

# Understanding the protective effects of wine components and their metabolites in the brain function

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**Abstract.** Moderate wine consumption has been suggested to exert a positive effect in prevention of neurodegenerative process and cognitive impairment. With the ultimate aim of achieving a better understanding of the molecular mechanisms behind this benefit, we have investigated the role of certain wine-derived phenolic metabolites and aroma compounds in the MAPK cascade (including ERK1/2, p38), one of the routes directly related to inflammation in neuronal cells. Some of the tested phenolic compounds, especially in the case of 3,4-dihydroxyphenylacetic acid, showed a significant neuroprotective effect against SIN-1-induced neuronal death. Regarding their effect over MAPK phosphorylation, immunoblotting technique revealed a beneficial and significant decrease on the phosphorylation of p38 and ERK1/2 kinases after incubation with wine constituents. In addition, activity of caspase-3-like protease, an executor of neuronal apoptosis and a downstream signal of MAPK, was significantly diminished by 3-(3-hydroxyphenyl) propionic acid and linalool, counterbalancing the increase produced by SIN-1. Altogether, these results suggest that wine aroma, phenolic compounds and their gut metabolites could exert neuroprotective actions by modulating MAPK signalling and caspase-3 proteases activation, which are known to play a key role in oxidative/nitrosative stress-induced response.

## 1. Introduction

Oxidative and nitrosative stress play important roles in the development of neurodegenerative diseases such as Alzheimer's and Parkinson's [1]. Several epidemiology studies have suggested that moderate wine intake (250 mL per day) can be beneficial in delaying the onset of cognitive impairments in aging and neurodegenerative diseases [2,3]. In agreement with this, evidences from human randomized controlled trials based on acute supplementations with specific wine compounds reinforce this benefit [4]. Also animal *in vivo* studies confirmed the profit of moderate wine consumption on cognitive function [5,6].

Wine is a complex matrix rich in polyphenols and aroma compounds. After consumption, flavan-3-ols, a representative type of polyphenols in wine, are metabolized in the gut by microbial catabolism reactions (i.e. hydrolysis, oxidation), originating different metabolites that include propionic, phenylacetic and benzoic acids derivatives. When these forms reach the liver through blood circulation, they are partially conjugated into glucuronides, sulfates and O-methyl derivatives [7]. Furthermore, these compounds had been detected in human urine and faeces after moderate and regular wine consumption [8,9], meanwhile several evidences have suggested the capacity of these compounds to go through the blood-brain barrier (BBB) [10,11].

Mechanisms by which these compounds might affect neuronal function have not been established but different studies suggest an ability to interact with signalling pathways such as the nuclear factor- $\kappa$ B (NF- $\kappa$ B) or mitogen-activated protein kinase (MAPK) pathways [1]. MAPK family (mitogenic extracellular signal-regulated protein kinase (ERK1/2) and p38, among others) is involved in neuronal stress-induced apoptosis [12] and activates downstream signals, such as caspase-3 proteases expression, a pro-apoptotic marker of cell death. Additionally, to date the effect of phenolic acids in stress-induced injury is poorly understood, as well as the role of aroma compounds in neuroprotection remains still unclear.

With the aim of going deep into the molecular mechanisms involved on the protective effect derived from moderate wine consumption, in this work the dopaminergic neuronal cell line SH-SY5Y has been applied as an approach model for the study of the interaction of wine-derived human metabolites, mainly phenolic acids: (3,4-dihydroxyphenylacetic (3,4DHPA), 3-(4-hydroxyphenyl)propionic (4HPP), 3-hydroxyphenylacetic (3HPA), and 3-(3-hydroxyphenyl) propionic (3HPP), salicylic acid, and  $\beta$ -D-O-glucuronide of salicylic acid, and aroma compounds (linalool and 1,8-cineole), with MAPK signalling route (ERK1/2, p38) in a stress-induced situation created by SIN-1 peroxynitrite generator. Caspase-3 protein expression, a downstream process of this route, has been analyzed too.

