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ORIGINAL RESEARCH

Direct and protective effects of single or combined addition of vincristine and ε-viniferin on human HepG2 cellular oxidative stress markers in vitro

Seda Tarhan · Filiz Özdemir · Zerrin İncesu · Emine Sütken Demirkan

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Abstract The objective of this study is to examine the direct effects of low doses and high doses of ε-viniferin, a substance known to be an antioxidant, and vincristine sulphate, a chemotherapeutic agent, alone and in combination [ε-viniferin + vincristine] on HepG2 cell strain, as well as evaluate oxidative stress after incubation periods of 3, 6, and 24 h. Direct effect was determined right after the incubation period; however, for protective effect, antioxidant protection response was determined after the treatment for 1 h with 500 μM H_2O_2, which is an oxidative stressor. For this purpose, superoxide dismutase was determined for enzyme activity, and lipid hydroperoxide (LPO) and reduced glutathione concentrations were studied as indicators of oxidative stress. Results show that low [3.63 μM vincristine + 3.75 μM ε-viniferin] and high [11.25 μM vincristine + 15.8 μM ε-viniferin] doses of combination groups showed similar direct antioxidant effect on LPO levels as protective when compared to the H_2O_2 control group ($p < 0.05$). Superoxide dismutase enzyme showed a direct antioxidant effect in low and high dose combination groups. In addition, when the incubation period was increased to 24 h, a protective effect was observed in both dose groups ($p < 0.05$). Reduced glutathione activities showed a direct effect in the low dose combination group, and a protective effect in both the low and high doses in the 24 h. These results show that combined usage of drugs in HepG2 cell strain possesses a protective effect against exogenically produced oxidative stress conditions.

Keywords Antioxidant · ε-Viniferin · HepG2 cell · Oxidative stress

S. Tarhan · E. S. Demirkan
Department of Biochemistry, Medical Faculty, Eskişehir Osmangazi University, Eskisehir, Turkey
e-mail: sedatarhan99@hotmail.com

E. S. Demirkan
e-mail: eczmine@yahoo.com

F. Özdemir (✉) · Z. İncesu
Department of Biochemistry, Faculty of Pharmacy, Anadolu University, 26470 Tepebası, Eskisehir, Turkey
e-mail: fozdemir3@anadolu.edu.tr

Z. İncesu
e-mail: zseller@anadolu.edu.tr

Introduction

Hepatocellular carcinoma (HCC) is among the five most frequently diagnosed cancers in the world (Xu et al. 2007b; Liu et al. 2012; Wang et al. 2013; Wu et al. 2013; Li et al. 2013) and is also the 3rd ranked cancer regarding mortality rates (Xu et al. 2007b). No effective treatment currently exists for HCC; development of more effective strategies for the treatment of hepatoma is necessary. Chemotherapy is among the most widely used methods in the treatment of HCC (Xu et al. 2007a). The primary goal of chemotherapy is to reduce side effects to a minimum while killing cancerous cells (Vitaglione et al. 2004). Many studies have demonstrated that

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antioxidants may be used in cancer treatment. In these studies, antioxidant substances decrease the proliferation of cancerous cells in vitro and lead to apoptosis, however, they minimize side effects of chemotherapy in vivo (Barjot et al. 2007; Do Amaral et al. 2008; Ganesaratnam et al. 2004; Ozben 2007).

ε-viniferin is an antioxidant derived from resveratrol via an oxidative process (Zghonda et al. 2011). ε-Viniferin also shows the direct cytotoxicity (Xue et al. 2014), anticancer, anti-inflammatory (Guschlbauer et al. 2013), and neuroprotective (Nopo-Olazabal et al. 2014) effects on various cancer cells. It was reported that ε-viniferin could kill C6, Hep G2, HeLa, and MCF-7 cancer cell lines in a dose-dependent manner (Xue et al. 2014). In addition, while ε-viniferin has antitumoral effects on lymphocytes, myeloid cells, HL-60, HepG2 and human breast cancer cells as an antioxidant, it also prevents necrosis caused by reactive oxygen species (ROS) in normal rat fibroblast cells (Morales et al. 2002; Piver et al. 2003; Privat et al. 2002; Zghonda et al. 2011). Oxidative stress status is known to have a role in the development and progress of HCC. Low levels of ROS are necessary for many physiological processes of the cell, including cell aging, blockage of cell cycle, apoptosis, and proliferation (Marra et al. 2011).

Studies on the use of ε-viniferin in combination with vincristine sulphate as an effective agent against oxidative stress in HepG2 hepatoma cells were not found in literature. Our study is comprised of two phases in order to evaluate both the direct and protective effects of [vincristine + ε-viniferin] combination treatment against oxidative stress. In the first phase, the objective is to carry out an examination of the effects of ε-viniferin alone and in combination with vincristine sulphate in HepG2 cells on cellular lipid peroxidation, SOD enzyme activation and reduced glutathione levels. In the second phase, oxidative stress formed in HepG2 cells via exogenically administered H_2O_2 was studied.

Materials and methods

Reagents

Dulbecco's modified eagle medium (DMEM), phosphate buffered saline (PBS), Tris HCl, vincristine sulphate, and etoposide (ETO) were purchased from Sigma Aldrich Co (St. Louis, MO, USA). ε-Viniferin was provided by Actichem SA (Montauban, France). H_2O_2, chloroform and methanol were purchased from Merck company (Darmstadt, Germany). Reduced Glutathione Kit "The Chemicon (Temecula, CA, USA)", Superoxide Dysmutase Kit "Cell Biolabs, Inc., (San Diego, CA, USA)" and Lipid Hydroperoxide Kit "Cayman Chemical Company (Ann Arbor, MI, USA)" were obtained from chemical suppliers.

