Olive Leaf Extracts Protect Cardiomyocytes against 4-Hydroxynonenal-Induced Toxicity *In Vitro*: Comparison with Oleuropein, Hydroxytyrosol, and Quercetin

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Abstract
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Olive (*Olea europaea*) leaf, an important traditional herbal medicine, displays cardioprotection that may be related to the cellular redox modulating effects of its polyphenolic constituents. This study was undertaken to investigate the protective effect of the ethanolic and methanolic extracts of olive leaves compared to the effects of oleuropein, hydroxytyrosol, and quercetin as a positive standard in a carbonyl compound (4-hydroxynonenal)-induced model of oxidative damage to rat cardiomyocytes (H9c2). Cell viability was detected by the MTT assay; reactive oxygen species production was assessed by the 2′,7′-dichlorodihydrofluorescein diacetate method, and the mitochondrial membrane potential was determined using a JC-1 dye kit. Phospho-Hsp27 (Ser82), phospho-MAPKAPK-2 (Thr334), phospho-c-Jun (Ser73), cleaved-caspase-3 (cl-CASP3) (Asp175), and phospho-SAPK/JNK (Thr183/Tyr185) were measured by Western blotting. The ethanolic and methanolic extracts of olive leaves inhibited 4-hydroxynonenal-induced apoptosis, characterized by increased reactive oxygen species production, impaired viability (LD$_{50}$: 25 μM), mitochondrial dysfunction, and activation of pro-apoptotic cl-CASP3. The ethanolic and methanolic extracts of olive leaves also inhibited 4-hydroxynonenal-induced phosphorylation of stress-activated transcription factors, and the effects of extracts on p-SAPK/JNK, p-Hsp27, and p-MAPKAPK-2 were found to be concentration-dependent and comparable with oleuropein, hydroxytyrosol, and quercetin. While the methanolic extract downregulated 4-hydroxynonenal-induced p-MAPKAPK-2 and p-c-Jun more than the ethanolic extract, it exerted a less inhibitory effect than the ethanolic extract on 4-hydroxynonenal-induced p-SAPK/JNK and p-Hsp27. cl-CASP3 and p-Hsp27 were attenuated, especially by quercetin. Experiments showed a predominant reactive oxygen species inhibitory and mitochondrial protecting ability at a concentration of 1–10 μg/mL of each extract, oleuropein, hydroxytyrosol, and quercetin. The ethanolic extract of olive leaves, which contains larger amounts of oleuropein, hydroxytyrosol, verbascoside, luteolin, and quercetin (by HPLC) than the methanolic one, has more protecting ability on cardiomyocyte viability than the methanolic extract or each phenolic compound against 4-hydroxynonenal-induced carbonyl stress and toxicity.

Abbreviations
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cl-CASP3:	cleaved caspase-3
DCF:	2′,7′-dichlorofluorescein
DCFH2-DA:	2′,7′-dichlorodihydrofluorescein diacetate
ΔΨ m:	mitochondrial membrane potential
HNE:	4-hydroxynonenal
Hsp27:	heat shock protein 27
H9c2:	embryonic rat heart-derived myogenic cell line
JC-1:	5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolo carbocyanine iodide
MAPKAPK-2:	MAP kinase-activated protein kinase 2
MTT:	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
OLE-1:	ethanolic extract of olive leaves
OLE-2:	methanolic extract of olive leaves
PMSF:	phenylmethanesulfonyl fluoride
ROS:	reactive oxygen species
SAPK/JNK:	stress-activated protein kinase/c-jun N-terminal kinases

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Table 1 Total phenolic content of the ethanolic (OLE-1) and methanolic (OLE-2) extracts of olive leaves. All values are presented as the means ± SD. [a] Significantly different from OLE-1 (p < 0.05); Student's *t*-test. GAE: gallic acid equivalent.

Sample (1 mg/mL)	Absorbance	SD	GAE (mg/mL)	%
OLE-1	0.78	0.03	0.14	14.1
OLE-2	0.55[a]	0.01	0.09	8.7

Extract	Concentration (mg/mL)	%	Retention time (min) HPLC
Oleuropein			
▶ OLE-1	2.047	20.47	21.740
▶ OLE-2	0.354[a]	3.54	21.753
Hydroxytyrosol			
▶ OLE-1	0.040	0.40	5.598
▶ OLE-2	0.023[a]	0.23	5.573
Verbascoside			
▶ OLE-1	0.060	0.60	16.968
▶ OLE-2	0.035[a]	0.35	16.979
Quercetin			
▶ OLE-1	0.039	0.39	25.051
▶ OLE-2	0.003[a]	0.03	25.737
Luteolin			
▶ OLE-1	0.167	1.67	26.186
▶ OLE-2	0.021[a]	0.21	26.543

Table 2 Major phenolic compounds of the ethanolic (OLE-1) and methanolic (OLE-2) extracts of olive leaves. [a] Significantly different from OLE-1 (p < 0.05); Student's *t*-test.

Introduction
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The interactions of proteins with ROS may result in numerous post-translational, reversible, or irreversible modifications, leading to a change in structure and/or function of the oxidized protein. 4-hydroxynonenal (HNE) is a major active aldehyde formed by lipid peroxidation reactions, which can react readily with histidine, cysteine, or lysine residues of proteins, leading to the formation of stable Michael adducts with a hemiacetal structure [1–3]. The modifications of proteins by reactive aldehydes act as signaling molecules and play multiple physiological functions; however, their excessive production with irreversible concentrations may contribute to cytopathological results including impaired signaling, RNA and DNA damage, cell cycle arrest, and apoptosis [4–6]. HNE is involved in various disease states including atherosclerosis [5], diabetes [7,8], and cardiac problems [9,10]. In cultured cardiomyocytes, HNE has been shown to mediate hypoxia/reoxygenation-induced injury and cell death inhibited by antioxidant treatment [11]. Despite the significance of HNE or carbonyl stress in cell survival or the death process, the effects of HNE incubation in cardiomyocytes remain uninvestigated. The aim of this study was to determine the mechanism of carbonyl (HNE)-induced apoptotic signaling and cardiotoxicity in H9c2 cells.
Olive leaves contain several groups of constituents, including polyphenols, flavonoids, flavones, iridoids, and carbohydrates [12–14]. Oleuropein, the major phenolic secoiridoid in *Olea europaea* L. (Oleaceae), has been characterized as a powerful antioxidant in *in vitro* [14,15] and *in vivo* [16] experiments. Olive leaves or oleuropein have been shown to attenuate cardiac, hepatic, and metabolic changes in high-carbohydrate-, high-fat-fed rats [16, 17] and protect heart function against ischemia and reperfusion [18]. In recent studies, we showed that the ethanolic extracts of olive leaves, oleuropein, or hydroxytyrosol exhibited the protective and regenerative effects on redox balance and related signaling pathways in insulin releasing pancreatic β-cells exposed to H_2O_2 [19] or a cytokine cocktail [20]. Thus, in this study, we sought to explore if olive leaf extracts or polyphenolic compounds (oleuropein, hydroxytyrosol and quercetin) can protect H9c2 cardiomyocytes *in vitro* against carbonyl (HNE) stress-induced toxicity and apoptosis.