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## 2. Material and methods

### 2.1. Cell culture

SH-SY5Y human cells (ATCC<sup>®</sup> CRL2266<sup>™</sup>) were routinely grown in 75 cm<sup>2</sup> flasks in a mixture of Dulbecco's modified Eagle Medium and Ham's F12 (1:1 v/v) supplemented with 10% foetal bovine serum, antibiotics (100 IU/mL penicillin and 100 µg/mL streptomycin) and 1% non-essential aminoacids (37°C and 5% CO<sub>2</sub>).

### 2.2. Assessment of cell viability

Neuronal cells were seeded on 96-well plates 24 h prior to the incubation with the phenolic acids and aroma compounds (0.1–10 µM). After 18 h, neurons were exposed to freshly prepared 1 mM SIN-1 for 0–15 h. Then, MTT reagent (0.5 mg/ml) was added and plates were returned to the incubator (37°C, 5%CO<sub>2</sub>) for 3 h. Formazan crystals were dissolved with pure DMSO before absorbance was measured (570 nm). Control was considered as maximum of percentage of viability (100%), and the sample values were calculated as: % viability =  $Abs_{sample}/Abs_{control} \times 100R$ . For MEK (PD98059), p38 (SB203580) and ERK (FR180204) inhibitors, the same procedure was applied with the exception that neurons were pretreated for 1 hour with different concentrations of inhibitor (0.5–50 µM) prior the exposition to freshly prepared SIN-1 (1 mM).

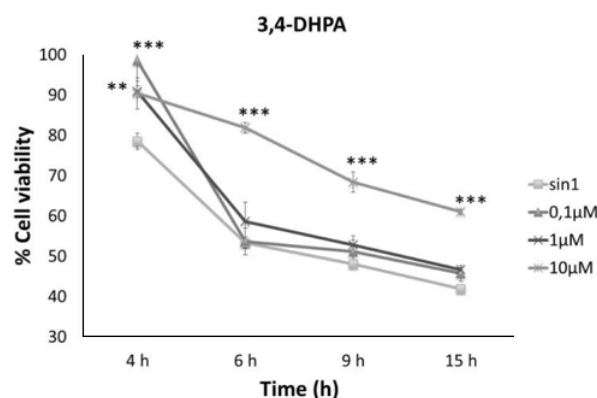
### 2.3. Western immunoblotting

#### 2.3.1. Protein extraction

After pre-treatment with wine compounds and SIN-1 stimulation, neuronal cells were washed with ice-cold PBS with 200 µM EGTA and 200 µM EDTA, and lysed on ice using 50 mM Tris, 0.1% Triton X-100, 150 mM NaCl and 2 mM EGTA/EDTA, containing Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktail. Lysed cells were scraped, left on ice to solubilize for 45 min and total protein concentration was determined by BCA assay to normalize protein level.

#### 2.3.2. Immunoblotting

Samples (20 µg protein/lane) were run on 10% SDS-polyacrylamide gels and proteins were transferred to nitrocellulose membranes by semi-dry electroblotting. Blots were incubated overnight at 4°C, with the primary antibodies (pERK1/2, ERK1/2, p-p38, p38, GADPH) in Tris-Tween Buffered Saline (TTBS) containing 1% (w/v) skimmed milk powder antibody buffer, on a three-dimensional rocking table. Then, blots were washed with TTBS, incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP) secondary antibody for 45 min, washed with TTBS and then exposed to ECL-reagent for 1.5 min and developed using ImageQuant<sup>™</sup> LAS mini 4000 (GE Healthcare). Bands were analyzed using ImageQuant<sup>™</sup> Software (GE Healthcare). Molecular weights of the bands were calculated from comparison with prestained molecular weight markers (MW 10–250 kDa), which were run in parallel with the samples. The equal loading and efficient transfer of proteins was confirmed by using GADPH as internal control.



