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Identification of naturally derived compounds for reducing excitotoxicity.

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Introduction

Excitotoxicity is defined as the injury and death of neurons, due to excessive or prolonged exposure to excitatory amino acids. Glutamate is the most important amino acid acting to mediate excitatory neurotransmission and its signaling is partially stopped by re-uptake into presynaptic terminals of neurons. Glial cells play a predominant role in scavenging free glutamate via high-affinity glutamate transporters (Verma et al; 2022). Glutamate is transformed into glutamine within glial cells, before being released for neuronal uptake, as initial component to replace both excitatory (glutamate) and inhibitory (GABA) neurotransmitters. All subcellular compartments are affected by the excitotoxic process, with changes in the cytosol, mitochondria, endoplasmic reticulum (ER), and nucleus. Excitotoxicity has been shown to play a pivotal role in neurodegenerative diseases, in fact, is well-demonstrated to be linked to chronic neurodegenerative disorders including epilepsy, Alzheimer's dementia, amyotrophic lateral sclerosis, multiple sclerosis, Parkinson's disease, and others (Binvignat et al; 2020). After a stroke, a traumatic brain injury or in neurodegenerative disorders, the consequent oxidative stress provided, induce cells to be more sensitive also to the normal level of glutamate. The binding of glutamate to α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors and kainate receptors (KAR), all glutamate receptors, during excitotoxic stimulus, induce a massive influx of Na+ ions. The consequence is membrane depolarization and opening of voltage-dependent Ca^{2+} channels (VDCC). AMPA receptors are also permeable to Ca^{2+} and glutamate can also bind, under depolarizing condition, to N-methyl-d-aspartate receptors (NMDAR) inducing an additional influx of Ca^{2+}. Metabotropic glutamate receptors (MetR) are also activated; inducing production of inositol trisphosphate (IP_3) that activates IP_3 and ryanodine receptor in endoplasmatic reticulum (ER) resulting in the release of Ca^{2+} into cytoplasm. The excessive level of calcium in the cells, inhibits the ATP synthase, stopping the production of ATP and has, as a consequence, the destruction of the electrochemical gradients of neurons and the alteration of metabolic enzymes dependent on ATP production (Stavrovskaya et al.; 2005). In excitotoxicity, this ATP depletion and consequent reduction of glutamate transporter activity, resulting in further elevation of extracellular glutamate, causes a massive influx of sodium into neurons via NMDARs. It is still a reversible process, but the consequential increase of calcium, leads to irreversible excitotoxic injury; because Ca^{2+} influx into cells activates a series of enzymes; endonuclease, phospholipases and nucleases that damage cellular structures

(Choi; 2020). In neurons, Ca^{2+} damages dendrites and induces cell death, by activating cysteine proteases called calpains that degrade a variety of substrates, including cytoskeletal proteins, membrane receptors, and metabolic enzymes (Mattson; 2019). Other mechanisms are involved in an increase of the level of cytosolic calcium, contributing to neuronal cell death; one of this is depending on function of mitochondria. They are implicated in calcium dysregulation, downstream of synaptic hyperactivity because they provide rapid, post-stimulatory calcium recovery by taking up massive amounts of calcium and then releasing calcium more gradually back into the cytosol. When the level of calcium is elevated, these organelles absorb high levels of calcium, inducing mitochondria to swell and release reactive oxygen species (ROS) that can activate apoptosis. The most important proteic complex, involved in mitochondrial calcium uptake, is the mitochondrial calcium uniporter (MCU). It ensures a rapid uptake of calcium into mitochondria and it is controlled by other accessory proteins like mitochondrial calcium uptake 1 (MICU1) or MICU3 isoform, expressed specifically in brain (Ashrafi et al.; 2020). Mitochondrial processes are controlled, in turn, by the level of calcium, in order to prevent neural toxicity and transportation of calcium. For example, the knockout of Liver kinase B1 (LKB1) involved in mitochondrial buffering, in neurons leads to neuron hyperexcitability reducing axonal branching (Marland et al.; 2013). Oxidative stress is one of the most important consequences induced by glutamate neurotoxicity. Nevertheless, there is no possibility to define an unidirectional cause/effect relationship between the two phenomena. Oxidative stress and consequent intracellular ROS production may also induce excitotoxicity, by stimulating extracellular glutamate release and releasing calcium from mitochondria into the cytosol (Armada-moreira. et al.; 2020). The intracellular mechanisms responsible for neuronal death due to excitotoxicity, despite the intense research activity, is not completely elucidated. The main obstacles are the heterogeneity of neurodegenerative diseases, because no single pathway has emerged as dominant; but also, the heterogeneity of neurons, that are affected and go into apoptosis during glutamate excitotoxicity, makes more difficult to understand the phenomenon (Lau et al.;2010). Excitotoxicity also occurs after a brain injury, in fact damaged neurons, in response to the traumatic stimulus increase the release of additional glutamate (Hulsebosch et al.; 2009). Among neurodegenerative disorders, the disease that relies upon excitotoxicity as pathological component, is epilepsy. In fact, the “glutamate hypothesis” explains that an excessive release of glutamate, causes over excitation of several classes of ionophore-linked postsynaptic receptors including NMDA, AMPA and

kainate receptors. All these receptors, are linked to the development of epileptic seizures. The exact cause of all forms of epilepsy is unknown, but it is thought, that the main feature underlying epilepsy, is an imbalance between excitation and inhibition resulting in epileptogenic networks. In the healthy brain, there is a balance between excitatory and inhibitory neurotransmitters. Excitatory neurotransmitters, such as glutamate, promote the firing of neurons, while inhibitory neurotransmitters, such as GABA, inhibit the firing of neurons. In epilepsy, this balance is disrupted, leading to an increase in excitatory activity and a decrease in inhibitory activity. Excessive levels of glutamate during epilepsy, at synapses, lead to the generation of oxidative stress which further leads to mitochondrial dysfunction, lipid peroxidation and oxidation of proteins and DNA. A consequence of this process is neuronal cell death due to excessive oxidative stress (Khatri et al.; 2018). Oxidative stress can be defined as a disruption of the balance between oxidants and antioxidants in the organism. It is a state of excess of pro-oxidant species, primarily reactive oxygen species (ROS) and reactive nitrogen species (NOS), compared to the antioxidant defenses (enzymatic and non-enzymatic) in a living cell or an organism. ROS are also known as important down-stream effectors of the increased intracellular level of calcium. Many studies, in fact, indicated the pathogenic role of calcium dysregulation also in epilepsy, but the precise process and role remain unclear. This over excitation and consequent calcium influx, results in stimulation of nitric oxide (NO) synthetase activity, with formation of NO molecules that interacts with super oxide to form peroxynitrite anions; able to damage proteins and nucleic acids. Other enzymes are deregulated during excitotoxicity and epilepsy; superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (Vishnoi et al.; 2016). Additionally, oxidative stress can lead to inflammation, which can further damage neurons. Precisely ROS are, also, known as pro-inflammatory mediators, which can activate microglia and cause chronic neuroinflammation, resulting in the onset of neurodegenerative diseases. In some neurodegenerative diseases, as Alzheimer's (AD), Parkinson's (PD), and epilepsy, inflammatory response is involved in neuronal death and brain tissue damage. Neuroinflammation is the most important player in the pathophysiology of epilepsy, due to neuroglial activation and cytokine production that induce an increase of seizure and consequentially neurotoxicity. Astrocyes and microglia under inflammatory stimuli secrete high levels of interleukin-1 β (IL-1 β) and tumor necrosis factor-α (TNF-α), that damage neurons and originate an inflammatory cascade; increasing the probability of seizure production (Ambrogini et al.; 2019). A further confirmation, of how

neuroinflammation is an important consequence of excitotoxicity in neurodegenerative diseases, is that people with epilepsy often have higher levels of pro-inflammatory cytokines in their blood than people without epilepsy. So, it is well confirmed that neuroinflammation and epilepsy are functionally interconnected and reinforce each other. Inflammation can damage neurons and leads to epilepsy, for example, a head injury can cause inflammation in the brain, which can lead to the development of epilepsy, but inflammation can also occur after a seizure. In our study, we focused our attention on a model of excitotoxicity that is widely used to mimic and study epilepsy. Epilepsy is one of the most common brain condition, that affects over 70 million people worldwide and has numerous neurobiological, cognitive, and psychosocial consequences. High mortality in people with epilepsy, poses a great public health problem, as some deaths are preventable. The features of epilepsy are an initial brain damage with hippocampal cell loss, followed by collateral axonal sprouting and a reorganization of synaptic circuitry (Thijs et al.; 2019). Current antiepileptic drugs mainly provide symptomatic relief from seizures, have multiple adverse effects, and fail to control seizures in up to 30% of people. Epileptogenesis, is a complex multifactorial process, including both the development of an epilepsy condition and its progression after its establishment. Research on epilepsy and other neurodegenerative diseases, in order to find good in-vitro and in-vivo models, are essential for elucidating the molecular and cellular processes involved in the disorders of the nervous system. Among *in vitro* models of excitotoxicity, we chose one of the most reproducible, achieved by the use of a strong oxidative stressor. It is obtained using Kainic acid (KA), a cyclic analog of L-glutamate and an agonist of the ionotropic KA receptors (KARs), on SH-SY5Y. It is a largely immortalized cell line, used in in vitro models of neurological disorders and allows to explore the pathophysiological mechanisms of neurodegenerative diseases. SH-SY5Y cells are human-derived, express several human-specific proteins and isoforms that are not present in primary rodent cell cultures. This cell line is useful for an efficient screening of drugs for treating the neurodegenerative diseases (Hoffman et al.; 2023). To better understand the mechanisms behind excitotoxicity and development of neurodegenerative diseases, also microglia activation needs to be investigated during excitotoxicity, thus we reproduced the same model of stimulation through KA on human microglia clone 3 (HMC3) cells. The mechanisms that control excitotoxicity and epileptogenesis are complex and need to be understand through in vivo models. Administration of KA in rats is one of the most used because it mimics the temporal lobe epilepsy (TLE), the most common type of epilepsy in adult. Moreover,

this type of epilepsy is often associated with treatment resistance, where approximately 30% of patients taking antiepileptic drugs present drug resistance that prevents them from living seizure-free (Peterson et al.; 2019). This model represents a validated animal model to study the onset and development of chronic epilepsy (De Bertoglio; 2017). One of the main features of this disease is the damage of the hippocampal pyramidal neurons, in fact, the histologic findings revealed neuronal degeneration and gliosis in the CA3 field of the hippocampus. KA administration will target all types of kainate receptors, with a unique effect in the hippocampus. The molecular mechanism includes a cascade of molecular interactions, that lead to osmotic imbalance, excessive depolarization and rupture of the postsynaptic membrane. The common aspect, is always the intracellular accumulation of Ca^{2+}, following the excessive activation of glutamate receptors. Following the systemic KA administration in rats, there is a latent seizure-free period and then a development of chronic epilepsy with abnormal and prolonged seizures. Seizures start after one hour from the administration and last for 5-6 hours. In fact, this model has long-term consequences such as neuronal death and injury and modification of neuronal networks (Trinka et al., 2015). In adult rats, a single dose of KA induces different behavioral changes according to Racine scale; and mortality in this model is relatively high about 22% (Racine; 1972). Extensive neurodegeneration in the hippocampus, mossy fiber sprouting, and spontaneous recurrent seizures (SRSs) with the same frequency, makes this a reproducible model (Rusina et al.; 2021).