Results
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The amount of total phenolic content of olive leaf extracts determined using the gallic acid calibration curve (data not showed) was found to be higher in OLE-1 (ethanolic extract) than OLE-2 (methanolic extract) (○ **Table 1**). Larger amounts of oleuropein, which is the major phenolic constituent of olive leaves [12–14, 19,20], were detected in both extracts than the amounts of other phenolic constituents. The quantity of oleuropein was found to be more in OLE-1 than in OLE-2. OLE-2 also contains less hydroxytyrosol, verbascoside, luteolin, and quercetin than OLE-1 (○ **Table 2**). High-performance liquid chromatography (HPLC) chromatograms of the ethanolic and methanolic extracts of olive leaves are demonstrated in ○ **Fig. 1**.
H9c2 cells were preincubated with increasing concentrations (0.1, 10, 25, and 50 μg/mL) of each extract, phenolic compound, or standard quercetin for 24 h. At 0.1, 10, 25, and 50 μg/mL concentration ranges, cell viability was 87.07 ± 2.01%, 91.16 ± 8.09%, 85.89 ± 6.81%, and 77.49 ± 6.87% for OLE-1, respectively; 96.02 ± 1.88%, 96.02 ± 2.75%, 96.02 ± 0.944%, and 88.76 ± 4.30% for OLE-2, respectively; 80.54 ± 2.82%, 81.26 ± 3.07%, 83.50 ± 1.36%, and 76.20 ± 2.87% for oleuropein, respectively; 68.55 ± 4.47%, 68.22 ± 7.50%, 74.88 ± 2.77%, 66.11 ± 10.32%, and 45.93 ± 2.74% for hydroxytyrosol, respectively; and 89.0 ± 6.80%, 92.0 ± 5.50%, 89.70 ± 3.40%, and 95.20 ± 11.30% for quercetin, respectively. According to these findings, the minimal effective two concentrations of the extracts, phenolic compounds, and the standard quercetin were selected for testing their protective effect on HNE toxicity by the MTT method.
○ **Fig. 2** shows that HNE (0–50 μM) induced inhibition on the viability of H9c2 cells in a dose-dependent manner. The IC_{50} value of 25 μM of HNE was obtained at 6 h. We used non-toxic doses
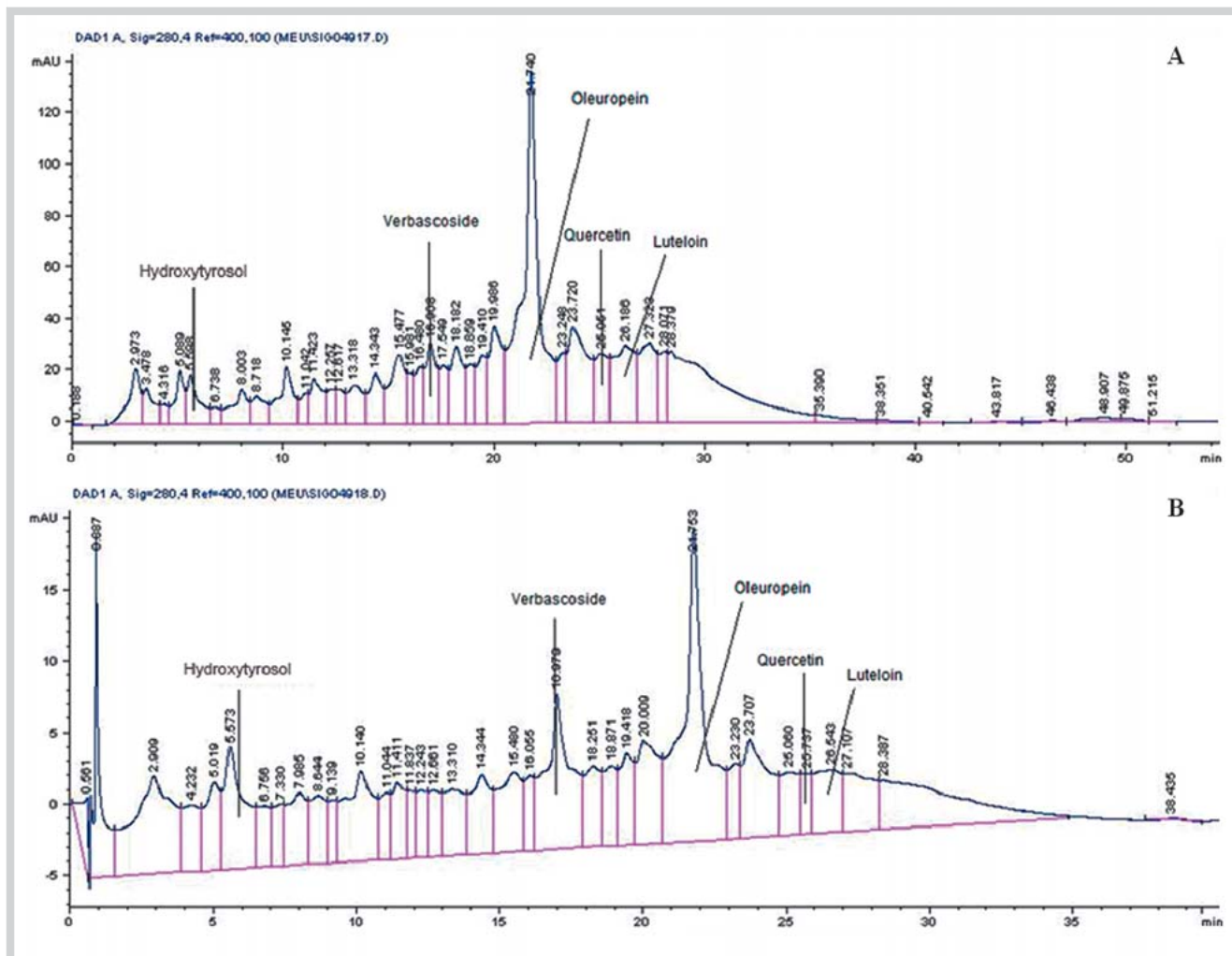
Fig. 1 HPLC profile of the ethanolic (**A** OLE-1) and methanolic (**B** OLE-2) extracts of olive leaves. Detection was performed using a diode array detector (DAD), and the chromatographs were recorded at $\lambda = 280$ nm (for polyphenols and oleuropein). Conditions: mobile phase, (**A**) acetic acid/water (2.5 : 97.5), (**B**) acetonitrile; flow rate, 1 mL·min^{-1}; column temperature, 36 °C; injection volume, 10 μL. A linear gradient was run from 95% (**A**) and 5% (**B**) to 75% (**A**) and 25% (**B**) during 20 min; it changed to 50% (**A**) and (**B**) in 20 min (40 min, total time); in 10 min, it changed to 20% (**A**) and 80% (**B**) (50 min, total time), after reequilibration, in 10 min (60 min, total time) to initial composition. (Color figure available online only.)