Cell culture

Human liver hepatoma (HepG2) (Cat. No HB-8065) cells were obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH/Braunschweig, Germany) and were cultivated in Petri dishes at 1 x 10^6 cells/ml and suspended in a DMEM medium containing 10 % FBS, penicillin/streptomycin, at 37 °C in an incubator containing 5 % CO_2. IC_{50} (a dose of 50 % mortality) and 80 % viability doses (doses obtain 80 % cell viability) that have been used in this study were performed by using values measured as per MTT method (Ozdemir et al. 2014). Briefly, cells (2×10^4 cells/well) were seeded in 96-well plates and incubated with etoposide (dose of 80 % cell viability—7 μM, IC_{50}—55 μM), vincristine (dose of 80 % cell viability—6 μM, IC_{50}—52.5 μM), ε-viniferin (dose of 80 % cell viability—80 μM, IC_{50}—98.3 μM) or combination of vincristine and ε-viniferin (dose of 80 % cell viability—3.63 + 3.75 μM, IC_{50}—11.25 + 15.8 μM, respectively).

Cell treatment conditions

Cells were incubated with the vincristine and ε-viniferin [vincristine + ε-viniferin] combination and etoposide's IC_{50} and 80 % viability dose values for 3, 6 and 24 h. Since etoposide is a widely used chemotherapeutic agent, it was used as control group for vincristine sulphate in all phases of the experiment. In order to evaluate both the direct and also protective effect of combination treatment [vincristine + ε-viniferin] against oxidative stress, the experiment was designed in two parts. Direct effects of the combination [vincristine + ε-viniferin] treatment on cellular markers were detected after 3, 6 and 24 h. In order to evaluate its protective effect, 500 μM H_2O_2 were added to the medium exogenically and the cells were incubated in an incubator for 1 h. At this phase, in

order to examine the direct effect of H_2O_2 on cellular lipid peroxidation, SOD activation and reduced glutathione levels, in addition to the substance groups used in the first phase and to observe the protective effects of the substances, another control group was used, in which only 500 µM H_2O_2 was added and was left for incubation at 37 °C for 1 h. This group was named the H_2O_2-control group (H_2O_2-C group).

Lipid peroxidation measurement method

LPO measurements were determined by 'Cayman's Lipid Hydroperoxide Experiment Kit'. According to the manufacturer, cells after incubation with drugs were sonicated and then 500 µl crystalline solid used for extraction of samples were added to each tube. After centrifugation, 500 µl of the collected chloroform was transferred to another glass tube and 450 µl chloroform–methanol solution was added. After the addition of 50 µl of chromogen, measurement was performed at 492 nm using microplate reader. Measured absorbance results were compared with a standard curve, and expressed as hydroperoxide concentrations (µmol) in samples.

SOD measurement method

SOD activities were determined using commercially available enzyme assay kit (Cell Biolab Inc, Cat. No STA-340-T). According to the manufacturer's protocol, the cells were lysed with 500 µl of lysis buffer on ice, and centrifuged at 12,000 rpm at 4 °C for 10 min. The supernatants were used for measurement of SOD activity using an SOD kit. SOD activity was read in a microplate reader at 490 nm (optical density—OD).

The results were expressed as percentage of SOD activity (% activity) and were calculated as follows:

$$\text{SOD Activity (inhibition\%)} = [(\text{OD blank} - \text{OD sample})/(\text{OD blank})] \times 100$$

Detection of reduced glutathione

GSH measurements were performed using the 'Chemicon Glutathione (GSH) Measurement Kit' in a fluorometer device. Cells were incubated with drugs depending on time and dose. No drugs were administered to the control group. After incubation, the cells were lysed on ice for 10 min, then scraped and collected in Eppendorf tubes. 90 µl cell lysate were withdrawn from each tube and added to 96 well plate. 10 µl of previously prepared MCB solution was added to the wells to prepare a total volume of 100 µl, and the 96 well plate was mixed. This was then incubated at room temperature for 1 h, avoiding light, and measurements were performed with a fluorometer having a 460 nm filter. Results were obtained in reference to a GSH standard curve.

Statistical analysis

Statistical analysis was carried out using the Statistical Package for the Social Science for Windows (SPSS, version 20.0). Data were expressed as the mean ± standard deviation (SD). In the comparison of the groups, a one way (ANOVA) variance analysis and for multiple comparisons LSD test were used. GraphPad Prism 5 software was used for the formation of graphics. p value <0.05 was accepted to indicate statistical significance.

Results

Direct effect

Lipid peroxidation is one of the well-known indicators for evaluating the role of oxidative stress in cell membranes. A main focus of the study was to observe direct effect of vincristine, ε-viniferin and [vincristine + ε-viniferin] combination group when IC_{50} and 80 % viability doses were administered. There were no direct significance effects on HepG2 cells at the 3rd h with the test groups. However, when the incubation period was increased to 6 h, LPO levels of HepG2 cells were increased, compared to the control group. This increase was neither statistically significant nor depended on the dose ($p > 0.05$) (Table 1). In addition, when the incubation period was increased to 24 h, LPO levels of HepG2 cell treated with 80 µM ε-viniferin (6.05 ± 0.73) was increased significantly, when compared to the control group (4.82 ± 0.47) and the other groups ($p < 0.05$) (Table 1).

Superoxide dismutase (SOD) is an enzyme that catalyzes dysmutation of superoxide radicals ($O\bullet_2^-$) (Jimenez-Del-Rio and Velez-Pardo 2012) and has important functions in the defense system against harmful effects of free radicals and ROS in biological

Table 1 Direct and protective effects of vincristine sulphate (VCR), etoposide (ETO), ε-viniferin (ε-VNF) and combined application (VCR + ε-VNF) on lipid peroxidation (μM) of HepG2 cells