With these experimental setting, we want to also explore the effective relationship between oxidative stress and excitotoxicity, finding a possible treatment, based on reduction of oxidative stress. Moreover, there is a topic on which, attention of researchers is growing: autophagy and its role in neurodegenerative diseases. The mechanisms behind excitotoxicity remain incompletely understood, but surely, the excess of glutamate induces production of ROS, which leads to dysfunction of mitochondria and molecular damage, activates apoptotic factors, and results in both apoptotic and excitotoxic cell death (Rusina et al.; 2021). That said, let’s step back to apoptosis, a mechanism of cell death that can be induced by a wide range of stimuli. It can be triggered by both internal stressors, as well as extracellular stimuli, including autophagy (Fairlie et al.; 2020). Autophagy is a catabolic process that degrades substances via the actions of intracellular lysosomes, this process modulates cellular homeostasis, in fact, abnormal autophagy can lead to cellular damage. Many studies have shown that mitochondrial ROS are the

primary mediators of autophagy and can increase the production of autophagy-related proteins, functioning as a unique kind of signaling molecule. The accumulation of autophagy is a crucial part of the response to ROS, in fact, other studies reported as natural antioxidants such as Tanshinone IIA and Hyperoside significantly reduced the levels of intracellular ROS and consequently inhibited autophagy (decreased level of LC3I/II and Beclin-1), via the activation of the PI3K/Akt/mTOR pathway to protect cells. Another study, demonstrated that overexpression of mitochondrial autophagy induces neuronal death, but the use of antioxidants restored the protective effects of autophagy (Cao et al.; 2020, Fan et al.; 2021). Therefore, the balance between ROS and autophagy is an important mechanism of homeostasis in neurological disorders. So, accumulation of ROS resulted in autophagy in neurons, as previously demonstrated, enhancing antioxidant levels and reducing the levels of over-activated autophagy. This seems essential for neuron survival. LC3 and Beclin1, regulators of autophagy, can promote the development of spontaneous seizures in mouse brain (Gao et al., 2012). A previous study, confirmed that the status of the autophagy-associated proteins, revealed that autophagy was activated in rats with epilepsy, moreover autophagy was induced before neuronal death during epilepsy (Li et al.; 2018). Going more into detail, autophagy is a kind of intracellular degradation process that remove damaged proteins and organelles in different conditions. The main feature is the formation of autophagosomes, by membrane wrapping part of the cytoplasm and surround the organelles and proteins that need to be degraded. Autophagy has an important significance in physiological and pathological conditions, in particular aging, inflammation, malignant tumor and neurodegenerative diseases. There are important evidence that autophagy is activated under stress conditions, such as nutritional starvation or energy exhaustion. Autophagy is generally divided into three categories: *macroautophagy* by endoplasmic reticulum-derived membrane wrapping, *small autophagy* phagocytosis by lysosome, *chaperone-mediated autophagy* when proteins bind molecular chaperones and transported to lysosomes to be digested by lysosomal enzymes. Based on nutritional condition, autophagy is classified in non-selective autophagy, under starvation conditions, and selective autophagy in nutrient-rich conditions, such as mitochondrial autophagy (Cao et al.; 2021). The initiation of autophagy is a complex process that is regulated by a large number of factors including nutrient starvation, growth factors and stress. These signals, activate a number of proteins, including the ULK1 complex, involved in the early stages and its kinase activities are regulated by mTORC1 (target of rapamycin complex 1). ULK1 has been shown to have,

also, an autophagy-independent effect in ROS-induced damage, in fact, cells try to protect themselves from ROS-induced damage in different ways (Joshi A. et al.; 2016). However, ULK mechanisms may be important in the prevention of a number of diseases, that are caused by ROS, such as cancer and neurodegenerative disorders. In the formation of phagosome one of the most important proteins is Beclin-1, a scaffold protein that binds a number of other proteins involved in autophagy, including ULK1, ATG14, and ATG16L1. These proteins work together to form the phagophore, which is the initial autophagosomal precursor. It is well-recognized that Beclin-1, has a role, also, in inflammation and apoptosis. The formation of autophagosomes is a tightly regulated process, many proteins are involved in regulation of autophagy and AMBRA1 is a key factor. AMBRA1 binds Beclin 1 and combines with Vps34/PI3KC3 to form a class III PI3K complex. This complex further promotes autophagy. Bcl-2 is a central regulator of autophagy and apoptosis; Beclin-1 binds to Bcl-2 to regulate both apoptosis and autophagy. Stress induced by autophagy disrupt Bcl-2-Beclin-1 complex and triggers autophagy. AMBRA1 also binds to Bcl-2 in mitochondria to modulate Beclin1-dependent autophagy and apoptosis (Levine et al.; 2008). The second stage of autophagy is elongantion, once the phagophore is formed, it can be elongated by the recruitment of other membranes. LC3-I is essential for this step, in fact it is conjugated to phosphatidylethanolamine (PE) lipids, which enables membrane fusion events, leading to autophagosome biogenesis. The process of membrane fusion leads to the elongation of the phagophore, which eventually forms the mature autophagosome. LC3-I, in particular when conjugated to phosphatidylethanolamine lipids became LC3-II. This process is mediated by the ATG12-ATG5 complex. Once LC3-II is formed, it is recruited to the phagophore, which is the initial autophagosomal precursor membrane structure. LC3-II then helps to fuse the phagophore with other membranes, such as the cytoplasmic membrane or the endoplasmic reticulum. The amount of LC3-II in a cell can be used as a marker of autophagy. Increased levels of LC3-II indicate that autophagy is active, while decreased levels of LC3-II indicate that autophagy is not active (Singh et al.; 2019). The last phase is the maturation of the autophagosome, it involves the fusion of the phagophore with the lysosome and the degradation of the contents, by the lysosomal enzymes. The outer membrane of the autophagosome will then fuse with the lysosomal membrane to form an autolysosome. Beclin-1 also, helps to recruit other proteins to the phagophore, such as ATG5 and ATG12. These proteins are involved in the fusion of the phagophore with the lysosome, which completes the formation of the

autophagolysosome. This protein is also involved, not only, in the formation of autophagolysosome, but also in the process of material transport. In some instances, the autophagosome may fuse with an endosome, forming an amphisome, before fusing with the lysosome. This process is mediated by several proteins, including ATG16L1 and ATG17. The contents of the autolysosome are then degraded and exported back into the cytoplasm for reuse by the cell (Parzych et al.; 2014). A protein that regulates autophagy is, also, AMPK involved in the regulation of metabolism, cell growth, and cell death (Villanueva-Paz et al.; 2016). It activates autophagy by phosphorylating the ULK1 complex, which is a key regulator of autophagy initiation. AMPK also inhibits mTor, which is a protein kinase that inhibits autophagy. But mTor is activated, in turn, through phosporilation by the serine/threonine protein kinase p-Akt. SQSTM1/p62 is another protein involved in the regulation of autophagy, it is a ubiquitin binding protein, which means that it binds to ubiquitinated proteins. Activating autophagy, reduces the expression of p62, since p62 accumulates when autophagy is inhibited, and decreased levels can be observed when autophagy is induced; thus p62 may be used as a marker to study autophagic flux (Bjørkøy et al.; 2009). In conclusion, autophagy is involved in the normal metabolism of cells by eliminating intracellular waste, it is necessary for cell survival. The activation autophagy is indicated, in many pieces of literatures by the elevated levels of autophagy markers such as LC3, Beclin1, and reduction of p62. Autophagy has two contrasting roles in cells and its effects depend on stages of disease. In normal conditions, autophagy has low levels in cells. But stimulation by external factors, can initiate autophagy, protecting cells from further damage. An important aspect is the over-activation of autophagic processes. It is probably one of the causes of neuronal damage, but this process in hippocampus and other parts of the brain is complex and not well understood. So, the neuroprotective effects of antioxidant substances, in KA-induced epileptic neuronal damage, is also related to the reduction of autophagy and contemporary activation of antioxidative systems. Understanding the role of oxidative stress in excitotoxicity and epileptogenesis is essential to delineate appropriate therapeutic strategies. Many strategies to combat oxidative stress have been proposed, in particular great attention was received by food rich in antioxidants. Pomegranate, berries, and walnuts have been recognized as “superfood”, particularly for their neuroprotective effects. Pomegranate (Punica granatum) is an ancient and adaptable fruit original from Western Asia that belongs to the Punicaceae family. The common characteristic of this and other fruits is the high content of ellagitannins that during digestion, are converted

mainly to Ellagic acid (EA), that represents the main component of the pomegranate fruit. EA recently was recognized as emerging oxidative stress modulators, inhibiting lipid peroxidation and reducing ROS production (Djedjibegovic et al.; 2020). It is a chromene-dione derivative (C14H6O8) that has four hydroxyl groups, two lactones, and two hydrocarbon rings, which give to EA the ability to accept electrons from different substrates and participate in antioxidant reactions. In particular, EA with its basic structure containing 4 hydroxyl groups that are responsible for a further antioxidant effect, by scavenging free radicals (a ROO· and an O2·) but also O2− and OH· to reduce the peroxidation of lipids, chelating metal ions, such as iron and copper and regulating several signaling pathways (Zhu H. et al.; 2022). In fact, it possesses, in addition to potent antioxidative activities, also anti-inflammatory and consequent recognized neuroprotective effects. There is evidence that thanks to its powerful antioxidative and anti-inflammatory properties, EA has protective effects on different diseases of the nervous system characterized by excessive oxidative stress. It is demonstrated that EA improves the level of expression of the Nrf2, in a mice model of PD. This activation of the Nrf2, resulted in the significant mitigation of apoptosis of hippocampal neurons, acting also on the Nrf2-ARE signal to increase HO-1 expression, resulting in an antioxidant effect. When Nrf2 is inhibited in mice, the protective effect of EA on neurons is significantly mitigated (Wang et al.; 2022). Through its anti-oxidative and anti-inflammatory properties, EA reduces or even eliminates inflammatory responses. It seems that the maintaining of redox homeostasis depends on Nrf2. This latter was also controlled by PPAR-γ and was established a reciprocal transcriptional regulation between Nrf2 and PPAR-γ. Nrf2 induces PPARγ expression, by binding to at least two ARE sequences in the upstream promoter region of the nuclear receptor and may act synergically in the activation of antioxidant genes (Kvandovà et al.; 2016). Another study described that the effects of EA were modulated by PKC-ERK-PPAR-γ signaling pathway. EA reduced the oxLDL-induced phosphorylation of extracellular signal-regulated kinase (ERK) and NF-κB activation. Cytokines production is also, in many cases, regulated by ERK pathway (Wang et al.; 2004). Moreover, EA ameliorated the oxLDL-induced suppression of PPAR-γ expression resulting in the suppression of ROS generation (Kuo et al.; 2011). In conclusion, data obtained by various literature sources, suggest that Ellagic acid acts trough PKC-α/ERK/PPAR-γ/NF-κB pathway. Researchers have also demonstrated that EA, can also have anti-inflammatory effects, reducing the phosphorylation of IkB and the

subsequent translocation of NF-κB into the nucleus, downregulating the levels of iNOS, TNF-α, COX-2, and IL-6. This is the rational for the use of this compound in the treatment and prevention of nervous system diseases. For example, the continuous activation of pro-inflammatory responses damages the brain and worse the state of Alzheimer disease (AD). A study revealed that in a model of AD, EA can regulate the production of IL-1β and TNF-1α to protect the damaged neurons (Sanadgol et al.; 2016). There is, also the evidence that EA might act as an inhibitor of caspase, protecting DNA from damage in a model of epilepsy caused by Pentylenetetrazol (PTZ). The targets evaluated were Bax, Caspase-3 and 9 that were increased in PTZ-harmed mice brain and the antiapoptotic protein Bcl-2 that was suppressed. In this experiment, EA coated in calcium-alginate nanoparticles (Ca2+-EA-ALG NPs) displayed a potent antiepileptic activity. In other chronic pathology, such as the development of colonic inflammation, EA demonstrated that dietary ellagic acid, as well as pomegranate enriched with or without ellagic acid reduced pro-inflammatory cytokines, such as TNF-α production (Derosa et al.; 2016). In a model of Huntington disease (HD), EA at various doses improved motor and cognitive functions but also reduce oxidative stress and neuroinflammation, improving mitochondrial functions. EA has also demonstrated an anti-fibrotic effect in a model of cardiac fibrosis, but also in other organs such as pancreas and lung thanks its anti-oxidative properties (Mannino et al.; 2023). Pomegranate Seed Oil contains 14 fatty acids and it represents around 20% of the total weight of seed, the most abundant acid contained is Punicic acid (PA) 50–80%. PA, also known as octadecatrienoic acid is an omega-5 isomer of conjugated α-linolenic acid and exhibits structural similarities to conjugated linoleic acid. PA represents a wide spectrum of beneficial effects such as anti-inflammatory, anti-diabetic, anti-obesity and anti-carcinogenic properties. PA is metabolized into circulating conjugated linoleic acid (CLA), via a saturation reaction; fatty acid translocase is involved in the passage of CLA through the blood–brain barrier. Studies confirmed the presence and metabolism of PA in the brain of both rats and humans (Pereira de Melo et al.; 2019). Various concentrations of PA were tested on rats, as supplementation in the diet, in order to evaluate the anti-oxidative potential of PA against lipid peroxidation. Compared to a control diet, positive results were obtained; for example, a significant decreased of the total cholesterol and low density lipoproteins (LDL-C) together with a reduction of membrane peroxidation (Shabbir et al.; 2017). It is demonstrated that it acts through peroxisome proliferator-activated receptors (PPARs),

involved in the modulation inflammation, in fact, PA can act as an agonist of PPARγ, increasing its mRNA expression. PPAR-γ is involved in neuron biogenesis, neuroinflammation and neurodegeneration. The evidence show that PA decreases inflammation, induced by pro-inflammatory cytokines TNF-α and Interleukin 6 (IL-6) (Guerra-Vázquez et al.; 2022). Particularly, in Alzheimer disease, the activities attributed to PPAR-α include the reduction in oxidative stress, neuroinflammation and tau hyperphosphorylation. Moreover, PA works also as an inhibitor of calpain, which is involved in the ROS production due to mitochondrial ROS generation and HDL degradation. Calpain-1 and 2 are abundant in the brain and it is involved in synaptic function and neuroplasticity; its hyper activation is implicated in late stages of neurodegenerative diseases (Frid et al.; 2020). Nano-formulation of Pomegranate Seed Oil was tested in a model of neurodegenerative disease TgMHu2ME199K mice, results demonstrated a strong reduction of lipid oxidation and neuronal death, indicating a strong neuroprotective effect (Mizrahi et al.; 2014). Numerous pieces of research have shown that PA can significantly improve neurological pathologies.

