis* leaves were

taken from plants growing in their natural environment (a region close to the village of Bachkovo, Bulgaria, 41.9520 N, 24.8587 E). The botanical identification was made at the Department of Pharmacognosy, Faculty of Pharmacy, Sofia Medical University. To comply with the rule that the amount of active substances directly depends on the conditions under which medicinal plants grow, the samples were collected in the same season (May and June) and in the same place. The fresh leaves were cut and dried at room temperature for one month in the dark. The dry leaves were refined to 1 mm particles. Following the leaves grinding, the mixture was macerated in 70% ethyl alcohol for 48 hours (Bulgarian Pharmacopoeia Roll 3, p.218, $d^{20} = 0.887$), after which the ethanol was distilled in a vacuum vaporizer to a drug/liquid phase ratio of 5:1. Next, the primary extract was concentrated in a vacuum-distillation apparatus on Ulbricht at a residual pressure of 0.3 atmospheres and temperature to 50°C until an azeotropic mixture of 5% ethanol was obtained, the distillation being terminated at a volume ratio of 1:1 extract to drugs. The filter paper removed the emulsified non-polar chemicals, chlorophyll, etc., from the resulting extract, leaving a clear liquid phase with 5% ethanol. The extract was standardized by comparing the mass of a given volume of the extract to the mass of a comparable amount of water measured at 20°C using an analytical balance with an accuracy of 10^{-4} g. This was accomplished using the formula for determining the relative density, d^{20}. The differences in the relative densities of the extract and the water and the number of total extracts were determined in g/cm^3. The extracted substances ranged between 9.8×10^{-3} and 11.3×10^{-3} g/cm^3 (average 10.5×10^{-3} g/cm^3). Stock extract with a 100 mg/mL concentration was diluted and used in the experiments.

2.2 Cell line culturing, treatment with HRE and exposure to gamma irradiation and H_2O_2

HeLa cells (human cervix epithelial carcinoma, HeLa ATCC® CCL-2™) were cultured in six-well plates with 2 mL growth medium (DMEM + 10% FBS) at an initial concentration of 4×10^5 cells/well. Incubation at 37°C, 5% CO_2 was allowed till reaching cell culture confluence (about 1.6×10^6 cells/mL). Three sets of cell samples were prepared (i) genotoxin-free, designated as HRE^+ $H_2O_2^-$ / 2 Gy^-, (ii) cells preincubated with HRE and then treated with 10 mM H_2O_2 for 30 min, designated as HRE^+ $H_2O_2^+$, and (iii) cells incubated with HRE before exposure to 2 Gy γ-IR, named HRE^+ 2 Gy^+. We applied the HRE extract in five different concentrations, i.e. (i) no HRE, (ii) HRE at 10 µg/mL, (iii) HRE at 25 µg/mL, (iv) HRE at 50 µg/mL, and (v) HRE at 100 µg/mL final concentrations (f.c.). For all HRE-treated samples, cells were cultured for 1 hour at 37°C with the respective HRE dose. In addition, each set of genotoxin-treated samples included a control not supplemented with HRE but exposed to H_2O_2 or γ-IR alone.

2.3 Analyzing the activity of catalase

The activity of catalase was determined by the UV spectrophotometric method, as described previously by Aebi (1984). In addition, the decrease of absorption at 240 nm caused by the enzymatic dissociation of H_2O_2 was assessed. Therefore, CAT activity was conveyed in units of ΔA_{240}/min/mg protein.

2.4 Total RNA preparation and cDNA synthesis

Following pre-incubation with HRE and exposure to genotoxic stress, cells were immediately scraped and collected for total RNA preparation in the case of hydrogen peroxide treatment or cultured at 37°C for 120 min before harvesting in the case of γ-irradiation.

