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**“Bioactive molecules isolated from olive pomace  
extract protect murine cortex neurons from NMDA-  
mediated cell death”**

**Candidate:**

Dott.ssa Alice Franchi

**Tutor:**

Prof.ssa Bianca Sparatore

**Course Coordinator:**

Prof. Giambattista Bonanno

# ABSTRACT

Changes in intracellular calcium concentration are crucial events during signal transduction processes involved in several cellular physiological functions. Moreover, even a limited dysregulation in intracellular calcium homeostasis can promote the onset and the progression of neuropathological processes. We have shown previously that the olive pomace extract, obtained in condition of high pressure and high temperature (HPTE), contains bioactive molecules able to prevent calcium-induced cell damages on human neuroblastoma (SK-N-BE) and mouse brain endothelioma (bEnd5) cell lines, operating through the regulation of the cell dynamics that involve the calcium ions.

In this thesis, we report the effect of HPTE on primary cell culture of murine cortical neurons used as a cellular model. Specifically, in order to induce a cytotoxic influx of  $\text{Ca}^{2+}$  through the opening of the NMDA (N-Methyl-D-Aspartic-Acid) receptor (NMDAR), we treated neurons with NMDA in the presence or in the absence of HPTE for 24 hours. We demonstrate that HPTE 1) significantly reduces the cytotoxic effect of NMDA, evaluated as percentage of cell death, and 2) operates preventing the increase of the intracellular calcium concentration mediated by the NMDAR.

To identify the chemical nature of the active factors in the olive pomace extract, we have fractionated HPTE by RP-HPLC, followed by the assay of the cell protective effect. Successively, on the fractions containing the bioactivity and using mass spectrometry techniques, we established that the relevant molecules could be derivatives of the epigallocatechin or of the loganin.

The ultimate aim of this work is to provide new information about the structural and functional specificity of molecules present in the HPTE in order to propose new possible strategies for therapeutic interventions in neurodegenerative diseases.

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# 1. INTRODUCTION

## *1.1. Extra virgin olive oil in Mediterranean diet*

Growing evidence shows that a dietary pattern inspired by Mediterranean Diet (MD) principles is associated with numerous health benefits. A Mediterranean-type diet has been demonstrated to exert a preventive effect toward cardiovascular diseases, in both Mediterranean and non-Mediterranean populations. These properties may in part depend on the positive action on the cardiometabolic risk, by decreasing the risk of diabetes and metabolic-related diseases. There is also evidence of a potential role of the MD in preventing certain cancers. A high adherence to the MD has been associated with a slower cognitive decline, with reduced risk of mild cognitive impairment conversion to Alzheimer's disease (AD) and with reduced risk of AD [1].

Older adults appear to be at high risk for cognitive impairment and the increased life expectancy observed in developed countries is accompanied by increasing prevalence of dementia and of its major cause, Alzheimer's disease. Among lifestyle-related factors recently proposed as the first line of defense against the development and progression of cognitive impairment, nutrition constitutes an interesting approach. Beyond the potential impact of specific nutrients such as antioxidants, *n*-3 polyunsaturated fatty acids (PUFA) and homocysteine-related vitamins (vitamin B12 and folate), dietary patterns allow to investigate their additional synergistic effects [1].

Most nutrients that have been individually associated with prevention of cognitive decline in preclinical or epidemiological studies are present in the MD: MUFA (monounsaturated fatty acids), found in large amounts in olive oil, long-chain *n*-3 PUFA, mainly provided by fish and seafood, vitamins B12, folate, and antioxidants (vitamins C and E, carotenoids, flavonoids and selenium) from plant foods.

The MD is characterized by abundant consumption of plant foods such as fresh fruits, vegetables, bread, other forms of cereals, potatoes, beans, nuts and seeds; extra virgin olive oil (EVOO) as the main source of fats, providing notably monounsaturated lipids; a low-to-moderate intake of dairy products in the form of cheese and yogurt; a low-to-

moderate consumption of fish depending of the proximity of the sea; a low-to-moderate consumption of poultry; low amount of red meat and wine consumed in low-to-moderate amounts, normally during meals [2].

*Olea Europea L.* is a fruit tree native to Asia Minor and Syria, which today is cultivated in the entire Mediterranean area; nowadays, the major producers of olives and olive oil are Spain, Italy and Greece. EVOO, physically extracted from the fruit, is known for its nutritional properties and health effects. These properties are due to the presence of high levels of fatty acids (98-99% of the total weight of EVOO), in particular of monounsaturated acids such as oleic, as well as of other valuable components like phenolics, phytosterols, tocopherols, and squalene even if present in low percentages (1-2%). Only EVOO, and not all seed oils, has a high percentage of fatty acids with a correct balance of unsaturated fatty acids stabilized by minor polar compounds, with antioxidant character. Among the minor components, the phenolic ones are relevant for the health effects attributed to EVOO and are the main antioxidants found in virgin olive oil, which contains both hydrophilic and lipophilic phenols. The main phenolic subclasses present in olive oil are phenolic alcohols, phenolic acid, flavonoids, lignans and secoiridoids. Despite their low concentration, they are responsible for numerous health effects in humans [3].

The chemical characteristics, functional or medical food properties, biological and biomedical activities of these compounds have been described to demonstrate how the olive tree can be considered a food species of great scientific and health interest. Recent circular economy models, promoting green technologies for the recovery of active compounds from by-products and waste, have been already established in the olive oil industry. Biological and biomedical activities of many secondary metabolites from *Olea Europaea L.* have been scientifically proven. For this reason, the olive tree is an unmatched sustained resource for unique bioactive compounds with diverse health benefit [3].

## ***1.2. Recovery of phenolic compounds present in olive oil by-product***

By-products from olive oil production are the starting material to obtain bioactive compounds by green technologies. Olive tree cultivation is particularly widespread in the Mediterranean basin and provides a strong contribution as a source of polyphenols. After the production of olive oil, the large quantities of olive pulp and olive oil waste-waters obtained as by-products represent a great environmental problem given their high toxicity. Olive oil by-products, such as olive mill and leave waste are allowed for production of feed, cosmetics, food, and nutraceuticals, whereas olive oil waste-waters are only allowed for use in agronomy [3].

Although the high phenol, lipid and organic acid content its responsible for phytotoxicity, olive mill waste also contains valuable resources such as a great amount of interesting phytochemicals that could be recovered. Indeed, although the olive fruit is very rich in phenolic compounds, the largest portion of these compounds is lost in the olive mill by-products during the milling process. In particular, phenolic fraction in oil is below 2% of the total phenolic content of the olive fruits, with the remaining 98% being lost in olive mill waste. These huge quantities of olive mill by-products are potential rich sources of phenol compounds, endowed with a wide array of biological activities [4].

Currently, EVOO can be obtained by two main milling processes: the three-phase system, which is widely used in Italy, Greece and other Mediterranean countries; and the two-phase system, which is mainly used in Spain. In the first case, olive oil is separated from two other by-products, olive mill waste-water and a solid olive residue (olive pomace) whereas, in the latter system, only a semi-solid waste containing both water and solid residue is obtained. The production of large quantities of these by-products, especially in the 3-4 months of intensive olive oil production, and the variability of the waste composition are the main problems in residue management, even today [4].

Olive pomace can reach up to 30% of olive oil manufacturing, depending on the milling process, which, after oil extraction, is generally distributed by means of

controlled spreading in agricultural soil. This solid waste is of heterogeneous nature and contains many chemical compounds, such as alkaline (potassium) and alkaline earth (calcium and magnesium) metals, sugars and polyphenols. Since polyphenols are bioactive compounds with antioxidant, neuro-sedative, anti-inflammatory, anti-viral and anti-cancer properties, olive pomace can be considered an interesting source of phenolic compounds [3].

The disposal of by-products of the olive oil industry creates a major environmental problem in the main olive-producing countries. Therefore, a suitable use of these olive residues could not only improve the economic status of olive oil producers, but could also reduce an environmental problem. Thus, it is of great interest to evaluate the possibility of recovering an extract enriched with phenolic compounds, from a low-cost and widely available by-product, especially in the Mediterranean area [4].

An innovative extraction technique exploits the performance of a high pressure-high temperature (HPHT) reactor for the phenolic extraction from olive pomace. High temperatures decrease the viscosity of liquid solvents, thus allowing better penetration of matrix particles and enhancing extraction. Increased temperatures can also disrupt the strong solute-matrix interactions caused by van der Waals forces, hydrogen bonding, and dipole attractions between the solute molecules and the active sites on the matrix. The high pressure should facilitate extraction of the analytes trapped in matrix pores. Indeed, the pressure forces the solvent into areas of the matrices that would not normally be reached by solvents under atmospheric conditions. The purpose of pressurizing the extraction cell is to prevent the solvent from boiling at the extraction temperature and to ensure that the solvent remains in intimate contact with the sample throughout the extraction time, granting a higher solubility to the molecules and accelerating the solvent desorption from the matrix [5].

### ***1.3. Protective effect of phenol compounds on neurodegenerations***

Neurodegenerative diseases represent a heterogeneous group of disorders that share common features like abnormal protein aggregation, perturbed  $\text{Ca}^{2+}$  homeostasis, excitotoxicity, impairment of mitochondrial functions, apoptosis, inflammation, and oxidative stress. Despite recent advances in the research of biomarkers, early diagnosis and pharmacotherapy, there are no treatments that can halt the progression or reverse the brain changes of any age-associated neurodegenerative disease nowadays. This is likely due to the multifactorial nature of these pathologies that arise from a confluence of multiple toxic insults [6]. Furthermore, these diseases are associated with a high morbidity and mortality and represent a primary health problem especially in the aging population [7].

In excitotoxic conditions, mitochondrial dysfunction associated with altered  $\text{Ca}^{2+}$  homeostasis and enhanced cellular oxidative stress, plays a major role in cell damage [8]. Excitotoxicity is involved in the pathogenesis of several central nervous system disorders, including Alzheimer's disease, amyotrophic lateral sclerosis, Huntington's disease and the AIDS dementia complex. This process can occur through over-activation of the N-Methyl-D-Aspartate receptor (NMDAR), with subsequent influx of  $\text{Ca}^{2+}$ , activation of both neuronal nitric oxide synthase (nNOS) and inducible nitric oxide synthase (iNOS), and excessive generation of nitric oxide (NO) [8].

Under these circumstances, stimulation of ionotropic glutamate receptors causes massive  $\text{Ca}^{2+}$  entry and is highly involved in the process of neuronal death. Energy depletion and increased oxidative damage affect the function of several synaptic proteins such as  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase and may result in a loss of local  $\text{Ca}^{2+}$  homeostasis and depolarization of the plasma membrane. Consequently, synaptic degeneration occurs. In addition, the increase of cytosolic  $\text{Ca}^{2+}$  concentration promotes the activation of several intracellular enzymes that can elicit generation of endogenous reactive oxygen species (ROS). Moreover, an increase in mitochondrial  $\text{Ca}^{2+}$  can also promote ROS generation, and intracellular  $\text{Ca}^{2+}$  overload associated with excitotoxicity can induce both apoptosis

and necrosis [7]. Therefore, elevation of intracellular  $\text{Ca}^{2+}$  and increased ROS production are the major causes of neuronal death in excitotoxicity [9].

In the recent years, many studies have been focused on natural phytochemicals present in food as important bioactive molecules against age-related chronic illnesses as neurodegenerative diseases [10] [11]. A large body of evidence supports the beneficial effects of the MD in preventing neurodegenerations, possibly due to its richness in phenols content. Furthermore, natural phenols are able to modulate the redox state of cells through direct action on enzymes, receptors, and different signaling pathways [7].

Recent papers have demonstrated the beneficial effects of polyphenols on the regulation of  $\text{Ca}^{2+}$  dynamics, such as the protective effect of oleuropein and its metabolite hydroxytyrosol in the dysregulation of  $\text{Ca}^{2+}$  dynamics occurring via T-type  $\text{Ca}^{2+}$  channels [12], or the protective activity of quercetin against mitochondrial dysfunction and cell death caused by the increase in the  $[\text{Ca}^{2+}]_i$  [13].

