Medicinal chemistry

Kim et al., Med chem 2015, 5:4
DOI: 10.4172/2161-0444.1000261

Research Article Open Access

Neuroprotective Effect of (-)-Linalool against Sodium Nitroprusside-Induced Cytotoxicity

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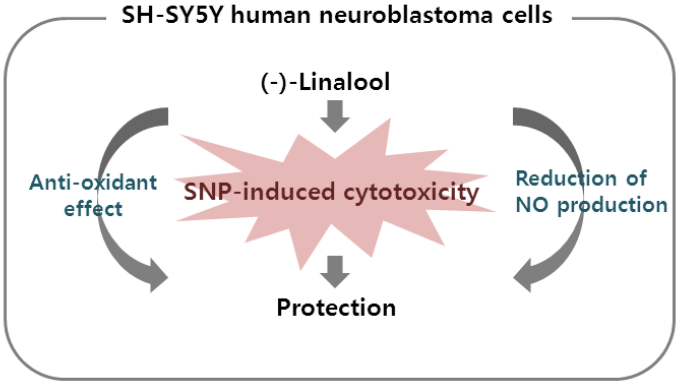
Abstract

(-)-Linalool has various pharmacological effects in humans and animal models, especially in the central nervous system. This study investigated whether (-)-linalool and linalyl acetate, the major components of lavender, can protect SH-SY5Y cells against sodium nitroprusside (SNP)-induced cytotoxicity. Cell viability and nitric oxide (NO) production in the presence of SNP and (-)-linalool was assessed by MTT and nitrite assays, and the free radical-scavenging activity of (-)-linalool was assessed by DPPH assay. (-)-Linalool, at low, non-toxic concentrations of 1 μM (p=0.003), 2.5 μM (p=0.001), and 5 μM (p=0.008), significantly enhanced neuronal cell viability in the presence of SNP. The protective effect of (-)-linalool against SNP-induced cytotoxicity was also confirmed by Hoechst staining. SNP-induced NO production was significantly decreased (p<0.001 each), and antioxidant levels significantly increased (p≤ 0.001), by 1, 2.5, and 5 μM (-)-linalool. These findings, showing that (-)-linalool protected SH-SY5Y cells against SNP-induced cytotoxicity by decreasing the production of NO, suggested that (-)-linalool has anti-oxidant activity in the central nervous system and may be a potential therapeutic drug in patients with neurodegenerative diseases.



Keywords: Linalool; Neuron; Nitric oxide; Sodium nitroprusside

Introduction

Aromatherapy is a form of alternative medicine that has been traditionally used for pain relief, psychological comfort, and prevention of disease. More recently, aromatherapy was reported to have therapeutic effects in several diseases, including neurodegenerative, cardiovascular and respiratory diseases [1-3]. For example, lavender oil has been found to possess therapeutic properties, including anti-oxidant, anti-inflammatory and anti-infective properties [4-6], and to have bidirectional effects in stress-induced changes [5]. Understanding the therapeutic effects of aroma essential oils requires determining the mechanisms of action of their major constituents. (-)-Linalool and linalyl acetate, the principal constituents of lavender, act together or individually. For example, (-)-linalool and linalyl acetate were found to induce anti-inflammatory activities in rats [7]. Although (-)-linalool showed no genotoxicity, linalyl acetate was genotoxic toward peripheral human lymphocytes, suggesting that the mutagenic activity of lavender may be due to the effects of linalyl acetate [8].

(-)-Linalool has been shown to have various pharmacological activities, including anti-inflammatory, antihyperalgesic and antinociceptive effects, in humans and animal models, especially in the central nervous system (CNS) [9]. Inhalation therapy using essential oils with high linalool content was effective in altering the profile of mood states (POMS) and parasympathetic nerve activity in pregnant women [10]. Functional brain imaging using functional near-infrared spectroscopy (fNIRS) showed that high concentrations of linalool increased the activation of olfaction-associated brain regions in patients with attention deficit hyperactivity disorder (ADHD) [11].

Sodium nitroprusside (SNP) has been found to induce caspase-dependent apoptosis in SH-SY5Y human neuroblastoma cells [12,13], causing nitrosative and oxidative stress. Oxidative stress is associated with several neurodegenerative diseases, including Alzheimer's and Parkinson's diseases.