Thermo Scientific's Gene JET™ RNA Purification Kit was used to prepare total RNA from control cells that had not been treated and cells that had been given HRE, H_2O_2, and/or 2 Gy IR, following the manufacturer's instructions. Using an ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE), the amount and purity of the isolated RNA were assessed at A_{260} and A_{280}. To eliminate the remains of genomic DNA, isolated total RNAs were treated with RNase-free DNase I (Fisher Scientific) at a f.c. of 1 U/µg RNA in the presence of 1 U/µL ribonuclease inhibitor at 37°C for 35 min. Subsequently, EGTA to f.c. of 2 mM was added to the reactions, followed by incubation at 65°C for 10 min for endonuclease deactivation. Reactions for copy DNA (cDNA) synthesis contained oligo $(dT)_{18}$ primer, 450 ng total RNA and Revert Aid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific). One microliter of the reverse transcriptase reaction was applied for the subsequent qPCR assay. Water replaced cDNA in the "no template controls" (NTC). The "reverse transcriptase minus" controls (RT-) contained all necessary reagents for the reverse transcription reaction except for the RT enzyme.

2.5 RT-qPCR analysis

To investigate the transcription of the TP53 gene, a reverse transcription quantitative PCR was performed (Carson et al. 2019) in Rotor-Gene™ 6000 Real-Time PCR cycler (Corbett Life Science, Qiagen) with 2X Maxima SYBR Green Master Mix (Thermo Scientific™) according to the protocol provided by the manufacturer (https://www.thermofisher.com/order/catalog/product/K0221). The optimized reaction contained 1 µL cDNA, 0.2 µM of the appropriate forward and reversed primer and 1× Maxima® SYBR Green qPCR Master Mix in a total volume of 20 µL. The conditions for PCR included initial heating at 95°C for 10 min and 45 cycles of 15 sec at 95°C and 60 sec at 60°C. For assessing TP53 gene transcription, the activity of the GAPDH (glyceraldehyde phosphate dehydrogenase) housekeeping gene served as a reference. Oligonucleotide primers are described in

Table 1 Nucleotide sequences of the oligonucleotide primers

Primer	Sequence	Amplicon, bp
TP53 forward	5'-AACAGCTTTGAGGTGCGTGTTTGTG-3'	144
TP53 reverse	5'-AGAGGAGCTGGTGTTGTTGGGCA-3'	
GAPDH forward	5-ACCAGGTGGTCTCCTCTGACTTCAA-3'	136
GAPDH reverse	5'-ACCCTGTTGCTGTAGCCAAATTCG-3'	

Table 2 Effect of HRE on catalase activity (ΔA_{240}/min/mg protein) in HeLa cells

	HRE [µg/mL]				
	0	10	25	50	100
CAT activity	6.10±0.09	5.65 ± 0.23[&]	5.42 ± 0.03[&]	5.97 ± 0.18[&]	6.10 ± 0.01[&]

[&]p>0.05

Table 1 and were designed on target templates NCBI RefSeqs NM_000546.5 and NM_002046.7 for TP53 and GAPDH mRNAs, respectively.

The polymerase reaction's specificity and the amplified products' correct size were examined by melting curve (dF/dT) analysis and electrophoretic analysis of DNA fragments in agarose gel. Melting curves analyses were performed with ramping from 55 to 95°C, increasing by 1°C per step and waiting for 5 sec before acquiring fluorescence.

For analyses of RT-qPCR results, we used the Rotor-Gene 6000 Series Software 1.7. The CT values were used to calculate the samples' relative gene expression (ratio). To this end, two comparative quantification methods were applied: the two standard curves method and the DeltaDelta Ct ($2^{-\Delta\Delta Ct}$) method (Livak and Schmittgen 2001). Both methods gave similar results. In general, the gene expression in a sample of interest is considered to be changed (up- or down-regulated) if there is at least a two-fold difference compared to the calibrator.

2.6 Statistic evaluation of the obtained results

The IBM SPSS Statistics (Statistical Package for Social Sciences) version 19.0 software was used for the statistical analysis. Data were reported as mean ± SD of three repeats. A paired Student t-test with a two-tailed distribution was applied to compare the experimental groups. Values of $p \leq 0.05$ were considered statistically significant, while those above 5% were denoted as insignificant.