(0.1–10 μg/mL, < IC$_{50}$) of each extract and olive leaf phenolic compounds to test their effects on HNE-induced cell death. MTT findings confirmed that each leaf extract (OLE-1 and OLE-2) has a protective effect on HNE-induced toxicity in H9c2 cells even at a 0.1-μg/mL concentration, which is comparable with the protective effect of oleuropein, hydroxytyrosol, or quercetin (○ **Fig. 3**). OLE-1 has a significantly more protective effect than OLE-2 on the viability of H9c2 cells (○ **Fig. 3**). The degree of the protective effect of quercetin was similar with the degree of protective effect of OLE-1. In comparison with OLE-1, oleuropein or hydroxytyrosol alone demonstrated a lesser protective effect on the viability in HNE-exposed H9c2 cells (○ **Fig. 3**).

A cell membrane permeable fluorescent dye, DCFH2-DA, that is sensitive to oxidation was used to assess the levels of intracellular oxidative stress after exposure to HNE and also following pretreatment with OLE-1, OLE-2, oleuropein, hydroxytyrosol, and quercetin for 24 h. OLE-2 and oleuropein at 0.1 and 10 μg/mL concentrations, and OLE-1 and hydroxytyrosol at 10 μg/mL concentrations completely suppressed the ROS production induced by HNE (○ **Fig. 4**). However, OLE-1, hydroxytyrosol, and quercetin at a 0.1 μg/mL concentration were found to have a relatively less
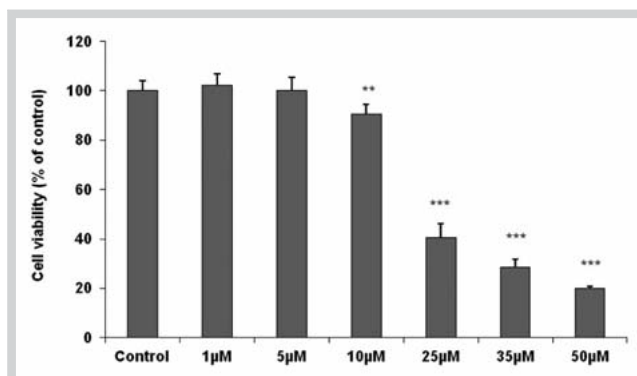

Fig. 2 Cytotoxicity of 4-hydroxynonenal (HNE) in H9c2 (6 h) cells assessed by the MTT viability test. * P < 0.05, ** p < 0.01, *** p < 0.001 vs. control, ANOVA.

suppressive effect than those observed with their 10 μg/mL concentrations against HNE-induced ROS production (○ **Fig. 4**). Ex-
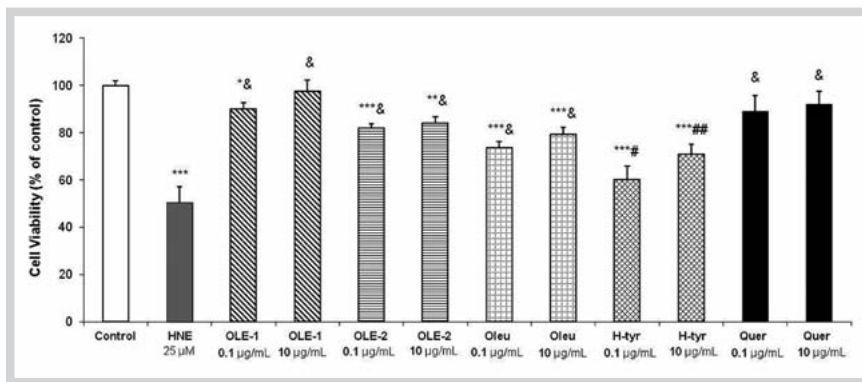
Fig. 3 The protective effects of olive leaf extracts OLE-1 and OLE-2, oleuropein [Oleu], hydroxytyrosol [H-tyr], or the standard compound quercetin [Quer] at 0.1 and 10 µg/mL concentrations on HNE-induced toxicity (25 µM; for 6 h) in H9c2 cells assessed by the MTT viability test. Data represent the results of three independent experiments performed in triplicate (means ± SD). * P < 0.05, ** p < 0.01, *** p < 0.001 vs. control; # p < 0.05, ## p < 0.01, & p < 0.001 vs. HNE, ANOVA.
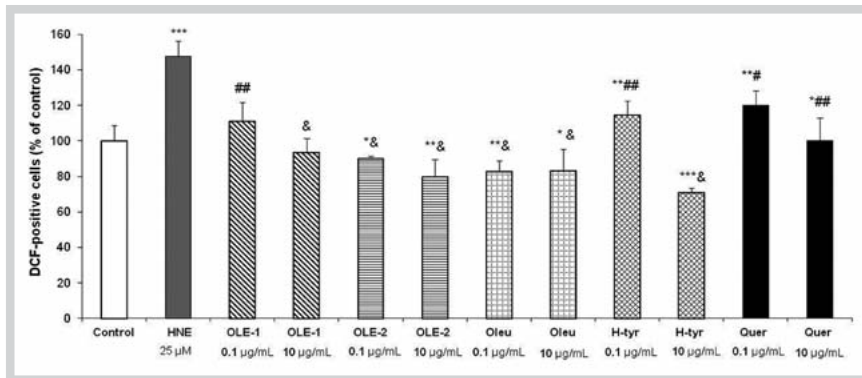