	H_2O_2	C	IC_{50} values				80 % cell viability			
			VCR (52.5 μM)	ε-VNF (98.3 μM)	ETO (55 μM)	(VCR + ε-VNF) (11.25 + 15.8 μM)	VCR (6 μM)	ε-VNF (80 μM)	ETO (7 μM)	(VCR + ε-VNF) (3.63 + 3.75 μM)
Lipid peroxidation (μM)										
3 h										
Direct effect	–	4.566 ± 0.672	4.594 ± 0.782	4.492 ± 0.850	4.919 ± 0.505	5.000 ± 1.213	5.454 ± 0.954	4.749 ± 0.982	5.258 ± 1.793	4.567 ± 0.471
Protective effect	20.908 ± 1.587[#]	3.774 ± 0.348	19.153 ± 3.171	18.842 ± 6.699	16.802 ± 4.956	15.285 ± 3.464[b]	14.070 ± 2.823[i]	21.077 ± 3.057	22.236 ± 2.609	23.543 ± 5.789
6 h										
Direct effect	–	4.485 ± 1.304	6.843 ± 3.278	7.371 ± 3.360	7.365 ± 4.008	7.764 ± 4.316	7.276 ± 3.019	7.615 ± 3.120	7.310 ± 2.827	8.252 ± 3.451
Protective effect	22.37 ± 6.733[#]	4.851 ± 0.771	19.194 ± 1.534	23.720 ± 2.648	16.281 ± 4.161	16.924 ± 7.706	27.033 ± 6.752	12.771 ± 4.275[c]	21.843 ± 3.121	18.205 ± 6.841
24 h										
Direct effect	–	4.824 ± 0.479	4.553 ± 1.284	4.648 ± 0.400	5.034 ± 1.286	4.302 ± 0.708	4.661 ± 0.787	6.057 ± 0.735[a]	3.841 ± 0.444	4.207 ± 0.575
Protective effect	19.70 ± 7.954[#]	8.977 ± 4.869	16.23 ± 7.875	27.561 ± 3.057[d]	18.252 ± 1.003	20.508 ± 2.967	22.588 ± 7.167	20.217 ± 2.857	26.673 ± 5.692	31.558 ± 7.288[e]

C Control group

[#] $p < 0.05$ when compared to control H_2O_2

[a] $p < 0.05$ 80 μM ε-VNF when compared to control

[b] $p < 0.05$ when compared to H_2O_2 [11.25 μM VCR + 15.8 μM ε-VNF] + H_2O_2

[c] $p < 0.05$ when compared to H_2O_2 [80 μM ε-VNF + H_2O_2]

[d] $p < 0.05$ when compared to H_2O_2 [98.3 μM ε-VNF + H_2O_2]

[e] $p < 0.05$ when compared to H_2O_2 [3.63 μM VCR + 3.75 μM ε-VNF] + H_2O_2

[i] $p < 0.05$ when compared to H_2O_2 [6 μM VCR + H_2O_2]

systems (Zhou et al. 2013). Superoxide dismutase enzyme activities were determined after the HepG2 cells have been treated with the test compounds for 3, 6 or 24 h. The data showed that the SOD activity was reduced after 3 h incubation with 52.5 μM vincristine (18.71 ± 4.70) compared to the untreated control group (28.95 ± 0.60) (Table 2). High and low dose combination groups led to a significant decrease in SOD activation after 6 h incubation time, compared to the control group (29.17 ± 3.63 %; 32.91 ± 5.36 %; 48.836 ± 1.51 %, respectively) ($p < 0.05$) (Fig. 1a; Table 2). Both results from the combination groups were statistically significant, compared to the untreated control at 24 h ($p < 0.05$) (Fig. 1a).

GSH is an important cellular antioxidant. Among the groups, the effects of vincristine, ε-viniferin and combination [vincristine + ε-viniferin] groups on GSH levels in HepG2 cells with incubation periods of 3, 6 and 24 h were examined. The treatment of HepG2 cells with 98.3 μM ε-viniferin increased GSH levels 1.8 times after 3 h of incubation, compared to the untreated control group. 80 μM ε-viniferin and the low dose combination group [3.63 μM vincristine + 3.75 μM ε-viniferin] also significantly ($p < 0.05$) (Fig. 1c) increased GSH levels compared to the untreated control group. When cells were subjected to incubation with the drugs for 6 h, the high dose combination group [11.25 μM vincristine + 15.8 μM ε-viniferin] exhibited decreased GSH levels, compared to groups with treatment of 52.5 μM vincristine, and of 98.3 μM ε-viniferin. Differences were significant compared to 98.3 μM ε-viniferin group, but not significant compared to the untreated control group and the 52.5 μM vincristine group. The low dose combination group [3.63 μM vincristine + 3.75 μM ε-viniferin] was found to be parallel to the 80 μM ε-viniferin group and had statistically significant increased GSH levels compared to the control group ($p < 0.05$) (Fig. 1d; Table 3). After 24 h of incubation time, treatment of HepG2 cells with 80 or 98.3 μM ε-viniferin showed an increase compared to the untreated control group ($p < 0.05$) (Table 3).

The cells were also treated with etoposide, as a different chemotherapeutic agent, with two different concentrations (55 and 7 μM). The level of lipid peroxidation was not changed after treatment with etoposide at concentrations of 55 or 7 μM at the 3rd hour. After treatment of cells for 6 h, the level of lipid peroxidation was found higher than for the control groups but the difference was not statistically significant (Table 1). When the SOD levels are examined, it was observed that they were lower for both concentrations at the 3, 6 and 24 h compared to the control group, but they were not statistically significant (Table 2). HepG2 cells incubated with 55 or 7 μM etoposide showed effects on glutathione levels compared with the control group, however these effects were not statistically significant (Table 3).