3 Results and Discussion

3.1 HRE ameliorates the redox imbalance

The first step of the present study was to follow CAT activity, a main constituent of the cellular enzyme antioxidant system, as changes in CAT activity indicate redox imbalance caused by H_2O_2 excess. The model system was the HeLa cell line (HeLa (ATCC® CCL-2™), derived from cervical adenocarcinoma cells. At standard conditions, in the absence of an exogenous stressor, treatment with HRE did not significantly affect the level of CAT activity (Table 2). Although incubation with 10 and 25 µg/ml HRE slightly decreased enzymatic activity, the observed differences were insignificant ($p>0.05$). The average CAT activity in HRE-administrated samples was 5.78±0.28 U/mg protein.

Incubating HeLa cells with 10 mM H_2O_2 led to a statistically significant ($p<0.05$) rise in CAT activity (Figure 1). However, enzymatic activity decreased (Figure 1A) when the cells were treated with various amounts of HRE and then exposed to H_2O_2. The decrease was not so marked in cells pre-incubated with 10 and 25 µg/mL HRE before adding hydrogen peroxide. The values were statistically distinguishable compared to the control $HRE^- H_2O_2^-$ group and undistinguishable compared to cells treated only with H_2O_2 (without HRE).

Notably, a significant effect of HRE on the level of CAT activity was detected when cells were pre-incubated with 50 µg/mL HRE before oxidative stress. At this concentration of HRE (Figure 1A), the enzyme activity was significantly different from that of the H_2O_2 treated group ($HRE^- H_2O_2^+$) and most similar to that of the $HRE^- H_2O_2^-$ control. Similar to this, the activity of CAT has elevated significantly after the treatment of HeLa cells with 2 Gy (HRE^- 2 Gy^+) but pared down in the samples, being supplemented with HRE before exposure to IR, HRE^+ 2 Gy^+ (Figure 1B). Again, the increase in HRE amount resulted in a concentration-dependent decrease in enzyme activity. Specifically, HRE at 10 µg/mL seemed insufficient as this concentration did not statistically attenuate CAT activity after 2 Gy IR exposure. However, after supplementing cells with higher concentrations of HRE, the enzyme activity decreased significantly compared to the control and irradiated groups (Figure 1B). Therefore, it can be deduced that the effective concentration of the extract needed to cope with 2 Gy of irradiation and to bring the activity of CAT to the average level would be higher than 10 but lower than 25 µg/mL of HRE.