Fig. 4 HNE increases the intracellular production of ROS. Pretreatment of H9c2 cells for 24 h with OLE-1, OLE-2, oleuropein [Oleu], hydroxytyrosol [H-tyr], or the standard compound quercetin [Quer] at 0.1 or 10 µg/mL concentrations reduced ROS levels induced by HNE. ROS levels in cells were measured by analyzing the DCF intensity. Data represent the results of three independent experiments performed in triplicate (means ± SD). * P < 0.05, ** p < 0.01, *** p < 0.001 vs. control; # p < 0.05, ## p < 0.01, & p < 0.001 vs. HNE, ANOVA.

periments showed a predominant ROS scavenging activity at a maximum sample dose of each extract, olive phenolic compound, or standard quercetin (10 µg/mL) (p < 0.001).

Mitochondrial membrane potential was measured by fluorescence staining with JC-1. Stimulation with HNE caused a significant decrease of red fluorescence of JC-1 in H9c2 cells showing mitochondrial dysfunction (○ **Fig. 5a** and **b**). Treatment with all preparations significantly prevented the drop of red fluorescence (○ **Fig. 5a** and **b**). OLE-1 was found to be less effective than OLE-2 and each olive leaf phenolic compound at 0.1 µg/mL (○ **Fig. 5b**). Western blot analysis of H9c2 cells exposed to HNE revealed expression levels of various proteins, which were partly or markedly downregulated by olive leaf extracts or the components of the extracts represented by commercial oleuropein, hydroxytyrosol, or quercetin as the standard (○ **Fig. 6**). HNE exposure to H9c2 cells resulted in significant increases (p < 0.001) in stress-activated proteins including p-SAPK/JNK (○ **Fig. 7a**), p-MAPKAPK-2 (○ **Fig. 7b**), p-Hsp27 (○ **Fig. 7c**) p-c-Jun, (○ **Fig. 7d**), and cl-CASP-3 (○ **Fig. 7e**). The HNE-induced increase on p-SAPK/JNK was partly but significantly and concentration-dependently blocked by both extracts and the polyphenolic compounds. At a concentration of 10 µg/mL, oleuropein and hydroxytyrosol were found to be more effective than other studied materials to inhibit HNE-induced p-SAPK/JNK (○ **Fig. 7a**). In comparison with OLE-1, oleuropein or quercetin and OLE-2 or hydroxytyrosol produced an upwards inhibition on p-MAPKAPK-2 protein levels (p < 0.001) (○ **Fig. 7b**). In response to carbonyl stress, HSP27 (Ser82) expression increases several folds to create a cellular adaptation to the adverse environmental stimuli. While HSP27 expression was significantly inhibited by all studied materials, OLE-1, oleuropein, and quercetin at a concentration of 10 µg/mL were found to be more effective (p < 0.001) (○ **Fig. 7c**). Olive leaf extracts and the phenolic compounds have an inhibitory effect on c-Jun phosphorylation at their nontoxic concentrations (p < 0.05 or

p < 0.01), and OLE-2 showed the most significant inhibition on HNE-induced c-Jun phosphorylation at 10 µg/mL (p < 0.001) (○ **Fig. 7d**). The upregulation of cl-CASP3 by HNE was decreased in the presence of phenolic olive leaf constituents. The degrees of attenuations after the treatment of each extract or phenolic phytochemical on HNE-induced cl-CASP3 were found to be similar (0.1 or 10 µg/mL, p < 0.05 or p < 0.01) and smaller than the inhibitory effect of quercetin (p < 0.001) (○ **Fig. 7e**).