Protective effect

H_2O_2 is a non-radical ROS usually formed in living cells as a result of cellular metabolism. To assess the protective effect, HepG2 cells were incubated with vincristine, ε-viniferin and/or a combination [vincristine + ε-viniferin] followed by incubation with 500 μM H_2O_2 for 1 h. According to this, the LPO levels in the presence of H_2O_2 at the 3 h period in the high dose combination treatment with 6 μM vincristine decreased in comparison to the H_2O_2 group (15.28 ± 3.46 μM; 14.07 ± 2.82 μM; 20.90 ± 1.58 μM, respectively) ($p < 0.05$) (Fig. 2a; Table 1). After the 6 h incubation, a significant decrease was observed between the H_2O_2-C group and the 80 μM ε-viniferin group ($p < 0.05$) (Table 1). The incubation of cells with the low dose combination treatment [3.63 μM vincristine + 3.75 μM ε-viniferin] led to a significant increase in the LPO levels, compared to the H_2O_2-C group at 24 h ($p < 0.05$) (Fig. 2b). As a protective effect, after 3 h incubation with 80 μM or 98.3 μM ε-viniferin, the activity of SOD enzyme was increased to 85.90 ± 2.63 and 89.14 ± 7.88, respectively, as compared to the H_2O_2-C group (69.96 ± 12.96) ($p < 0.05$) (Table 2). Except for vincristine and etoposide treatment, the activity of SOD was also increased after 24 h incubation with either high low dose combination, 80 μM ε-viniferin or 98.3 μM ε-viniferin treatments as compared to the H_2O_2 group (87.82 ± 3.56 %; 89.73 ± 4.14 %; 94.65 ± 0.95 %; 73.29 ± 9.01 %; 58.72 ± 0.00 %, respectively). ($p < 0.05$) (Fig. 2c; Table 2). After induction with H_2O_2, a decrease was also observed in the GSH levels at 3 h in the 80 μM ε-viniferin, 98.3 μM ε-viniferin and both high and low dose combination groups, compared to the H_2O_2-C group ($p < 0.05$) (Fig. 2d; Table 3). However, after 6 h of treatment for the 98.3 or 80 μM viniferin groups, there was an increase, compared to the H_2O_2-C group ($p < 0.05$)

Table 2 Direct and protective effects on the HepG2 cell line of vincristine sulphate (VCR), etoposide (ETO), ε-viniferin (ε-VNF) and combined application (VCR + ε-VNF) on SOD activation (%) in HepG2 cells

	H_2O_2	C	IC_{50} values				80 % cell viability			
			VCR (52.5 μM)	ε-VNF (98.3 μM)	ETO (55 μM)	(VCR + ε-VNF) (11.25 + 15.8 μM)	VCR (6 μM)	ε-VNF (80 μM)	ETO (7 μM)	(VCR + ε-VNF) (3.63 + 3.75 μM)
Superoxide dismutase (%)										
3 h										
Direct effect	–	28.959 ± 0.600	18.711 ± 4.705[f]	23.590 ± 2.832	25.198 ± 8.264	29.693 ± 0.233	21.842 ± 5.974	26.118 ± 3.949	26.738 ± 3.072	23.494 ± 1.292
Protective effect	69.96 ± 12.967[#]	32.333 ± 8.876	57.617 ± 4.498	89.146 ± 7.889[d]	68.014 ± 5.556	59.873 ± 4.297	75.029 ± 7.843	85.904 ± 2.639[c]	69.596 ± 4.821	81.378 ± 0.222
6 h										
Direct effect	–	48.836 ± 1.511	39.345 ± 10.607	39.017 ± 0.017	21.665 ± 2.080	29.171 ± 3.633[g]	48.314 ± 8.738	50.870 ± 0.555	35.446 ± 1.249	32.919 ± 5.364[h]
Protective effect	76.14 ± 0.940[#]	21.194 ± 0.697	64.528 ± 1.179	71.267 ± 4.854	22.30 ± 0.441	78.329 ± 0.217	49.168 ± 18.641[i]	66.396 ± 0.667	69.309 ± 2.085	70.690 ± 2.687
24 h										
Direct effect	–	99.022 ± 0.477	85.227 ± 2.905	52.121 ± 7.079	87.357 ± 2.064	43.792 ± 13.431[g]	94.328 ± 1.044	51.649 ± 17.517	90.073 ± 3.184	48.570 ± 15.203[h]
Protective effect	58.721 ± 0.000	64.077 ± 1.095	55.610 ± 1.100	73.297 ± 9.010[d]	73.812 ± 2.905	87.829 ± 3.565[b]	78.007 ± 16.304	94.657 ± 0.956[c]	73.015 ± 0.854	89.733 ± 4.149[e]

[#] $p < 0.05$ against control H_2O_2

[b] $p < 0.05$ when compared to H_2O_2 [11.25 μM VCR + 15.8 μM ε-VNF] + H_2O_2

[c] $p < 0.05$ when compared to H_2O_2 [80 μM ε-VNF + H_2O_2]

[d] $p < 0.05$ when compared to H_2O_2 [98.3 μM ε-VNF + H_2O_2]

[e] $p < 0.05$ when compared to H_2O_2 [3.63 μM VCR + 3.75 μM ε-VNF] + H_2O_2

[f] $p < 0.05$ when compared to control 52.5 μM VCR

[g] $p < 0.05$ when compared to control [11.25 μM VCR + 15.8 μM ε-VNF]

[h] $p < 0.05$ when compared to control [3.63 μM VCR + 3.75 μM ε-VNF]

[i] $p < 0.05$ when compared to H_2O_2 [6 μM VCR + H_2O_2]