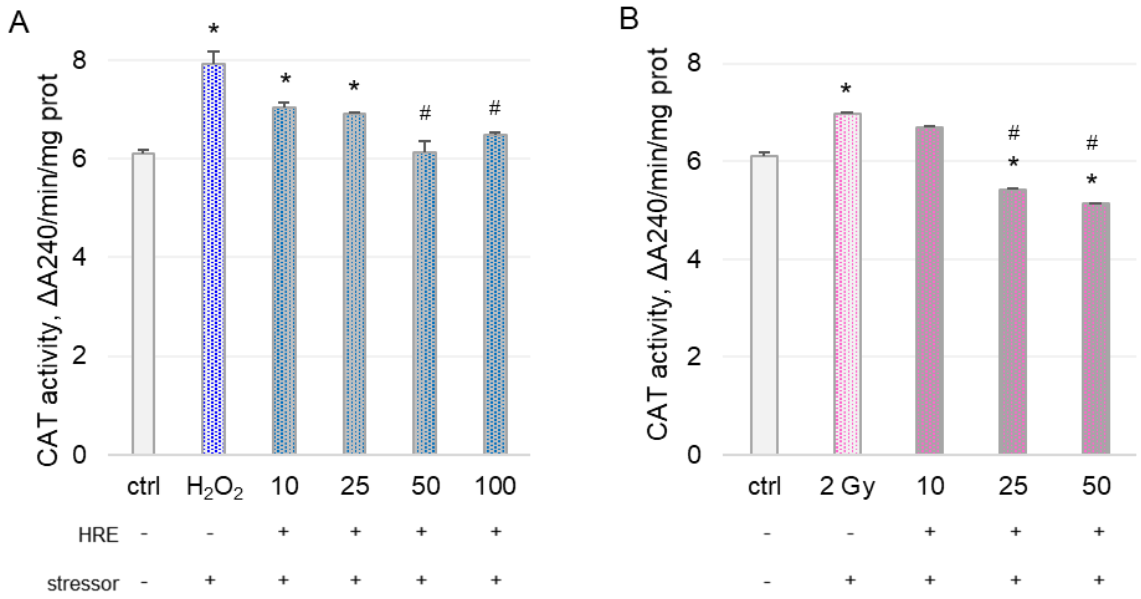

Figure 1 The activity of catalase in stressed HeLa cells, (A) 10 mM H_2O_2 and (B) 2 Gy irradiation, with or without HRE pre-incubation. *Haberlea* extract was administrated to cell cultures to f.c. of 10, 25, 50 or 100 µg/ml. The presence or absence of the specified therapy is shown by the + and - marks. Catalase activity varied statistically from that of the control HRE^- $H_2O_2^-$ / 2 Gy^- group (*$p<0.05$ versus ctrl) or the stressor-treated HRE^- $H_2O_2^+$ / 2 Gy^+ groups ($^{\#}p<0.05$); values are provided as average ±SD.

Catalase (EC 1.11.1.6), one of the most abundant and essential cellular antioxidant enzymes, converts hydrogen peroxide (H_2O_2) into molecular oxygen (O) and water (H_2O). Its vital role is neutralizing excess ROS, which is involved in many physiological processes and pathological conditions, including cancer, atherosclerosis, cataract, diabetes, nutritional deficiency, inflammation, and ageing (Vendemiale et al. 1999; Checa and Aran, 2020). Previous reports showed that administration of H_2O_2 induces the intracellular generation of ROS, which is accompanied by alterations in the synthesis and activities of cellular antioxidant enzymes, e.g. CAT, glutathione peroxidase (GPX) and superoxide dismutase (SOD), as well as sestrin 1, sestrin 2, etc. (Valko et al. 2007; O'Connor et al. 2008; Zhang et al. 2020). For example, in Hep G2 cells treated with H_2O_2 for 4 h, the excess ROS reduced the activity of CAT, GPX and SOD and enhanced lipid peroxidation (Zhang et al. 2020). However, it was shown that the catalase gene in the gills of the crab *Macrophthalmus japonicus* linearly increased in response to exposure to persistent organic pollutants, which cause oxidative stress (Park and Kwak 2022). Furthermore, decreased ROS production and inhibited p53-mediated apoptosis were established in various systems upon overexpression or exogenous addition of CAT (Johnson et al. 1996; Bai and Cederbaum 2003; Hussain et al. 2004; Popowich et al. 2010). We identified that shortly (40 min) after the addition of H_2O_2 to the cells, the intracellular CAT activity increased by 30% relative to the control group ($p<0.05$). Pre-incubation with HRE abrogated this impact and restored the enzymatic activity to the basal level of the controls. The observed effect is most likely due to the well-proven potential of HRE to scavenge ROS and thereby reduce the level of oxidants in cells.

3.2 HRE abrogates the alterations in TP53 gene transcription caused by stress conditions

One of the molecules studied extensively in stress-response research is p53 because of its critical role in the physiological stress response (Kastenhuber and Lowe 2017). Logically, we continued our studies by assessing HRE's impact on the activity of the gene coding for the p53 protein – TP53 gene in human cells cultivated under normal conditions. It is worth noting that although the gene encoding the p53 protein is changed by numerous mutations in different cancerous cell lines, the TP53 sequence is not altered in HeLa cells (HeLa p53+/+) (Hoppeseyler and Butz 1993; Leroy et al. 2014). The levels of TP53 gene transcription upon incubation of HeLa cells with the studied concentrations of *Haberlea* extracts are shown in Table 3. The incubation with four increasing concentrations of HRE did not evoke dramatic alterations in the relative amount of mRNA transcribed from the TP53 gene compared with the non-HRE control (HRE^+*vs* HRE^-). Although we have observed slight differences in the relative amount of the TP53 transcript, these variations were considered insignificant as they were below 2-fold change and $p>0.05$. This proves that at the concentrations tested, *H. rhodopensis* extract does not significantly affect the level of TP53 transcript in HeLa cells under non-stress conditions.