Nitric oxide (NO) is an important signaling molecule involved in the regulation of cerebral blood flow, thrombogenesis, and modulation of neuronal activity, as well as in various other physiological and pathological processes, with both deleterious and protective properties

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Received March 25, 2015; **Accepted** April 16, 2015; **Published** April 18, 2015

Citation: Kim KY, Lee HS, Min SS, Seol GH (2015) Neuroprotective Effect of (-)-Linalool against Sodium Nitroprusside-Induced Cytotoxicity. Med chem 5: 178-182. doi:10.4172/2161-0444.1000261

[14]. Excessive or inappropriate NO production has been found to cause cell injury and death, including damage to proteins and DNA resulting from nitrosative and oxidative stress. Oxidative stress is the major cause of damage associated with elevated NO [15-17]. NO is synthesized in several cell types, including endothelial cells, neurons, and glia, by three isoforms of the enzyme nitric oxide synthase (NOS)—neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS)—the latter of which has multiple functions in the brain. The production of NO and the expression of iNOS have been reported to be increased in patients with neurodegenerative diseases [18,19]. The link between NO and (-)-linalool suggested that the latter may protect SH-SY5Y cells from SNP-induced cytotoxicity. This study therefore determined whether (-)-linalool has neuroprotective activity against SNP-induced cytotoxicity in SH-SY5Y cells.

Materials and Methods

Chemicals

(-)-Linalool, linalyl acetate, and sodium nitroprusside (SNP) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). (-)-Linalool and linalyl acetate were dissolved in dimethyl sulfoxide (DMSO); and SNP and L-NG-nitroarginine (L-NNA) (Enzo Life Sciences Inc., NY, USA) were dissolved in distilled water (DW).

Cell culture

SH-SY5Y cells were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) (Welgene, Daegu, Korea), supplemented with 10% fetal bovine serum (FBS) (Biowest, Nuaillé, France) and 1% penicillin/streptomycin (PAA Laboratories, Pasching, Austria), at 37°C in a humidified incubator with 5% CO_2.

MTT assay

Cell viability was measured using MTT assays. Briefly, SH-SY5Y cells were seeded in 96-well plates at a density of 1 x 10^4 cells per well and cultured overnight. The cells were incubated with various concentrations of (-)-linalool for 24 h, followed by incubation for 24 h with 2.5 mM SNP. Cell viability was determined by incubation with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Amresco, Solon, OH, USA), at a final concentration of 0.25 mg/ml, for 3 h at 37°C. The absorbance of each well at 540 nm was determined using a microplate reader (BMG Labtech, Ortenberg, Germany).

Hoechst staining

SH-SY5Y cells were cultured on coated cover slips in 12-well plates. The cells were fixed with 4% paraformaldehyde for 30 min. After washing twice with phosphate-buffered saline (PBS), the cells were stained with 5 μg/ml of Hoechst 33342 (Sigma-Aldrich Co.) for 5 min. Images of stained cells were acquired using a fluorescence microscope (Leica DM 2500, Wetzlar, Germany), and apoptosis was calculated from pixel intensity values.

Nitrite assay

Accumulation in the culture medium of extracellular nitrites (NO_2), an indicator of NO synthase activity, was measured by the Griess reaction. Griess reagent includes 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride and 1% sulfanilamide. Following incubation with (-)-linalool and/or SNP, Griess reagent was added to 100 μl of cell culture medium, and the solution was mixed and incubated for 10 minutes at room temperature. The optical density was measured at 540 nm in a microplate reader (BMG Labtech).

DPPH assay

The free radical-scavenging ability of (-)-linalool was analyzed using the stable 1,1-diphenyl-2-picrylhydrazyl radical (DPPH). In brief, linalool (1, 2.5, 5 μM) was mixed with DPPH solution (23.6 μg/ml in ethanol) and incubated for 30 min at 37°C in the dark. The absorbance was measured at 517 nm using a microplate reader (BMG Labtech). Scavenging activity was calculated as background (blank)-subtracted values expressed as a percentage of values obtained with vitamin C, used as a positive control.

