Neuroprotective effect of (R)-(-)-linalool on oxidative stress in PC12 cells

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ABSTRACT

Background: Oxidative stress plays an important role in neurodegeneration, pain and inflammation. (R)-(-)-linalool (LIN) is endowed with neuroprotective, anti-nociceptive and anti-inflammatory properties.

Purpose: The present study aims at investigating the hypothesis that LIN's neuroprotective, antinociceptive and anti-inflammatory properties descend from its ability to act as antioxidant. The study challenges this hypothesis by verifying whether LIN may counteract hydrogen peroxide (H_2O_2)-induced oxidative stress in PC12 cells.

Methods: In H_2O_2-exposed PC12 cells, LIN was tested on a) cell viability, measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), b) damage of plasma membrane, measured by lactate dehydrogenase (LDH) release, c) intracellular levels of reactive-oxygen-species (ROS), d) apoptosis and e) cell cycle distribution.

Results: Under H_2O_2-induced cell viability reduction, LIN protects PC12 cells. Likewise, LIN protects cells from oxidative damage by preventing the H_2O_2-dependent increase of LDH release, counteracts intracellular ROS overproduction and reduces H_2O_2-induced apoptosis. Finally, the results of the cell cycle analysis from cells exposed to H_2O_2 indicate that LIN incubation reduces the number of cells induced into quiescence by H_2O_2 in the G2/M phase.

Conclusions: These findings indicate that LIN protects PC12 cells from H_2O_2-induced oxidative stress. This mechanism could justify the neuroprotective, anti-nociceptive and anti-inflammatory effects of this compound and suggest LIN as a potential therapeutic agent for the management oxidative stress-mediated pain.

Abbreviations

ANOVA	analysis of variance
H_2O_2	hydrogen peroxide
LDH	lactate dehydrogenase
LIN	(R)-(-)-Linalool
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PC12	rat pheochromocytoma cells
ROS	reactive-oxygen-species

1. Introduction

Linalool is a monoterpene compound present in essential oils from aromatic plants. Due to the presence in its structure of an asymmetry center, linalool exists in nature as racemate of the two enantiomers,

(R)-(-)-linalool and (S)-(+)-linalool (Peana et al., 2002). Linalool, as racemic form, has been suggested by *in vitro* studies to possess anti-inflammatory effects (Kim et al., 2019) as well as, by *ex vivo* studies and in immortalised neuronal HT-22 cells, antioxidant properties (Celik and Ozkaya, 2002; Sabogal-Guáqueta et al., 2019). Moreover, as reported by Sabogal-Guáqueta et al. (2018), linalool possesses neuroprotective effects in different cerebral ischemia models.

On the other hand, also the enantiomer (R)-(-)-linalool (LIN) possesses neuroprotective effects (Park et al., 2016) shown by its ability to decrease intracellular oxidative stress during oxygen-glucose deprivation/reoxygenation-induced injury, as well as to scavenge peroxyl radicals. Interestingly, LIN's anti-inflammatory activity has been studied both in acute (Peana et al., 2002) and in chronic inflammatory and neuropathic hypersensitivity (Berliocchi et al., 2009; Batista et al., 2008, 2010) and it has described that LIN, upon acute treatment, pos-

sesses anti-hyperalgesic and anti-nociceptive effects in different animal models of pain (Peana et al., 2003, 2004a, 2004b). The anti-nociceptive and anti-hyperalgesic properties of LIN have been ascribed to many factors including its capacity of stimulating the opioidergic, cholinergic and dopaminergic systems as well as its interaction with $K+$ channels (Peana et al., 2004a, b) or its negative modulation of glutamate transmission (Peana et al., 2004a; Batista et al., 2008). Furthermore, other studies have demonstrated that the effects of LIN on pain responses are also, at least partially, mediated by an activity onto adenosine A1 and A2A receptors (Peana et al., 2006a) and by the reduction of nitric oxide production/release mainly due to its activity on the enzyme NOS (Peana et al., 2006b). Notably, the anti-inflammatory properties of linalool and LIN significantly differ for pharmacokinetic reasons. In particular, although both the racemate and the (R)-(-)-enantiomer were reported to have the same anti-inflammatory properties their actions differ as LIN elicits a delayed and prolonged action with respect to that caused by the racemic form (Peana et al., 2002).