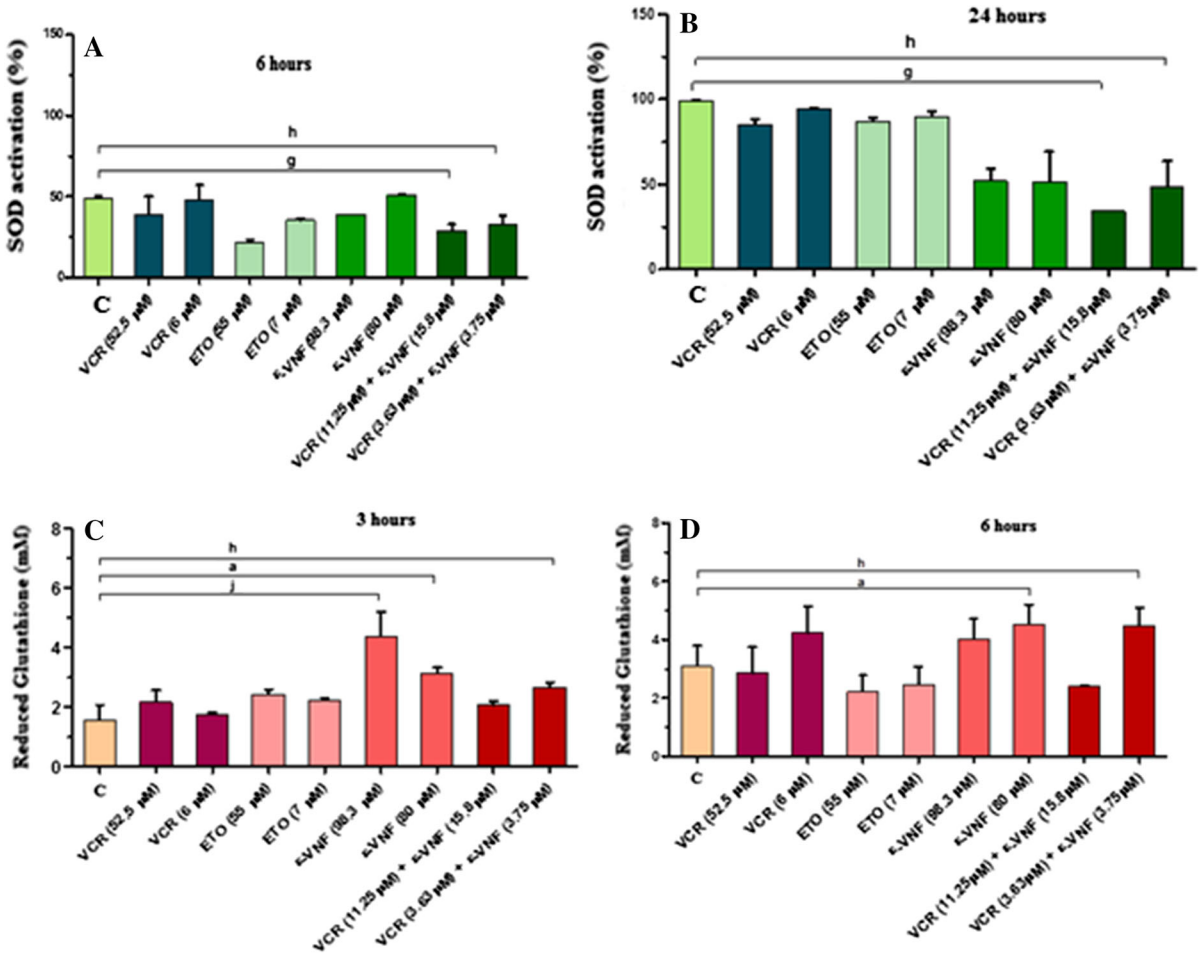


Fig. 1 The direct effects of the addition of vincristine sulphate (VCR), etoposide (ETO), ε-viniferin (ε-VNF) or a combined application (VCR + ε-VNF) on SOD activity (inhibition %) of HepG2 cells in dependence of incubation time (6 and 24 h) (**a**, **b**) and reduced gluthathione levels (mM) of HepG2 cells at 3 and 6 h (**c**, **d**). Superoxide dismutase activity was expressed as percentage of SOD activity (activity %). *Different letters* show statistically significant difference ($p < 0.05$) in comparison with the control group

(Table 3). After 24 h, the 80 μM ε-viniferin, the low dose [3.63 μM vincristine + 3.75 μM ε-viniferin], and the high dose [11.25 μM vincristine + 15.8 μM ε-viniferin] combination groups showed a significant increase in the GSH levels compared to the H_2O_2-C group ($p < 0.05$) (Fig. 2e; Table 3).

The data obtained from the protective activity studies with etoposide showed no significant differences with all test compounds (Tables 1, 2, 3).

The SOD H_2O_2-C group displayed lower values in comparison with the untreated control group at the 3rd and 6th hours, however, it was higher at the 24th hour and the difference was statistically significant. There was no statistically significant difference among the other groups (Table 2). Similar values were determined when the reduced glutathione levels at the 3, 6 and 24 h for H_2O_2-C group were compared with the untreated group, and there was no statistically significant difference (Table 3).

Discussion

There are many studies indicating the facts that dietary antioxidants suppresses the growth of a wide variety of tumor cells, maintain the integrity of normal cells, repair induced cellular damage, and remove the effect of free radicals (Barjot et al. 2007; Do Amaral et al. 2008; Ganesaratnam et al. 2004; Ozben 2007; Sarrias

Table 3 Direct and protective effects of vincristine sulphate (VCR), etoposide (ETO), ε-viniferin (ε-VNF) and combined application (VCR + ε-VNF) on reduced glutathione levels (mM) in HepG2 cells