Statistical analysis

All samples were analyzed in triplicate, and each assay was repeated over 6 times under identical conditions. Results were expressed as means ± SEMs and compared using one-way analysis of variance (ANOVA), followed by the Tukey HSD post hoc test. All statistical analyses were performed using the SPSS 20.0 software package (SPSS Inc., Chicago, IL, USA), with results considered statistically significant at $p < 0.05$.

Results

Effect of SNP on SH-SY5Y cell viability and NO production

At concentrations of 1 to 3.5 mM, SNP reduced cell viability, with statistically significant differences observed at 2 to 3.5 mM ($p<0.001$, Figure 1a). Moreover, at these concentrations, SNP significantly increased NO production ($p<0.001$, Figure 1b).

Effects of (-)-linalool and linalyl acetate on SNP-induced cytotoxicity in SH-SY5Y cells

At concentrations of 1 to 5 μM, both (-)-linalool and linalyl acetate alone had no cytotoxic effects on SH-SY5Y cells, as determined by MTT assays (Figure 2a, white bar), and did not significantly induce NO (Figure 2b, white bar). To test the effects of (-)-linalool on SNP-induced cell death, cells were pre-treated with 1 to 5 μM (-)-linalool for 24 h, followed by incubation with 2.5 mM SNP for 24 h, a concentration at which SH-SY5Y cells show about 60% cell viability. Interestingly, 1 ($p=0.003$), 2.5 ($p=0.001$), and 5 ($p=0.008$) μM (-)-linalool significantly increased neuronal cell viability (Figure 2a, black bar), as well as significantly reducing NO production ($p<0.001$ each, Figure 2b, black bar). In contrast, 1 to 5 μM linalyl acetate had no effect on SNP-induced cell death (Figure 2a, black bar) or on SNP-enhanced NO production (Figure 2b, black bar). As a positive control, the effects of L-NG-nitroarginine (L-NNA), a NOS inhibitor, on the viability of and NO production by SNP-treated SH-SY5Y cells were investigated. At a concentration of 0.5 mM, L-NNA significantly increased cell viability ($p<0.001$) and reduced NO production ($p<0.001$). The protective effect of (-)-linalool against SNP-induced cytotoxicity was confirmed by Hoechst staining. SNP effectively reduced cell viability, measured by the intensity of Hoechst staining, to approximately 33% of that in untreated controls. These effects were significantly attenuated by 5 μM (-)-linalool, which increased Hoechst staining in the presence of SNP to 73% of control values ($p=0.021$) (Figure 3).

DPPH scavenging activity of (-)-linalool

Assessments of the free radical-scavenging activity of 1, 2.5, and 5 μM (-)-linalool, as measured by DPPH assays, showed that (-)-linalool increased antioxidant levels in a concentration-dependent manner compared with the control group ($p\leq0.001$). As expected, the positive control, vitamin C (100 μM), also significantly scavenged free radicals ($p=0.002$).