The imbalance between oxidant and antioxidant systems produces an oxidative stress which is deleterious to cells (Sies et al., 2017) whereby the maintenance of normal physiological functions depends on the equilibrium among reactive oxygen species (ROS) and intracellular antioxidant agents. There is growing evidence indicating that oxidative stress, both central (spinal) and peripheral, plays an important role in pain also of inflammatory origin (Berliocchi et al., 2009; Shim et al., 2019). Accordingly, several endogenous pathways that initiate the inflammatory response depend on the overproduction of ROS (Medzhitov, 2008). Similarly, oxidative stress subsequent from ROS increase, shows a key role in neuropathic pain (Kuthati et al., 2021).

Having the above evidence in mind, the present study aims at challenging the hypothesis that LIN's neuroprotective, antinociceptive and anti-inflammatory properties may descend from its ability to act as antioxidant by counteracting hydrogen peroxide (H_2O_2)-induced oxidative stress in rat pheochromocytoma (PC12 cells) a well-known model for studying neuronal signaling pathway (Zhang et al., 2010). To this end we have evaluated the activity of LIN on H_2O_2-affected cell viability, by the 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide (MTT) assay, as well as on a number of other measures related to cells' exposure to oxidative stress such as lactate dehydrogenase (LDH) release, intracellular levels of ROS, apoptosis and changes of cell cycle distribution.

2. Material and methods

2.1. Cell culture

PC12 derived from a pheochromocytoma cell line (ATCCCRL-1721) (passages 12–25) of the rat adrenal medulla were maintained at 37°C in a 95% humidified atmosphere and 5% CO_2 in 100 mm plastic culture petri in Dulbecco's-modified eagle medium (DMEM)/F12 supplemented with 10% horse serum, 5% fetal bovine serum, and 1% of penicillin/streptomycin as reported by Rassu et al. (2020).

2.2. Drug treatments

PC12 cells were treated for 24 h with LIN (0.1, 10 and 100 μM) alone or with H_2O_2, (100μM, applied 20 min after LIN). LIN concentrations were chosen on the basis of literature data (Park et al., 2016; Sabogal-Guáqueta et al. 2019; Varia et al., 2020). All experiments were performed in triplicate.

2.3. Cell viability measurement by MTT assay

The cell viability was assessed with the 3-(4, 5-dimethyl-thiazol-2-yl)−2, 5, diphenyltetrazolium bromide (MTT; Sigma-Aldrich, Italy) assay in which only the viable cells convert the soluble dye MTT in insoluble (in aqueous media) blue formazan crystals by the action of mito-

chondrial reductase. In brief, at the end of the exposure time, 1 mg of MTT (200 µl of a 5 mg/ml stock solution) was added for each sample and incubated for 4 h at 37 ˚C. After the incubation the MTT solution was removed, the cells were washed with phosphate-buffered saline (PBS) and centrifuged, and the pellet was dissolved in 2 ml of isopropanol. The absorbance value were detected by a Beauty Diagnostic Microplate Reader at 578 nm. All experiments were performed in 24-well plates (1×10^5 cells/well) and repeated in triplicate as reported by Rassu et al. (2020). All experiments were performed in triplicate.

2.4. LDH assay

This assay is based on measurement of LDH release which is a stable enzyme normally found in the cytosol of all cells but that is rapidly released into the supernatant upon damage of plasma membrane. Cellular LDH release was measured using CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega Corporation, USA) as described in the user's manual instructions and as reported by Langasco et al. (2019). All experiments were performed in triplicate.

2.5. ROS detection

The intracellular ROS content was evaluated using Total ROS Detection Kit (ENZ-51,011, ENZO). PC12 cells were seeded in 6-well plates. After 24 h of treatment, cells were harvested by adding 400 μl of 0.25% trypsin and centrifuged at 1200 rpm for 10 min. The pellets were resuspended in PBS and stained with ROS Detection Reagent for 30 min in dark. After incubation, intracellular production of ROS was detected by FACSCANTO cytometer (Becton & Dickinson, USA) as reported by Langasco et al. (2019). All experiments were performed in triplicate.