Table 3 Relative concentration of TP53 mRNA in HeLa cells after treatment with *H. rhodopensis* ethanol extract (HRE)

	HRE [μg/mL]				
	0	10	25	50	100
The relative concentration of TP53 mRNA	1.00	1.43 ± 0.54[&]	1.15 ± 0.63[&]	0.78 ± 0.72[&]	1.02 ± 0.07[&]

[&]p>0.05

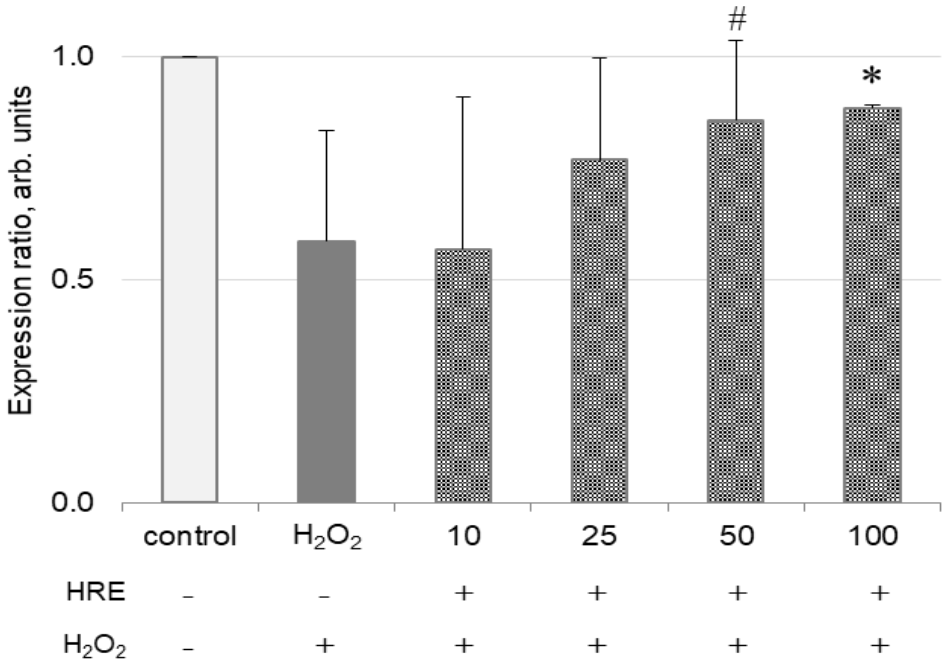

Figure 2 Relative expression of TP53 gene in cells incubated with different concentrations of HRE and exposed to 10 mM H_2O_2; the control sample has not been subjected to any treatment (HRE^- $H_2O_2^-$); Value is presented as mean ± SD; *p<0.05 compared to the control; [#]p<0.05 vs the group treated with H_2O_2 alone (HRE^- $H_2O_2^+$)

Hydrogen peroxide is essential for cell viability as one of the most reactive oxygen species. It is well evidenced that exogenous and endogenously present H_2O_2, especially in higher than physiological concentrations, can inflict various types of damage on cellular DNA. This study used hydrogen peroxide as a genotoxin with well-described genotoxic activity (Benhusein et al. 2010). After being exposed to the studied HRE doses for an hour, HeLa cultures were treated with 10 mM H_2O_2 to induce oxidative stress. The TP53 transcription levels were studied by RT^2-PCR (Figure 2). To evaluate the combined effect of the stressor and pre-incubation with the extract, the level of TP53 transcript in non-treated control samples (HRE^- $H_2O_2^-$) was used as a calibrator. The immediate response of HeLa cells to the direct H_2O_2-induced oxidative stress was a decrease in TP53 gene expression (Figure 2, compare HRE^- $H_2O_2^+$ *vs* HRE^- $H_2O_2^-$).

Interestingly, despite the hydrogen peroxide treatment, the amount of TP53 mRNA steadily raised in cells pre-incubated with HRE with the increment of extract concentration (Figure 2). The addition of 50 μg/mL of HRE to the cells normalized the expression of p53 transcript to the level close to that of non-treated control cells (HRE^- $H_2O_2^-$) and the genotoxin-free (HRE^+ $H_2O_2^-$), i.e. HRE-only supplemented, cells (1.0 *vs* 0.86±0.18 and 0.78±0.72 *vs* 0.86±0.18; Figure 2 and Table 3, respectively). Furthermore, the increase of TP53 transcription in cells pretreated with 50 μg/mL HRE was statistically significant compared to those treated with H_2O_2 alone, HRE^- $H_2O_2^+$. This demonstrates the beneficial effect of HRE supplementation. Therefore, the obtained results reveal that *H. rhodopensis* extracts modulate the expression of TP53 mRNA in HeLa cells under oxidative stress.