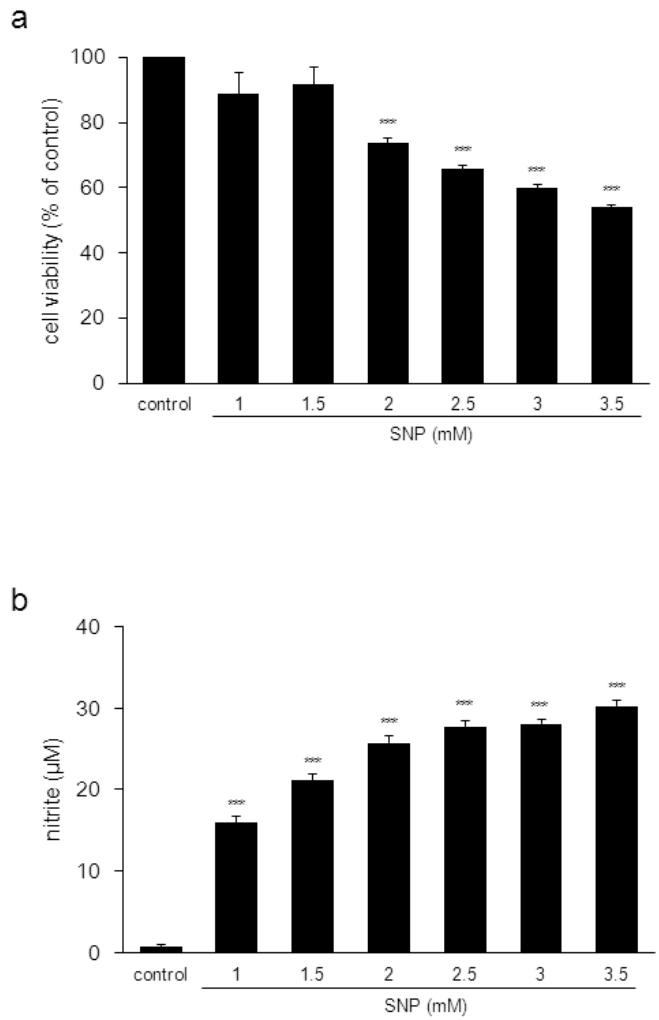

Figure 1: Effects of SNP on SH-SY56 cell viability and NO production. SH-SY5Y cells were treated with 1, 1.5, 2, 2.5, 3, or 3.5 mM SNP for 24 h. (a) Cell viability was measured by the MTT assay, and (b) NO production was measured by the nitrite assay. Data represent the mean, and the bars represent the standard error of the mean (SEM). *** $p < 0.001$ compared with control (untreated SH-SY5Y cells).

Discussion

Lavender has been widely used as an alternative medicine. It was shown to have the CNS depressant properties, with sedative, anticonvulsive, anxiolytic, motor inhibitory and spasmolytic effects [20]. However, its exact mechanism of action has not been fully investigated. This study, which investigated the major components of lavender, showed that linalyl acetate did not play an essential role in neuroprotection against SNP-induced cytotoxicity. In contrast, (-)-linalool protected SH-SY5Y cells against SNP-induced cytotoxicity by decreasing the production of NO. (-)-Linalool has been reported to have dose-dependent sedative effects on the CNS of mice, indicating its psychopharmacological activity [7]. Excess stimulation by glutamate has been found to cause excitotoxicity, with (-)-linalool inhibiting glutamate release *in vitro* and *in vivo* [21]. Furthermore, (-)-linalool showed anti-inflammatory effects in LPS induced RAW 264.7 cells, reducing iNOS and cyclooxygenase-2 (COX-2) mRNA and protein levels as well as pro-inflammatory cytokine production. Moreover, (-)-linalool suppressed the phosphorylation of IκB-α protein in an LPS induced inflammation model [22]. These findings suggest that (-)-linalool may inhibit iNOS by suppressing NF-κB activation, thereby reducing NO production. Moreover, these findings indicate that (-)-linalool is the major neuroprotective component of lavender, being responsible for its antioxidant effects, with these antioxidant activities associated with reduced NO production in neuronal cells.

Aromatherapy requires special caution because of the potential toxicities of the essential oils. It is therefore important to determine the safe and non-toxic doses of aroma essential oils. At concentrations of 1 to 100 μM, (-)-linalool was not cytotoxic to neuronal cells (data not shown). Although (-)-linalool itself had no effect on NO production by neuronal cells, it reduced the increase in NO induced by SNP. NO, nitrative stress, and oxidative stress have been found to damage neuronal cells. Treatment of LPS-stimulated macrophages with (-)-linalool reduced the production of NO, as well as cyclooxygenase-2 (COX-2), prostaglandin E_2 (PGE_2) and pro-inflammatory cytokines, including interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) [22-25]. PGE_2 formation and COX-2 expression have been associated