**Figure 1.** Neuroprotective effect of 3,4DHPA against SIN-1-induced damage in SH-SY5Y cells. \*\*, \*\*\* indicates values significantly different ( $p < 0.01$ ,  $p < 0.001$ , respectively) from sin-1 group.

### 2.4. Caspase-3 activity

Cells were lysed and collected as previously described. The activity of caspase-3-like proteases in the lysates was determined using the caspase-3 colorimetric assay kit (Sigma-Aldrich) according to the manufacturer's protocol, with the exception that 30 µl of cell lysate was used in assays. Absorbance data (405 nm) obtained using the caspase-3 inhibitor, were subtracted from the absorbance obtained without caspase-3 inhibitor to correct for any non-specific hydrolysis.

### 2.5. Statistical analysis

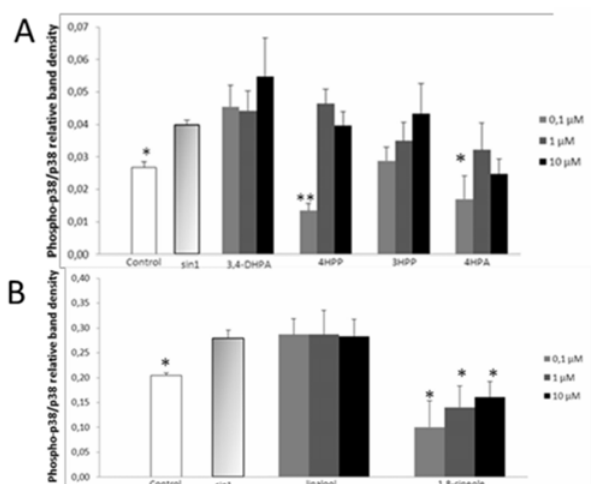
Assays were carried out in triplicate, and data were expressed as the mean  $\pm$  standard error (SEM). One-way ANOVA and post-Hoc Dunnett analysis test were performed. The STATISTICA program for Windows version 7.1 was used for data processing (StatSoft, Inc., 2005, www.statsoft.com).

## 3. Results and discussion

In the present work, we showed a protective effect on cell viability of wine-derived aroma compounds as well as wine phenolic compounds, and its gut derived metabolites, against SIN-1-induced stress neuronal death. Besides, an interaction of these compounds with ERK1/2 and p38 MAPK signalling route kinases, and also and with a downstream point of this stress-induced pro-apoptotic response, caspase-3 protein, has been reported. Although the obtained results do not let to establish a general trend of the protective behavior of these wine compounds on this situation, and further studies are required, the data presented here proposes the first evidences about the molecular mechanisms involved in the neuroprotective role of wine components, specifically in an oxidative process.

### 3.1. Neuroprotection against SIN-1-induced cell death

Pretreatment with 10 µM 3,4DHPA acid resulted in a significant increase in cell viability against SIN-1-induced neuronal death (Fig. 1) when compared to the SIN-1 control group ( $p < 0.001 - p < 0.01$ ), meanwhile a weaker protective action was also described for the



**Figure 2.** Inhibition of p38 phosphorylation in SIN-1-activated SH-SY5Y cells after pretreatment with A) 3,4-DHPA, 4HPP, 3HPP and 3HPA and B) linalool and 1,8-cineole. \*, \*\* indicates values significantly different ( $p < 0.05$ ,  $p < 0.01$ , respectively) from SIN-1 group.