The aim of our study was to evaluate the effects of EA and PA, in an *in vitro* and *in vivo* model of excitotoxicity KA-induced. It is of great significance to inhibit oxidative stress, scavenging free radicals and reduce or even eliminate the consequent inflammatory responses. Conflicting opinions emerged from literature search, on the role of autophagy in neurodegenerative disorders. We want to elucidate the interconnection between oxidative stress, resulting inflammation and activation of autophagy. In addition, the purpose is, to understand if, natural antioxidant compounds as EA and PA, can be used as treatment or as supplementation of diet, to treat and prevent excitotoxicity, the major aspect of neurodegenerative diseases.

Methods

Cell cultures and treatments

Human neuroblastoma cell line (SH-SY5Y) and human microglial cells (HMC3) were purchased by ATCC (ATCC Manassas, Manassas, VA, USA). SH-SY5Y were maintained in cultured with formulated Eagle's minimum essential medium and F12 medium (EMEM/F12) in a 1:1 mixture; HMC3 cells were cultured with EMEM (Sigma-Aldrich, St. Louis, MO, USA). Then 1% of penicillin/streptomycin antibiotic (Sigma-Aldrich, St. Louis, MO, USA) and 10% fetal bovine serum (FBS) (ATCC Manassas, Manassas, VA, USA) were added to both culture media and cells were incubated at 37 ◦C with a percentage of 5% CO2. The culture's medium was substituted every two days. SH-SY5Y cells and HMC3 cells were challenged with KA at the dose of 50 and 100 µM respectively to induce excitotoxicity. After 1 hour from the stimulation with KA alone, EA and PA were added to the medium for 24 hours, to evaluate their anti- oxidative and anti-inflammatory effects. At the end of the treatment period, SH-SY5Y cells and HMC3 cells were collected and used for further molecular analyses.

Animals and ethics

Excitotoxicity was induced using a standard protocol of oxidative stress model. For this experiment, the animal procedures were in accordance with the Principles of Laboratory Animal Care, authorized by the ethical committee of University of Messina and following guidelines (n° of the ministerial authorization 521/2022-PR). Sprague-Dawley male rats, 6-week-old, pathogen-free, were purchased from Charles River (Italy). Animals were maintained in plastic cages with the proper enrichment, standard environmental conditions and fed ad libitum in the Animal Facility of the Department of Clinical and Experimental Medicine of the University of Messina, Messina, Italy. Animals were allowed to acclimatize for 1 week before the beginning of the experiments.

Excitotoxicity Induction and Animal Grouping

Seizures were induced by intra peritoneal injection of rats with KA (15 mg/kg), which was dissolved in distilled water. The dose of KA was based on previous studies in KA-

treated seizure rats. Ellagic acid (50 mg/kg) and Punic acid (150 mg/kg), instead in combination the doses were halved. EA and PA were orally administered 30 minutes before the injection of 15 mg/kg KA intraperitoneally (i.p.). In control animals, KA was replaced with the same volume of saline. The dose and schedule of administration were chosen based on other studies. After injections, the rats were placed individually in cages and observed for 6 h for the development of seizures. Seizure severity was rated by the Racine scale: category 1, immobility and facial twitch; category 2, head nodding; category 3, forelimb clonus; category 4, rearing; and category 5, rearing and falling. Rats (n=38) were randomly divided in five groups including control: Kainic acid (n=8; KA), Kainic acid plus Ellagic acid (n=8; KA+EA), Kainic acid plus Punicic acid (n=8; KA+PA), Kainic acid plus Ellagic acid and Punicic acids (n=8; KA+EA+PA) and SHAM (n=6). 72 h after KA injection, the rats were sacrificed by decapitation to obtain the brain tissue samples. Histology was performed on the fixed brain tissue of five rats per group. Fresh brain tissue of rats was stored for successive Western blotting and Real-time PCR analysis.

MTT assay

Cell viability was evaluated by the MTT assay (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) to investigate KA, EA and PA cytotoxicity. In particular, SH-SY5Y and HMC3 cell lines were seeded at a density of 1×10^5 cells/well in a 96-well plate; upon reaching confluence, cells were treated with KA (10, 25, 50, 100 µM), and the anti-oxidant substances, EA and PA, at different doses (0,1, 1, 5, 10 and 20 µM) alone and in combination with KA for 24 h. Then 5 mg/mL of tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma Aldrich, St. Louis, MO, USA) was thawed in sterile PBS and 20 µL was added into each well, five hours before the end of the treatments. Twenty-four hours after starting treatments, formazan crystals were thawed using 200 µL dimethyl sulfoxide (DMSO). Cytotoxicity was quantified using a VICTOR Multilabel Plate Reader (Perkin Elmer; Waltham, MA, USA) at λ 540 and 620 nm. Data are expressed as the percentage of cell viability compared to control cells.

Evaluation of intracellular ROS Production

To evaluate the anti-oxidant effects of natural compounds EA and PA, in a model of excitoxicity induced by KA, the production of intracellular ROS was assessed in both cell lines using a 5-(and-6)-chloromethyl-20,70-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) probe. Cells were stimulated with KA and treated for 24 h with EA, PA and their combination. Fluorescein diacetate (FDA) is a substrate of esterase and it is useful to measure enzymatic activities (hydrolyzation of the substrate and formation of fluorescence product) and the integrity of cell membrane, in fact, this fluorescent product is retained in the cells. The increase in fluorescence due to the oxidation of this probe can be detected with a fluorescence microscope, using excitation sources and filters appropriate for fluorescein (FITC). At the end of the treatment period, cells were incubated with 5 µL of CM-H2DCFDA probe (Thermo Fisher, Carlsbad,CA, USA) for 1 h at 37 ◦C with a percentage of 5% CO2. After three washing with sterile PBS, cells were observed with a fluorescent microscope and the fluorescent intensity was quantified using ImageJ 1.53e software for Windows (Softonic, Barcelona, Spain).

Comet assay

The comet assay (Abcam, ab238544) is an electrophoresis-based technique that allows to determinate the damages of DNA. At first, the slide and various solutions are required: Lysis Buffer (LB) (including NaCl, EDTA solution, 10X Lysis Solution, DMSO, H2O), Alkaline Solution (AS), (including NaOH, EDTA Solution and H2O) and the Electrophoresis Running Solution (ERS) (including Tris Base, EDTA, DI H2O and Boric Acid). These solutions need to be prepared before starting the assay. To create a base layer on the slide, the comet Agarose was warmed at 90-95°C and added to the comet slide. The protocol provides that cells were scraped, centrifuged at 700g for 2 minute and the supernatant was discarded. Later, cells were suspended in PBS and combined with Agarose. The slide was then incubated first in darkness at 4 °C for 15 minutes. Thereafter, with LB (30-60 minutes 4°C) and AS (4°C 30 minutes) in the dark. Lately, the slide was equilibrated in ERS and transferred into the electrophoresis chamber (10-15 min 1volt/cm). After electrophoresis, it was added the Vista Green DNA Dye for 15 minutes and then the results have been examined with a fluorescence microscope. DNA damage

is quantified by measuring the displacement between the genetic material of the nucleus ('comet head') and the resulting 'tail'.

Hematoxylin- eosin staining of cell cultures

SH-SY5Y cells were plated at density of 2.5×10^4 cells/well in 8-well chamber slides and stimulated with KA for 1h before the treatment with EA, PA and combination of these two compounds. At the end of treatments, cells were fixed with 4% of paraformaldehyde (PFA), cells were washed with sterile phosphate-buffered saline (PBS) and then stained as standard protocol like and scanned with a light microscope.

Immunofluorescence

SHSY-5Y cells were maintained in culture at a density of 2.5×10^4 cell/well in 8-well chamber slides. Cells were treated with Ellagic and Punicic acids alone and in combination for 24 h following KA stimulation as previously described. At the end of the treatments, cells were fixed with 4% of paraformaldehyde (PFA) in 0.2 M phosphate buffer (pH 7.4) for 10 min at RT; cells were washed 3 times for 10 min with sterile phosphate-buffered saline (PBS). Cells were, also, incubated with 0.3% triton X-100 in PBS for 10 min to induce permeabilization of the membranes and with 1% bovine serum albumin (BSA) in PBS, for 1 h at RT, in order to block non-specific binding sites. At the end of incubation, cells were incubated with a rabbit polyclonal anti-Beclin 1 antibody (1:100 dilution) (Abcam, ab62557, Cambridge, UK) overnight at 4 ◦C. The day after, cells were rinsed with PBS and a FITC conjugated IgG anti-rabbit antibody was added (GeneTex, Irvine, CA, USA) for 1 h at RT, in order to detect the primary antibody. Nuclei were stained with DAPI diluted to 1:1000 in PBS (Thermo Fisher Scientific, Carlsbad, CA, USA) for 10 min RT. At the end of this process, cells were rinsed in PBS, the coverslips were mounted on slides and fluorescence was monitored using a fluorescent microscope. Digital images were cropped, and figure montages were prepared.

Autophagy/cytotoxicity dual staining

The autophagy/cytotoxicity dual staining kit (Abcam, ab133075) allowed the detection of autophagy and apoptosis of cells. The kit uses a fluorescent compound (monodansylcadaverine- MDC) that permits to evaluate the presence of autophagic vacuoles, by interacting with membrane lipids and exploiting the ion trapping mechanism. The kit also contains propidium iodide, used as a marker of cell death and a positive control for autophagy tamoxifen (TA). At first, approximately 50.000 cells of both SH-SY5Y and HMC3, were plated into a plate of 96 wells and incubated overnight to support growth. The following day, cells were treated with, KA, EA and PA diluted in culture medium, following the timing previously decided. Subsequently, the plate was centrifuged for five minutes at 400 x g. Later, the PI solution was added and cells were incubated for two minutes. Then, the plate was centrifuged and the assay buffer used for a wash. The MDC solution was added at last. Cells were incubated for 10 min. At the end, was added 100µl of assay buffer and the plate was immediately analyzed by fluorescent microscopy. Dead cells are stained by PI and can be detected with a fluorescent filter usually designed to detect rhodamine (excitation/emission = 540/570 nm) or Texas Red (excitation/ emission = 590/610 nm). Autophagic vacuoles stained by MDC, can be detected with a UV filter usually designed to detect DAP1.

RNA extraction and Quantitative RT-PCR

At the end of the treatment period, extraction of total RNA was done from SH-SY5Y and HMC3 cells and from brain samples using Trizol reagent, according to the standard protocols and quantified with a spectrophotometer (NanoDrop Lite; Thermo Fisher Scientific). Total RNA (1 µg) was reverse transcribed in the cDNA using Superscript IV Master Mix (Invitrogen, Carlsbad, CA, USA). The obtained cDNA (1 µL) was added to the BrightGreen qPCR Master Mix (ABM, Richmond, Canada) to measure the mRNA level of IL-1 β, IL-6, TNF-α, Caspase-3 and 9. qPCR was monitored by using the QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems, CA, USA) and the GAPDH gene was used as the housekeeping gene. The qPCR reaction was monitored by using QuantStudio 6 Flex (Thermo fisher Scientific, Monza, MB, Italy), and the results were quantified using the $2^{-\Delta\Delta CT}$ method, using GAPDH as a housekeeping gene and

the control group as a calibrator. The primers for targets and reference genes are listed in Table 1 and 2.