However, flavonoids have been proposed to play a useful role in protecting central nervous system against oxidative and excitotoxic stress [14]. In addition to the antioxidant properties of the natural phytochemicals, the inhibitory effect exerted by some of these compounds on specific excitotoxic processes, such as  $\text{Ca}^{2+}$  influx, provides additional evidence for the potential beneficial health effects of polyphenols on excitable tissues, particularly the central nervous system [15].

#### ***1.4. Excitotoxicity and overactivation of NMDA receptor***

The excitatory amino acids (EAAs) primarily L-glutamate (Glu), L-aspartate (Asp) and related derivatives, act through multiple classes of receptors that are located on virtually all neurons in the vertebrate CNS. Receptors for EAAs belong to two major categories: metabotropic and ionotropic receptors. Ionotropic receptors control ion channels, whereas metabotropic receptors are coupled to G proteins. The mammalian family of ionotropic glutamate receptor encodes 18 gene products that form ligand-gated ion channels containing an agonist recognition site, a transmembrane ion permeation pathway, and gating elements that couple agonist-induced conformational changes to the opening or closing of the permeation pore. The glutamate ionotropic receptors

mediate fast excitatory synaptic transmission in the central nervous system but they are also localized on non-neuronal cells and regulate a broad spectrum of processes in brain, spinal cord, retina, and peripheral nervous system. These integral membrane receptors are composed of four large subunits (>900 amino acid residues) that form a central ion channel pore. Sequence similarity among different ionotropic glutamate receptor subunits, including the AMPA ( $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid), kainate, NMDA (N-Methyl-D-Aspartate) and  $\delta$ -receptors; suggest that they share a similar architecture [16].

NMDAR, one of the ionotropic glutamate receptors, consists of several distinct recognition sites. The primary neurotransmitter binding-site can interact with Asp, Glu and the Asp analog NMDA that acts as a selective agonist for this glutamate receptor. The other sites include: 1) a cation channel that permits the conductance of calcium and sodium, 2) a voltage-dependent site that binds magnesium and, at resting membrane potential, blocks channel conductance, 3) a co-agonist site that binds glycine (Gly), 4) an inhibitory site that binds phencyclidine (PCP), ketamine, MK-801 and other non-competitive antagonists, and 5) an allosteric modulatory site that recognizes polyamines [17]. The molecular structure of NMDAR shows a heterotetrameric assembly, that typically contains two NR1 subunits and other two subunits including NR2 (A, B, C & D) or NR3 (A & B) variants. The various combination of NR2 subunits produce multiple subtypes of NMDAR. NR2A- and NR2B-containing receptors generate high conductance opening, whereas NR2C- and NR2D-containing receptors have low conductance opening. Expression of the NR2 subunits varies across different brain regions, developmental stages and physiological responses to environmental stimulation. Meanwhile, NR2 subtypes provide different physiological function. Over activation or upregulation of NR2B seems involved in neurological diseases including stroke, ischemia, chronic pain, Alzheimer's disorder and Huntington's disease. In agreement with these observations, NR2B-selective antagonists display protective effects against these diseases in animal models. NR2C- and NR2D-containing NMDARs are critical for ischemia-induced myelin damage that is prevented by NR2C/D antagonists. Essentially, selective NMDAR antagonists for distinct subtypes have more efficient therapeutic and lower side effects for specific neurological disorders compared to non-selective NMDAR antagonists [16].

Activation of the NMDAR results in an influx of  $\text{Ca}^{2+}$  that acts as a second messenger in the regulation of neurotransmitter release, synaptic plasticity and gene expression. In addition to stimulating physiological intracellular events, the excessive activation of these receptors may damage the neuron, causing excitotoxic cell death. This phenomenon seems a common outcome following brain insults of different origin, such as trauma, epileptic seizures and cerebral ischemia. The excitotoxic process is primarily mediated by elevated concentrations of intracellular  $\text{Ca}^{2+}$  and ROS and it can be manifested as apoptosis or necrosis [18].

Glutamate-induced excitotoxicity, caused by an excessive cell exposure to glutamate or to excitatory concentrations of NMDA, leads to influx of  $\text{Ca}^{2+}$  through the NMDA subtype receptor, triggering the generation of NO and superoxide anions, and altering the regulation of gene expression, with the final result of cell death. Indeed, excessive stimulation of the NMDAR is implicated in the development of several neurological diseases such as stroke, Alzheimer's disease and depression. Therefore, NMDAR is an important pharmacological target for the treatment of neurological diseases and disorders [19].

### ***1.5. Activation of proteolytic calcium-dependent enzyme calpain-1***

A rise in the ( $[\text{Ca}^{2+}]_i$ ) has been identified as a primary trigger of different pathologies.  $\text{Ca}^{2+}$ -dependent processes, which represent points of convergence among a heterogeneous set of degenerative mechanisms, are therefore logical targets for intervention strategies, and have the potential to generate valuable markers of pathogenesis [20].

Pathological activation of NMDARs, with consequent alteration in intracellular calcium homeostasis, determines the activation of several protein such as calpain-1 a ubiquitous calcium-dependent cysteine protease that plays a key role in cell survival [20].

Calpains are a group of intracellular endopeptidases present in almost all eukaryotes and bacteria, but not in archaeabacteria. In humans, this group of enzymes includes 15

isoforms, some of which are present ubiquitously and other ones show a tissue-specific pattern of expression [21]. Calpains operate in many pathological and physiological processes such as cytoskeletal remodeling, cell proliferation, migration, invasion, apoptosis and signal transduction [21] [22]. Following activation, calpains can catalyze the proteolytic processing of a large number of intracellular proteins leading to modification of cell behavior and viability. Because these enzymes are involved not only in normal intracellular signal transduction cascades, but also in various aberrant pathways, investigations on the molecular mechanisms for the control of calpain activity has aroused great interest in wide areas of life sciences in both basic and clinical research in the last decades [23]. Calpains show peculiar functional properties not overlapping to those played by the ubiquitin-proteasome system, the autophagy-lysosomal system and the caspases. First of all, the digestion of the substrates mediated by calpains generally occurs in a limited and selective manner. Since the target protein does not undergo extensive degradation but often acquires novel functional properties, this irreversible protein modification has been termed "proteolytic processing". Furthermore, calpains do not require any protein modification or tag to recognize and hydrolyze the susceptible peptide bonds present in the consensus sequences of their substrates [22].

The conventional members in the superfamily of calpains are heterodimers consisting of different 80 kDa catalytic large L-subunits and 30 kDa regulatory small S-subunits. Calpain-1 and calpain-2, are the best characterized isozymes of the calpain family. These heterodimers share the same 30 kDa regulatory subunit, but each one has a different 80 kDa catalytic subunit. These two calpains are ubiquitous (with the exception of erythrocytes that lack calpain-2) and are distinguished by the calcium requirement to undergo *in vitro* activation. Indeed, calpain-1 (also known as  $\mu$ -calpain) needs 5-20  $\mu\text{M}$   $\text{Ca}^{2+}$ , while calpain-2 (also known as m-calpain) needs 400-800  $\mu\text{M}$   $\text{Ca}^{2+}$  [22]. The catalytic subunits of calpain-1 and calpain-2 contain four domains/regions: the N-terminal anchor helix region, the CysPc Protease Core Domain (PC1 and PC2), the C2 like domain (C2L), and the penta-EF-hand domain (PEF(S)) [22]. This domains structure of the conventional calpains is defined "classical". Alternatively, "non-classical" calpains exclude C2L and/or PEF domains [22].

The ubiquitous calpains, play an essential role in all cells and defects in the expression of these isoforms may be lethal [22] [24]. Conversely, other forms of the enzymes are

involved in more specific cellular functions and their defects may cause tissue-specific altered phenotypes [22] [25].

The catalytic mechanism of calpain is common to other cysteine-proteases, serine-proteases and threonine-proteases and depends on three amino acids collectively named "catalytic triads"[26]. In calpain, the residue used as a nucleophile is the oxidized sulfur of the cysteine. In addition, the basic and acid residues needed for catalysis are provided by an histidine and an aspartate, respectively [27].

The physiological role of classical calpains is related to extracellular signals transduction mediated by changes in the permeability of the plasma membrane to  $\text{Ca}^{2+}$  or by the mobilization of this ion from intracellular stores. Indeed, the  $\text{Ca}^{2+}$  ions trigger the structural transitions of these calpains observed during the activation of the enzyme [28]. The crystallographic analysis of the enzyme structure and the studies of the kinetics of  $\text{Ca}^{2+}$  binding have demonstrated that the onset of proteolytic activity appears only after a delay. This delay has been attributed to a  $\text{Ca}^{2+}$ -induced conformational change making the active site of the enzyme accessible to the substrate. Furthermore,  $\text{Ca}^{2+}$  causes the dissociation of the heterodimeric native calpain into its constitutive L- and S-subunits, resulting in an increased affinity for  $\text{Ca}^{2+}$  of the catalytic subunit compared to the native form, thereby yielding the active form of the protease [28] [29]. In the absence of  $\text{Ca}^{2+}$  the amino acid residues of the active site, which are crucial for the proteolytic activity, are not correctly positioned with respect to the substrates binding site. Hence, the binding of  $\text{Ca}^{2+}$  causes an electrostatic switch of the L-subunit producing the conformational changes necessary for the correct positioning of the catalytic triad [28]. Indeed, the primary event in the activation process corresponds to the binding of  $\text{Ca}^{2+}$  to eight interacting sites, four in each of the two subunits, localized in calmodulin-like regions. Progressive binding of  $\text{Ca}^{2+}$  ions is linearly correlated with the dissociation of the heterodimeric enzyme, which reaches completion when all the  $\text{Ca}^{2+}$  binding sites are occupied [29].

Exposure to  $\text{Ca}^{2+}$  also induces autoproteolysis of classical calpains. In these conditions, the 80 kDa calpain subunit, undergoes intramolecular autoproteolysis that, by the removal of a peptide from the N-terminus, generate a 75 kDa species with an increased affinity for  $\text{Ca}^{2+}$ . Thus, such autoproteolytic event contributes to make the active site accessible to the substrate [29] [30]. An important question in the field of calpain

research concerns the remarkable inconsistency of  $\text{Ca}^{2+}$  requirement necessary to activate the enzyme *in vitro* and the intracellular availability of this metal ion. Indeed although  $\mu\text{M}$  concentrations of  $\text{Ca}^{2+}$  are sufficient to activate purified calpain-1, the physiological amounts of cytosolic  $\text{Ca}^{2+}$  are in the range of 50-300 nM [31]. Since *in vitro* experiments have demonstrated that phospholipids, the most abundant type of lipid constituents in the cell membranes, lowered the  $\text{Ca}^{2+}$  requirement necessary to activate the protease, it has been hypothesized that cell microdomains close to the plasma membrane could present favorable locations for the calpain activation. It has been also proposed that the activation of a limited amount of total calpain, whose localization coincides with the spots where cell  $\text{Ca}^{2+}$ -concentration substantially increases, is sufficient to carry-out the calpain-dependent physiological function of a cell [31]. Although the isolated 80 kDa catalytic subunit of calpain shows a 2-fold increased affinity for  $\text{Ca}^{2+}$  than the native heterodimeric form, only the autoproteolyzed 75 kDa form of the catalytic subunit shows a 20-fold increased affinity for  $\text{Ca}^{2+}$ , reaching a  $K_d$  closer to the physiological  $\text{Ca}^{2+}$  concentration [29].

An extensive literature suggests that classical calpains participate in a variety of cell functions including the remodeling of cytoskeletal/membrane attachments during cell fusion and motility, processing of proteins involved in the control of the cell cycle, gene expression and apoptotic pathways [31]. Furthermore, over-activation of calpains has been linked to a variety of degenerative conditions in the brain and several other tissues. Therefore, calpain activation is considered a primary contributor to the onset and progression of degenerations in vulnerable populations of neurons and other cell types [20]. Particularly, pathological activation of calpain in condition of calcium-overloading causes excessive proteolysis and consequently degradation of different substrates crucial for neurons survival [20].