Next, we evaluated the levels of TP53 gene transcription in HeLa cell cultures incubated with increasing concentrations of HRE before γ-irradiation. The results indicated up to two-fold increased transcription from the TP53 gene two hours after exposure of HeLa cells to 2 Gy -IR (Figure 3, p<0.05). Furthermore, we have observed that the expression of TP53 was in reverse order following the increase of HRE concentrations. Namely, the higher the extract concentration, the lower the expression of TP53 was (Figure 3).

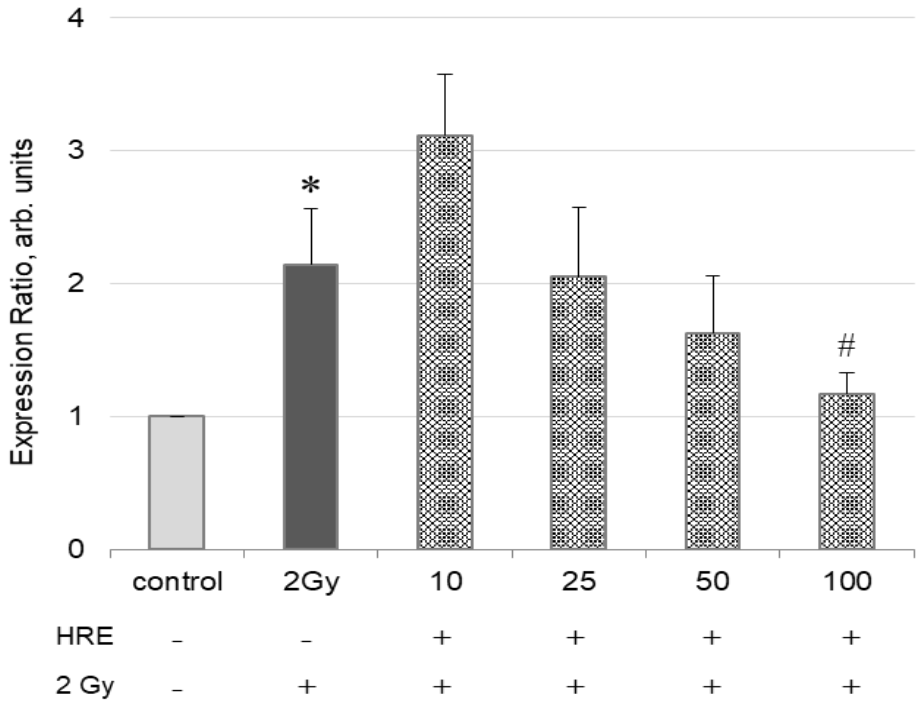

Figure 3 The average fold change of TP53 gene expression in HeLa cells incubated with HRE and exposed to 2 Gy γ-IR. The control cells were treated neither with the extract nor with γ-radiation (HRE^- $2Gy^-$) and used as a calibrator. Results are presented as mean ± SD. *$p<0.05$ from the control group; $^{\#}p<0.05$ relative to the 2 Gy γ-IR treated group (HRE^- 2 Gy^+)

When cells were supplemented with 100 µg/mL of HRE, TP53 transcription returned to the basal level, showing values close to those of the two control samples: the one without treatment (1.17±0.16 *vs* 1.0, $p>0.05$) and that with 100 µg/mL HRE alone (1.17±0.16 to 1.02±0.07), $p>0.05$. Compared to cells exposed to 2 Gy of radiation, pretreating the cells with 100 µg/mL HRE caused a nearly two-fold reduction in the amount of TP53 mRNA, and this difference was significant ($p=0.05$), as shown in Figure 3. Therefore, pre-supplementation of cells with ethanol HRE diminished the expression of the TP53 gene upon 2 Gy-induced genotoxic stress. The effect on TP53 transcription most probably resulted from reduced radiation/oxidation-induced DNA damage in HRE preincubated cells due to the potent antioxidant activities of HRE (Popov et al. 2010b; Georgieva et al. 2013; Popov et al. 2013).