Western blot analysis

After treatments, cells were collected using RIPA buffer (25 mM Tris/HCl, pH 7.4; 1.0 mM EGTA; 1.0 mM EDTA) with NP40 (1%), phenyl methylsulfonyl fluoride (PMSF, 0.5%), aprotinin, leupeptin and pepstatin (10 µg/mL each) and centrifuged at 15,000 rpm for 15 min at 4 ◦C to obtain the supernatant from each sample. Brain samples were previously homogenated in RIPA buffer and then centrifuge following protocol. The total protein content was quantified in cell supernatants using the Bradford method. Then, 30 µg of proteins was separated via electrophoresis on an SDS polyacrylamide gel (10%) and transferred to PVDF membranes (Amersham, Little Chalfont, UK) using a specific transfer buffer. Following washes in TBS-0.1% Tween buffer and incubation with 5% non-fat dry milk, membranes were incubated with specific primary antibodies for β- actin (Cell Signaling, 4967S, Danvers, MA, USA), Nrf2(Abcam, ab31163, Cambridge, UK), PPARγ (Cell Signaling, 2443S, Danvers, MA, USA), PGC-1α (Thermo scientific, PA1-31202, Carlsbad, CA, USA), p-ERK (Cell Signaling, 9101S Danvers, MA, USA), BAX (BIOVISION, 3032-100), BCL2 (Abcam, ab59348, Cambridge, UK), Caspase-3(Cell Signaling, 9662S, Danvers, MA, USA), phospho-AKT (Cell Signaling, 40656, Danvers, MA, USA), p-mTor (Abcam, ab109268 Cambridge, UK), AMBRA 1(Cell Signaling, Danvers, MA, USA), LC3B (Abcam, ab192890, Cambridge, UK), P62 (Abcam, ab109012, Cambridge, UK) diluted in TBS-0.1% Tween as required by data sheet and incubated overnight, at 4 °C. The day after, the membranes were washed three times with TBS-0.15% Tween buffer and a secondary peroxidase-conjugated goat anti-rabbit or anti-mouse antibody (GeneTex, Irvine, CA, USA), according to antibody, was used for 1 h at room temperature (RT) to bind and detect the target proteins. Following three washes with TBS-0.15% Tween buffer, membranes were analyzed by chemiluminescence system (LumiGlo reserve; Seracare, Milford, MA, USA). Images were obtained and quantified via scanning densitometry using a bio-image analysis system (C-DiGit, Li-cor, Lincoln, NE, USA). Results were expressed as relative integrated intensity using β-actin (Cell Signaling, Danvers, MA, USA) as a control for the equal loading of samples. For the in-

vivo model, two samples of animals for each group were loaded in the same western blot gel.

Nissl staining of rat brain samples

72 hours after KA challenge, animals of each group were sacrificed by decapitation. The brains were harvested and preserved in a solution of 10% buffered formalin in 0.1 M phosphate buffer (PB). To observe morphologic changes, samples were dehydrated, cut in coronal sections and stained with Nissl staining, following a standard protocol. A set of sections, regularly spaced through the brain, was mounted for light microscopic observations. Histological identification of nervous structures was made by analogies with the brain of the rat, according to the atlas of Paxinos and Watson (1986). Corresponding topographical areas were selected in each animal of the specific groups. Three sections per animal were normally analyzed.

Statistical Analysis

All data are expressed as mean and standard deviation (mean ± SD) for each group. Statistical significance of the difference between group of control and treatment was obtained by using one-way or two-way ANOVA. The probably of error was set at $p<0.05$. Graphs were drawn using GraphPad Prism software version 5.0 for Windows.

Results

Evaluation of cytotoxicity of KA, EA and PA on cell cultures

Toxicity of KA, EA and PA was evaluated using MTT assay in SH-SY5Y and HMC3 cell lines. In particular, KA stimulation reduced cell viability starting from the dose of 50 µM in SH-SY5Y (Figure 1A) instead in the HMC3 cell line the reduction of cell viability starts from 100 µM (Figure 2A). In SH-SY5Y, EA and PA alone reduce viability from the dose respectively of 5 and 20 µM (Figure 1B, C). We also evaluate the cytotoxicity of EA and PA after KA challenge. The doses, selected for treatment with EA and PA alone, after KA stimulation (50 µM), were 1 µM (Figure 1D, E); when combined EA and PA, the doses that give the best result are 1 µM for EA and 0,5 µM for PA (Figure 1F). In HMC3, EA and PA alone did not show toxicity up to 10 µM (Figure 2B, C), the doses chosen for the treatments with EA and PA alone, after stimulation with KA (100 µM) were 0.5 µM for both compounds (Figure 2 D, E). Instead, the doses chosen for EA and PA in combination was 1µM (Figure 2 F). The selected doses for the treatments were the ones that improve cell viability after KA challenge.

Ellagic and Punicic acid show anti-oxidant effects

Excessive production of ROS is a principal mechanism by which KA was able to induce oxidative stress and consequentially inflammation. The increase of oxidative stress was strong after stimulation with KA compared to untreated cells, it is demonstrated by the accumulation of intracellular ROS in both cell lines. It was significantly increased in SH-SY5Y and HMC3 cells challenged with 50 µM (Figure 3B) and 100 µM (Figure 4B) of KA, while ROS accumulation, was significantly reduced by the treatment with EA and PA alone (Figure 3-4 C, D) and in combination (Figure 3-4 E) at the tested doses in two cell lines. Best results were obtained with combination of natural compounds in both cell lines. The same doses of KA, EA and PA were tested to verify the damage at DNA. It was verified by Comet assay, demonstrating as, in both cell lines, the stimulation of KA is capable to induce strong DNA damages, represented by the long tails formed, visible at the fluorescence microscope in SH-SY5Y and HMC3 (Figure 5-6 B). EA and PA alone and in combination, significantly, reduced the number and the length of DNA tails in neuronal and microglial cell lines (Figure 5- 6 C, D, E) compared to cells stimulated by

KA, demonstrating an effect of protection against KA-induced DNA damages. Nrf2, an important antioxidant transcription factor, is involved in redox-modulated signaling pathway. It was significantly reduced when cells were challenged with KA (Figure 7-8 A), reducing its protective effects. EA and PA treatments promoted the increase of Nrf2 protein level and also its nuclear translocation with consequent upregulation of anti-oxidant genes and inhibition of inflammation. Best results were obtained with the combination of these two natural compounds tested in HMC3 cells, instead in SH-SY5Y Nrf2 level was increased more with only PA treatment. Exist a crosstalk between Nrf2 and Peroxisome proliferator-activated receptor gamma (PPAR-γ), for this reason we evaluate also the level of this nuclear receptor in this experimental setting. In particular, PPAR-γ were significantly reduced when cells were stimulated with KA compared to untreated cells (Figure 7-8 B). The same trend was followed by peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α; Figure 7-8 C), confirming its inhibition. The levels of both PPAR-γ and PGC-1α, were restored with the treatments on both cell lines, exerting its anti-oxidant and anti-inflammatory activities. Best results were obtained once again with PA and its combination with EA in both cell lines. In in-vivo model of excitotoxicity, obtained by administration of KA in rats, the evaluation of oxidative stress in hippocampus of rats was based on Nrf2 target (Figure 9 A). Results demonstrated a strong reduction of Nrf2 in the group of animals with administration of KA only. The treatment with EA, PA and their combination restored the levels of Nrf2 with no great differences between groups of treated animals. These results assessed the efficacy of both naturally-derived compounds in reducing oxidative stress in hippocampus of treated animals maybe acting through Nrf2 signaling pathway.

Histological evaluation of SH-SY5Y morphology in a model of excitotoxicity

For the purpose of monitoring the morphological change of simile neuronal cells, we set up a hematoxylin/eosin staining. This staining revealed that SH-SY5Y cells exhibited morphological changes visualized under light microscope when challenged with KA. In detail, they showed a shrinkage of cell bodies, nuclear swelling and a marked reduction of cellular extensions compared to control cells SH-SY5Y that showed a neuronal phenotype. This morphological change was due to the exposure to pro-oxidative stimulus

produced by KA and the consequent induction of apoptosis visible by the fragmentation into membrane-bound apoptotic bodies. Cell membrane began to show blebs and spikes, typical features of this process (Figure 10 B). Treatment with EA and PA inhibited the morphological alterations induced by KA and cells returned to the similar aspect of the untreated cells (Figure 10 C, D, E). We could say that apart from molecular change previous analyzed, a morphological change in structure of cells was extremely visible in this experimental setting.

Ellagic and Punicic acids decrease the inflammatory targets in in vitro and in vivo models of excitotoxicity

In order to further examine the protective effects of EA and PA, inflammatory pathway was evaluated. KA stimulus of SH-SY5Y and HMC3 produced a significant increase of pro-inflammatory cytokines, demonstrated by the upregulated mRNA levels of IL-1β (Figure 11-12 A), IL-6 (Figure 11-12 B) and TNF-α (Figure 11-12 C) compared to control cells. Instead, the treatments with EA, PA and their combination reduced the levels of the same targets, demonstrating as these potent anti-oxidative substances induce a reduction of inflammatory targets in this model of excitotoxicity. Particularly effective is PA and its combination with EA in both cell lines. ERK 1/2 pathway activation is important in pro-inflammatory cytokine production, for this reason we also evaluate it. Results demonstrated that in both cell lines, induction with KA increased levels of phosphorylated ERK (p-ERK) (Figure 7-8 D), so the increase of cytokines may be linked also p-ERK. On the other hand, the treatment with these tested compounds induce a strong reduction of p-ERK. In in-vivo rat model of excitotoxicity, the administration of KA induces an important increase of mRNA levels of IL-1β (Figure 13 A), IL-6 (Figure 13 B) and TNF-α (Figure 13 C) in hippocampus of rat administrated with KA compared to Sham. Administration of EA and PA in rats with epilepsy, significantly decreased the expression of these three proinflammatory cytokines in the hippocampus compared with the only KA-treated group. These data indicated a decrease of neuroinflammation after the treatments with tested anti-oxidative compounds, especially combination of EA and PA treatment. An additional confirmation of inflammatory activation is given by results on other targets in vivo model. They showed that administration of KA only, determinate a significant increase in the activities of respectively p- ERK and p-Nf-Kb (Figure 9 B, C),

while treatments with EA and PA significantly decreased the activity of these two, in particular the best results were obtained in the group of animals treated with the combination of EA and PA.

Ellagic and Punicic acids decrease the apoptotic pathway in vitro and in vivo models of excitotoxicity

SH-SY5Y and HMC3 cells challenged with KA exhibited the activation of apoptotic process; demonstrating by a significant down-regulation of Bcl-2 protein level in SH-SY5Y (Figure 14B) and HMC3 cells (Figure 15B) and simultaneous increase of Bax (Figure 14-15 A) compared to untreated cells. An additional confirmation of the activation of the apoptosis is the up-regulation of executive factors of apoptosis Caspase-3 and 9 in two cell lines, confirmed by protein level of Caspase-3 and mRNA expression of both Caspase-3 and 9 (Figure 14-15 C, D, E). On the other hand, the treatments with EA and PA alone and in combination are able to reduce all pro-apoptotic factors and increase the level of anti-apoptotic target, in particular treatment with PA. In rat model of excitotoxicity, the level of Bax and Caspase- 3 are increased in the brain samples of KA group compared with Sham group (Figure 16 A, B, C). Contrary, Bcl2 the anti-apoptotic target, is decreased in the same group. Animals treated with EA and PA alone and in combination demonstrated a reduction of all pro-apoptotic targets, these data confirmed how oxidative and inflammatory pathway induce a potent activation of apoptosis in the brain of animals of KA group, instead all the treatments with tested anti-oxidative compounds are able to modulate the apoptotic pathway, down-regulating it.

Behavioral analysis of animals

The effect of EA and PA on seizure activity induced by KA (15 mg/kg, intraperitoneally (i.p.)) was investigated by Racine scale after administering of antioxidant compounds EA and PA 30 min before KA injection. Stimulation with KA was well-demonstrated to induce seizures typical of epilepsy. In our experiments, all animals appertaining to KA group showed an intense seizures pattern that was monitored by Racine Scale reaching stage 5 (Figure 17). In general, epileptic seizures were characterized by continuous motor-limbic seizures accompanied by intermittent rearing and falling, two rats of this group

died during the behavioral monitoring. Contrary, statistical analysis (one-way ANOVA) demonstrated that EA and PA treatments reduced KA-induced seizure activity compared to KA group, decreasing seizure score in almost all treated animals. The combination of these two tested compounds and the treatment with EA alone showed, significantly, good results in evaluation of animal behavior with an important attenuation of seizures activity and consequentially an improvement in the stage of Racine scale.