## 2. AIM OF THE WORK

The aim of this thesis consists in the assessment of the possible biological effects of the olive pomace extract purified at high temperature and high pressure (HPTE) and containing concentrated olive oil polyphenols. Specifically we have investigated the cell response and the underlying biochemical processes modulated by HPTE in conditions of altered  $[Ca^{2+}]_i$ . First we have set up the experimental conditions on human neuroblastoma (SK-N-BE) and mouse brain endothelioma (bEnd5) cell lines. Subsequently we have used primary cultures of murine cortical neurons as a cell model considerably most suitable to investigate the pathological role of calcium in neurodegenerative processes. We have evaluated the specificity of the bioactive molecules present in HPTE during cell stimulation with excitotoxic concentrations of various agonists, directed toward the different glutamate receptors. In these conditions we have analyzed the activity of HPTE by measuring changes of cell viability and by exploring structural and functional aspects of intracellular pathways activated by cell calcium overloading.

### 3. MATERIALS AND METHODS

#### *3.1. Reagents and antibodies*

Ca<sup>2+</sup>-ionophore A23187, NMDA, AMPA, glutamate, MK-801 hydrogen maleate powder and mouse monoclonal anti-non-erythroid spectrin antibody, were purchased from Sigma-Aldrich. ECL Select<sup>TM</sup> Western Blotting Detection Reagent was obtained from GE Healthcare. Calcium Green<sup>TM</sup>-1 and t-BOC (t-butyloxycarbonyl)-Leu-Met-CMAC (7-amino-4-chloromethylcoumarin), a fluorogenic calpain substrate, were purchased from Molecular Probes (Invitrogen). Mouse monoclonal anti-nNOS antibody was purchased from BD Transduction Laboratories. Horseradish peroxidase (HRP)-linked anti-mouse and anti-rabbit secondary antibodies and Protease and Phosphatase Inhibitor Cocktail were purchased from Cell Signaling. Mouse monoclonal anti  $\beta$ -actin antibody was from Santa Cruz.

#### *3.2. Cell culture*

Human neuroblastoma SK-N-BE cells (Interlab Cell Line Collection ICLC, HTL96015, Italy) were maintained in continuous culture at 37°C (5 % CO<sub>2</sub>) with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum, 10 U/ml penicillin, 100  $\mu$ g/ml streptomycin and 2 mM L-glutamine.

Mouse brain endothelioma bEnd5 cells were kindly provided by L. Riboni (Department of Medical Chemistry, Biochemistry and Biotechnology, University of Milan, LITA-Segrate, Milan, Italy) and maintained in continuous culture at 37°C (5% CO<sub>2</sub>) with DMEM containing 10% foetal bovine serum, 10 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 2 mM L-glutamine. All products used for cell culture were purchased from Euroclone S.p.A.

Primary neuron cultures were obtained by the collaboration with Prof. Franco Onofri (Department of Experimental Medicine, section of Physiology, University of Genova)

and were prepared from cerebral cortices of E18.0 day-old mouse embryos recovered from CO<sub>2</sub> anaesthetized pregnant C57BL/6J. Briefly, embryos were removed, microdissected and brain cortex pieces were dissociated by enzymatic digestion in Trypsin 0.25% for 20 min at 37 °C and then triturated with a fire-polished Pasteur pipette. Dissociated neurons were plated onto 0.1 mg/ml poly-L-lysine coated cell culture supports. Cells were maintained in a culture medium consisting of Neurobasal (Invitrogen), supplemented with B-27 (1:50 v/v, Invitrogen), Glutamax (1% v/v, Invitrogen), 10 U/ml penicillin, 100 µg/ml streptomycin, and kept at 37°C in a 5% CO<sub>2</sub> humidified atmosphere. The 50% medium was changed weekly. No antimitotic medium agent was used to control glial proliferation, because application of serum-free medium limits the growth of non-neuronal cells. Neurons were allowed to grow functional and structural mature networks (approximately 14 days *in vitro*).

### ***3.3. Extraction of bioactive molecules from olive pomace of Taggiasca cultivar***

HPTE was kindly provided by Prof.ssa Patrizia Perego (Department of civil, chemical and environmental engineering, University of Genova). Bioactive molecules were extracted from olive pomace of Taggiasca cultivar by means of a green and non-conventional extraction technique using a high pressure and temperature (HPTP) reactor (model 4560, PARR Instrument Company, Moline, USA). Extractions parameters were selected based on previous work [5] [32].

Particularly, the temperature of the extraction is 180 °C and authors observed that, despite the high temperature, no production of 5-hydroxymethylfurfural occurred [5]. During the extraction, the pressure inside the reactor reached 25 bar. After extraction, the liquid phase rich in biophenols, was separated from the exhausted olive pomace by centrifugation at 6.000 ×g for 10 min. Then, the extract of olive pomace, after a preliminary concentration by rotary evaporator (Laborota, Heidolph, Germany), was submitted to solid phase extraction using a C18 cartridge (Sep-Pak C18 5 mL, Water, Milan, Italy) in order to remove proteins or sugars. Briefly, 1 mL of sample was filtered in a C18 cartridge after its activation with a solution of H<sub>2</sub>SO<sub>4</sub> 5 mM. The cartridge was

washed with 5 mL of H<sub>2</sub>SO<sub>4</sub> 5 mM, than the adsorbed material was eluted by the addition of 5 mL of methanol. Methanol was removed and the material suspended in the same solvent used for the HPTE extraction. The HPTE extract was stored at -20°C until further analysis. On HPTE extract, total biophenol concentration was determined using Folin-Ciocalteu assay and was expressed as mg of caffeic acid equivalents (CAE) per mL of solvent (mgCAF/mL). Single phenolic compounds were quantified using HPLC methodology using the corresponding phenol standard solutions following the methodology described in [32] (see **Table 1**). Different preparations of HPTE were performed, but the method of extraction was the same [32] [33].

**Table 1.** Concentration (mg/mL) in total biophenols and single phenolic compounds in HPTE after the extraction at high pressure and high temperature.

Total Biophenols (mg <sub>CAE</sub> /mL)	19.2
Tyrosol (mg/mL)	0.51
Caffeic acid (mg/mL)	0.23
Coumaric acid (mg/mL)	0.18
Oleuropein (mg/mL)	3.59
Apigenin (mg/mL)	0.08

### ***3.4. Cell viability assay***

Cell viability was measured using the Neutral Red Uptake (NRU) assay, as reported by Repetto *et al* [34]. Briefly, the neutral red uptake assay provides a quantitative estimation of the viable cells in a culture. It is based on the ability of viable cells to incorporate neutral red dye in the lysosomes. Cells were seeded in 96-well plates and treated for the appropriate period. The plates were then incubated for 1 h at 37 °C with a medium containing neutral red. The cells were then washed and the dye was extracted in each

well with a destaining solution (48% EtOH 1% CH<sub>3</sub>COOH). The absorbance was finally read using a spectrophotometer set at a wavelength of 540 nm [34]

### ***3.5. Immunoblot analysis***

Murine cortical neurons ( $3 \times 10^5$ ), seeded in 35 mm culture dishes and treated as described elsewhere, were lysed by sonication in 150  $\mu$ L of 50 mM Tris/HCl, pH 7.4 containing 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and Protease and Phosphatase Inhibitor Cocktail diluted 1:100. The lysates were centrifuged at  $10,000 \times g$  for 10 minutes at 4 °C, the supernatants were collected, and protein quantification was assayed by the method of Lowry. Samples were then heated for 5 minutes at 95°C. Proteins (20  $\mu$ g/lane) were separated by SDS/PAGE (8% or 10%) and transferred onto nitrocellulose membranes. The nitrocellulose membranes were blocked in 5% non-fat dry milk, 0.1% Tween 20 and incubated for 16 h at 4 °C with a primary antibody: anti-non erythroid spectrin (1:500), anti-nNOS (1:2500), anti- $\beta$ -actin (1:1000), anti-phospho-ERK1/2 (1:1000). Peroxidase-conjugated secondary antibodies (1 h at 22 °C) were anti-rabbit (1:5000), and anti-mouse (1:5000). Immunoreactive signals were developed using ECL Select™ Western Blotting Detection Reagent (GE Healthcare), acquired and quantified using ChemiDoc XRS equipped with the Quantity One Image Software (Bio-Rad).

### ***3.6. $[Ca^{2+}]_i$ assay***

$2 \times 10^4$  SK-N-BE cells were incubated in 200  $\mu$ L of HEPES buffer (NaCl 128 mM, KCl 2.4 mM, MgSO<sub>4</sub> 1.2 mM, CaCl<sub>2</sub> 1.2 mM, KH<sub>2</sub>PO<sub>4</sub> 1.2 mM, glucose 10 mM, HEPES 10 mM, pH 7.3–7.4) containing 10  $\mu$ M Calcium Green™-1, AM. After 40 min at 37°C cells were washed with HEPES buffer and calcium ionophore A23187 and HPTE were added in 100  $\mu$ L of HEPES buffer. The fluorescence intensity (Excitation 485 nm; Emission 535 nm) was measured before (F<sub>0</sub>) and 3 min after the addition of stimuli (F) using the top reading mode in the fluorescence multilabel reader LB 940 Mithras

(Berthold Italia). Variations of the fluorescence values were calculated as the difference between F and F<sub>0</sub>.

Neurons ( $2.5 \times 10^4$ , seeded in 96-well microplate) were incubated in HEPES buffer containing 10  $\mu$ M Calcium Green™ -1, AM. After 40 min at 37°C, cells were washed twice with HEPES buffer and then exposed to 0.5 mM NMDA, in the absence or in the presence of HPTE extract. The fluorescence intensity (Excitation 485 nm; Emission 535 nm) was measured every 10 seconds for 20 minutes using the top reading mode in the fluorescence multilabel reader LB 940 Mithras (Berthold Italia). Variations of the fluorescence values were calculated as the difference between each fluorescence value recorded and that one measured at time zero. The values obtained were then subtracted of the relevant control values.

### ***3.7. Assay of intracellular Calpain-1 activity***

Human neuroblastoma SK-N-BE cells and murine cortical neurons ( $2.5 \times 10^4$ , seeded in 96-well microplate) were incubated at 37°C for 20 min-with 50  $\mu$ M t-Boc-Leu-Met-CMAC fluorogenic calpain substrate in HEPES buffer. Cells were then washed twice with HEPES buffer to remove substrate excess, exposed to 0.5 mM NMDA, in the absence or in the presence of HPTE extract, and the fluorescence emission was monitored every 2 minutes for 1 hour with a Mithras LB 940 plate reader (Berthold Technologies). The excitation/emission wavelengths were 355/485 nm, respectively.

### ***3.8. HPLC and mass spectrometry analysis***

The HPLC and mass spectrometry analysis were performed in collaboration with Prof. Gianluca Damonte (Center of Excellence for Biomedical Research, University of Genova). Firstly, HPTE was submitted to a pre-treatment: 10 minutes at 37°C, 2 minutes of vigorous agitation on vortex, and finally 10 minutes at 6.000  $\times$ g to separate the insoluble fraction of the extract. After that, 20  $\mu$ l of the supernatant was put together with 30  $\mu$ l of water and the resulting 50  $\mu$ l was injected in analytical HPLC

Agilent 1260 with manual injection. The operative parameters of the separations are reported in **Table 2**:

Column	Temp.	Eluents	Gradients		Flow	Vol. of injection	$\lambda$
Luna C <sub>18</sub> (pore size 300 Å, particle size 5µ) 3.9 mm × 150 mm (ID × L).	30° C	A:H <sub>2</sub> O + 1% Acetic acid B:CH <sub>3</sub> CN/CH <sub>3</sub> OH (50:50)	t	%B	1ml/min	50 µl	220 nm 254 nm
			5	0			
			10	5			
			30	30			
			40	40			
			45	48			
			55	70			
			60	100			

Fractions were collected every 60 seconds, dried with centrifugal vacuum concentrator and conserved at -20 °C.