	H_2O_2	C	IC_{50} values				80 % cell viability			
			VCR (52.5 μM)	ε-VNF (98.3 μM)	ETO (55 μM)	(VCR + ε-VNF) (11.25 + 15.8 μM)	VCR (6 μM)	ε-VNF (80 μM)	ETO (7 μM)	(VCR + ε-VNF) (3.63 + 3.75 μM)
Reduced glutathione (mM)										
3 h										
Direct effect	–	1.564 ± 0.519	2.162 ± 0.398	4.368 ± 0.835[j]	2.430 ± 0.177	2.073 ± 0.132	1.760 ± 0.068	3.142 ± 0.191[a]	2.223 ± 0.096	2.676 ± 0.161[h]
Protective effect	2.69 ± 0.448[#]	1.985 ± 0.240	1.138 ± 0.067	1.397 ± 0.043[d]	1.199 ± 0.195	1.151 ± 0.031[b]	1.083 ± 0.051	1.642 ± 0.088[c]	1.146 ± 0.092	1.263 ± 0.141[e]
6 h										
Direct effect	–	3.083 ± 0.711	2.852 ± 0.908	4.016 ± 0.717[j]	2.223 ± 0.561	2.395 ± 0.058	4.234 ± 0.915	4.521 ± 0.683[a]	2.459 ± 0.627	4.495 ± 0.612[h]
Protective effect	1.491 ± 0.241	1.726 ± 0.382	1.499 ± 0.039	3.050 ± 0.188[d]	1.824 ± 0.174	1.764 ± 0.042	1.700 ± 0.325	2.417 ± 0.183[c]	1.496 ± 0.149	1.760 ± 0.087
24 h										
Direct effect	–	2.523 ± 0.104	2.029 ± 0.175	3.767 ± 0.498[j]	2.317 ± 0.104	2.935 ± 0.459	2.313 ± 0.764	3.759 ± 0.717[a]	2.376 ± 0.324	2.093 ± 0.081
Protective effect	0.609 ± 0.332	0.748 ± 0.008	0.839 ± 0.164	0.565 ± 0.118	0.208 ± 0.063	1.230 ± 0.228[b]	0.174 ± 0.078[i]	1.214 ± 0.099[c]	0.477 ± 0.083	1.151 ± 0.167[e]

[#] $p < 0.05$ against control H_2O_2

[a] $p < 0.05$ when compared to control 80 μM ε-VNF

[b] $p < 0.05$ when compared to H_2O_2 [11.25 μM VCR + 15.8 μM ε-VNF] + H_2O_2

[c] $p < 0.05$ when compared to H_2O_2 [80 μM ε-VNF + H_2O_2]

[d] $p < 0.05$ when compared to H_2O_2 [98.3 μM ε-VNF + H_2O_2]

[e] $p < 0.05$ when compared to H_2O_2 [3.63 μM VCR + 3.75 μM ε-VNF] + H_2O_2

[h] $p < 0.05$ when compared to control [3.63 μM VCR + 3.75 μM ε-VNF]

[i] $p < 0.05$ when compared to H_2O_2 6 μM VCR + H_2O_2

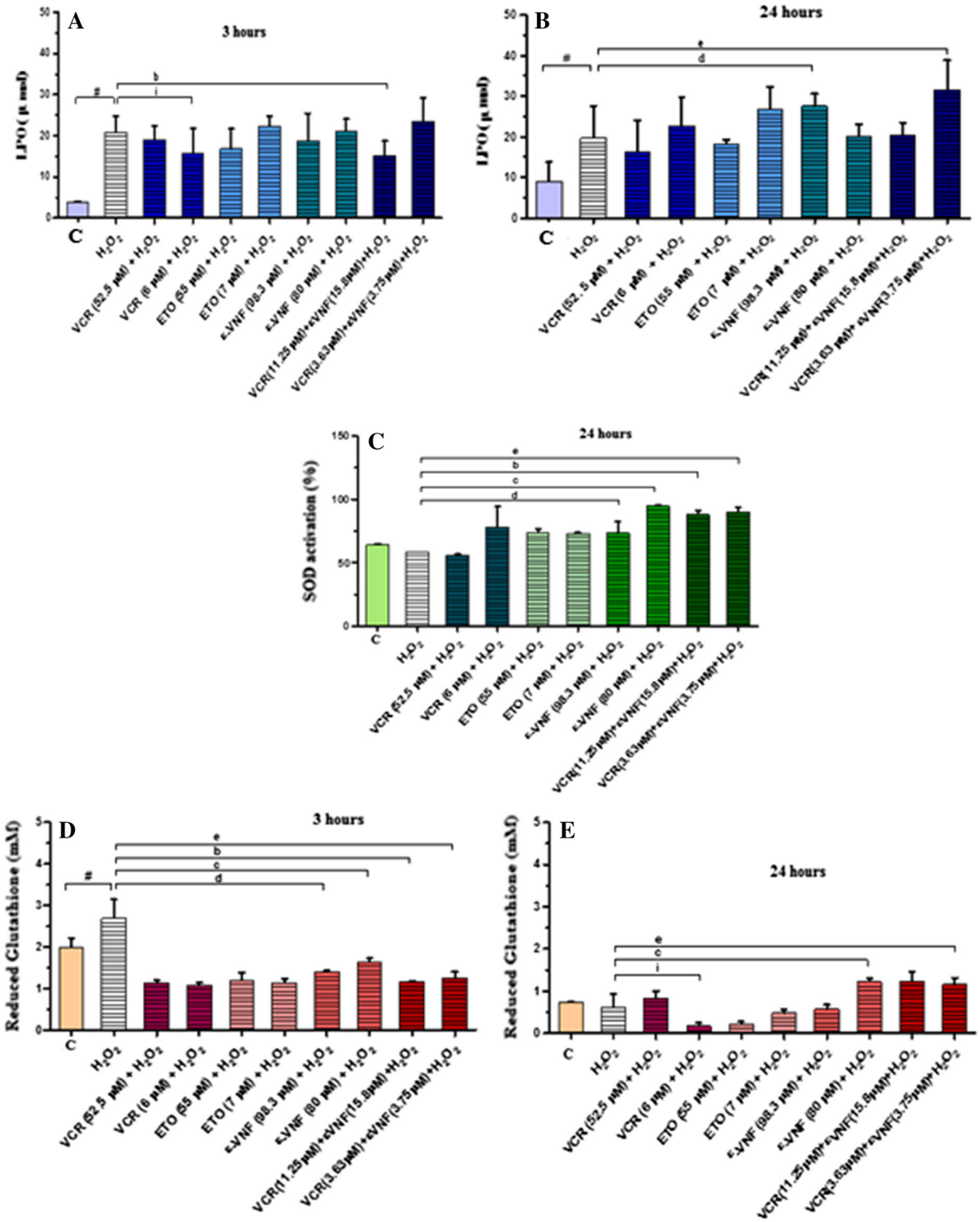
[j] $p < 0.05$ when compared to control 98.3 μM ε-VNF