Discussion
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In this study, an HNE-induced toxicity model was used to investigate the effects of carbonyl compound-induced redox stress and the protective ability of olive leaf phenolic compounds and extracts on cardiomyocyte viability and the apoptotic process in H9c2 cells *in vitro*. We found that (1) a carbonyl compound, HNE, inhibits viability and accelerates apoptosis in a concentration-dependent manner and (2) OLE-1, OLE-2 and each phenolic component of olive leaves, like oleuropein, hydroxytyrosol, and quercetin, exert a significant antiapoptotic effects against HNE-induced toxicity in H9c2 cells. The protective action mechanisms of the extracts or the phenolic compounds were accompanied by a decrease of the induction of the apoptotic stress markers (p-MAPKAPK-2, p-SAPK/JNK, p-HSP27, p-c-JUN, and cl-CASP3). The findings were related to the cellular protective effects of the extracts, and oleuropein and hydroxytyrosol were comparable with the effects of positive standard [21,22]. The protective action mechanisms of the extracts and the phenolic constituents of olive leaves include the reduction of ROS production and the amelioration of the mitochondria membrane potential in H9c2 cells. These results suggest that the ethanolic extract of olive leaves and also its polyphenolic constituents have a key role in cardiomyocyte protection.
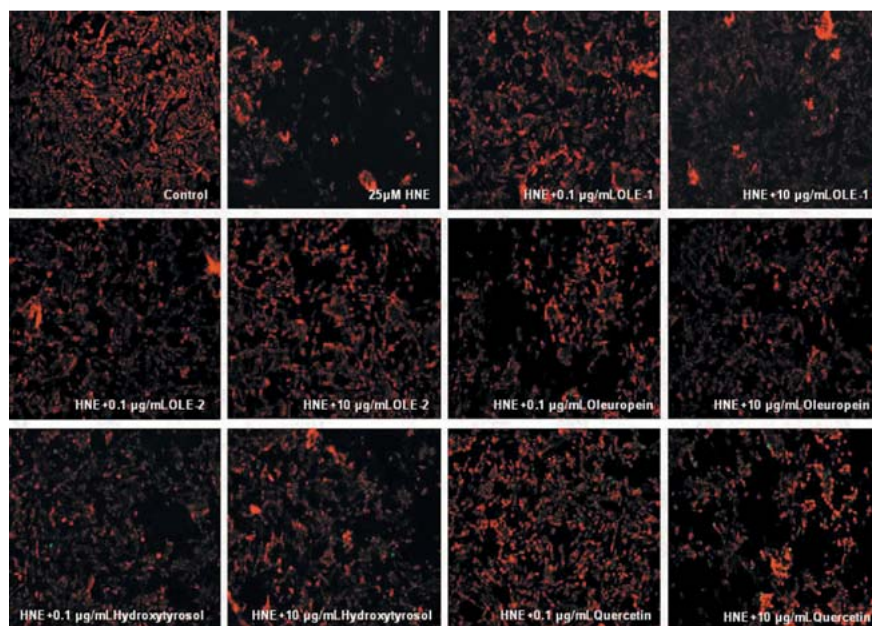
Fig. 5 a JC-1 staining for 4-hydroxynonenal-induced dissipation of mitochondrial membrane potential in H9c2 cells. The cells were treated with 0.1 µg/mL and 10 µg/mL doses of OLE-1, OLE-2, oleuropein [Oleu], hydroxytyrosol [H-tyr], or the standard compound quercetin [Quer] for 15 minutes and stained with JC-1, a mitochondrial potential sensitive dye. **a** Fluorescence pattern of different treatment groups as indicated in the pictures appearing as an increment of red JC-1 aggregates; decreasing green fluorescence means the diminution of mitochondrial depolarization decreases. (Color figure available online only.)
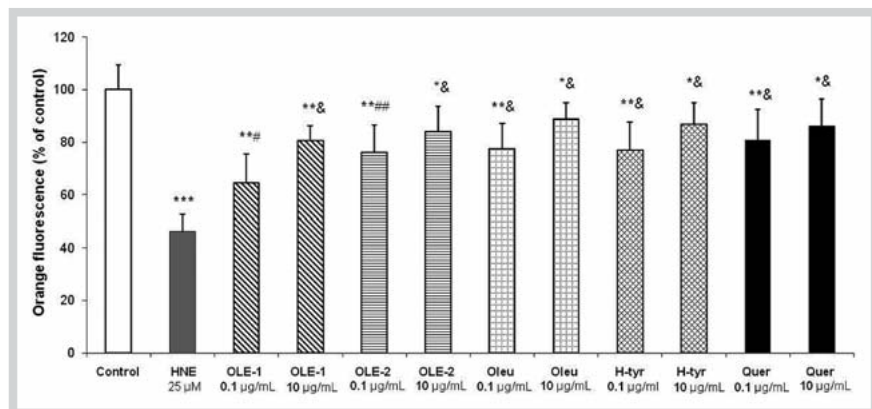

Fig. 5 b Quantitative presentation of the data as (mean ± SD of three samples) percent of positive JC-1 stained cells. * $P < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control; # $p < 0.05$, ## $p < 0.01$, & $p < 0.001$ vs. HNE, ANOVA.

HNE, a highly reactive α,β-aldehydic product generated from polyunsaturated fatty acid peroxidation, is able to accumulate in the heart in response to chronic oxidant infestation [23]. Long-term HNE exposure leads to an increase in HNE-protein adduct formation and dysfunctional proteins, mediating molecular mechanism of carbonyl stress-induced cardiotoxicity [24]. Although carbonyl-induced redox stress is known to aggravate cardiac abnormalities [7,8], there is no report showing stress-induced signaling pathways after HNE exposure *in vitro* in cardiomyocte cell lines except for one, which reported a link with increased endoplasmic reticulum stress [25].

Upon stimulation by carbonyl stress, p38MAPK is phosphorylated. It then phosphorylates MAPKAPK2 to phosphorylate and activate HSP27 [26,27]. The phosphorylation of HSP27 is under the control of the intracellular ROS level [28]. We observed that HSP27 is phosphorylated during HNE exposure in H9c2; on the other hand, the pretreatment with olive leaf phenolic compounds led to a decrease in this phosphorylation of HSP27. This indicates that olive leaf phenolic compounds contribute to H9c2 cell survival via decreasing lethal effects of HNE-induced ROS, which eliminates the requirement for HSP27-regulated prosurvival pathways. Quercetin has been shown to restrict the adaptation-induced MAPKAPK2 and HSP70 synthesis to HNE. The blockade of HSP27 synthesis by quercetin was demonstrated previously by authors in H9c2 cells [29].

c-Jun N-terminal kinase-1 (JNK-1) is activated by oxidative stress as a consequence of HNE induction [30] and thought to promote injury during severe acute myocardial infarction [26]. Several studies reported that JNK is another protein activated by oxidative stress, which is a consequence of HNE induction [31]. The present study demonstrated, for the first time, that JNK is a downstream signaling molecule in HNE-mediated cardiotoxicity in H9c2 cells. HNE-induced p-SAPK/JNK was downregulated partly by the extracts oleuropein, hydroxytyrosol, or quercetin as concentration-dependently. The results indicated that HNE induction in c-Jun, an important transcription factor that is activated by JNK, was attenuated by olive leaf extracts, especially by treatment with OLE-2 and hydroxytyrosol. Quercetin, which has been shown to be an inhibitor of c-Jun N-terminal kinase in ischemia/reperfusion injury in isolated rat hearts [32], attenuated HNE-induced c-Jun in H9c2 cells. Quercetin is the most abundant flavonoid [21] and is believed to attenuate cardiovascular disease and inhibit cardiac hypertrophy by blocking c-Jun [33].