After the cell viability test on neurons, only the active fractions was submitted to HPLC-MS analysis and the chromatographic separation in HPLC (Agilent 1100 µHPLC) with micro-autosampler was developed using a column Zorbax C18 (pore size 300 Å, particle size 3,5 µ) 0,5 mm (ID) x 150mm (L). In **Table 3** we reported the analysis parameters:

Column	Temp.	Eluents	Gradient		Flow	Vol. of injection	$\lambda$
Symmetry C18 (pore size 300 Å, particle size 3.5µ) 1mm x 150mm (ID × L).	30° C	H <sub>2</sub> O + 0.1% FOA CH <sub>3</sub> OH + 0.1% FOA	t	%B	20µl/min	8µl	220 nm 280 nm
			0	10			
			5	10			
			50	100			
			60	100			

All the parameters were established in order to obtain the best ionization of the fractions components. The instrument utilized is a mass spectrometry with orthogonal electrospray source (ESI) and high capacity ion trap (Agilent 1100 MSD xct ion trap). Analysis was performed in a ion-charged mode control with selected target at 100,000 and accumulation time of 300 ms. The operative parameters were: capillary voltage: 3,8 V; nebulizer pressure: 20 psi; drying gas: 8 L/min; dry temperature: 360°C; rolling averages: 3, fragmentation Amplitude 0,8 V.

All the mass spectra have been acquired in Full-scan and in MS-MS modality, breaking between the most abundant species under every peak. The acquisition was performed on negative and positive ions in the mass range of 50-1200 m. Finally, the HPLC was coupled with the mass spectrometry (HPLC-ESI-MS) to value quantitatively the compound of interest.

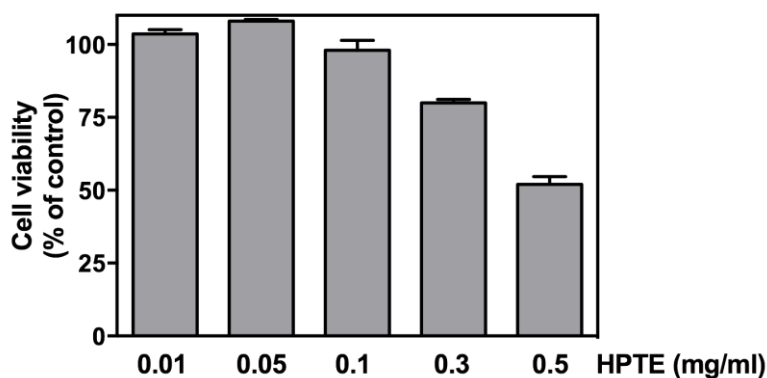
## 4. RESULTS

### *4.1. Evaluation of possible cytotoxicity of HPTE on human and murine cell lines*

In the first part of this study, in order to define our experimental conditions, we analysed the potentially toxic effects of the bioactive molecule(s) contained in HPTE on human and mouse cell lines. At this purpose, we performed cell viability experiments on human neuroblastoma SK-N-BE cells and murine endothelial bEnd5 cells by means of NRU assay.

Thus, we treated SK-N-BE and bEnd5 cells with increasing concentrations of HPTE for 24 hours to establish the maximum non-cytotoxic concentration useful for our experiments.

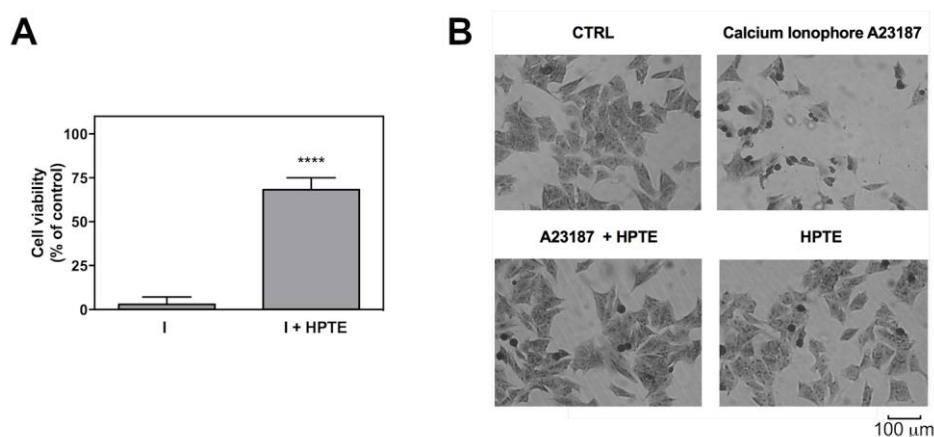
As shown in **Fig.1**, the highest HPTE concentration present in the culture medium which still maintained 100% of cell viability was 0.05 mg/ml. Indeed, in the presence of concentrations higher than 0.05 mg/ml the cells started dying. Similar results were obtained with bEnd5 cells.



**FIGURE 1:** Cell viability of SK-N-BE cells exposed to increasing concentrations of HPTE. SK-N-BE cells ( $2 \times 10^4$ ) were treated with the indicated concentrations of HPTE and cultured for additional 24 hours. Cell viability was then evaluated by NRU assay. Values represent means  $\pm$  SEM from three independent experiments in triplicate. Similar results were obtained with bEnd5 cells.

## 4.2. Effect of HPTE on cell viability of SK-N-BE and bEnd5 cells in condition of altered $[Ca^{2+}]_i$

In order to alter intracellular calcium homeostasis, we treated SK-N-BE and bEnd5 cells with cytotoxic concentrations of calcium ionophore A23187 (I), a mobile ion-carrier that forms stable complexes with divalent cations allowing their transportation across the membranes [35] in the absence or presence of 0.05 mg/ml HPTE. When cells were incubated with calcium ionophore alone, we observed a significant reduction in cells viability due to the alteration of the intracellular calcium homeostasis. However, when the same treatment was performed in the presence of HPTE, a significant recovered cells viability was observed, as shown in **Fig.2**. Similar results were obtained with bEnd5 cells.



**FIGURE 2: Cell viability of SK-N-BE cells exposed to calcium ionophore (I) A23187 and HPTE.**

(A) SK-N-BE cells ( $2 \times 10^4$ ) were treated for 1 h and 15 minutes with 5  $\mu$ M calcium ionophore A23187 in the presence ("I+HPTE") or in the absence ("I") of HPTE 0.05 mg/mL. Cells viability was evaluated by NRU assay. Values represent  $\pm$  SEM from three independent experiments in triplicate. Statistical analysis was performed with unpaired t test. \*\*\*\*  $p < 0.0001$  for I + HPTE vs I. Similar results were obtained with bEnd5 cells. (B) Sub-confluent SK-N-BE cells were treated as specified in A, fixed with ice-cold 100% methanol for 15 minutes at 4°C, rinsed three times in PBS for 5 minutes, and then stained for 2-3 minutes with 0.1% Toluidine blue.

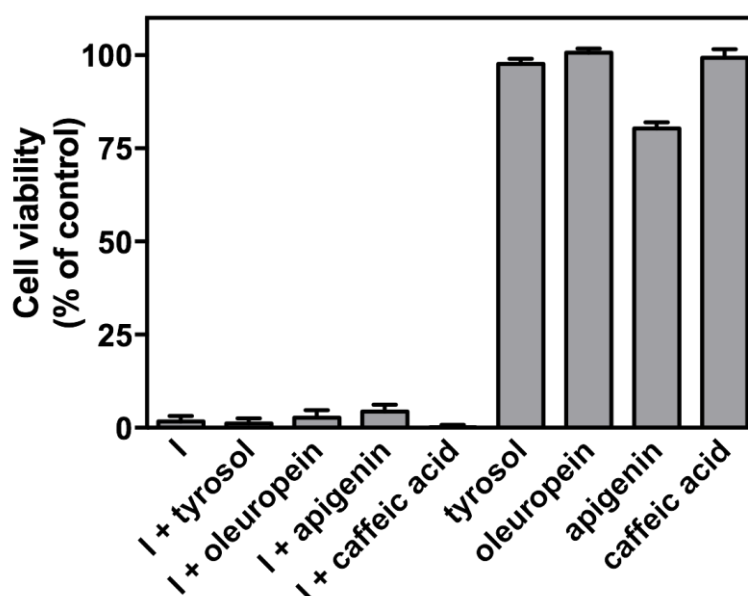
These data, obtained on two different cell lines, suggest that HPTE contains bioactive molecule(s) capable to counteract the toxic effect of an aberrant calcium influx.

#### 4.3. Effect of different olive oil polyphenols on viability of SK-N-BE and bEnd5 cells in condition of altered $[Ca^{2+}]_i$

It is well known that EVOO is rich in phenolic compounds that exert several biochemical and pharmacological beneficial effects [36]

To verify if the bioactive molecule(s) present in HPTE, and responsible for the protective effect from cell death induced by altered  $[Ca^{2+}]_i$  could belong to the same polyphenols concentrated in HPTE as previously reported [32] we analysed the effect of these polyphenols separately on cell viability.

As shown in **Fig.3**, when we treated cells with calcium ionophore in the presence of the polyphenols tested (tyrosol, oleuropein, apigenin, caffeic acid), we observed that none of this molecules were endowed with the ability to protect calcium-induced cell death. Similar results were obtained with bEnd5 cells.

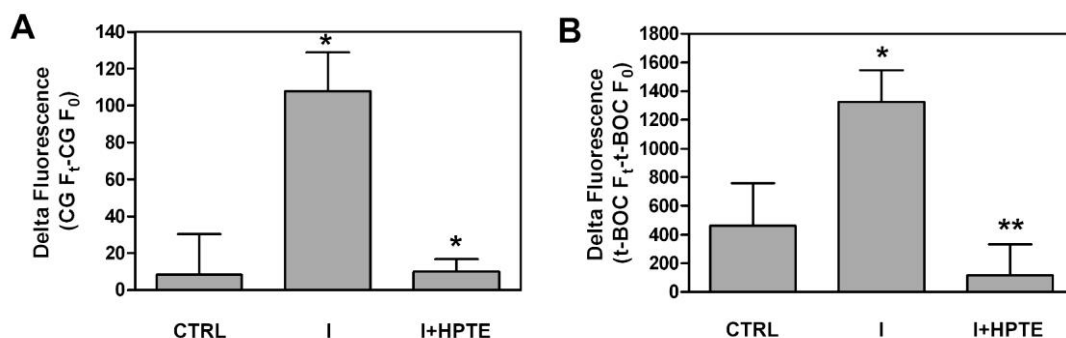


**FIGURE 3:** Cell viability of SK-N-BE cells exposed to calcium ionophore A23187 in the presence of olive oil biophenols. SK-N-BE cells ( $2 \times 10^4$ ) incubated in the presence (“I+tyrosol”, “I+oleuropein”, “I+apigenin”, “I+caffeic acid”) or in the absence (“I”) of 0.01 mg/mL of tyrosol or oleuropein or apigenin or caffeic acid, were treated for 1 h and 15 minutes with 5  $\mu$ M calcium ionophore A23187. Cells viability was evaluated by NRU assay and similar results were obtained with bEnd5 cells. Values represent means  $\pm$  SEM from three independent experiments in triplicate.

This result suggests that the bioactive molecule(s) responsible for the protective activity could belong to polyphenols not yet tested or different types of still unidentified molecules.

#### 4.4. Evaluation of $[Ca^{2+}]_i$ and calpain-1 proteolytic activity on SK-N-BE cell line

The results obtained with calcium ionophore and/or HPTE indicate that the bioactive molecule(s) were able to counteract calcium induced cell death. In order to better elucidate this process, we firstly performed different experiments using a fluorophore able to detect the intracellular calcium level and then, we performed different experiments using a fluorescent substrate of the calcium-dependent protease calpain-1, to monitor its proteolytic activity.



**FIGURE 4: Evaluation of  $[Ca^{2+}]_i$  increase and calpain activity in SKNBE cells exposed to HPTE and/or Calcium Ionophore A23187 (I).** Notes: A) Calcium Green™-loaded cells were pre-treated for 1 h with 0.01 mg/mL HPTE (“I+HPTE” and “HPTE”) or vehicle (“CTRL” and “I”). The cells were then left untreated (“CTRL” and “HPTE”) or exposed to 1  $\mu$ M I for 3 min (“I” and “I+HPTE”). Data are means  $\pm$  SEM from four independent experiments. Statistical analysis was performed with ANOVA followed by Tukey’s post-hoc test. \* $p < 0.05$  for I vs CTRL and I+HPTE vs I. B) t-BOC-loaded cells were pre-treated as in A) and then left untreated or exposed to 1  $\mu$ M I for 60 min. Data are means  $\pm$  SEM from three independent experiments. Statistical analysis was performed with ANOVA followed by Tukey’s post-hoc test. \* $p < 0.05$  for I vs CTRL and \*\* $p < 0.01$  for I+HPTE vs. I.