2.6. Apoptosis analyses

PC12 cells, were seeded in 6-well plate at 1.5×10^6 cells/well concentration. After 24 h of treatment, the apoptosis was evaluated using Annexin V-FITC (BD Pharmingen, USA) and propidium iodide (BD Pharmingen, USA). In detail, cells were incubated with Binding Buffer 1X containing Annexin V-FITC and PI for 15 min in dark. Cells were analysed using a FACSCANTO flow cytometer (Becton & Dickinson, USA), as reported by Virdis et al. (2020). All experiments were performed in triplicate.

2.7. Cell cycle distribution

PC12 cells (seeded in 6-well plates at 1.5×10^6 cells) were washed two times with PBS and centrifuged at 1200 rpm for 10 min. The pellets were fixed in 70% cold ethanol and then stained with propidium iodide (BD Pharmingen, USA) for 15 min. Subsequently, the samples were analysed by flow cytometer FACSCANTO (Becton & Dickinson, USA) as described by Langasco et al. (2019). All experiments were performed in triplicate.

2.8. Statistical analysis

All experimental data are expressed as mean values $\pm$ SEM. In the presence of overall significant main effects and interactions (p values <0.05) following One-Way-analysis of variance (ANOVA), Bonferroni multiple comparisons test as post hoc, was performed. All data were evaluated by using Graph-Pad Prism 10.0 software (Inc, San Diego, CA, USA).

3. Results

3.1. Effects of LIN on cell viability

The cell viability of PC12 cells was determined by the MTT assay, performed to evaluate the effect of 24 h exposure to LIN (0.1, 10 and
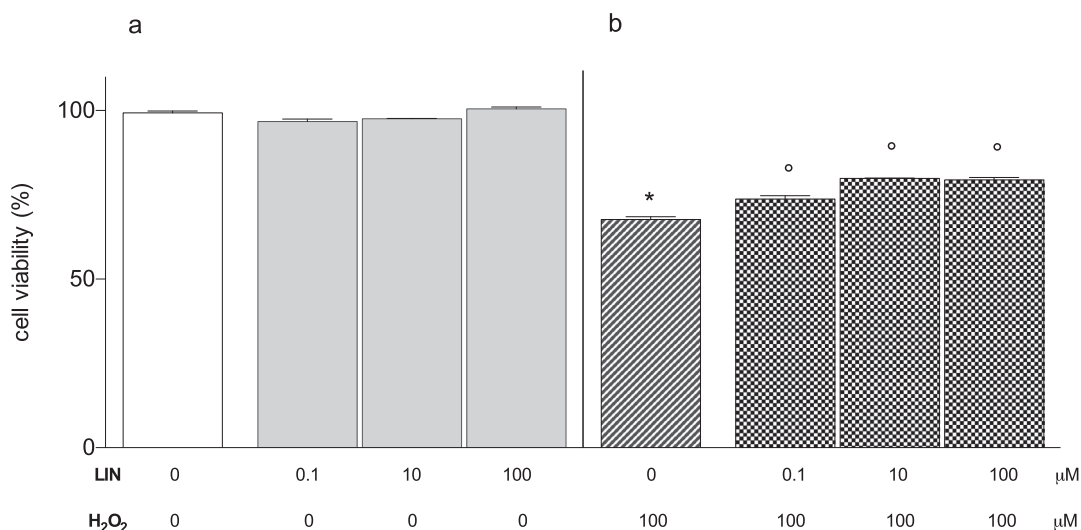
Fig. 1. Cell viability measurement by MTT assay. Effect of LIN (0.1, 10 and 100 μM) on the viability of PC12 cells. Panels a and b show the effect of LIN with or without H_2O_2 (100 μM), at 24 h of exposure. * ($p<0.05$) versus PC12 untreated cells (control); ° versus H_2O_2. As post hoc we used Bonferroni multiple comparisons test.
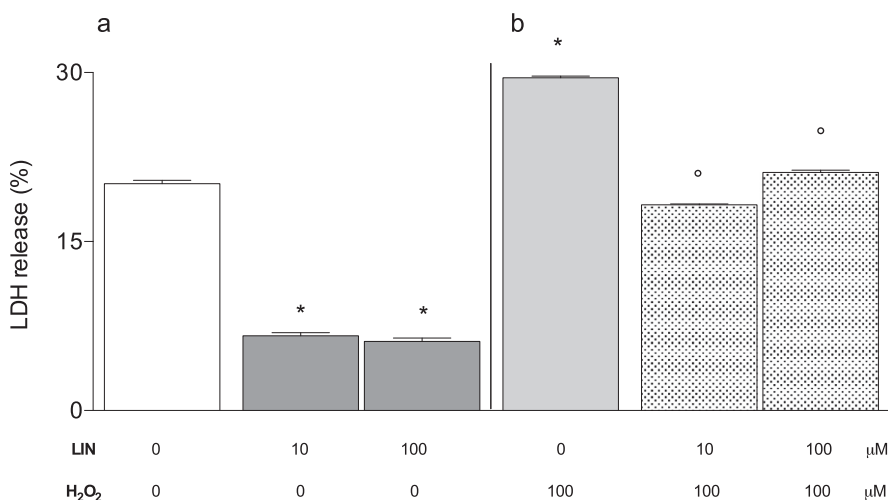