Ellagic and Punicic acids modulate the levels of activated autophagy, in in-vitro and in-vivo models of excitotoxicity stimulated with KA

Autophagy was monitored in in-vitro model of excitotoxicity, in both cell lines the strong stimulation with KA was able to hyper-activate the cascade of autophagy. In particular, the autophagy/cytotoxicity dual staining demonstrates the contemporary activation of apoptosis and autophagy (Figure 18-19 B, G), probably, as a result of a potent oxidative stress stimulus. Other targets were evaluated to better demonstrate the effective activation of autophagy. Protein level of AMBRA 1 and LC3B (Figure 20-22 C, D), mediators of the autophagic pathway, were increased when KA was added to SH-SY5Y and HMC3 media. Also Beclin- 1 (Figure 21- 22F), monitored by immunofluorescence and western blot, results in SH-SY5Y and HMC3 significantly increased; in particular, fluorescent pattern was mainly distributed around nuclei. On the other hand, mTor a well-known inhibitor of autophagy, and p-Akt its activator, resulted decreased in the same condition, further confirming the activation of autophagy (Figure 20-22 A, B). P62, whose decrease is a marker of activation of autophagy, results in our experiment drastically reduced in SH-SY5Y and HMC3 stimulated KA cells (Figure 20-22 E). EA and PA, potent anti-oxidant compounds, reducing the oxidative stress induced by KA, were able to reduce the hyper activation of autophagic pathway, in fact the levels of all pro-autophagic proteins, AMBRA 1, LC3B and Beclin-1 were marked reduced compared to cells stimulated with only KA. Contemporary, the inhibitor of autophagy p-mTor and its activator p-Akt showed an important increase with almost all treatments. In particular, treatment with PA resulted the best in reducing the level of activated autophagy compared to other treatments.

In in-vivo model of excitotoxicity, KA group compared to Sham demonstrated increased protein levels of the same targets evaluated in in-vitro experiments, AMBRA 1, Beclin-

1, LC3B in brain samples (hippocampus) of all animals analyzed in KA group (Figure 23 C, D, E). Protein levels of these targets were significantly attenuated in both the EA and PA treated groups compared to the KA group. Moreover, p-Akt, p-mTor and p62 (Figure 23 A, B, F) protein levels were strongly decreased in the KA group compared to the Sham group and increased in animals treated with these natural anti-oxidant compounds. These results indicate that the neuroprotective effect of Ellagic and Punicic acids against KA-induced excitotoxicity is closely related to the attenuation of autophagy probably mediated by the enhanced antioxidant responses and reduction of inflammation.

EA and PA treatments ameliorate neuronal loss in the hippocampus of KA-administered rat

To evaluate if the analyzed extracts, EA and PA, have a neuroprotective effect in this in vivo model of excitotoxicity, we performed Nissl staining that permit to observe the morphology of hippocampus. Brain tissue samples obtained from the Sham group, showed a normal neuronal density and a regular arrangement in the area of hippocampus (Figure 24A). Instead, results highlighted morphological modification in region CA1 and CA3 of hippocampus in rats administrated with KA compared with Sham (figure 24B). In particular, rats administrated with KA and not treated with anti-oxidant extracts, demonstrated a disorganization in neuronal structures and a remarkable loss of neurons in regions CA1 and CA3. Treatments with EA and PA and their combination reduced the neuronal loss and preserve the normal architecture compared to KA-administered rats (Figure 24 C, D, E).

Discussion

Excitotoxicity occurs when neurons are exposed to high levels of excitatory amino acids that cause a persistent activation of the NMDA, AMPA receptors and voltage-gated calcium channels. In particular, excitotoxicity refers, primarily, to the overactivation of glutamate receptors as a result of increased release and/or decreased uptake of glutamate (Wong et al; 2009). The consequence of this hyper-activation is an overload of calcium, that leads cells to death via the generation of free radicals, inhibition of protein synthesis and mitochondrial damage. The excessive activation of glutamate receptors, also leads to an imbalance between ROS and antioxidant defenses of cells, a phenomenon named oxidative stress. Oxidative stress, as known, induces damages of macromolecules in cells, that involves a gradual loss of tissue and organ function. It plays an important role in the pathogenesis of many brain chronic diseases, in fact, it is well-established to be a common denominator in neurodegenerative diseases (Armada-Moreira et al.; 2020). So, we can say that neuronal excitotoxicity and oxidative stress, produced by an excessive production of free radicals, may play a role in the initiation and progression of neurodegenerative diseases, in particular of epilepsy (Geronzi et al.; 2018). In epilepsy, oxidative stress can damage neurons and lead to seizures. This is because free radicals can damage the cell membrane, DNA and other structures within neurons. Several studies have demonstrated a strong association, between epilepsy and oxidative stress. Moreover, during epileptic seizures, the production of ROS is increased in the brain but the antioxidant defense mechanisms in the brain are particularly weak. Epilepsy is one of the most common neurological disorders, estimated to affect more than 70 million people worldwide it actually is a family of disorders which comprise several diseases and conditions (Fisher et al.; 2017). The kainic acid (KA) model is commonly used to mimic temporal lobe epilepsy (TLE), the most common form of epilepsy worldwide. The systemic administration of KA to animals induces tonic-clonic seizures and causes severe loss of neurons and synaptic dysfunction in the central nervous system (Lin et al.; 2022). Particularly, the part of brain that is affected is the hippocampus. KA is also used in cellular cultures for the purpose of simulating an in-vitro model of excitotoxicity and it is particularly useful to evaluate the effect of new drugs in this pathological condition. It is not easy studying neurodegenerative diseases and the mechanisms underlying them, such as excitotoxicity, because of the various pathways that are involved in these diseases, and even more difficult, finding treatments. However, a solution to this problem, could be to

look for a novel anti-seizure drug, from fruits and plants that contain potent and beneficial compounds. There is a number of anti-oxidant and anti-inflammatory drugs that are studied for the treatment of excitotoxicity and consequentially, ameliorate epilepsy and other pathological conditions. Precisely, some natural compounds are known to exhibit anti-seizure activity in animal models with fewer side effects. These compounds have primarily the potential to reduce oxidative stress and inflammation. Two potent and well-known anti-oxidant compounds are Ellagic and Punic acids (EA, PA), main components of the Punica granatum (pomegranate) fruit and seed oil respectively (Mannino et al.; 2023). EA exerts an important neuroprotective effect through its free radical–scavenging action, modulation of several cell signaling pathways and alleviation of mitochondrial dysfunction (Gupta et al.; 2021). Numerous in vitro and in vivo studies have demonstrated the neuroprotective effects of EA against various neurotoxins. Punicic acid is a polyunsaturated fatty acid with an 18-carbon, it is a bioactive compound of pomegranate seed oil and has received great attention for its therapeutic potential in various chronic diseases such as Alzheimer's diseases and diabetes but also in metabolic syndromes, as demonstrated by various in vivo experiment. The mechanism of action is based on its effectiveness against ROS production, but has also anti-inflammatory effect, reducing the activation of inflammatory cascades (Shabbir et al.; 2017). Previous approaches to targeting oxidative stress with new drugs in animal models of epilepsy, have met with some success in reducing neuronal damage following prolonged seizures (Lin et al.; 2022, Pardo-Peña et al.; 2022). For this reason, we have decided to use these two potent compounds, EA and PA acting as an acute antioxidant. Another aim of our research was to elucidate the precise mechanisms by which activation of autophagy promotes epilepsy-induced neuronal loss. According to some studies on rats, excessive activation of autophagy induces cell death in brain of animals, identifying this process, as a possible stimulus to activate neuronal apoptotic pathway in rats (Wen et al.; 2014). We try to evaluate the connection between oxidative stress and neuroinflammation, with the activation of autophagy and apoptosis, finding in use of EA and PA a positive effect in reducing excitotoxicity. Previous studies, reported the anti-oxidant and anti-inflammatory effect of EA and PA, in particular, for the treatment of chronic disorders and ROS-related diseases, such as Huntington's (Bains et al.; 2022), Alzheimer's and Parkinson's disease (Guerra-Vázquez et al.; 2022). Our results, in an *in vitro* model of excitotoxicity on SH-SY5Y and HMC3 cell lines, confirmed that KA significantly, increased oxidative stress and in particular ROS levels. Instead, EA and PA were able to reduce oxidative stress in

these two different cell lines challenged with KA, reducing the levels of intracellular ROS. Another effect was the consequent neuronal damage induced by oxidative stress, for example the damage at DNA as demonstrated by Comet assay. Both compounds seemed to demonstrate excellent effects in reducing this stressor stimulus, taking the cells back to optimal state. Nrf2, anti-oxidant target is well-described in literature, as a component in the anti-oxidant mechanism of these two compounds, involved in redox-modulated signaling pathway. In this experimental setting, SH-SY5Y and HMC3 cells challenged with KA demonstrated a decrease of Nrf2 expression compared to unstimulated cells. Both EA and PA increased the protein levels of Nrf2 confirming its involvement in the mechanism of action of these two natural compounds. PPAR-γ, important anti-oxidant mediator and its co-activator PGC-1α, seemed to follow the trend of Nrf2, maybe, for their interconnected pathways as previously demonstrated (Kvandovà et al.;2016). EA and PA exerted anti-oxidant and anti-inflammatory potentials on treated cells, modifying protein levels of PPAR-γ and PGC-1α. Results showed, also, that these compounds alone and in combination, reduced oxidative stress in brain of rats, during epileptogenesis, induced by KA; significantly improving pathological outcomes. These improvements are provided by a reduction of oxidative stress as demonstrated, also in this case, by the reduced levels of Nrf2, in particular in rats treated with PA. The ameliorative effect of EA and PA on oxidation in the hippocampus, might participate in its anti-seizure and neuroprotective effects in KA-treated rats. Other evidence, have confirmed the beneficial effect of EA and PA compounds are given from behavioral changes observed. In KA-treated group, all animals showed a stage 5 of Racine scale, in contrary, behavioral changes were markedly reduced in the majority of animals appertained to the EA and PA-treated groups. Signs that, oxidative stress reduction, is an important factor in maintenance of normal functionality of neurons and consequent behavioral aspect. Suppression of neuroinflammation in SH-SY5Y, HMC3 cells and in the hippocampus of KA-treated rats, was another beneficial effect exerted by EA and PA. In vivo studies have demonstrated that activation of glial cells produces a large amount of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6, that, in turn, cause neuronal damage in the hippocampus of rats with epilepsy (Lin et al.; 2022). In the present study, the obtained results in excitotoxicity condition showed an enhanced expression of TNF-α, IL-1β and IL-6 mRNA; both EA, PA and even more their combination, significantly, reduced expression of pro-inflammatory cytokines. This decrease of

inflammatory down-stream signal, in accordance with previous papers, confirm the anti-inflammatory effects of these natural compounds (Mafetu et al.; 2022, Mannino et al.; 2023). Also, in the animal model of excitotoxicity, our results have demonstrated an increase of inflammatory cytokines in the hippocampus of animals injected with KA. On the other hand, the anti-oxidant compounds alleviated inflammation, in fact, downregulation of TNF-α, Il-1β and IL-6 expression in rats treated with our compounds, was associated with a reduction of hippocampal neuronal inflammation. ERK 1/2 pathway activation is, in general, important in pro-inflammatory cytokine production, our results confirmed the probable involvement of this pathway in the production of cytokine. p-ERK trend, both in in vitro model and in animals analyzed, is up-regulated when KA administration was not contrasted by antioxidant effect of EA or PA. The opposite occurred when the two natural compounds were administrated to animals or added to cell media. Numerous clinical studies and animal experiments, have investigated that the inflammatory response, during excitotoxicity and neurological diseases, is linked to neuronal death and epileptogenesis (Murugesan et al.; 2018). On the other hand, previous studies and experiments have confirmed that reduction of the inflammatory response, can induce an improvement of pathological condition (Spigolon et al.; 2010). In our in-vitro model, SH-SY5Y and HMC3, stimulated with 50 and 100µM, respectively, of KA exhibited an increase of apoptotic markers. The stressor stimulus provided by this neurotoxic substances, may be strong trigger, not only of the activation of inflammatory pathway, but also of the apoptotic pathway (Li et al.; 2018). Our results revealed that, EA and PA treatments significantly reduce neuronal apoptosis in the KA-induced excitotoxicity model, including reductions of caspase-3 and Bax protein expression but increasing Bcl-2 protein level. We found that treatments with 50 mg/kg EA and 150 mg/kg PA alone and half doses in combination, significantly protected neurons against KA-induced neuronal damage, in accordance with previous studies in other model of neurodegeneration (Sanadgol et al.; 2017). In particular, the levels of protein expression of Bax, Caspase 3 and Bcl2 confirmed the protective action of EA and PA from the induction of apoptosis in hippocampal regions in KA-induced model. Our results, moreover, suggested that EA and PA can prevent KA-induced seizures and have preventive effects on cell apoptosis and dysfunction in the hippocampus. Therefore, the contemporary suppression of activated inflammation and cell apoptosis ameliorated seizures and neuronal death in KA-treated rats.