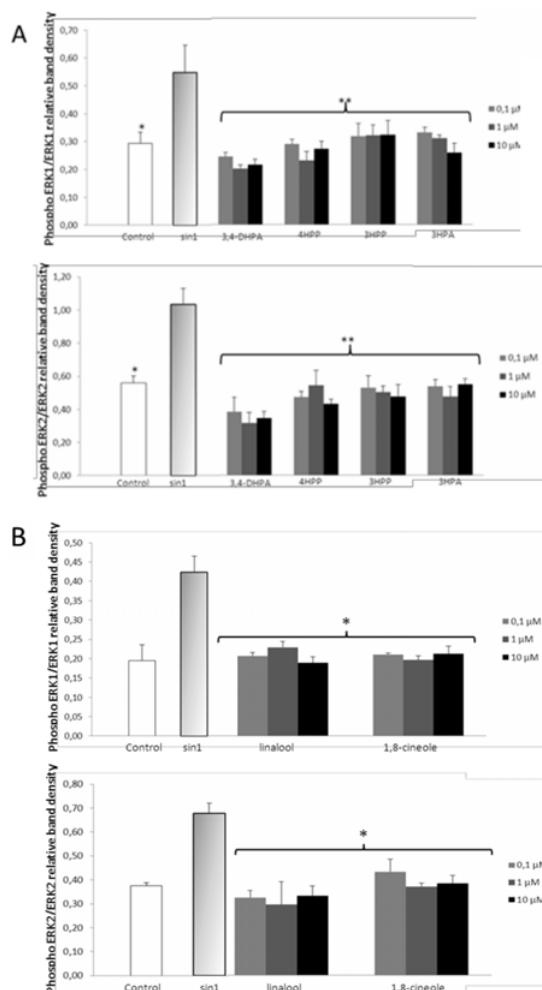
other assayed compounds at different concentrations and time points: glucuronide of salicylic acid (0.1 μM at 4h,  $p < 0.001$ ; 1–10 μM at 4h,  $p < 0.05$ ), 3HPP (10 μM at 6h,  $p < 0.05$ ; 0.1 μM at 15h,  $p < 0.05$ ), 3HPA (0.1 μM at 4h,  $p < 0.05$ ; 1 and 10 μM at 6h,  $p < 0.05$ ; 1 μM at 9h,  $p < 0.01$ ), 4HPP (1 μM at 6h;  $p < 0.01$ ), salicylic acid (1 μM at 4h;  $p < 0.05$ ), linalool (0.1 μM at 4h;  $p < 0.05$ ) and 1,8-cineole (1 μM at 9h;  $p < 0.01$ ).

3,4-DHPA is a colonic metabolite derived from quercetin microbial metabolism and several beneficial roles in oxidative stress have been described for it. For instance, a preventing action against oxidative stress, apoptosis and mitochondrial dysfunction of this compound, have been described [13]. Furthermore, 3,4-DHPA is able to completely block the peroxynitrite nitration effect in tyrosine hydroxylase enzyme, process that uses to take place on the beginning of Parkinson's disease [14].

### 3.2. Effect of wine constituents on p38 phosphorylation

MAPK intracellular pathway is involved in the transduction of extracellular signals into cellular responses. When pro-inflammatory signals become triggered after an oxidative/nitrosative stress stimuli, MAPK kinases become phosphorylated, and therefore activated, leading to the subsequent activation of transcription factors [1]. These molecules affect gene induction, favouring the expression of proteins related to cellular damage, inflammation and apoptosis, as pro- apoptotic caspase-3 protein.

Among MAPK, p38 is related to neuronal stress and the control of cell death and survival [15]. Pretreatment of the cells with wine compounds (0.1–10 μM, 24 h) prior to exposure to SIN-1 resulted in a significant inhibition of p38 phosphorylation status for 4HPP ( $p < 0.01$ ) and 3HPA ( $p < 0.05$ ) at the lower concentration tested (0.1 μM) (Fig. 2A). The aroma compound 1,8- cineole also exerted a significant suppressive effect on p38 activation at all concentrations tested ( $p < 0.05$ ) (Fig. 2B).