Fig. 2. LDH assay. Panel a shows the effect of the incubation with LIN (10 and 100 μM) on LDH release in PC12 cells after 24 h of exposure. Panel b shows the effect of treatment with LIN (10 and 100 μM) under H_2O_2. *$p < 0.05$ versus control; °$p < 0.05$ versus H_2O_2. As post hoc was used Bonferroni multiple comparisons test.

100 μM) (Fig. 1). As shown in Fig. 1a, LIN did not affect cell viability in comparison with the control group. Fig. 1b shows the effect of LIN on H_2O_2 (100 μM) induced-oxidative stress. Upon H_2O_2 exposure the cell viability undergoes a significant decrease (68%) as compared with the control (99%) but the concomitant treatment with LIN at all concentrations tested, significantly prevents it (74%, 80% and 79%, respectively). Nevertheless, this reduction (10%) was significantly different ($p<0.05$) with respect to the oxidative damage induced by H_2O_2 alone.

3.2. Effects of LIN on LDH release

LDH is an enzyme released upon damage of plasma membrane. The LDH assay was performed to further evaluate the neuroprotective properties of LIN on the cytotoxic effect of 24-hour exposure of PC-12 cells to H_2O_2 (100 μM) (Fig. 2). Considering that in the MTT test, LIN induces a greater effect with the two higher concentrations (10 and 100 μM), we decided to use them in this and the following assays. As shown in panel 2a, LIN incubation significantly reduces the levels (7% and 6%, $p<0.05$,

respectively) of LDH release from cytosol when compared with control group (20%; $p<0.05$).

In presence of H_2O_2 which induced a significant increase of LDH release to 29% (Fig. 2b), the incubation with LIN (10 and 100 μM) determines a significant ($p<0.05$) decrease of oxidative injury (18% and 21%, respectively) with respect to the H_2O_2 group (Fig. 2b).