Another important intracellular signaling pathway leading to ROS-mediated apoptosis is involved in the activation of the caspases. Activation of p38 MAPK then leads to cleavage of pro-CASP3, yielding active cleaved-CASP3, one of the key effectors of apoptosis [34]. It has been reported that, upon oxidant exposure, H9c2 cells exhibit increased activation of CASP3, which is considered an indicator of cell death [35]. In our study, oleuropein, hy-
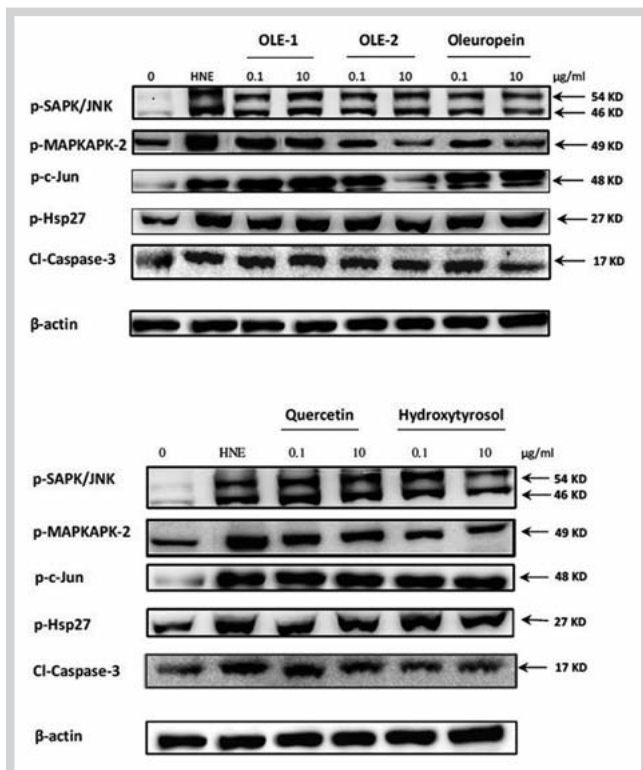
Fig. 6 Effects of ethanolic and methanolic extracts of olive leaves, OLE-1 and OLE-2, oleuropein [Oleu], hydroxytyrosol [H-tyr], and the standard compound quercetin [Quer] on the protein levels of H9c2 cell lines (Western blotting). After 24 h pretreatment with each extract or phenolic compounds, the cells were exposed to HNE (25 μM) for 30 min.

Fig. 7 Western blot analysis demonstrated that the expressions of some different stress-stimulated and apoptotic signaling proteins (p-SAPK/JNK, p-MAPKAPK-2, p-c-Jun, p-Hsp27, cl-Caspase-3) significantly increased after 25 μM 4-hydroxynonenal treatment of H9c2 cells. However, OLE-1, OLE-2, oleuropein [Oleu], hydroxytyrosol [H-tyr], or quercetin [Quer] (0.1 or 10 μg/mL) significantly decreased HNE-induced protein expression. The protein bands of p-SAPK/JNK, p-MAPKAPK-2, p-c-Jun, p-Hsp27, and Cl-Caspase-3 were obtained from Western blot analysis. They were from a representative experiment repeated three times with similar results. The expression level of the proteins was quantitated by densitometric analysis by using Image J Software Informer, and the mean densitometry values from three independent Western blot experiments are expressed relative to respective untreated controls (relative optical density = 1). Oleu: oleuropein; H-tyr: hydroxytyrosol; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control; # $p < 0.05$, ## $p < 0.01$, & $p < 0.001$ vs. HNE, ANOVA.

droxytyrosol, and both extracts reduced CASP3 activity by modulating the mitochondrial response to HNE. It has been demonstrated that quercetin exerts protective effects against H_2O_2 cardiotoxicity as confirmed by measurement of caspase-3 activity and that the mechanism of its action involved in the modulation of PI3K/Akt and ERK1/2 signaling pathways in H9c2 cardiomyoblasts [36]. Overall, the effects of different olive leaf extracts on apoptotic markers were different: OLE-1 exerted an inhibitory effect more than OLE-2 on HNE-induced p-SAPK/JNK and p-Hsp27, while p-MAPKAPK-2 and p-c-Jun were more downregulated by OLE-2 than OLE-1.

The cardiomyocyte protection observed in the present study by olive leaf extracts or their phenolic components against HNE toxicity are consistent with our previous findings showing a powerful protection of the ethanolic extract from olive leaves (~20% oleuropein content) in insulin-releasing cells against H_2O_2-induced [19] or cytokine-induced [20] toxicity and apoptosis. Based on the present findings, we suggest a model representing the action mechanisms of olive leaf phenolic compounds on HNE-induced apoptotic cell death and toxicity (● **Fig. 8**).