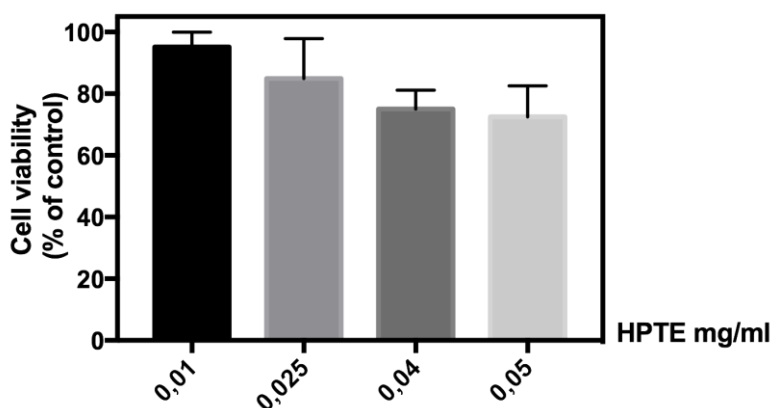
As shown in **Fig.4A**, treatment of SK-N-BE cells with HPTE maintained the intracellular level of the metal ion close to the physiological concentrations (CTRL).

We also observed that as a consequence, also the intracellular proteolytic activity of calpain-1 (**Fig.4B**) was maintained at basal level.

Altogether these results obtained on SK-N-BE cells indicate that HPTE contains bioactive molecule(s) able to counteract calcium induced cell death acting through the modulation of the  $[Ca^{2+}]_i$ .

#### ***4.5. Evaluation of possible cytotoxicity of HPTE on cortical neurons***

The promising results obtained on cell lines prompted us to further investigate the biological activity of HPTE. Thus, we used a primary cell culture of murine cortical neurons as a cellular model to test the protective effect of these molecule(s) in condition of calcium-mediated excitotoxicity. Indeed, primary neuronal culture from rodents is a well-established model to investigate cellular neurobiology *in vitro* [37]. After 14 days *in vitro* (DIV), we exposed mature neurons to increasing concentrations of HPTE to establish the maximum non-cytotoxic concentration to be used in our experiments.



**FIGURE 5:** Cell viability of cortical neurons exposed to increasing concentration of HPTE. After 14 DIV, neurons ( $2 \times 10^4$ ) were incubated for 24 hours with 0.01, 0.025, 0.04 and 0.05 mg/ml of HPTE. Cell viability was evaluated by NRU assay. Values represent means  $\pm$  SEM from three independent experiments in triplicate.

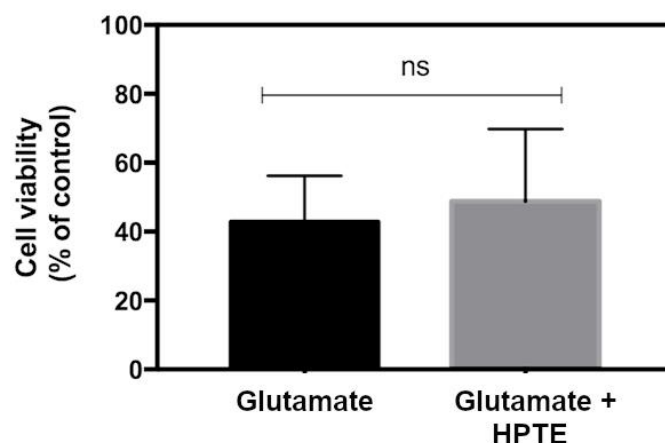
As shown in **Fig.5**, we observed that after 24 hours of incubation, the maximum non-cytotoxic concentration of HPTE which still maintained the highest cell viability was

0.01 mg/ml. Differently from the cell lines, cortical neurons started dying at concentrations immediately higher than 0.01 mg/ml.

#### ***4.6. Effect of HPTE on cell viability of cortical neurons exposed to excitotoxic concentration of glutamate***

It is well known that excitotoxicity mediated by glutamate receptors is implicated in many neurodegenerative processes [38]. Indeed, the vast majority of the excitatory neurotransmission in the central nervous system is mediated by vesicular release of glutamate, which activates both pre and post synaptic G-protein-coupled metabotropic glutamate receptors and ionotropic glutamate receptors [39]. Glutamate receptor activation results in an influx of  $\text{Ca}^{2+}$  that acts as a second messenger in the regulation of neurotransmitter release, synaptic plasticity and gene expression. This change in the  $[\text{Ca}^{2+}]_i$  stimulates a number of intracellular events and it can also trigger the cell death process [18].

Accordingly, we stimulated neurons with a cytotoxic concentration of glutamate in the absence or the presence of 0.01 mg/ml HPTE for 24 hours. As shown in **Fig.6**, when we treated neurons with glutamate, HPTE was not able to protect cells from calcium-induced cell death. This result suggests that the bioactive molecules could be not able to protect neurons from death caused by glutamate-mediated excitotoxicity or could act through a mechanism that involves only a precise molecular target.



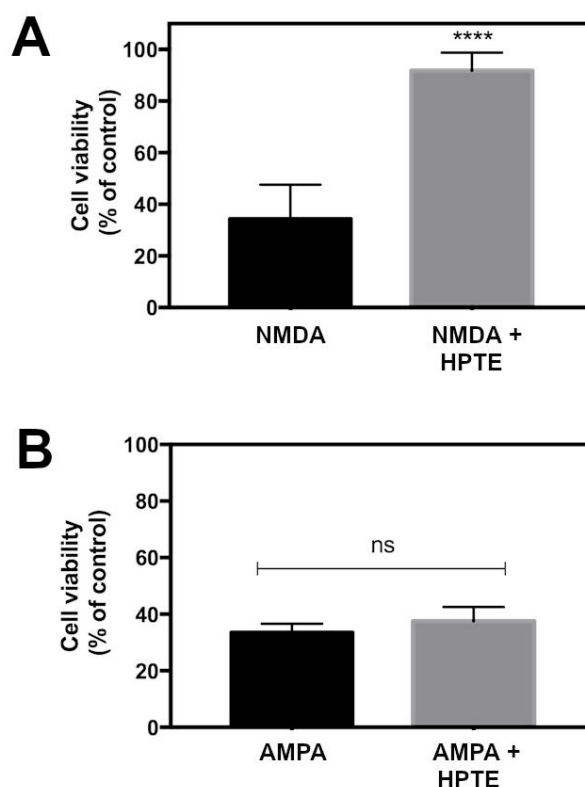
**FIGURE 6: Cell viability of cortical neurons exposed to glutamate and HPTE.** After 14 DIV, neurons ( $2 \times 10^4$ ) were treated for 24 hours with 300  $\mu$ M glutamate in the presence ("GLUT+HPTE") or in the absence ("GLUT") of 0.01 mg/mL HPTE. Cells viability was evaluated by NRU assay. Values represent means  $\pm$  SEM from seven independent experiments in triplicate. Statistical analysis was performed with unpaired t test. ns, not statistically significant.

#### ***4.7. Stimulation of cortical neurons with excitotoxic concentration of NMDAR and AMPAR agonists***

To better explain the failure of the bioactive molecule(s) in protecting neurons viability in condition of glutamate-mediated excitotoxicity, we decided to stimulate neurons in a more selective way by using the agonists of the two major expressed ionotropic glutamate receptors: NMDA, a selective agonist of NMDAR, and AMPA ( $\alpha$ -amino-3-hydroxy-5-methylisoxazole-4-propionic acid), a selective agonist of AMPAR. In this way, we excluded the metabotropic receptors from the stimulation and selected two alternative and specific targets able to promote the cell  $\text{Ca}^{2+}$  influx.

First, we stimulated cortical neurons with 300  $\mu$ M NMDA for 24 hours in the absence or in the presence of 0.01 mg/ml of HPTE. As shown in **Fig. 7A**, the results obtained demonstrate that, when neurons were selectively stimulated with NMDA, the bioactive molecule(s) contained in HPTE significantly attenuated the calcium influx through the receptor, avoiding the alteration of the intracellular calcium homeostasis and preventing calcium-induced neuronal death.

This result suggests that the bioactive molecule(s) operate through a molecular mechanism that involves NMDAR, one of the ionotropic glutamate receptors, as a possible specific target.



**FIGURE 7: Cell viability of cortical neurons exposed to NMDA or AMPA, and HPTE.** After 14 DIV, neurons ( $2 \times 10^4$ ) were treated for 24 hours with 300  $\mu$ M NMDA (A) or AMPA (B) in the absence or in the presence of 0.01 mg/mL HPTE. Cells viability was evaluated by NRU assay. Values represent means  $\pm$  SEM from fifteen independent experiments in triplicate. Statistical analysis was performed with unpaired t test. \*\*\*\*  $p < 0.0001$ ; ns, not statistically significant.

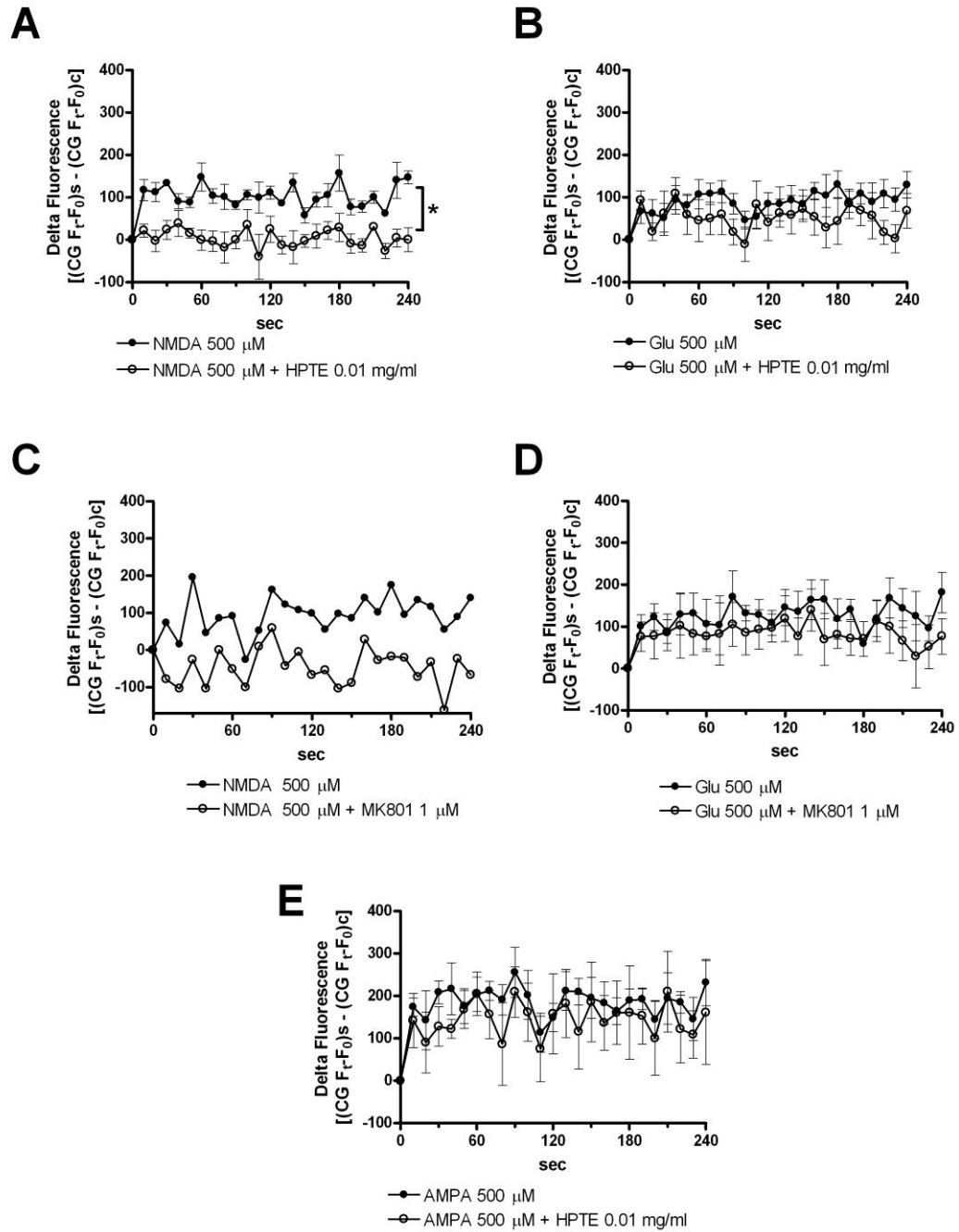
Furthermore, when we stimulated neurons in the same conditions but using AMPA instead of NMDA, as shown in **Fig.7B**, the bioactive molecule(s) were not able to prevent cells death.