3.3. Effects of LIN on ROS production

Data from ROS detection experiments were achieved to evaluate the antioxidant properties of LIN with or without H_2O_2 (24 h of exposure)-induced oxidative stress (Fig. 3). As shown in Fig. 3a, ROS levels in cells incubated with LIN did not significantly differ with respect to those in the control group. However, as shown in Fig. 3b, H_2O_2 significantly increases (68%, $p<0.05$) ROS production with respect to the control (48%). In these experimental conditions both LIN concentrations induce a significant decrease ($p<0.05$) of ROS levels from 68% (H_2O_2) to 32% (LIN 10 M) and 33% (LIN 100 μM).
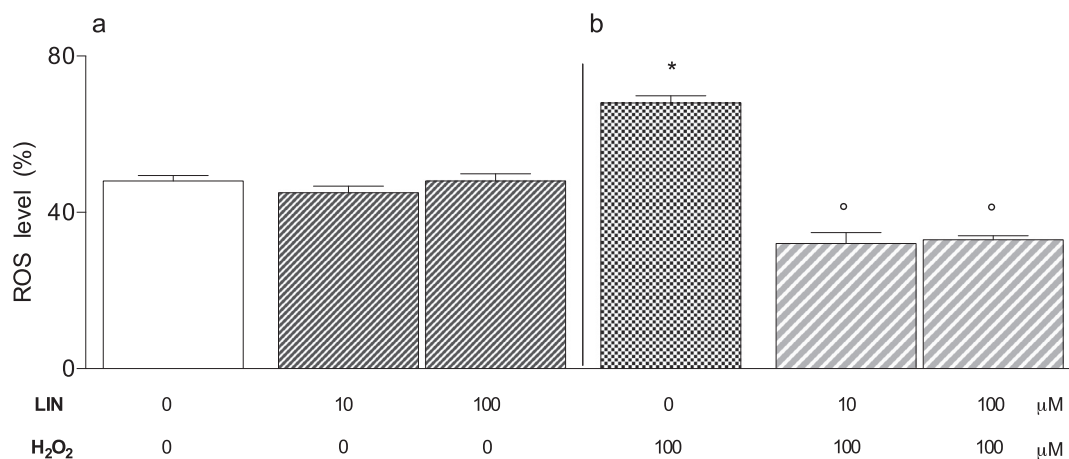
Fig. 3. *ROS detection*. Evaluation of effect of LIN (10 and 100 μM) on the ROS production with (panel b) or without H$_2$O$_2$ (panel a) on PC12 cells after 24 h of exposition. Bonferroni multiple comparisons test shows a significant difference for $p < 0.05$. $*$ *versus* control; $°$ *versus* H$_2$O$_2$.

3.4. Effects of LIN on apoptosis

The apoptosis studies reveal that, in the present experimental conditions, at the concentration of 10 but not of 100 μM, LIN significantly ($p<0.05$) raises PC12 cells' apoptosis (31%) with respect to the control group (11%) (Fig. 4a).

In addition, as shown in Fig. 4b, H$_2$O$_2$ significantly increases cells apoptosis (68%, $p<0.05$) with respect to the control (11%), and LIN significantly decreases ($p<0.05$) H$_2$O$_2$-dependent apoptosis levels from 68% (H$_2$O$_2$) to 54% (10 μM) and 47% (100 μM). Besides, Fig. 4c, shows the various steps of apoptosis in PC12 cells, treated with LIN (10 and 100 μM) with or without H$_2$O$_2$. These experiments were examined by flow cytometry using Annexin V FITC/PI in comparison with the control or H$_2$O$_2$ cells.

3.5. LIN effect on cell cycle analysis

The cell cycle analysis has been performed to evaluate, by flow cytometry, the effects of LIN on PC12 cells' cycle distribution phases with or without H$_2$O$_2$ (Fig. 5). Generally, the higher LIN concentration (100 μM), if compared with the control group, prompts an increase ($p<0.05$) of G0/G1 and a decrease ($p<0.05$) of S phase (Fig. 5a).

On the other side, under H$_2$O$_2$, a higher number of PC12 cells remain stagnate in the G2/M phase if compared with the control group ($p<0.05$) (Fig. 5b).

The amount of the stagnation in the G2/M phase in presence of both LIN concentrations (10 and 100 μM) was significantly ($p<0.05$) lower if compared with the number of cells induced into quiescence by H$_2$O$_2$. It is important to note that the incubation of LIN at both concentrations prompts the cells to a lower value than that of control ($p<0.05$) (Fig. 5b). Likewise, the same trend shown from LIN on G0/G1 and S phases as presented in Fig. 5a, appears in Fig. 5b, revealing an increase of G0/G1 and a decrease of S phase in the cell cycle distribution.

4. Discussion

The findings of the present study demonstrate antioxidant and neuroprotective effects of the enantiomer R-(-)- of linalool, LIN, in conditions of H$_2$O$_2$-induced oxidative stress. These effects were achieved, by a convergent and complementary approach, through an improvement of cell viability, a decrease of damage of plasma membrane, a reduction of intracellular overproduction of ROS, a decrease of apoptotic cells, and altering of distribution of cell cycle in PC12.