The present study established that OLE-1 has more ability than OLE2 or each phenolic compound to protect cardiomyocytes against HNE-induced stress and toxicity. The more effective protecting ability of OLE-1 on cardiomyocyte viability is likely due to the synergistic interactions of polyphenolics and other compounds of OLE-1 since OLE-1 also includes a larger amount of other phenolics (verbascosid and luteolin, as well as oleuropein, hydroxytyrosol, and quercetin) than OLE-2. In this respect, we highlight once more that the usage of a polyphenolic extract mixture can be preferred for cell protection in comparison with a
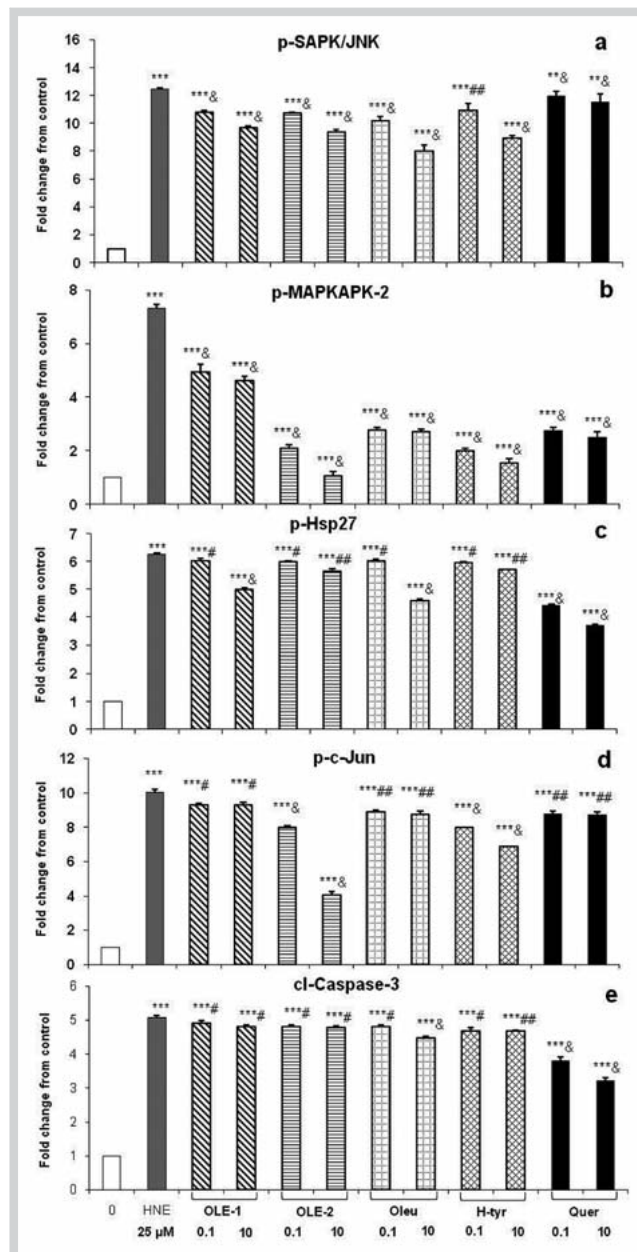
mono-component antioxidant compound [24]. The leaves of the olive plant (*O. europaea*) have been commonly used for centuries in folk medicine. Recently, the medicinal properties of olive products have focused on its polyphenols (particularly oleuropein and hydroxytyrosol), which, according to animal and *in vitro* studies,
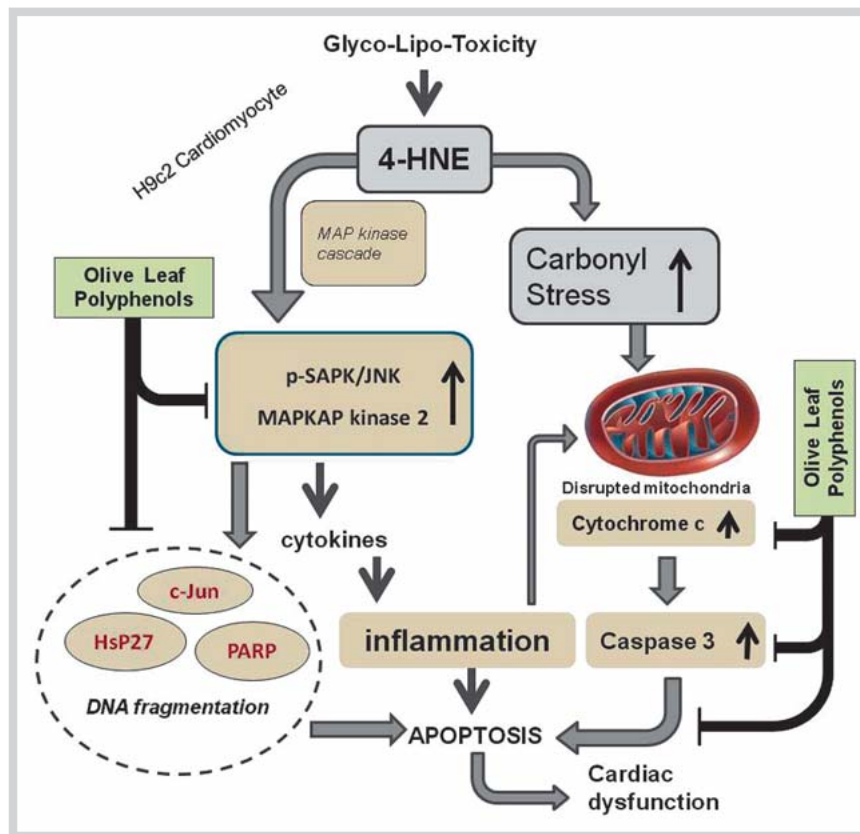
Fig. 8 Intracellular action mechanisms of olive leaf extracts and phenolic compounds exerting protective effects on cardiomyocytes following 4-hydroxynonenal exposure-induced carbonyl stress and toxicity. (Color figure available online only.)

have antioxidant, hypoglycemic, antihypertensive, and anti-atherosclerotic properties, and improve metabolic syndrome and cardiovascular risk factors in patients [37].

In conclusion, we provided the first evidence for cardiomyocyte protection by olive leaf phenolic extracts and the major phenolic components oleuropein and hydroxytyrosol against HNE-induced cytotoxicity. This study supports the results of animal or clinical studies demonstrating the cardioprotective effects of the olive leaf and its phenolic constituents along with elucidating their cellular action mechanisms and effects on downstream stress signaling pathways.

Materials and Methods
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Chemicals
Folin-Ciocalteau reagent and gallic acid were from Merck Co. All cell culture products were obtained from Lonza. HNE was purchased from Cayman Chemical. Oleuropein (HPLC grade ≥ 96%; Applichem A6909 Lot# 12 101 123) and hydroxytyrosol (HPLC grade > 98%; Applichem A5303 Lot# 11 120 406) were purchased from Applichem. Quercetin (HPLC grade ≥ 98%) was from Sigma-Aldrich (Q4951 Lot# 060M1196 V).

Extracts
The ethanolic and methanolic extracts of olive leaves prepared according to a modified method by the authors [12, 13] were obtained from Farmasens Co. as a gift. OLE-1 (Lot# 012–08) and OLE-2 (Lot# 005–10) were stored in light-protected glass bottles at 4 °C until further analyzed.

Determination of the total phenolic content
Total phenolic content was determined using the Folin-Ciocalteau assay [14], which is based on colorimetric oxidation and reduction reaction. An aliquot (0.5 mL) of each extract was reacted with freshly prepared 1.25 mL of 20% sodium carbonate and 0.5 mL of 1 N Folin reagent in a screw-capped test tube. Required dilutions were prepared with distilled water. Test tubes were vortexed and after 40 min, absorbance readings were recorded at 725 nm by a UV-Vis spectrophotometer (Shimadzu). The amount of total phenolic compounds was expressed as milligram of gallic acid equivalent per gram of dry weight of sample.