Altogether these results demonstrate that the protective activity on neurons viability exerted by the bioactive molecule(s) contained in HPTE is related to the specific action on the NMDAR-mediated excitotoxicity. Indeed, the treatment with AMPA receptor agonist confirmed that when the ion influx occurs through a different ionotropic receptor, the protective activity was not detectable.

#### ***4.8. HPTE preserves the intracellular calcium homeostasis following NMDA receptor over-stimulation***

To confirm the specific molecular target of the bioactive molecule(s), we measured the intracellular calcium concentration variations by means of CG fluorophore following cells exposure to 500  $\mu$ M glutamate, NMDA, and AMPA. The concentrations of the stimuli in these experiments were higher than in the cell viability assay because the experiments were performed for 20 minutes instead of 24 hours. Moreover, in this experiments we used also MK801, a selective non-competitive antagonist of NMDA receptor, that completely inhibits the receptor opening.

As shown in **Fig.8**, the stimulation of glutamate receptors with the indicated agonists resulted in an increase in  $[Ca^{2+}]_i$ , detectable as delta fluorescence. Interestingly, HPTE was able to significantly reduce the delta fluorescence only during NMDA receptor stimulation, being ineffective on cells stimulated with either glutamate or AMPA. These results confirm that the bioactive molecule(s) could act specifically on the calcium influx through NMDA receptor and not on the influx mediated by AMPAR or by the other glutamate receptors. Furthermore, the fact that also MK801 failed to block the calcium influx promoted by glutamate stimulation, demonstrate that NMDAR is not the only responsible of the glutamate-mediated neurons death (see **Fig.6**).

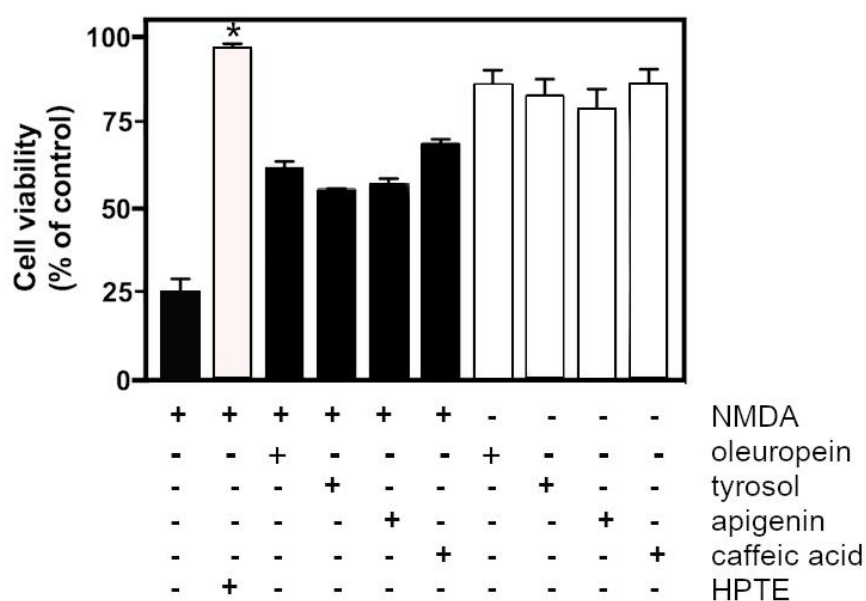


**FIGURE 8: Evaluation of  $[Ca^{2+}]_i$  increase in neurons exposed to excitotoxic stimuli.** CG-loaded neurons were treated with the indicated stimuli in the absence or in the presence of HPTE. Data are means  $\pm$  SEM from six independent experiments in triplicate, except for NMDA and MK801 (a single experiment in triplicate). Statistical analysis was performed with unpaired t test, analysing each fluorescence value recorded in the presence of the agonist with its corresponding one recorded in the presence of the agonist and HPTE (except for panel C). \*  $p < 0.05$

#### 4.9. Evaluation of different olive oil polyphenols on cortical neurons cell viability in condition of calcium-mediated excitotoxicity

Since we knew that some polyphenols could have a role in the regulation of cell calcium concentration and that excitotoxicity is mediated by an aberrant calcium influx through glutamate receptors, we replicated the experiments on neurons by using single polyphenols, that have been proven to be particularly concentrated in HPTE [32].

Hence, we treated neurons for 24 hours with an excitotoxic concentration of NMDA in the absence or in the presence of oleuropein, tyrosol, apigenin, caffeic acid, or HPTE, as a positive control followed by the assay of cell viability. As shown in **Fig.9**, on neurons stimulated with excitotoxic amounts of NMDA, HPTE demonstrated a protective activity significantly higher than the olive oil polyphenols did.



**FIGURE 9: Cell viability of cortical neurons exposed to NMDA and olive oil polyphenols.** Neurons ( $2 \times 10^4$ ) were incubated with the indicated stimuli for 24 hours. NMDA was used  $300 \mu\text{M}$ ; polyphenols and HPTE were used  $0.01 \text{ mg/ml}$ . Values represent  $\pm$  SEM from three independent experiments in triplicate. Statistical analysis was performed with ANOVA followed by Tukey's post-hoc test. \*  $p < 0.05$  for NMDA+HPTE vs NMDA+polyphenols.

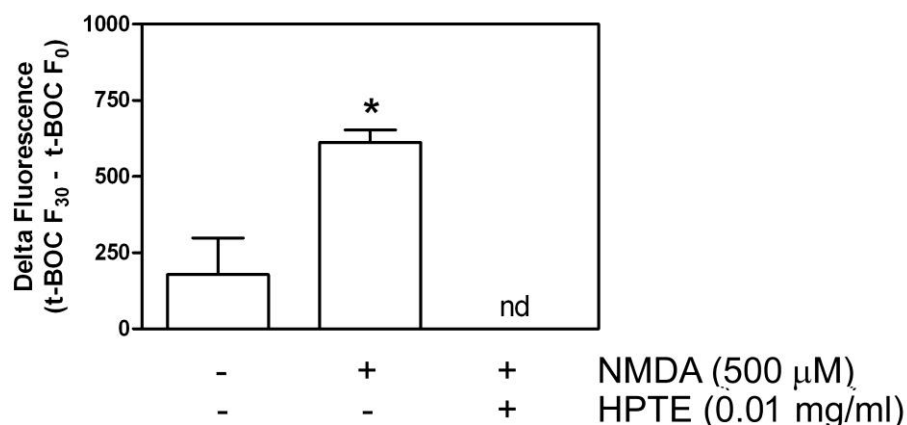
These results suggest that bioactive molecule(s) responsible for the protective activity in HPTE could belong to a different class of compounds or could be modified derivatives that act in a synergistic way.

#### ***4.10. HPTE prevent the aberrant proteolytic activity of calpain-1***

Controlled changes in intracellular calcium concentration are linked to cellular functions such as cell proliferation, motility, secretion, and expression of specific genes, but prolonged and abnormal alterations of this parameter have been related to a number of acute and chronic disease [40]. Since the bioactive molecule(s) were able to counteract neuronal death promoted by an intracellular calcium overloading, we investigated if the protective effect could also affect the proteolytic activity of the calcium-dependent protease calpain-1.

To this purpose, we loaded neurons with a fluorescent substrate of calpain-1 and detected the intracellular proteolytic activity of the enzyme in different conditions as shown in **Fig.10**. Particularly, we detected a basal proteolytic activity of the enzyme in untreated cells, whereas in condition of excitotoxicity, the increasing in intracellular calcium concentration due to the receptor opening resulted in a significant rise in this protease activity. Furthermore, when we treated neurons with NMDA in the presence of HPTE, the regulation of the calcium influx exerted by the bioactive molecules resulted in the maintenance of the intracellular calcium homeostasis and, as a consequence, the aberrant activation of calpain-1 was prevented.

These results provide further evidence that these molecules are endowed with the ability to maintain the intracellular calcium homeostasis protecting neurons from different calcium-induced adverse effects, including the aberrant calpain activation.



**FIGURE 10: Evaluation of calpain-1 activity in neurons exposed to HPTE and/or NMDA.** t-BOC-loaded neurons were treated with the indicated stimuli. Fluorescence was monitored as specified in Materials and Methods. Delta fluorescence was calculated subtracting the fluorescence values recorded at time zero from those recorded at 30 minutes. Data are means  $\pm$  SEM from three independent experiments in triplicate. Statistical analysis was performed with ANOVA followed by Tukey's post-hoc test. \*  $p < 0.05$ .

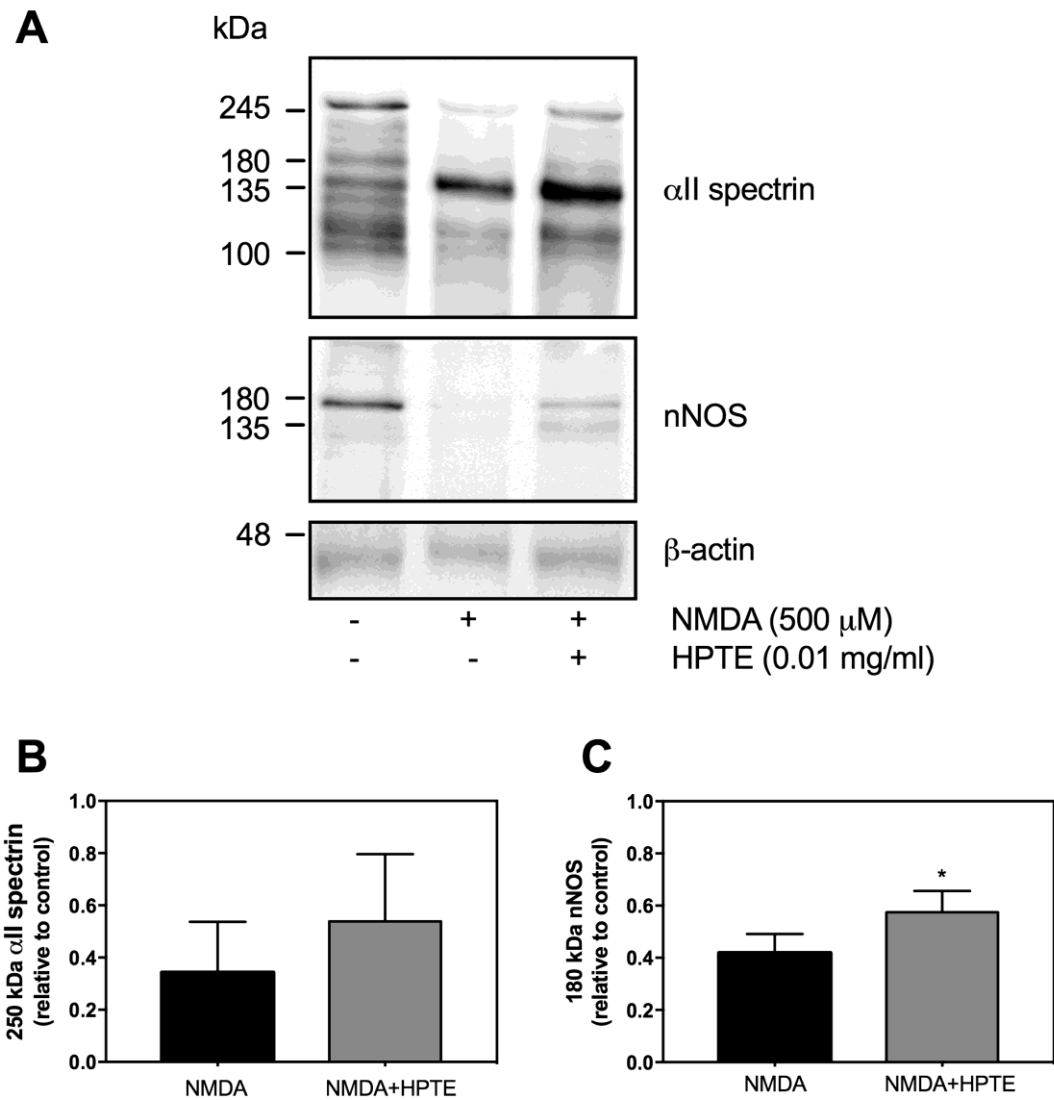
#### ***4.11. Protection of calpain-1 substrates from uncontrolled degradation following over-stimulation of NMDAR***

To further confirm that the aberrant proteolytic activation of the calcium-dependent protease calpain-1 was prevented, we analysed two characteristic substrates of the enzyme:  $\alpha$ II spectrin and nNOS.