These observations are in agreement, although in different experimental conditions, with previous studies supporting neuroprotective effects of LIN determined by the *in vitro* model of oxygen-glucose deprivation/re-oxygenation-induced cortical neuronal injury (Park et al., 2016). In this study, similar concentrations of LIN disclosed that this monoterpene exerts its protective effects by significantly decreasing intracellular oxidative stress and scavenging peroxyl radicals (Park et al., 2016).

Our study shows that LIN improves the viability of PC12 cells in conditions of oxidative stress. Several reports have described similar results although with the use of racemic linalool and different experimental conditions. Alinejad et al. (2013) reported that linalool counteracts the reduction of viability of PC12 cells against glucose/serum deprivation-induced cytotoxicity. In addition, Sabogal-Guáqueta et al. (2019) reported that linalool counteracts cell death mediated by glutamate-induced mitochondrial oxidative stress in immortalized neuronal HT-22 cells.

Our findings reveal that LIN decrease not only the release of LDH, showing a protective effect *on its own* but also the release of LDH during cells' exposure to H$_2$O$_2$. This result is partially in agreement also with the ability of racemic linalool to protect neurons and astrocytes by reducing LDH release in an *in vitro* model of glutamate excitotoxicity, as demonstrated by Sabogal-Guáqueta et al. (2018).

In PC12 cells, exposure to H$_2$O$_2$ produces an increase of ROS levels responsible to cause oxidative damage (Muthaiyah et al., 2011). In our experimental conditions, we found that a neuroprotective effect of LIN could be attributed to a reduction of detrimental levels of ROS. This effect is supported by the results of Sabogal-Guáqueta et al. (2019), in which linalool reduces ROS levels mediated by glutamate and NMDA toxicity in mitochondrial cells, and by those of Yuan et al. (2021), in which linalool decreases amyloid β-induced ROS overproduction. Antioxidant properties of the racemic linalool were also exhibited in a study, investigating oxidation reactions in unsaturated fatty acids obtained from guinea pig brains tested with H$_2$O$_2$ to stimulate oxidative stress (Celik and Ozkaya, 2002).