Autophagy is well-known to play a crucial role in neuron survival. Over overexpression of mitochondrial autophagy can induce neuronal death, but the use of antioxidant stress compounds may reduce it and have protective effects, as described in previous studies (Vidoni et al.; 2016). Autophagy activation has contrasting effect in cells, in normal circumstances, autophagy is activated at low levels. In contrast, provided stimuli by external factors, such as oxidative stress, induces autophagic processes, in order to eliminate damaged organelles, protecting cells from other damage; but the over activation of autophagy is likewise able to damage cells. Increasing antioxidant level and reducing the activation of autophagy seem to be essential for survival of neurons. Our results demonstrated that treatments show a significant inhibition of KA-induced autophagy both in in-vitro and in vivo models. Cell lines stimulated with KA show an important increase of all markers of autophagy analyzed AMBRA 1, Beclin-1, LC3B. High levels of these two last, are indicative of autophagy activation and it is well-established that promotes the development of spontaneous seizures in mouse brain neurons (Gao et al., 2012). In SH-SY5Y and HMC3 cells, in particular, the combination of EA and PA, more than these compounds alone, reduces markedly AMBRA 1, LC3B and Beclin-1. So, we could confirm that EA and PA inhibit over-activation of mitochondrial autophagy, probably, by reducing levels of oxidative stress. How supported by literatures, mTor is the most important inhibitor of autophagy, in KA model of excitotoxicity the level of mTor and p-Akt its activator, were extremely low, supporting the hypothesis of the hyper-activation of autophagy. Contrary, the level of this inhibitor was restored when cells were treated with EA and PA. Comparable results were obtained in rat model, hippocampal samples analyzed, confirmed that KA systemic administration activates autophagy in the brain excessively. The analysis of our results on compounds tested, helped to confirm the potentiality of these substances in modulating autophagy through the reduction of oxidative stress. Other previous studies, have demonstrated that excitotoxicity injury provided and epileptic seizures, were the cause of the accumulation of ROS and the stimulus for the hyper activation of autophagy. The purpose was the elimination of the damaged cellular components (Lin et al.; 2014). The autophagy/cytotoxicity kit used, allowed to demonstrate a contextual activation of both these two pathways, confirming that autophagy and apoptosis are both associated with neuronal loss subsequent to kainic acid-induced excitotoxicity. Particularly, activation of autophagy is one of the mechanisms that can also trigger apoptosis under certain circumstances via the activation of caspases and the depletion of endogenous apoptosis inhibitors (Su et al.; 2015). It

seemed that the effect of protection of EA and PA was given by the ability to maintain balance between oxidative stress and autophagy, preventing apoptosis of neurons and microglia. These results demonstrated that the neuroprotective effects of EA and PA against KA-induced excitotoxicity and neuronal apoptosis were markedly related to decrease of autophagy, driven by a reduction of oxidative stress. In summary, our study demonstrated that EA and PA treatments, alone and in combination, protected neurons against KA-induced neuronal damage both in vitro and in a rat model of excitotoxicity. Additionally, the neuroprotective effects of these antioxidant compounds, may have been closely related to the maintenance of antioxidant levels and autophagy. There was a correlation between molecular analysis carried out and the histological analysis. Hematoxylin/eosin staining of SH-SY5Y brought out the morphological changes that occurred when cells were stimulated with KA. As described in other studies, neurotoxic stimuli can induce significant morphological alterations (Enogieru A. et al.; 2018). In our images obtained by light microscope, was evident how the excitotoxic compound KA, at the chosen dose, causing not only a modification of molecular parameters analyzed, but also important changes in morphological structures of cells. These modifications emphasized how, oxidative stress and the consequent neuroinflammation provided by KA in this experimental model were able to induce massive changes in homeostasis of cells. Treatments, however, were capable to restore, not only molecular levels of targets but also morphological features, thanks to the potent anti-oxidant action of EA and PA. They reduced the stressful stimulus provided by KA, inducing cells to reacquire a normal morphology. In in- vivo model, was important to evaluate the morphological modification of hippocampus, the portion of brain particularly affected by KA. 72 hours following KA administration, we observed a marked neuronal loss in areas of hippocampus in brain of animals KA-administrated. In contrast, oxidative stress reduction, induced a significant neuroprotective effects and preserved a good neuronal architecture, as demonstrated by the images derived from animals of EA and PA groups. In the present study, EA and PA at the doses tested, exerted similar effects in the targets analyzed, with a slight improvement in results obtained with PA alone and in combination with EA. In summary, our present results demonstrated neuroprotective effects of these compounds on excitotoxicity. The modulation of multiple processes such as neuronal apoptosis, hippocampal degeneration, inflammatory and autophagic signaling by EA and PA promoted the maintenance of homeostatic condition, contrasting the neurodegenerative process induced by KA. Our findings indicated that EA and PA may serve as a promising

therapeutic agent for excitotoxicity, a common component of the majority of neurodegenerative diseases.

Conclusion

In conclusion, these preliminary data suggest that Ellagic and Punicic acids, in this experimental setting, are able to reduce inflammation and modulate autophagy thank to its antioxidant properties, leading to a reduced activation of the apoptotic pathway. The translational aspect of this research is based on reduction of excitotoxicity and consequentially neuroinflammation, common aspect of neurodegenerative disorders. In the experimental model reproduced, EA and PA may be able to prevent seizures and improve quality of life for people with epilepsy; but not only, excitotoxicity is a common component of the majority neurodegenerative diseases, therefore, finding a treatment that acts on excitotoxicity may mean, improving life of many patients that suffer from a neurodegenerative disease. However, more researches are needed to determine the safety and efficacy of these drugs in the possible treatment of neurodegenerative diseases.

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Figure legend

Figure 1. The graphs show the cytotoxicity assay performed by MTT assay following 24 h of incubation with KA (A), EA (B), PA (C), KA+EA (D), KA+PA (E) and KA+ EA+PA (F) at different doses in SH-SY5Y cells. Values are expressed as the means and SD

Figure 2. The graphs show the cytotoxicity assay performed by MTT assay following 24 h of incubation with KA (A), EA (B), PA (C), KA+EA (D), KA+PA (E) and KA+ EA+PA (F) at different doses in HMC3 cells. Values are expressed as the means and SD

Figure 3. Intracellular ROS accumulation evaluated by a CM-H2DCFDA fluorescent probe in SH-SY5Y cells (A) stimulated with KA (B) and then treated with EA (C) or PA (D) or EA+PA (E). Panel F shows the number of fluorescent cells. All images were captured at 10× magnification.

Figure 4. Intracellular ROS accumulation evaluated by a CM-H2DCFDA fluorescent probe in HMC3 cells (A) stimulated with KA (B) and then treated with EA (C) or PA (D) or EA+PA (E). Panel F shows the number of fluorescent cells. All images were captured at 10× magnification.

Figure 5. DNA damage evaluated by the comet assay in SH-SY5Y (A), cells stimulated with KA (B) and then treated with EA (C) or PA (D) or EA+PA (E).

Figure 6. DNA damage evaluated by the comet assay in HMC3 (A), cells stimulated with KA (B) and then treated with EA (C) or PA (D) or EA+PA (E).

Figure 7. The graphs represent Nrf2, PPAR-γ, PGC-1α and p-ERK protein level assessed by Western blot analysis in SH-SY5Y (A, B, C, D) cells treated with EA, PA and combination for 24 h following KA (50μM) challenge. The data are expressed as means ± SDs. * $p < 0.05$ vs. CTRL; # $p < 0.05$ vs. KA.

Figure 8. The graphs represent Nrf2, PPAR-γ, PGC-1α and p-ERK protein level assessed by Western blot analysis in HMC3 (A, B, C, D) cells treated with EA, PA and combination for 24 h following KA (50μM) challenge. The data are expressed as means ± SDs. * $p < 0.05$ vs. CTRL; # $p < 0.05$ vs. KA.

Figure 9. The graphs represent Nrf2, p-Nf-kB, p-ERK (A, B, C) protein level assessed by Western blot analysis in hippocampus of different groups of rats SHAM, rats

administrated with KA, rats treated with EA, PA and combination for 72 h with KA administration. The data are expressed as means ± SDs. * $p < 0.05$ vs. CTRL; # $p < 0.05$ vs. KA.

Figure 10. Images show the morphological change of SH-SY5Y cells (A), stimulated with KA (B), treated with EA (C), PA (D), EA+PA (E), evaluated with hematoxylin-eosin staining

Figure 11. mRNA level of IL-1β and IL-6 and TNF-α evaluated by qPCR analysis in SH-SY5Y (A, B, C) cells challenged with KA, cells treated with EA, PA alone and in combination, for 24 h following KA (50µM) stimulation. The data are expressed as means ± SDs. * $p < 0.05$ vs. CTRL; # $p < 0.05$ vs. KA.

Figure 12. mRNA level of IL-1β and IL-6 and TNF-α evaluated by qPCR analysis in HMC3 cells (A, B, C) challenged with KA, cells treated with EA, PA alone and in combination, for 24 h following KA (50µM) stimulation. The data are expressed as means ± SDs. * $p < 0.05$ vs. CTRL; # $p < 0.05$ vs. KA.

Figure 13. mRNA level of IL-1β and IL-6 and TNF-α (A, B, C) evaluated by qPCR analysis in hippocampus of different groups of rats SHAM, rats administrated with KA, treated with EA, PA alone and in combination, for 72 h with KA (50µM) stimulation. The data are expressed as means ± SDs. * $p < 0.05$ vs. CTRL; # $p < 0.05$ vs. KA.

Figure 14. The graphs represent BAX, BCL2, Caspase-3 protein level assessed by Western blot analysis (A, B, C) and Caspase- 3 and 9 mRNA levels assessed by Realtime-PCR (D,E) in SH-SY5Y cells treated with EA, PA and combination for 24 h following KA (50µM) challenge. The data are expressed as means ± SDs. * $p < 0.05$ vs. CTRL; # $p < 0.05$ vs. KA.

Figure 15. The graphs represent BAX, BCL2, Caspase-3 protein level assessed by Western blot analysis (A, B, C) and Caspase- 3 and 9 mRNA levels assessed by Realtime-PCR (D, E) in HMC3 cells treated with EA, PA and combination for 24 h following KA (50µM) challenge. The data are expressed as means ± SDs. * $p < 0.05$ vs. CTRL; # $p < 0.05$ vs. KA.

Figure 16. The graphs represent BAX, BCL2, Caspase-3(A, B, C) protein level assessed by Western blot analysis in hippocampus of different groups of rats SHAM, rats administrated with KA, rats treated with EA, PA and combination for 72 h with KA administration. The data are expressed as means ± SDs. * $p < 0.05$ vs. CTRL; # $p < 0.05$ vs. KA.

Figure 17. Seizure behavior analysis evaluated by Racine scale in the different groups (n = 8 rats/group). Statistical results showed a decreased seizure score (one-way ANOVA). *= $p < 0.05$ vs. KA-treated group.

Figure 18. The images show the evaluation of autophagy and cytotoxicity by a fluorescent label MDC and PI staining in SH-SY5Y without any treatment (A, F) , stimulated with KA (50µM) (B,G), treated with EA (C,H), PA (D,I), EA+PA (E,L) following stimulation with KA.

Figure 19. The images show the evaluation of autophagy and cytotoxicity by a fluorescent label MDC and PI staining in HMC3 without any treatment (A, F), stimulated with KA (100µM) (B,G), treated with EA (C,H), PA (D,I), EA+PA (E,L) following stimulation with KA.