$\alpha$ II spectrin is one of the best known and studied substrate of calpain-1 correlated to neurodegenerative processes [41] [31], whereas nNOS is related to cell damages associated to the abnormal production of NO due to the alteration of calcium homeostasis and subsequent abnormal calcium-dependent proteolysis. Indeed, unneeded activation of calpain, the protease of the  $\text{Ca}^{2+}$ -dependent proteolytic system, and NO synthase are considered the triggering events leading to cell death typical of several human neurodegenerative disorders [42].

Hence, we analysed the protein levels of these two calpain-1 substrates in our experimental conditions. As reported in **Fig.11**, treatment of neurons with NMDA for 4

hours in the presence of 0.01 mg/ml HPTE resulted in a preservation of both calpain-1 substrates: nNOS and  $\alpha$ II spectrin.



**FIGURE 11: Murine cortical neurons treated for 4 hours with NMDA and/or HPTE.** A) Neurons ( $3 \times 10^5$ ) were submitted to immunoblotting following the procedure described in the section materials and methods. Antibodies anti  $\alpha$ II spectrin and anti nNOS were diluted 1:1000 and incubated overnight at 4 °C. For each panel a representative blot of two ( $\alpha$ II spectrin) or three (nNOS) is shown. B) and C) Immunoreactive signals were quantified as described in the section materials and methods. All the values reported were normalized vs.  $\beta$ -actin signal, and reported as amount relative to control (untreated cells). Data are means  $\pm$  SEM from two ( $\alpha$ II spectrin) or three (nNOS) independent experiments. \*  $p < 0.05$ , according to paired t test.

The protection of the native form of  $\alpha$ II spectrin in condition of altered  $[Ca^{2+}]_i$  represents an evidence that the protective activity of the bioactive molecule(s) occurs through a mechanism that maintains the calcium homeostasis preventing the aberrant activation of the calpain-1 calcium-dependent protease.

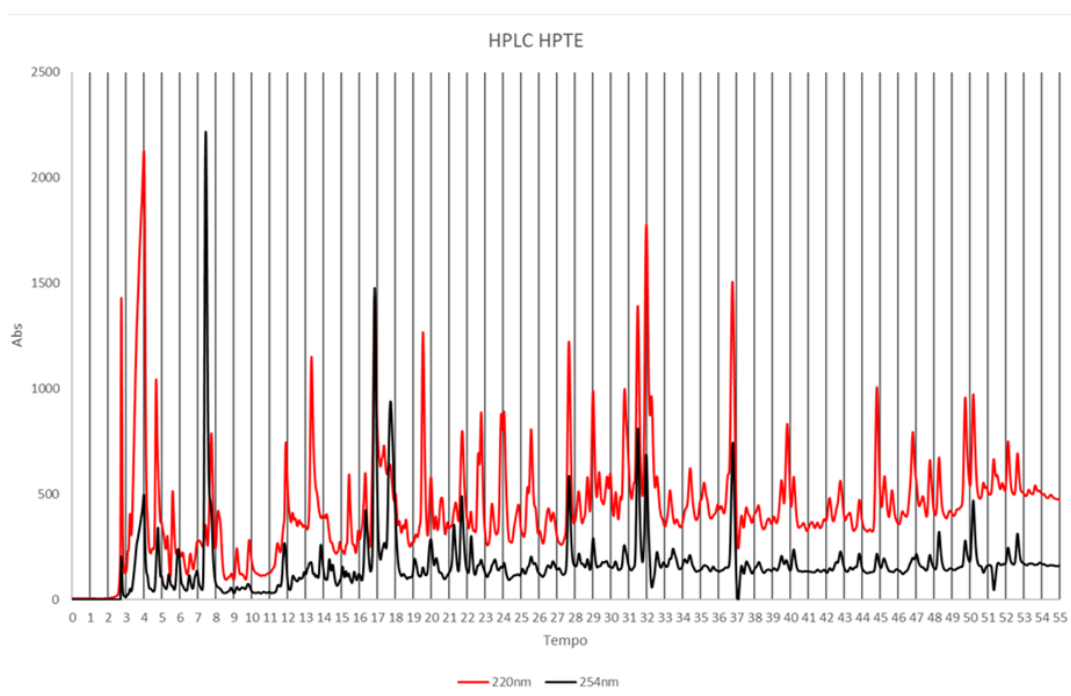
Furthermore, we have previously demonstrated that the conversion of the native form of nNOS into the activated form of 130 kDa is a calcium-dependent event mediated by the proteolytic activity of calpain-1 that allows the modulation of NO production [43]. Accordingly, in neurons treated with excitotoxic concentration of NMDA in the presence of HPTE, we observed the partial preservation of the native form of nNOS and the accumulation of the activated 130 kDa nNOS form. These data suggest that the proteolytic activity of the calcium-dependent protease calpain-1 occurs in a regulated way preventing its aberrant activation.

Taken together these results highlight once again that HPTE was able to protect neurons from calcium triggered harmful events.

#### ***4.12. Structural characterization of the bioactive molecules contained in olive pomace extract***

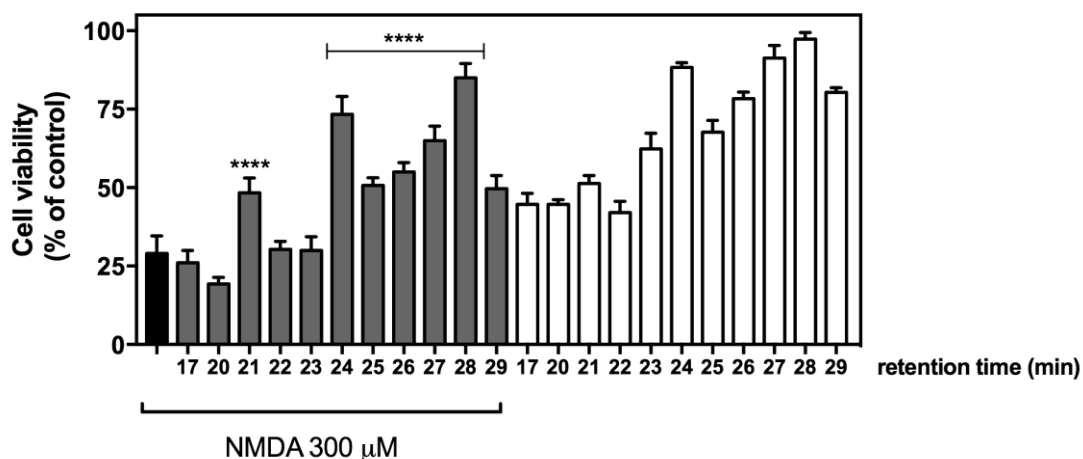
To identify the molecular nature and structure of the bioactive molecule(s) contained in HPTE, we performed several analysis by using High Performance Liquid Chromatography (HPLC) and mass spectrometry techniques.

We fractionated HPTE using HPLC as shown in **Fig.12** and we tested the biological protective activity of the fractions selected from the range of interest (17<sup>th</sup> minute and from 20<sup>th</sup> to 29<sup>th</sup> minutes), as we previously established.



**FIGURE 12: Chromathogram of HPTE fractionation.** We performed HPLC analysis on HPTE as we described in the section material and methods. In this figure, red line is referred to absorbance at 220 nm and black line at 254 nm. Vertical black lines are referred to the single fractions collected every 60 seconds.

The results presented in **Fig.13**, indicate that the active fractions were the one eluted at the 21<sup>st</sup> minute and those in the range between 24<sup>th</sup> and 28<sup>th</sup> minutes. Successively, we analysed these active fractions using HPLC MS-MS technique.



**FIGURE 13: Murine cortical neurons viability exposed to NMDA and/or chromatographic fractions of HPTE.** After 14 DIV, neurons ( $2 \times 10^4$ ) were incubated for 24 hours in presence of different chromatographic fractions of HPTE (corresponding to the indicated retention time) tested at 0.5 mg/ml in the presence (grey bars) or in the absence (white bars) of 300  $\mu$ M NMDA. Cells viability was evaluated by NRU assay. Values represent means  $\pm$  SEM from five independent experiments in triplicate. Statistical analysis was performed with ANOVA follow by Tukey's post hoc test. \*\*\*\*  $p < 0.0001$  vs NMDA (black bar).

In spite of accurate bibliographic and database library research, we have not yet succeeded in elucidating the precise molecular structure of these bioactive molecules. However, from the study of the fragmentation spectra, we observed that some of the molecules identified have a molecular skeleton resembling the one of other molecules found in olive pomace extract. Particularly, from the fragmentographic analysis of two signal at  $m/z$  407 and  $m/z$  529 we obtained fragmentation spectra compatible with two possible molecules: a derivative of *Loganin* (**Fig.14**) or of *Proanthocyanidins* (**Fig.15**), specifically dimers/trimers of Catechin/Epicatechin and/or Gallocatechin/Epigallocatechin.

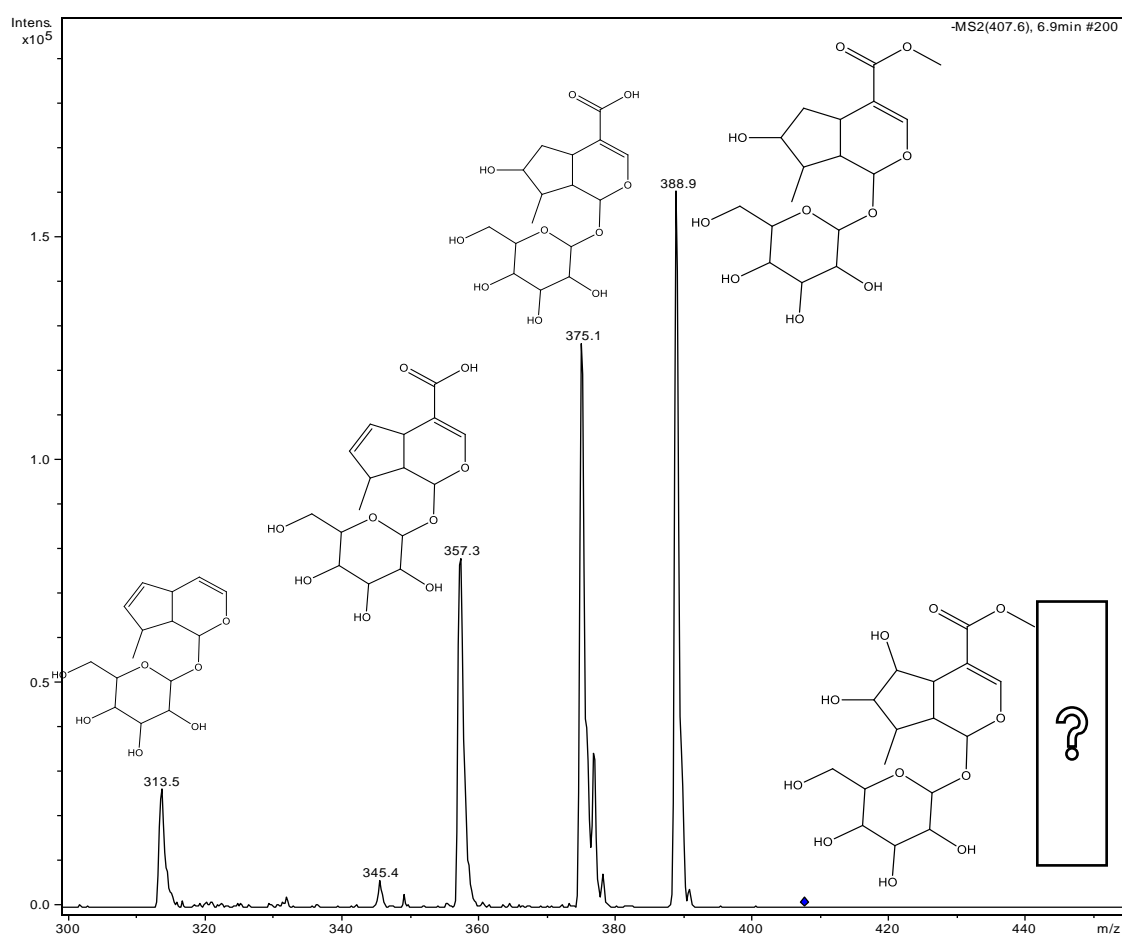


FIGURE 14: Fragmentation spectra of m/z 407 ion, possible derivative of *Loganin* molecule.