High-performance liquid chromatography determination
The modified HPLC analysis given in the literature [13, 14] was used for the quantification of phenolic compounds of the ethanolic and methanolic extracts of olive leaves. The HPLC equipment used was a Hewlett-Packard Series HP 1100 equipped with a diode array detector, and the chromatographs were recorded at $\lambda = 260$ nm (for hydroxytyrosol and oleuropein). The stationary phase was a C_{18} LiChrospher 100 analytical column (250×4 mm i. d.) with a particle size of 5 μm (Merck) thermostated at 30 °C. The HPLC chromatogram of the olive leaf extract was compared to the standard mix of identified compounds. The compounds in the olive leaf extracts were identified by comparison of their retention times with the corresponding standards.

Cell culture
The embryonic rat heart-derived myogenic cell line H9c2 was purchased from the American Tissue Type Collection (#CRL-1446). The H9c2 cells were cultured in 75-cm^2 TC flasks in DMEM supplemented with 10% FBS, 2 mM L-glutamine, and 1% penicil-

lin-streptomycin. The cells were cultured at 37 °C in a humidified atmosphere containing 95 % air/5 % CO_2.

Application of the extracts and phenolic compounds

Stock solutions of olive leaf extracts were dissolved in 10 % DMSO, and the concentrations of the extracts were diluted with DMEM. The extracts at the maximum concentration contained 0.02 % DMSO. Hydroxytyrosol, oleuropein, and quercetin were dissolved in media.

Cell viability assay

The effect of each extract or phenolic compound on the viability of H9c2 cells in the presence of HNE was detected by the MTT method [38]. The cells (1×10^4/well) were seeded in 96-well plates and incubated for 24 h. Later, fresh medium containing 0.1, 10, 25, and 50 μg/mL concentrations of each extract or phenolic compound was added and the cells were incubated for 24 h followed by incubation with HNE (25 μM) for 6 h. Then 10 μL of MTT stock solution (5 mg/mL) was added, and the plates were incubated at 37 °C for 4 h. Culture medium was removed and the resultant formazan crystals were dissolved in 100 μL DMSO, and the absorbance values were read on a microplate reader (PowerWave XS2; BioTek Instruments) at the wavelength of 572 nm. The cells were assayed in quadruplicate, and at least two independent experiments were carried out.

Measurement of reactive oxygen species

The accumulation of intracellular ROS was determined by measuring DCF fluorescence and evaluated with a modified method described by [39]. ROS causes oxidation of DCFH2-DA to the fluorescent product DCF in the cell. In brief, the cells (2×10^4/well) were pretreated with each OLE or the phenolic compounds in doses of 0.1 and 10 μg/mL and then exposed to HNE for 15 min at 37 °C in darkness. Cell culture plates were washed and incubated with 10 μM DCFH2-DA (Molecular Probes) in medium. The DCFH2-DA-containing medium was removed; the cells were washed twice with PBS, and DCF fluorescence was quantified (Ex 485 nm/Em 535 nm) using a multimode microplate reader (PowerWave XS2; BioTek Instruments).

Determination of mitochondrial membrane potential

ΔΨ m was indirectly determined using a JC-1 assay kit (Cayman Chemical Company). JC-1, a fluorescent dye, and the lipophilic cationic probe 5,5′,6,6′-tetrachloro-l,l′,3,3′-tetraethyl benzimidazol carbocyanine iodide were used for investigating ΔΨ m in living cells. An unincorporated dye exists in a monomeric form, emitting at 527 nm after excitation at 490 nm [40]. To assess the integrity of ΔΨ m, the cells were pretreated with each extract or phenolic compounds in 0.1 and 10 μg/mL concentrations for 24 h. After pretreatment, the cells were incubated with 25 μM HNE for 15 min and then stained with 10 μM JC-1 in medium at 37 °C for 30 min in darkness. Then cells were washed twice with PBS. The JC-1 fluorescence was measured by fluorescence microscopy or spectrofluorometry. Fluorescence images were captured and processed using a Leica DM6000 microscope with a Leica DFC420 camera controlled via Leica LAS AF7000 software. Fluorescence signals were also measured by spectrofluorometry in 485 nm excitation, 525 nm, and 590 nm for emission of green and red fluorescence, respectively. The ratio of red/green fluorescence of JC-1 was used as an indicator of mitochondrial function. In healthy cells with high mitochondrial ΔΨ m, JC-1 forms multimers called J-aggregates that produce red fluorescence. On the other hand, in apoptotic or unhealthy cells with low ΔΨ m, JC-1 remains in the monomeric form, which shows green fluorescence. The results are illustrated as the percentage change in fluorescence values in comparison with the untreated controls.

Western blotting

H9c2 cells cultured in 60-mm petri dishes (Sarstedt) were lysed in 250 μL of lysis buffer (1 % Triton, 20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, and 1 μg/mL leupeptin) supplemented with 1 mM PMSF (Roche Diagnostics). Protein concentrations were determined by using the BCA protein assay (Pierce). Protein lysates (20 μg) were heated for 5 min at 94 °C in Laemmli sample buffer containing 5 % β-mercaptoethanol and loaded on 4–15 % Tris-glycine SDS-PAGE gels, then transferred electrophoretically to nitrocellulose membranes. Membranes were blocked with 5 % nonfat dry milk for 1 h and incubated overnight at 4 °C with phospho-MAPKAPK-2 (Thr334), phospho-HSP27 (Ser82), phospho-SAPK/JNK (Thr183/Tyr185), phospho-c-Jun (Ser73), and cleaved Caspase-3 (Asp175) antibodies (Cell Signaling Technology). Protein bands were detected with horseradish peroxidase-conjugated anti-rabbit secondary antibodies (Cell Signaling Technology) and visualized by West-Femto ECL reagents (Pierce). Chemiluminescent signals of immunoblots were documented by using Gel Logic 2200 Pro (Carestream Health). The intensity of specific proteins was quantified by using Carestream Molecular Image Software and ImageJ Software (v. 1.4; National Institutes of Health).

Statistical analysis

Experiments were performed at least three times (more than three times for MTT and DCF experiments), and statistical analysis was performed with the unpaired Student's t-test using XL-Plot v. 2.81. One-way ANOVA tests were performed using program Past, v. 2.16. All values are expressed as means ± SD, and $p < 0.05$, $p < 0.01$, and $p < 0.001$ were considered statistically significant.

Conflict of Interest
▼

The authors declare no conflict of interest.

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