et al. 2011). ε-Viniferin used in this study is a sample of dietary antioxidants (Barjot et al. 2007). ε-Viniferin, a dimer of resveratrol, is a polyphenolic substance. Polyphenols may influence carcinogenesis via many mechanisms. They may especially prevent the formation of oxidative stress (Porrini et al. 2005; Yao et al. 2004). While resveratrol among polyphenols is known to possess anti-inflammatory, antioxidant, and anticarcinogenic properties (Athar et al. 2007, 2009; Fremont 2000; Leonard et al. 2003; Santandreu et al. 2011; Sun et al. 2002), the properties of its dimer, ε-viniferin, have not been examined in detail. In an earlier study, ε-viniferin was shown to have hepatoprotective, antioxidant, and apoptosis-inducing properties in leukemia B cells (Conklin 2004). Antiproliferative and pro-apoptotic effects of dimers of resveratrol, including ε-viniferin, were demonstrated in human hepatoma, HepG2 (Colin et al. 2008) and human colon cancer cells (Delmas et al. 2005).

In the present study, vincristine sulphate was used as a chemotherapeutic agent. Vincristine sulphate prevents the formation of mitotic spindles, stops the cell cycle in the G2/M phase, and stimulates apoptosis (Harmsma et al. 2004; Ricci and Zong 2006). However, in the present study, ε-viniferin was used as an antioxidant to stop the cell cycle in the G2/M phase leading to apoptosis (Barjot et al. 2007; Khan et al. 2007). Since these two agents affect the cell cycle at the same phase, their usage in combination may increase each other's effects, leading to the consideration that they may be useful in decreasing the dose of vincristine sulphate acting as a chemotherapeutic agent. With this concept, the present study was conducted in two phases. In the first phase, ε-viniferin was used alone and in combination with vincristine sulphate, and its direct effects on oxidative stress were examined. In the second phase, oxidative stress was formed in HepG2 cells with exogenically administered H_2O_2 and the objective was to examine their protective effects alone and in combination doses on lipid peroxidation, SOD enzyme activation, and reduced glutathione levels.

In the first phase of the study, the direct effects of ε-viniferin, vincristine sulphate, and combination doses on HepG2 cells during incubation periods of 3, 6, and 24 h were examined. The cells treated with 98.3 and 80 μM ε-viniferin for 6 h that was administered as antioxidant increased LPO level and showed pro-oxidant effect, however, values had no significance in comparison to untreated control group. High dose [11.25 lM vincristine ? 15.8 μM ε-viniferin] and low dose [3.63 lM vincristine ? 3.75 μM ε-viniferin] combination groups showed an effect similar to the ε-viniferin groups after 6 h. The LPO levels after incubation with the test compounds for a longer time (24 h), except for 80 μM ε-viniferin (6.05 ± 0.73) treatment, were found to range between 3.84 ± 0.44 and 5.03 ± 1.28, however, the values were not significant as compared to untreated cells (4.82 ± 0.47). Filomeni et al. (2007) had also observed that 50 μM of resveratrol had a pro-oxidant effect on MCF-7 cells which is quite similar to our results. In addition, Santandreu et al. (2011) have used HT-29 and SW-620 colorectal cancer cells to analyze 5-Fluorouracil (5-FU), cisplatin (cDDP), and etoposide (ETO) agents used alone and administered in combination as [5-FU + resveratrol], [cDDP + resveratrol], [ETO + resveratrol] in the treatment of tumors. It was observed that these combinations decreased cell viability in both cell lines when compared to control groups, and also compared to groups in which the agents were administered alone. In this study, the level of MDA was increased in the combined treatment of 5-FU and resveratrol in both types of cancer cells in comparison with the treatment of 5-FU alone. This may be due to the fact that intracellular ROS with the combined treatment accumulates more and increases cellular lipid peroxide levels more and strengthens the effects of the chemotherapeutic agent. The effects of combined treatment of vincristine and ε-viniferine on LPO levels were similar to the 52.5 and 6 μM vincristine groups. Taking this into consideration, when vincristine sulphate and ε-viniferin agents are used for treatment in combination, we believe that a similar effect at low doses is generated as compared to 52.5 μM vincristine sulphate.

In the present study, when the direct effects of the groups on total SOD activation during an incubation period of 3 h was examined, the 52.5 μM vincristine group showed decreased SOD activation only, when compared to the untreated control group. However, during incubation periods of 6 and 24 h, both the high-dose [11.25 μM vincristine + 15.8 μM ε-viniferin] and low dose [3.63 μM vincristine + 3.75 μM ε-viniferin] combination groups showed significantly decreased SOD activation, compared to the untreated control group ($p < 0.05$). In a structure–activity


A
3 hours
LPO(µ mol)
B
24 hours
LPO (µ mol)
C
24 hours
SOD activation (%)
D
3 hours
Reduced Glutathione (mM)
E
24 hours
Reduced Glutathione (mM)
C
H2O2
VCR (52.5 µM) + H2O2
VCR (6 µM) + H2O2
ETO (55 µM) + H2O2
ETO (7 µM) + H2O2
ε-VNF (98.3 µM) + H2O2
ε-VNF (60 µM) + H2O2
VCR(11.25 µM)+ε-VNF(15.8µM)+H2O2
VCR(3.63µM)+ε-VNF(3.75µM)+H2O2

◀**Fig. 2** The protective values of vincristine sulphate (VCR), ε-viniferin (ε-VNF), etoposide (ETO) and combined application (VCR + ε-VNF) on LPO (μM) levels of HepG2 cell line at 3 and 24 h (**a**, **b**), SOD (inhibition %) at 24 h (**c**) and reduced glutathione (mM) at 3 and 24 h (**d**, **e**) activation induced by H_2O_2. *Different letters* show statistically significant difference ($p < 0.05$) in comparison to the control and H_2O_2 groups

relationship study performed by Farines et al. (2004), it was interpreted that *trans*-resveratrol inhibited SOD in vitro and the anticarcinogenic property of *trans*-resveratrol was dependent on SOD. SOD inhibition leads to the accumulation of cellular superoxide radicals, resulting in the apoptosis of cancer cells through free radical damage. In addition, Farines et al. (2004) showed that the presence of OH groups in the structure of 4 and/or 4′ carbon in stilbene derivatives was effective in the inhibition of SOD activation. SOD enzyme activation was observed twofold–fourfold higher in HepG2 cells compared to normal liver cells (Lee et al. 2002; Hanif et al. 2005). According to the results of these studies, HepG2 cells contain higher antioxidant enzyme values than the human normal hepatic cell line (Chang). This may suggest that HepG2 becomes more resistant to oxidative stress. Furthermore, this may aid the survival of HepG2 cells.