Figure 20. The graphs represent mTor (A), p-Akt (B), AMBRA 1 (C), LC3B (D), P62 (E) protein levels (Western blot analysis) in SH-SY5Y cells stimulated with KA (50µM) and treated with EA (1 µM), PA (1 µM) and EA+PA (1 and 0,5 µM). The data are expressed as the means and SD. *$p < 0.05$ vs CTRL; #$p < 0.05$ vs KA.

Figure 21. Compound panel of immunofluorescence reactions using an anti- Beclin 1 antibody (green fluorescence) in SH-SY5Y cells (A1–A3) treated with EA (C1–C3) or PA (D1–D3), and combination of EA and PA (E1-E3) for 24 h following KA (B1–B3) stimulation.

Figure 22. The graphs represent mTor (A), p-Akt (B), AMBRA 1 (C), LC3B (D), P62 (E), Beclin-1 (F) protein levels (Western blot analysis) in HMC3 cells stimulated with KA (50µM) and treated with EA (1 µM), PA (1 µM) and EA+PA (1 and 0,5 µM). The data are expressed as the means and SD. *$p < 0.05$ vs CTRL; #$p < 0.05$ vs KA.

Figure 23. The graphs represent mTor, p-Akt, AMBRA 1, Beclin-1, LC3B and p62 (A, B, C, D, E, F) protein level assessed by Western blot analysis in hippocampus of different groups of rats: SHAM, rats administrated with KA, rats treated with EA, PA and

combination for 72 h with KA administration. The data are expressed as means ± SDs. * $p < 0.05$ vs. CTRL; # $p < 0.05$ vs. KA.

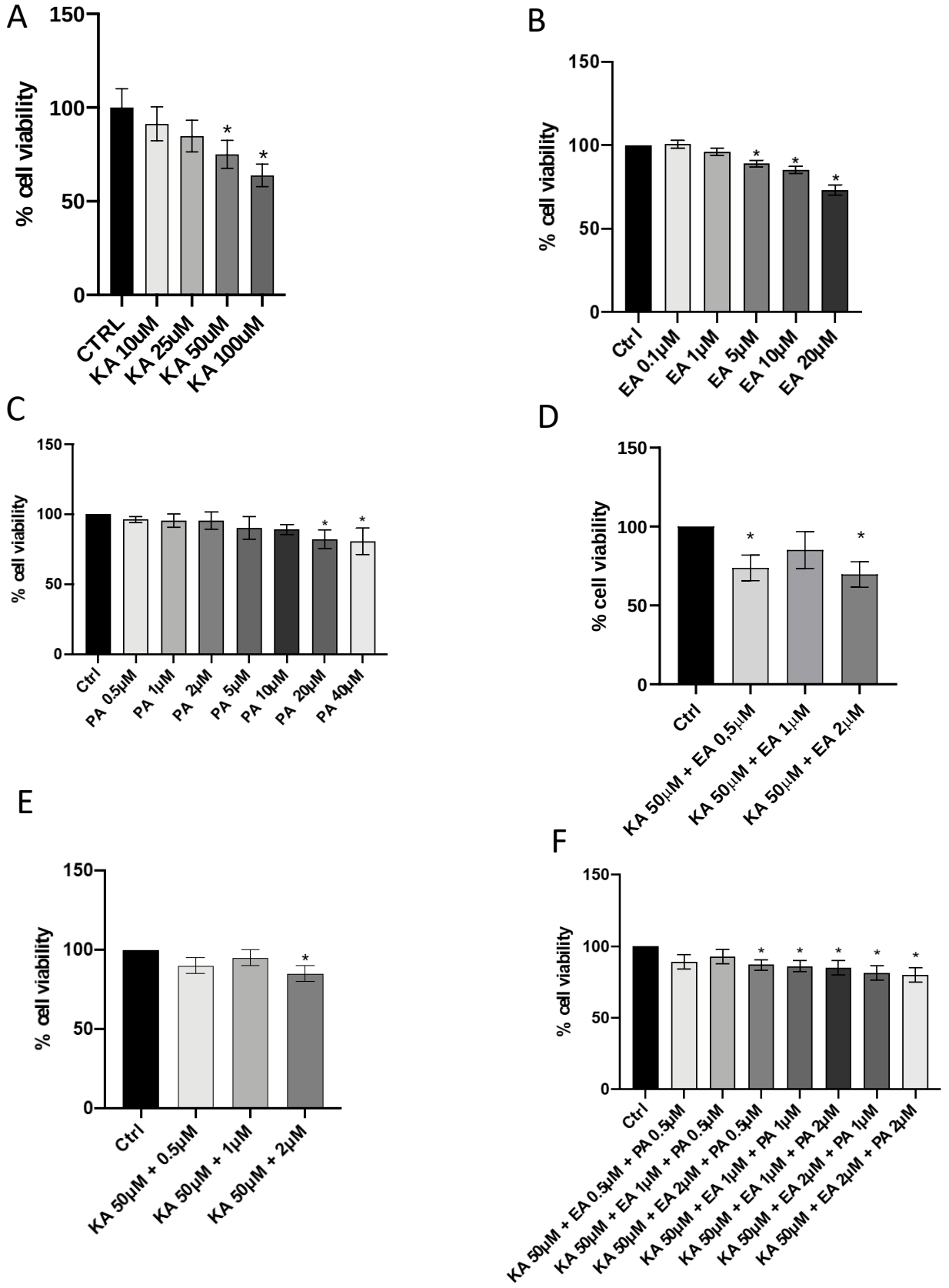
Figure 24. Images show Nissl staining performed on brain sections. SHAM group (A), KA group (B), KA+ EA group (C), KA+PA(D), KA+ EA+ PA (E).

Table 1. Primer list. Human primer used in this experiment.

Table 2. Primer list. Rat primer used in this experiment.