As it is well-known, the oxidative stress is also involved in the induction of apoptosis and several reports have described that the overproduction of ROS leads to apoptosis (Kannan and Jain, 2000). In our experimental conditions, we have found that treatment with LIN at the lower dose (10 μM) significantly increases apoptotic cells if compared with control conditions. These results are partially supported by the results of other researchers like Zhao et al. (2020) and Chang and Shen (2014) reporting that racemic linalool induces apoptosis in 22Rv1 and T-47D cells. However, in our experimental conditions LIN, at both
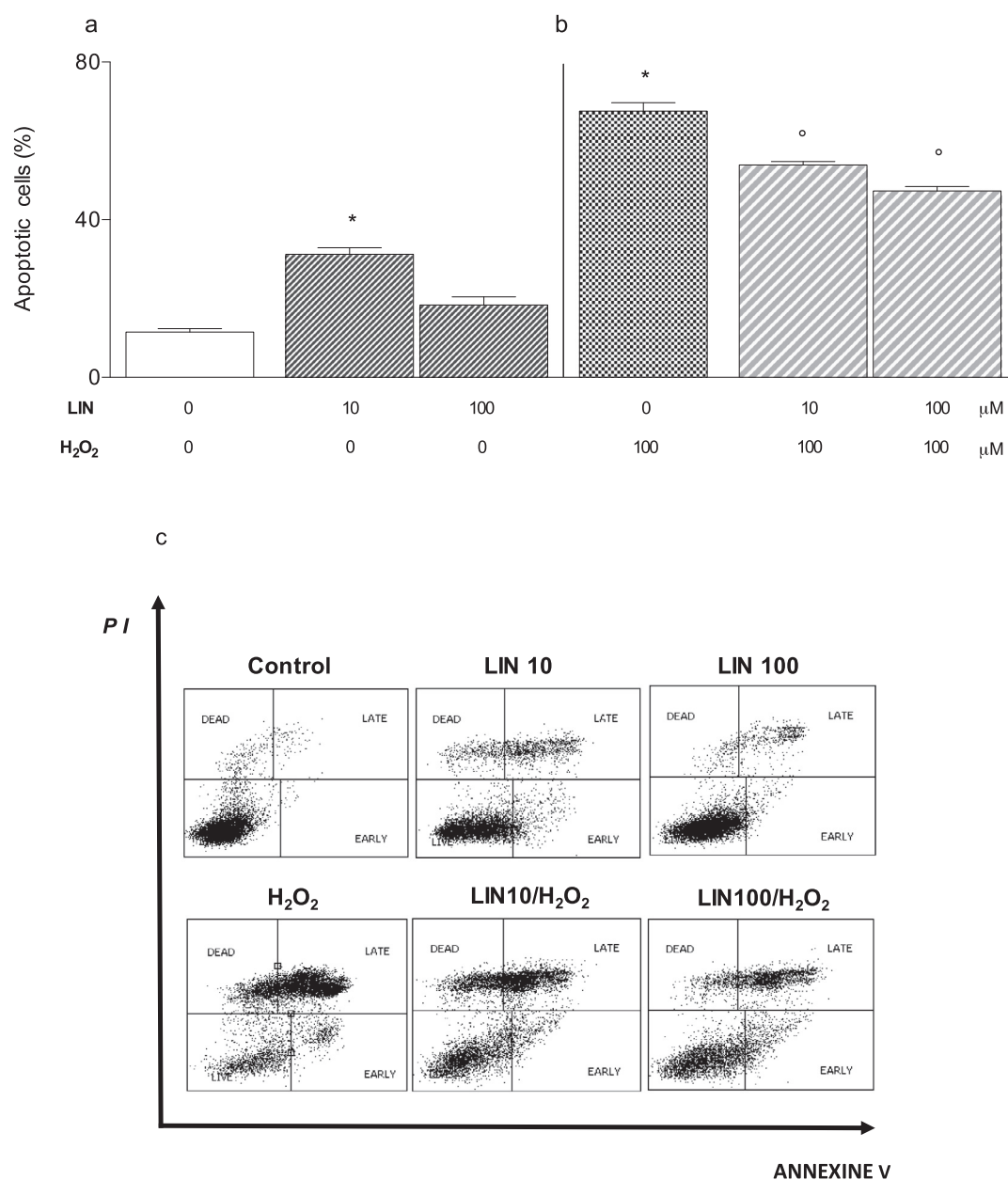
Fig. 4. *Apoptosis analyses.* Evaluation of apoptotic cells of PC12 cells treated with LIN (10 and 100 μM) with (panel b) or without H_2O_2 (panel a) for 24 h of exposure. Bonferroni multiple comparisons test reveals a significant difference for $p < 0.05$; *versus control; ° versus H_2O_2. Dot spot graph of the apoptotic analysis represents one out of three independent experiments (panel c).

concentrations tested, decreases H_2O_2-induced apoptosis in PC12 cells. These results are consistent with the results of previous studies showing that linalool is able to decrease the apoptosis induced by an increase of amyloid β in eye discs as well as in larval brain (Yuan et al., 2021). Thus, on the basis of what above discussed, our results are coherent with the fact that LIN, by decreasing ROS levels, prompts a reduction of apoptotic cells. As a result, these activities could be the main mechanisms underlying the neuroprotective effect of LIN.

From the results of cell cycle experiments at which LIN at the highest concentration induces a cell arrest in G0/G1 phase as well as a significant decrease in S phase, the present data appear in accordance with the results of a recent study of Rodenak-Kladniew et al. (2018) in HepG2 cells, in which racemic linalool at highest concentrations, was suggested as promising anticancer agent. On the other side, our results show that

LIN decreases the percentage of cells stagnant in G2/M phase, increased by oxidative damage.

Overall, our results provide novel valuable information that the enantiomer LIN could be an effective therapeutic option to control oxidative stress. Particularly, its antioxidant effect is attained through *a*) a protection of cell viability from H_2O_2-induced reduction; *b*) a reduction of LDH release; *c*) a reduction of ROS overproduction, *d*) a reduction of apoptotic cells; and *e*) a reduction of G2/M phase on cell cycle distribution, in PC12 cells.