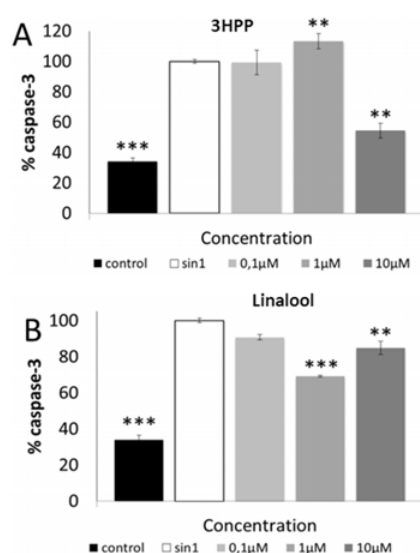
**Figure 3.** Inhibition of ERK1/2 phosphorylation in SIN-1-activated SH-SY5Y cells after pretreatment with A) 3,4-DHPA, 4HPP, 3HPP and 3HPA, and B) linalool and 1,8-cineole. \*, \*\* indicates values significantly different ( $p < 0.05$ ,  $p < 0.01$ , respectively) from SIN-1 group.

### 3.3. Effect of wine constituents on ERK1/2 phosphorylation

ERK1/2 activation has been generally associated with pro-survival signalling [16]. Nevertheless, several lines of evidence suggest that it also mediate oxidative stress-induced apoptosis [17]. Pretreatment with 3,4-DHPA, 4HPP, 3HPP and 3HPA resulted in a significant decrease of ERK1/2 phosphorylation, reaching levels of activation similar to the control condition (Fig. 3A) ( $p < 0.01$ ). Both aroma compounds, linalool and 1,8-cineole exerted a significant reduction in ERK1/2 activation for all concentrations tested ( $p < 0.05$ ) in comparison to the SIN-1 group (Fig. 3B). On the other side, not significant modulation of either ERK1/2 or p38 by salicylic acid or its glucuronide has been observed. This can be due to this compounds acting by other mechanisms or interaction with other related signalling routes, such as NF-κB.

### 3.4. Effect of wine constituents on Caspase-3 activation

The phenolic acid 3HPP at the highest concentration (10 μM) significantly ( $p < 0.01$ ) reduces caspase-3



**Figure 4.** Relative expression of Caspase-3 protein (%) in SH-SY5Y cells after SIN-1-induced damage and pretreatment with A) 3HPP and B) linalool. Data are indicated as % of caspase-3, considering SIN-1 group expression of caspase-3 the maximum (100%). \*\*, \*\*\* indicate values significantly different ( $p < 0.01$ ,  $p < 0.001$ , respectively) from SIN-1 group.

activation, in spite of the significantly increasing trend that is observed at 1  $\mu\text{M}$  concentration ( $p < 0.01$ ) (Fig. 4A). The aroma compound linalool (1, 10  $\mu\text{M}$ ) also showed a significant reduction ( $p < 0.01$  and  $p < 0.001$ , respectively) in the activation of caspase-3 in relation to SIN-1 treated cells (Fig. 4B).

Furthermore the use of specific MEK, p38 and ERK inhibitors with phenolic-like structure suggested a possible protective effect of these compounds based on ERK1/2, p38 and caspase-3 modulation (data not shown). Overall, this suggests that wine-derived phenolic acids might prevent neuronal death induced by SIN-1 by modulating the activation of ERK1/2 and p38 proteins. This is in agreement with previous results that indicated that both ERK and p38 were the MAPK involved in apoptotic cell death induced by peroxynitrite damage [18]. Both linalool and 1,8-cineole, significantly reduced ERK1/2 phosphorylation, in a similar manner to the wine-derived phenolics tested (Fig. 3A). 1,8-cineole also significantly modulates p38 phosphorylation, suggesting that this might be one potential mechanism by which it can exert a protective effect, in accordance with previous observations (Fig. 3B) [19]. Despite several of the phenolic compounds being able to modulate ERK and p38 phosphorylation, only 3HPP was effective at reducing caspase-3 activation (Fig. 4A). The fact of MAPK-mediated protection not being accompanied by caspase-3 decrease was also perceived in SH-SY5Y cells after induced-endoplasmic reticulum stress [20]. This suggests that the majority of these phenolics are not able of affecting this point, but also that caspase-3 can become activated by other related damage routes, since it is known that caspases expression can be stimulated in several scenarios, such as mitochondrial damage, and cytochrome c release [21]. Despite reported evidences, no trend can be inferred and future studies are suggested.

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