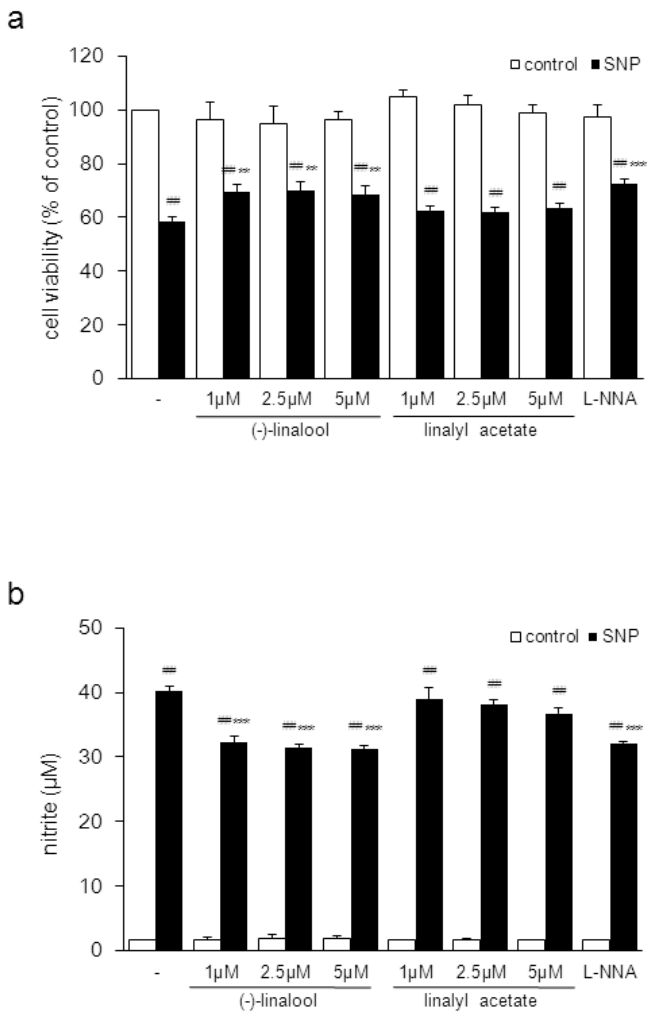

Figure 2: Effects of (-)-linalool and linalyl acetate on SNP-induced cytotoxicity in SH-SY5Y cells. SH-SY5Y cells were incubated with 1, 2.5, or 5 μM (-)-linalool or linalyl acetate, or with 0.4 mM L-NNA (0.5 mM) for 24 h, followed by incubation without (white bar) or with (black bar) 2.5 mM SNP for 24 h. (a) Cell viability was measured by the MTT assay and (b) NO production was measured by the nitrite assay. Data represent the mean, and the bars represent the standard error of the mean (SEM). ### $p < 0.001$ compared with control, untreated cells; ** $p < 0.01$, *** $p < 0.001$ compared with cells treated with SNP alone.