5. Conclusion

The properties of LIN against oxidative stress hereby disclosed add on the many mechanisms of action proposed by the scientific literature
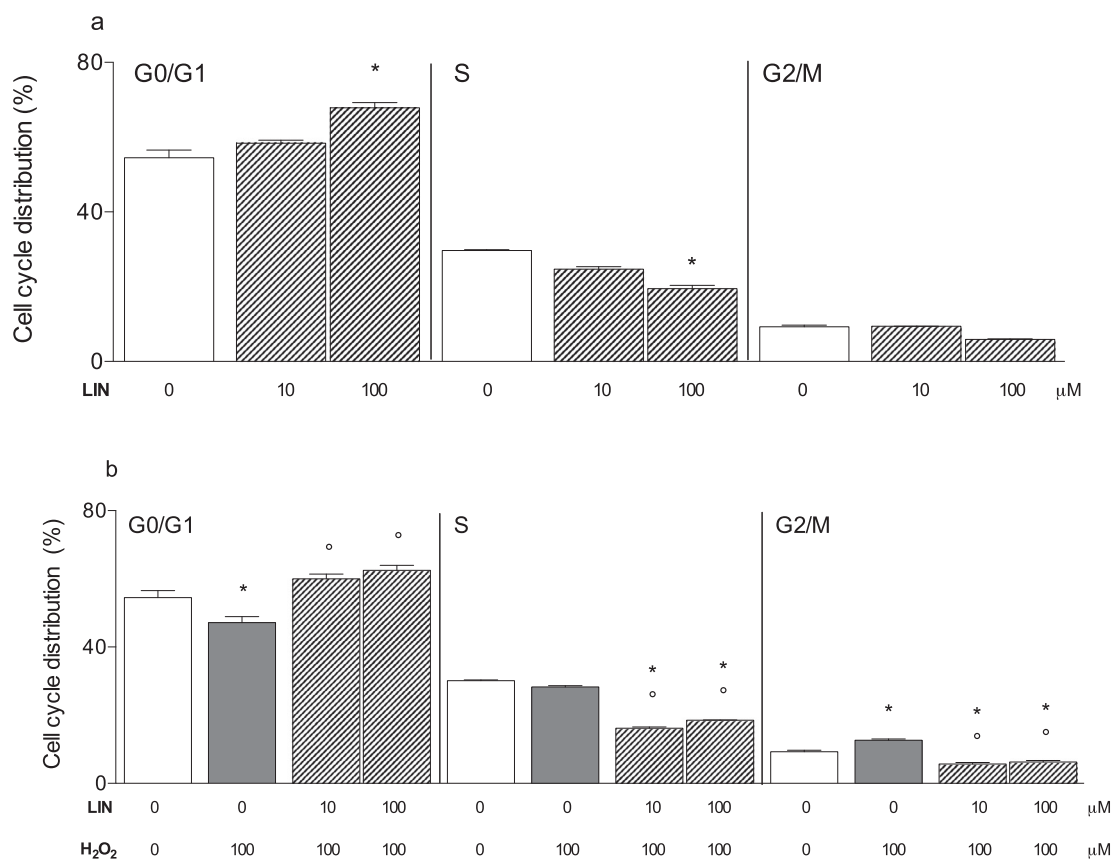
Fig. 5. *Cell cycle distribution*. Distribution of cell cycle after LIN (10 and 100 μM)) incubation, with (panel b) or without H_2O_2 (panel a) for 24 h of exposure. Bonferroni multiple comparisons test reveals a significant difference for $p < 0.05$; *versus control; ° *versus* H_2O_2.

on this compound. Moreover, in turn, these observations provide support to explain the mechanism of neuroprotective, analgesic and antiinflammatory properties of this monoterpene compound and overall emphasize that LIN could be of possible interest as useful drug in the clinical management of the types of pain in which oxidative stress plays a major contribution.

Declaration of Competing Interest

All the authors declare to not have any potential conflict of interest.

Acknowledgments

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