Along these lines, in the present study performed with HepG2 cells, the decrease in SOD activation with ε-viniferin in the 24 h incubation groups is due to the fact that it possessed 4′-OH structure as a dimer of resveratrol, and HepG2 cells are subjected to free radical damage with decreased SOD activation. In the combination groups, SOD activation was decreased in a time-dependent (6 and 24 h) manner because of pro-oxidant effects of the compounds that might disturb the balance in the cellular antioxidant system and may leave the cell unprotected against ROS, possibly having a similar effect like ε-viniferin.

As the last part of the first phase of the study, direct effects of the agents on cellular GSH levels in hepatoma cells were examined. After 3 h, all test groups had pro-oxidant effects on GSH levels of HepG2 cells compared to the untreated control group. However, treatment of cells in the low-dose combination [vincristine and viniferin] group increased the GSH level after 6 h, and appears to be the most effective dose and period. In a study previously performed with resveratrol in HepG2 cells, resveratrol was observed to increase the expression of γ-glutamyl cysteine synthase enzyme related to GSH synthesis, showing increased cellular GSH level (Sekhar et al. 2002).

In order to explain the mechanisms regulating antioxidant defense, it is better to examine the response of external signals regulating intracellular pro-oxidant-antioxidant balance in these defense systems. Unfortunately, due to the complexity of organ and tissue organization in in vivo experiments, along with sudden hormonal changes and other factors, it is difficult to prove the molecular mechanism of a specific regulation. One of the most commonly used external signals increasing intracellular oxidative stress for an investigation like this one in cultured cells is H_2O_2 (Alia et al. 2005; Fukui et al. 2010; Kim et al. 2008; O'Brien et al. 2000). H_2O_2 damages DNA, lipids, and other macromolecules in the cell, and may lead to oxidative damage (Aherne and O'Brien 1999). When not metabolized, it can react with transition metal ions like Fe^{2+} and Cu^{2+} to form the highly reactive hydroxyl radical ($^{\bullet}OH$) and this may lead to the spreading of oxidative damage in the cells (Guiardelli et al. 1997; Halliwell and Gutteridge 1999). Therefore, in order for viable cells to maintain their viability, any excess amount of H_2O_2 should be reduced (Meneghini and Martins 1993).

In order to examine the protective effects of the substances against LPO levels in an oxidative stress environment, an H_2O_2-C group was maintained along with the control group. The protective substances against oxidative stress formed with H_2O_2 was examined. The incubation period was prolonged, along with the fact that the lower dose of combination group induced an LPO increase in comparison with the H_2O_2-C group during 24 h of incubation. This may be related to metabolization of ε-viniferin by cells in a time-dependent manner. In a study performed by Do Amaral et al. (2008), cisplatin-induced nephrotoxicity in rats, when administered the antitumoral drug cis-platine alone and in combination with the antioxidant resveratrol, MDA levels were found to be significantly higher in the cis-platine alone group, compared to the control groups. However, MDA levels of [resveratrol + cDDP] combined group decreased, compared to the cis-platine alone group. In another similar in vivo study, resveratrol treatment in gentamycin-

formed nephrotoxicity significantly prevented the formation of MDA (Morales et al. 2002).

In our study, protective effects of the test compounds on oxidative stress generated after treatment of HepG2 cells by H_2O_2 with the test compounds against SOD activation in the presence of oxidative stress generated by H_2O_2 was observed via SOD activation at 24 h. The activity of SOD increased significantly after incubation with 98.3 μM ε-viniferin, 80 μM ε-viniferin, the low dose and the high dose combination groups, compared to the H_2O_2 group (73.29 ± 9.01 %; 94.65 ± 0.95 %; 89.73 ± 4.14 %; 87.82 ± 3.56 %; 58.72 ± 0.00 %, respectively). However, the SOD activity in HepG2 cells did not change after treatment with both concentrations of vincristine. These data suggested that the combined treatment with ε-viniferin increased the protective effects of vincristine against oxidative damage.

Incubation of cells with all test substances for 3 h decreased GSH levels significantly, but this result was not obtained for the longer incubation periods. In our study, HepG2 cells showed pro-oxidant effects against an increase in GSH level induced with H_2O_2 during a 3 h incubation period. In a study performed by Alia et al. (2005), oxidative stress induced by t-BOOH decreased GSH levels in HepG2 cells compared to the controls. This increase was prevented by a pre-treatment with quercetine, and it was found that quercetine protected HepG2 cells from probable oxidative stress.

ROS is a potential double-edged sword in the progression and prevention of cancers. Temporary changes in ROS concentration in the body can affect activity of signal transduction pathways leading to either cell proliferation, or to apoptosis and necrosis, depending on the dosage and duration of ROS, as well as the type of cell (Kardeh et al. 2014).

In conclusion, there are novel anti-tumor strategies for hepatic cancer based on a growing interest in using antioxidants as potential cancer therapeutics or cancer preventive agents. ε-viniferin is an antioxidant and opens possibilities to selectively develop the beneficial health properties of those natural compounds for the prevention and treatment of human diseases such as hepatic cancers. As a result, further studies are needed to unravel the role of ROS in redox regulation and the potential outcome of antioxidant administration such as ε-viniferin on cellular responses.

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