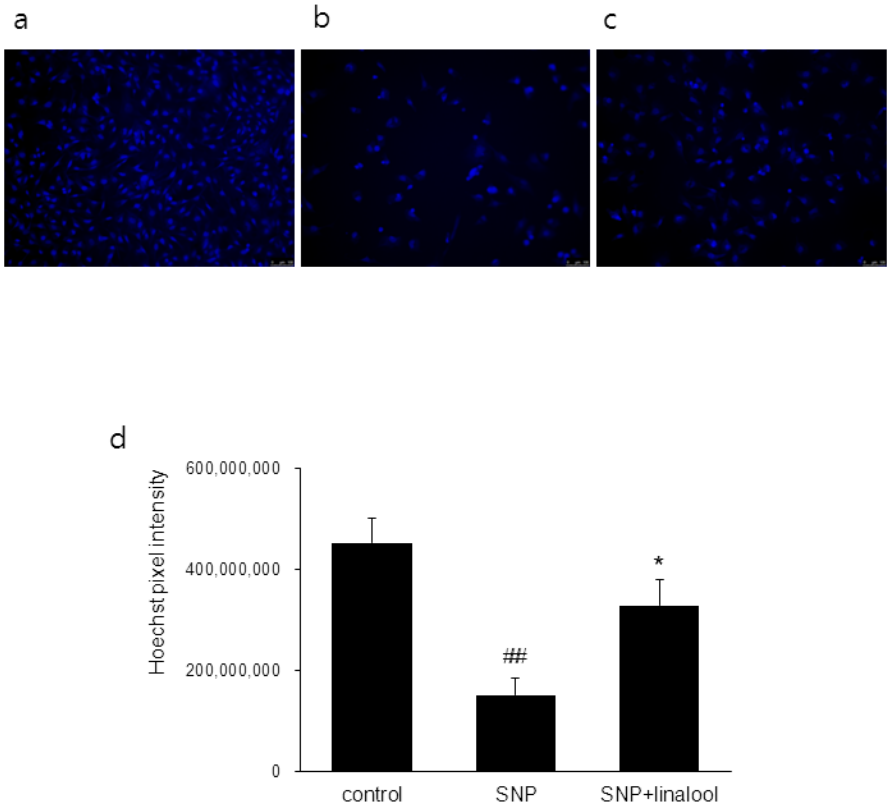

Figure 3: Effects of (-)-linalool on SNP-induced cytotoxicity in SH-SY5Y cells. SH-SY5Y cells were incubated with 5 μM (-)-linalool for 24 h prior to treatment with SNP (2.5 mM) for 24 h. Apoptosis was assessed by Hoechst 33342 staining. (a) Control, (b) SNP only, (c) SNP + linalool, and (d) quantification of Hoechst staining, calculated from intensity values. Data are presented as means ± SEMs (error bars). ### $p < 0.001$ compared with the control group; * $p < 0.05$ compared with the SNP-only group.

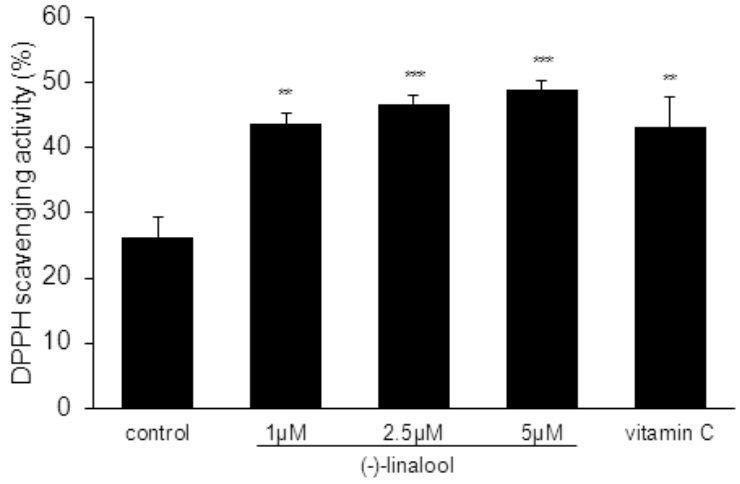

Figure 4: DPPH scavenging activity of (-)-linalool. The free radical-scavenging activity of (-)-linalool was assessed by DPPH assay. Compared with control cells, 1, 2.5, and 5 μM (-)-linalool increased antioxidant levels in a concentration-dependent manner. Vitamin C (100 μM) was used as a positive control. Data are presented as means ± SEMs (error bars). ** $p < 0.01$, *** $p < 0.001$ compared with controls.

with high concentrations of (-)-linalool in LPS-stimulated macrophages [24]. The results presented here expand these findings, showing that neuronal cells subjected to oxidative stress conditions were similarly affected by non-toxic concentrations of (-)-linalool (Figure 4).

SNP has been reported to mimic NMDA-induced neurotoxicity [26] and to induce oxidative stress [27]. Similarly, the present study found that the protective effects of (-)-linalool were associated with an increase in antioxidants, as measured by DPPH assays. It was also previously shown that, at concentrations of 2, 4 and 8 mM, SNP did not increase intracellular calcium [28]. Similarly, we found that 2.5 mM SNP did not induce an increase in intracellular calcium levels

(data not shown). Oxidative stress has been associated with several neurodegenerative diseases, including Alzheimer's and Parkinson's diseases. Biomarkers such as reactive oxygen species (ROS) and NO, which cause oxidative damage, are considered important in the pathogenesis of these diseases [29-31]. Rutin, a dietary flavonoid, was reported to inhibit Aβ-induced neurotoxicity in SH-SY5Y cells by decreasing the formation of ROS, NO, glutathione disulfide (GSSG), and malondialdehyde (MDA), compounds closely related to the etiology of Alzheimer's disease [32].

Conclusion

(-)-Linalool protected SH-SY5Y cells against SNP-induced cytotoxicity by decreasing the production of NO and by having antioxidant properties. Moreover, SNP-induced neuronal cell death associated with high production of NO may be a useful model for assessing the neuroprotective effects of various compounds. (-)-Linalool may be a potential therapeutic drug for patients with neurodegenerative diseases.

Acknowledgments

This research was supported by a grant from the National Research Foundation of Korea (NRF) funded by the Korea government (MEST) (No. 2012R1A2A2A02007145) and a Korea University Grant.

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