Low physiological amounts of intracellularly produced ROS are not harmful because cells have an efficient system to maintain cellular redox equilibrium and prevent oxidative damage. However, exogenous factors such as ionizing radiation or artificial hydrogen peroxide generate excess ROS production and trigger oxidative stress. Moreover, these factors exhibit strong genotoxicity by causing further damage to the genomic DNA. They thus could direct cellular fate to proliferation, cell-cycle arrest, differentiation, transformation, senescence, autophagy or apoptosis (Campisi, 2013). All this could imperil organismal health, eventually leading to cancer or premature ageing.

The transcription factor p53 is a crucial regulator of cellular response to various stressors, e.g. ionizing radiation, ROS, genotoxins and carcinogens. By activating different downstream pathways, p53 could direct cellular fate to cell cycle arrest, senescence or apoptotic death (Liu et al. 2008; Santoro and Blandino 2010; Cao et al. 2014; Pflaumf et al. 2014; Shi et al. 2021; Park and Kwak 2022). Here, we have demonstrated that while H_2O_2 down-regulates the transcription of the TP53 gene, the addition of *Haberlea* extract to the cells is helping the transcription machinery to increase its expression. Thus, concerning TP53 expression, pre-incubation of the cells with HRE at concentrations above 50 µg/mL is efficient for maintaining the gene's activity at an average level and alleviating the attack of 10 mM H_2O_2 by increasing the TP53 transcription to the level of the non-treated control. This effect can be explained by the richness of the extracts with many biologically active compounds such as polyphenols, polysaccharides, vitamins etc. Previous studies demonstrated that the presence of *H. rhodopensis* extract (i) increased the transcription of collagen VI, collagen XVI and elastin genes in H_2O_2-stressed human dermal fibroblasts (Dell'Acqua and Schweikert 2012), (ii) reduced the H_2O_2-induced oxidative stress and (iii) prevented cells from apoptosis and cell death (Hayrabedyan et al. 2013). Therefore, it is unsurprising that HRE modulated the expression of a significant stress-responsive gene like TP53 under stress conditions.

3.3 Interlinks between p53 and redox enzymes, including CAT

Numerous investigations have demonstrated that *H. rhodopensis* alcohol extracts exhibit antigenotoxic and radiation-protective properties both *in vivo* and *in vitro*. The high concentration of physiologically active chemicals in HRE that have antioxidant, immunomodulating, antiaging, and anticancer activities can account for these characteristics (Popov et al. 2010b; Mihaylova et al. 2011; Popov et al. 2011; Hayrabedyan et al. 2013; Kondeva-Burdina et al. 2013). It should be noted that to apply these extracts properly and successfully, the molecular mechanisms of their action must be thoroughly understood. However, the precise details of these mechanisms are yet unclear. To reveal the details of HRE effects at a molecular level, we have incubated HeLa cells with increasing concentrations of *H. rhodopensis* ethanol extracts and studied the activity of the antioxidant enzyme CAT and the expression of the TP53 stress-responsive gene under optimal and stress conditions.

Gamma radiation causes ionization which can disrupt macromolecules and cellular structures; that is why gamma rays pose a significant challenge to radiation protection, especially during radiotherapy. Data show that HRE pre-treatment could significantly decrease chromosome aberrations, micronuclei frequencies, and the level of blood plasma malondialdehyde and increase SOD and CAT activity in erythrocytes of *in vivo* irradiated rabbits (Georgieva et al. 2013). Previous studies reported that γ-IR did not affect TP53 protein levels but induced p53-serine 15 phosphorylation and increased transcriptional targets p21 and MDM2 (Siliciano et al. 1997; Cao et al. 2014). Conversely, it was shown that upon radiation exposure, the cells respond with increased p53 protein by inhibiting protein degradation and protein translation enhancement (MacCallum et al. 1996; Lee et al. 2013). However, the rationality of these effects regarding gene transcription regulation remained obscure. In line with the above data, we detected a statistically significant increase in CAT activity and two times increased transcription from the TP53 gene after irradiation of HeLa cells with 2 Gy gamma rays.