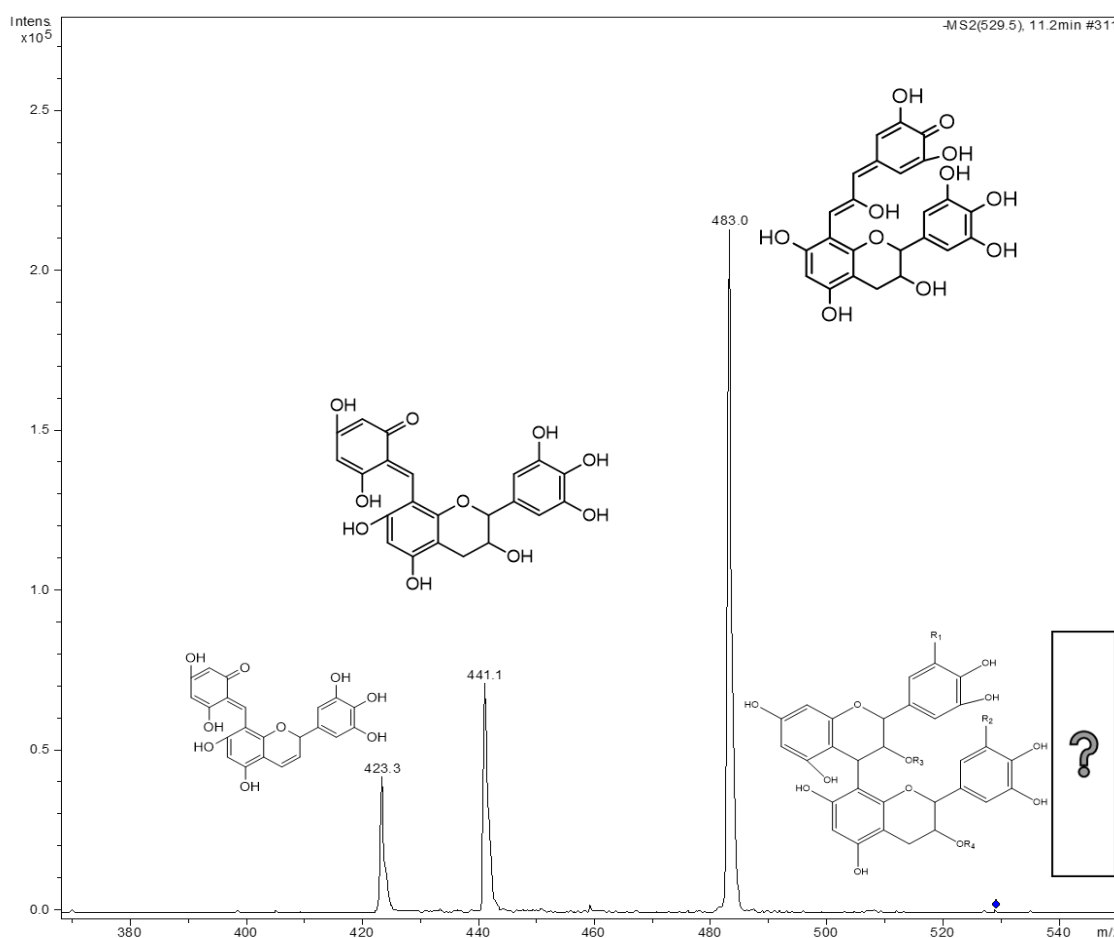
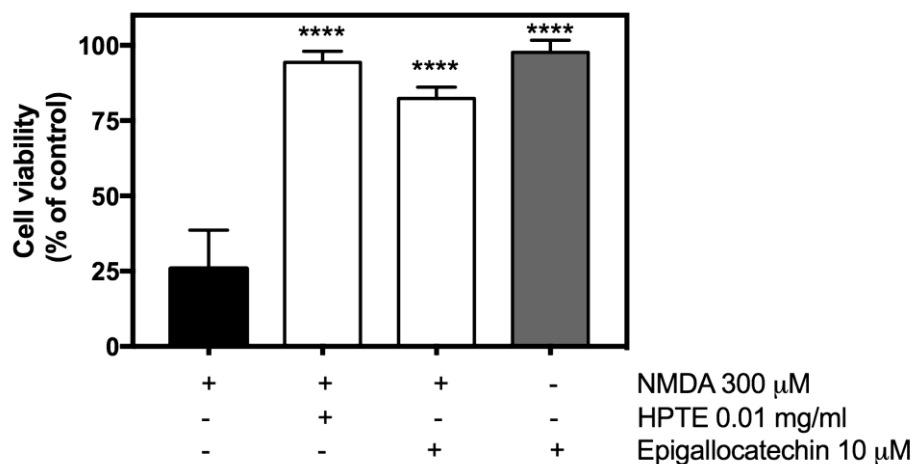


FIGURE 15: Fragmentation spectra of m/z 529 ion, possible derivative of *Proanthocyanidins*

#### 4.13. Neurons viability in condition of NMDAR-mediated excitotoxicity in presence of epigallocatechin molecule

On the basis of the data obtained using mass spectrometry techniques, we performed different experiments to evaluate neurons viability in condition of NMDAR-mediated excitotoxicity in the presence or in the absence of epigallocatechin. This compound is a member of the family of *proanthocyanidins* identified as a possible constituents of the bioactive molecule(s) contained in HPTE extract.

The results shown in **Fig.16** indicate that epigallocatechin was able to protect neurons from death induced by calcium-mediated excitotoxicity. However, we observed a trend of a protective activity less effective than the one exerted by the HPTE.



**FIGURE 16: Cell viability of cortical neurons exposed to NMDA and epigallocatechin.** After 14 DIV, neurons ( $2 \times 10^4$ ) were incubated with the indicated stimuli for 24 hours. Values represent  $\pm$  SEM from six independent experiments in triplicate. Statistical analysis was performed with ANOVA followed by Tukey's post-hoc test. \*\*\*\* $p < 0.0001$  for "NMDA+HPTE"; "NMDA+epigallocatechin" and "Epigallocatechin" vs NMDA.

This data suggests that *proanthocyanidins* could be the right family of compounds to study for the identification of the bioactive molecule(s) responsible of the effect of HPTE.

## 5. DISCUSSIONS

In this study we found that bioactive molecules extracted from olive pomace by means of a very reproducible high pressure and temperature extraction method prevent the death of murine cortical neurons induced by the intracellular  $\text{Ca}^{2+}$  overloading triggered following cell stimulation with NMDA. However, HPTE failed to protect the viability of neurons treated with toxic concentrations of glutamate or AMPA.

In the extract, despite the high extraction temperature (180 °C) no traces of 5-HMF were detected by HPLC-MS analysis. The absence of this furanic compound, formed during Maillard reaction, could indicate that no degradation reactions occurred during the extraction process [44]. This could be due to the presence of high ethanol concentrations (50% v/v) and to the absence of oxygen in the reaction vessel.

Detailed investigation of the effect of HPTE treatment showed that it limited the increase in intracellular  $\text{Ca}^{2+}$  as well as the over-activation of calpain occurring in NMDA-stimulated neuron cells. Altogether, these results demonstrate that HPTE selectively reduces the cell  $\text{Ca}^{2+}$  influx mediated by NMDAR activation on cells exposed to cytotoxic concentrations of the glutamate analogue NMDA, resulting in a significant protection of neuronal viability.

Since calpains undergo activation following calcium dependent cell injury, and the uncontrolled activation of calpains is clearly lethal to neurons as well as to other cell types [45] [46] [47], we can hypothesize that the preservation of neuronal viability by cell treatment with HPTE could depend by a reduced activation of calpain. In this context, our results provide evidence that the levels of two natural intracellular calpain substrates, nNOS and  $\alpha$ II-spectrin, both dramatically decreased in NMDA-treated neuron cells, are partially preserved when the cells are stimulated in the presence of HPTE. These data confirm that HPTE prevents an aberrant activation of calpain induced by cytotoxic NMDA amounts. Of interest, the proteolytic degradation of both calpain substrates is directly related with neuronal cell death [48] [49]. This observation suggests that, by evaluating the occurrence of a specific pattern of degradation of these calpain targets, it could be possible to find a relationship between calpain activity and neuronal cell death. Particularly, when we treated neurons with HPTE we could observe not only a recovery of the native nNOS, but also a protection of the active 130 kD form produced by

calpain against further degradation. This limited proteolytic processing and activation of nNOS has been proposed previously to represent a neuroprotective mechanism that prevents overproduction of NO [43]. Hence, we hypothesize that one of the molecular events involved in the preservation of the neurons viability operated by HPTE consists in the regulation of the extent of calpain activation to ensure the accumulation of the 130 kD form of nNOS. However, further studies are needed for complete insight into the protective pathways triggered by the biomolecules contained in HPTE able to prevent the onset of excitotoxicity promoted by NMDA. Although our results suggest that these bioactive olive pomace-derived molecules act directly on NMDA receptor, additional investigations are in progress to characterize this interaction. Moreover, the issue we give the highest priority is to identify the molecular nature of the relevant bioactive compounds present in HPTE. Our analyses identified two possible molecules: one could be a derivative of *Loganin* and the other one of *Proanthocyanidins* (specifically dimers/trimers of Catechin/Epicatechin and/or Gallocatechin/Epigallocatechin). Since it is well known that derivative molecules of *Proanthocyanidins* play neuroprotective effects against glutamate-induced excitotoxicity [50] [18], we tested the potential effect of epigallocatechin on neuronal cell viability in conditions of NMDAR over-activation. Our preliminary results indicate that this monomer promotes a protective effect on neurons similar to that exerted by HPTE. Hence, we hypothesize that the molecules contained in HPTE could be derivative of *Proanthocyanidins*. However, we have planned further experiments, carried out by using dimers/trimers of *Proanthocyanidins*, to establish if these compounds actually correspond with the bioactive molecules characterized in our study.

The alteration in  $[Ca^{2+}]_i$  and the pathological activation of calpain are considered among the most important neurodegenerative factors [51] [52]. Considering the widespread and devastating effects that derive from excitotoxicity, at present the development of effective and reliable approaches to prevent intracellular calcium overloading and the consequent aberrant calpain activation represents a major challenge to counteract neurodegenerative diseases. In our opinion, the identification in HPTE of bioactive molecules endowed with neuroprotective activity on cultures of primary neurons, represents a promising starting point for new prophylactic applications and therapeutic interventions in these diseases. To obtain a comprehensive information about the molecular mechanisms underlying this cell protection from cytotoxicity

mediated exclusively by the NMDA glutamate receptor, we are now investigating about the physico-chemical, structural and biochemical properties of the relevant molecules contained in HPTE, including the evaluation of possible side effects dependent on their overdose.

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