Here we demonstrated that supplementation with HRE in a concentration above 50 μg/mL before radiation exposure normalized the TP53 mRNA synthesis to the control group level. It seems that 100 μg/mL of the ethanol HRE can abolish the effect of radiation to trigger TP53 gene activation and keep its expression at the level of the non-irradiated cells. Thus, HRE's observed *in vivo* radioprotective property is associated with a change in the transcription of the essential stress-response modulator p53.

Interlinks between p53 and redox enzymes, including CAT, have been previously reported in regulating the cellular antioxidant response. It was demonstrated that p53 regulates the transcription of catalase and some other antioxidant enzymes encoding genes (e.g. GPX1, ALDH4, MnSOD), and these genes are direct p53 targets. On the human MnSOD, GPX, and rat CAT promoters, consensus sequences for binding the p53 transcription factor have been found (Hussain et al. 2004; O'Connor et al. 2008). On the other hand, several authors have reported that upregulation of the CAT gene defends against apoptotic cell death induced by genotoxins (Johnson et al. 1996; Hussain et al. 2004; Popowich et al. 2010). This correlated with an increase in the protein degradation of p53 and a decrease in its phosphorylation (Bai and Cederbaum, 2003). A linear correlation between the expression patterns of p53 and catalase was detected in rat retinal ganglion and *Macrophthalmus japonicas* crab cells (O'Connor et al. 2008; Park and Kwak 2022). However, in human fibroblasts, CAT protein expression remained unaffected after p53 induction and consensus p53 binding elements were not identified on the CAT promoter (Hussain et al. 2004). Kang and co-authors have shown that the opposing roles of p53 in regulating intracellular ROS levels via tuning CAT activity, both up and down, depended on the type and degree of stress and the cellular environment (Kang et al. 2013). Under physiological conditions or mild stress, p53 functions as an antioxidant and upregulates catalase activity through its downstream target p53R2, thereby protecting against ROS. Upon activation following genotoxic stress, p53, in cooperation with PIG3 (another p53-inducible gene), inhibits CAT activity, disturbs intracellular redox homeostasis and could result in p53-mediated apoptosis (Kang et al. 2013). Following earlier findings, our results showed a linear association between p53 expression and CAT activity after cells were exposed to 2 Gy irradiation and an inverse correlation in the case of 10 mM H_2O_2 treatment. Thus, the different responsiveness of the stress-sensing TP53 gene and CAT activity that we observed could be explained by the diverse nature of the applied stressors, the short period after the stress in which the effect was examined and the activation of specific p53 downstream targets. Importantly, one-hour incubation of HeLa cells with HRE in a concentration range between 25-100 μg/mL before stress conditions maintained TP53 gene expression and catalase activity at basal levels characteristics of untreated controls.

At first sight, the opposite effects of H_2O_2 and γ-IR on the TP53 gene activity can be startling. H_2O_2 and gamma rays can produce single and double-strand cuts in DNA and oxidize proteins and lipids. However, behind their similar effects, these two agents possess some very well-pronounced differences in action on the biological macromolecules. H_2O_2, as one of the ROS, directly cuts DNA and oxidizes proteins, while gamma radiation ionizes cellular cytoplasm and produces ROS, but simultaneously can modify chemically macromolecules. For instance, the reductive stress brought on by IR causes protein methionine and cysteine residues to lose sulphur, and DNA-protein adducts are created as a result (Reisz et al. 2014).

Conclusions

Our findings unequivocally demonstrate that *H. rhodopensis* alcoholic extracts alter the TP53 gene transcription in a way that distinguishes the genotoxic effects of H_2O_2 and gamma rays. Shortly after being exposed to stress, these effects were discovered. One of the molecular mechanisms of the well-established radioprotective effects of HRE *in vivo* can be attributed to the observed alterations in the activity of catalase, a major cellular antioxidant enzyme, and the expression of TP53, a significant stress-responsive gene. More extensive future trials will reveal the specifics of the phenomena seen in the present investigation.

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Conflict of interest

The authors declare they have no competing interests.

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