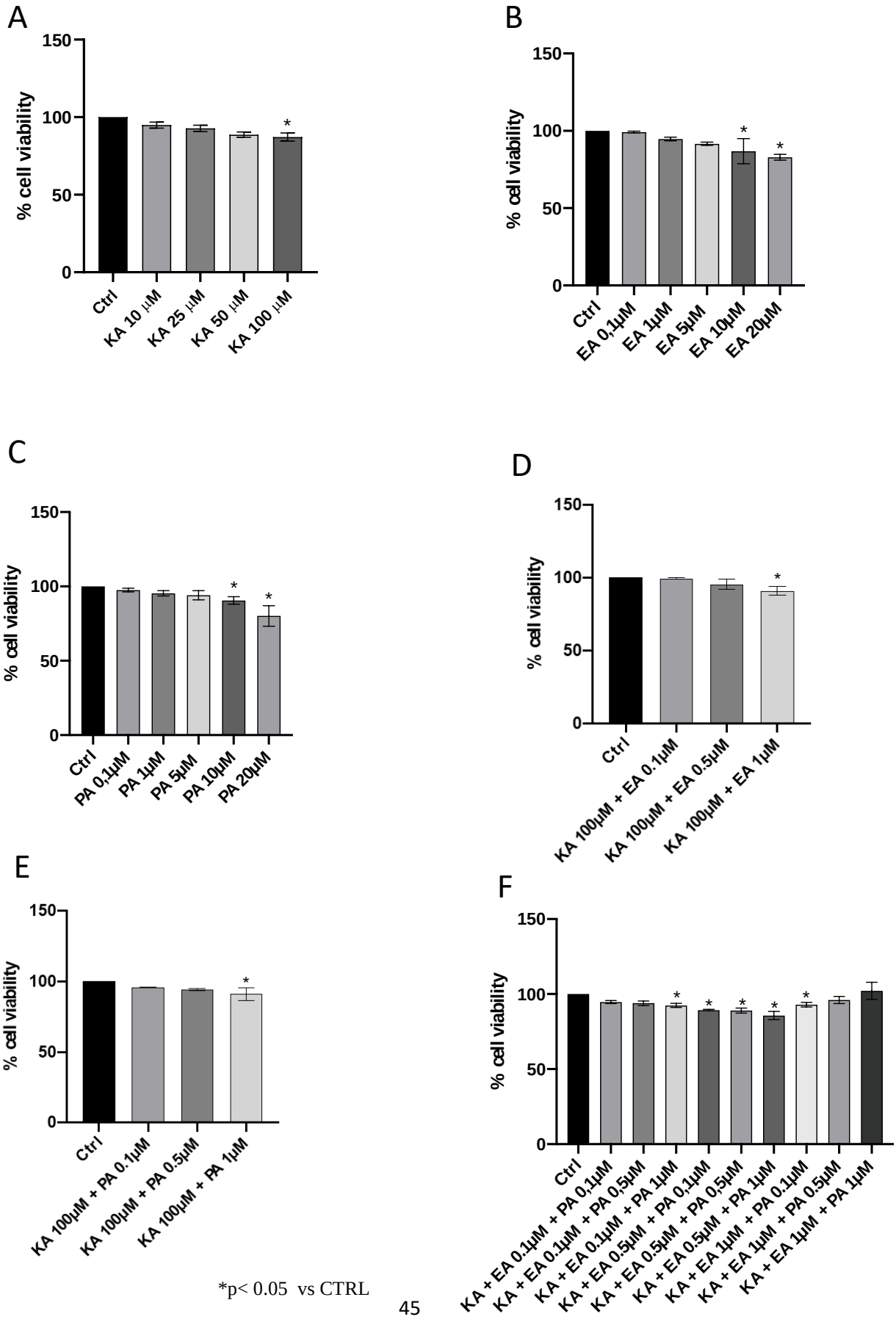
Figures and tables

Figure 1



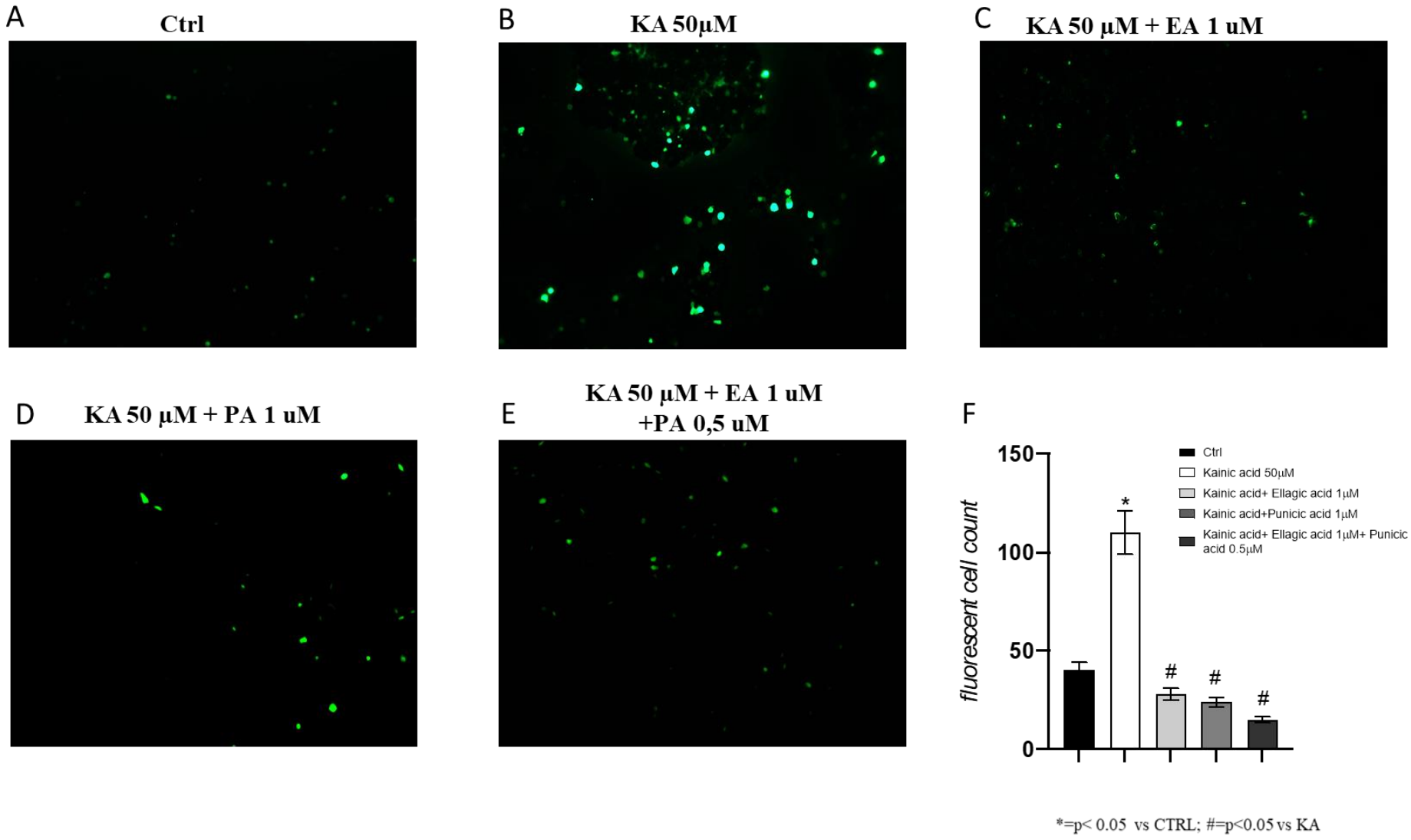
*p< 0.05 vs CTRL

Figure 2



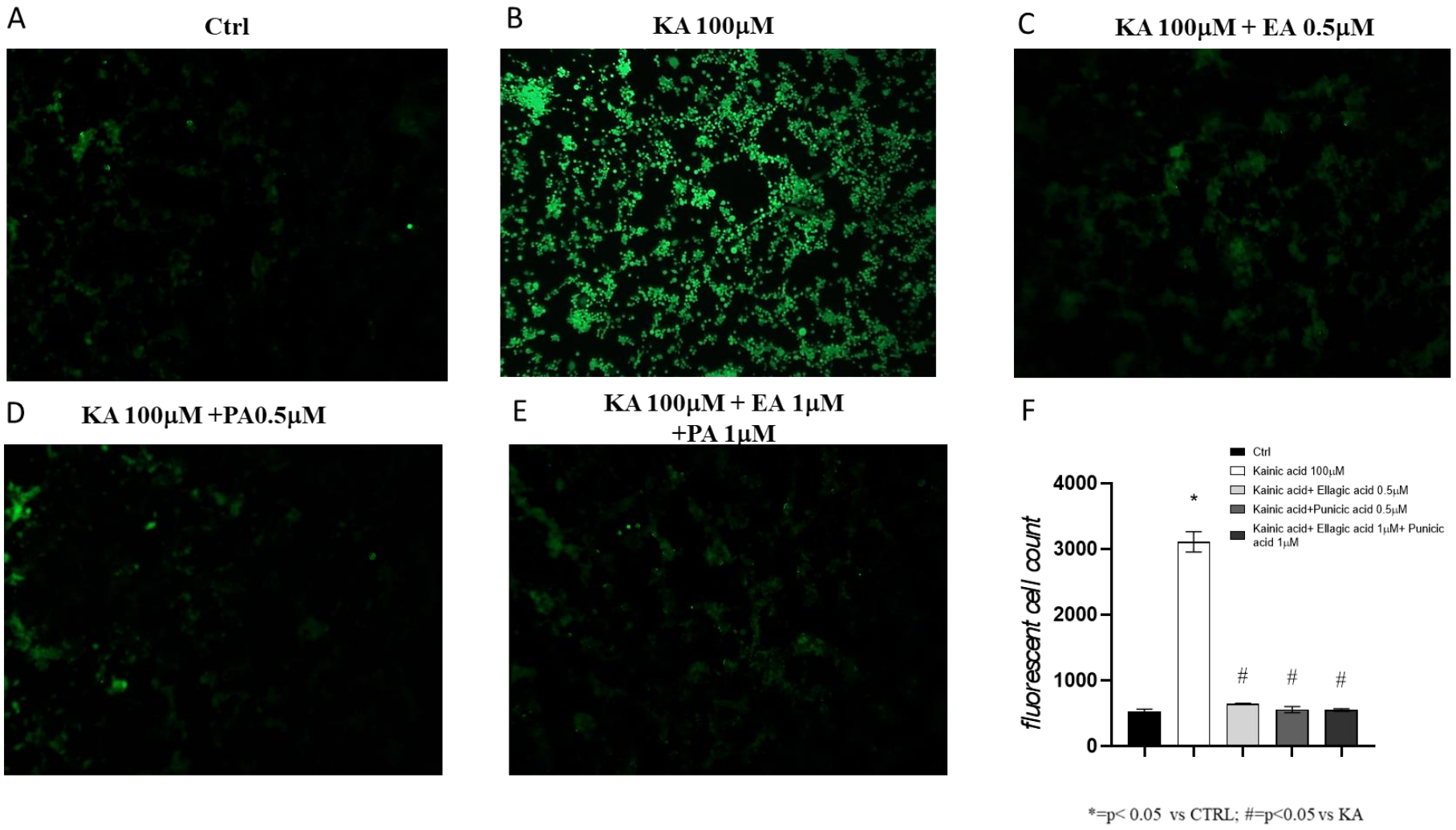
*p< 0.05 vs CTRL

Figure 3



*=p< 0.05 vs CTRL; #=p<0.05 vs KA

Figure 4



*=p< 0.05 vs CTRL; #=p<0.05 vs KA

Figure 5

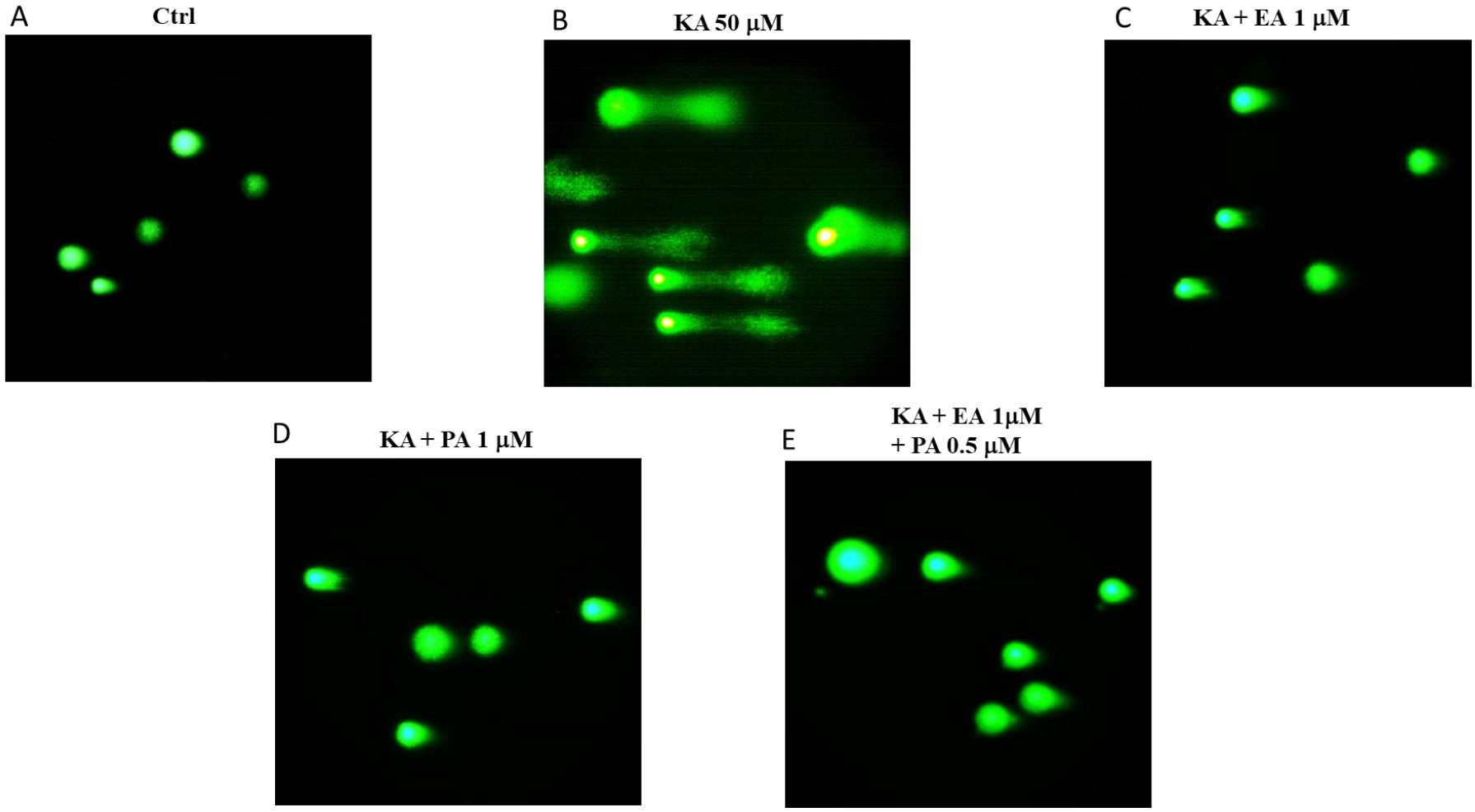


Figure 6

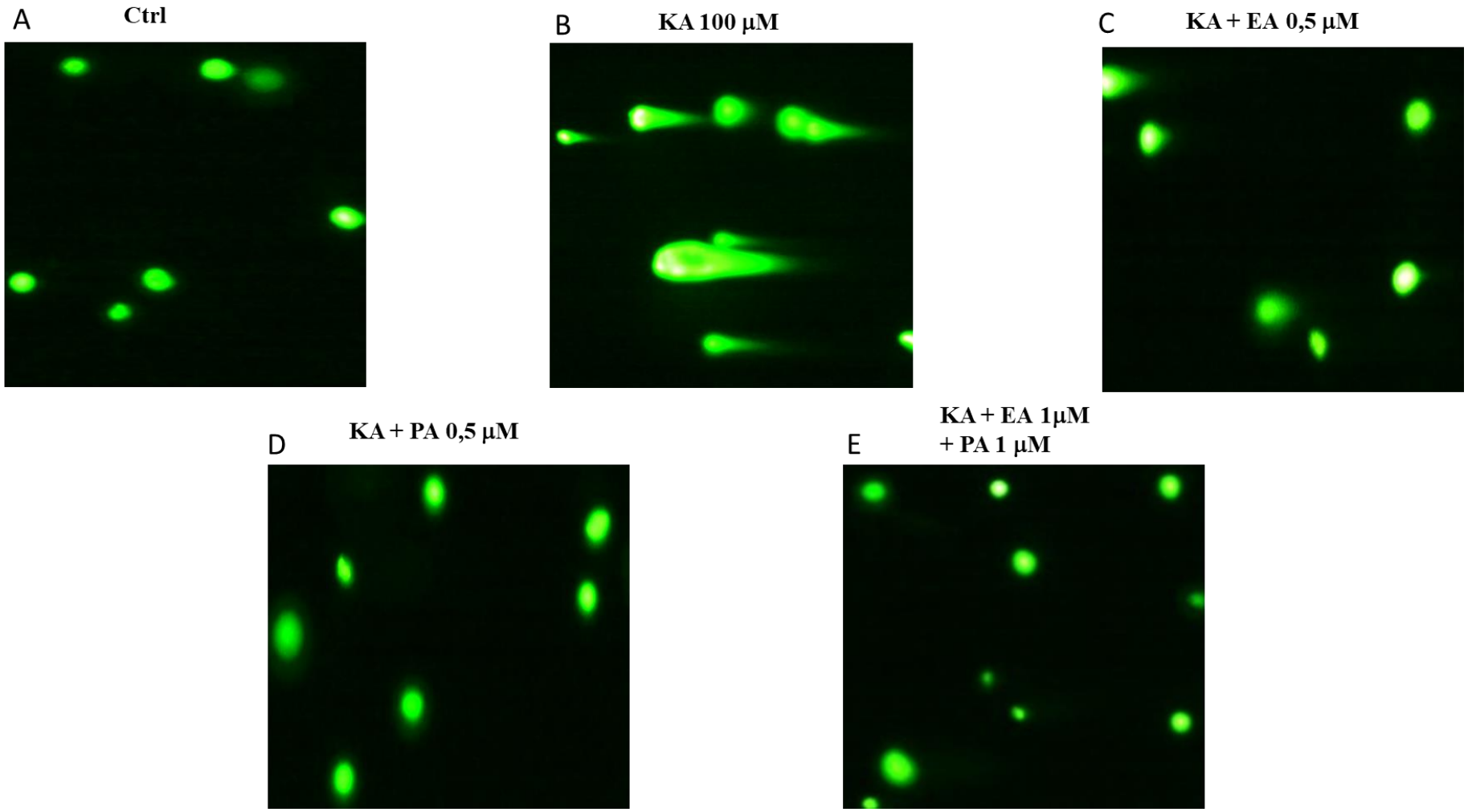


Figure 7

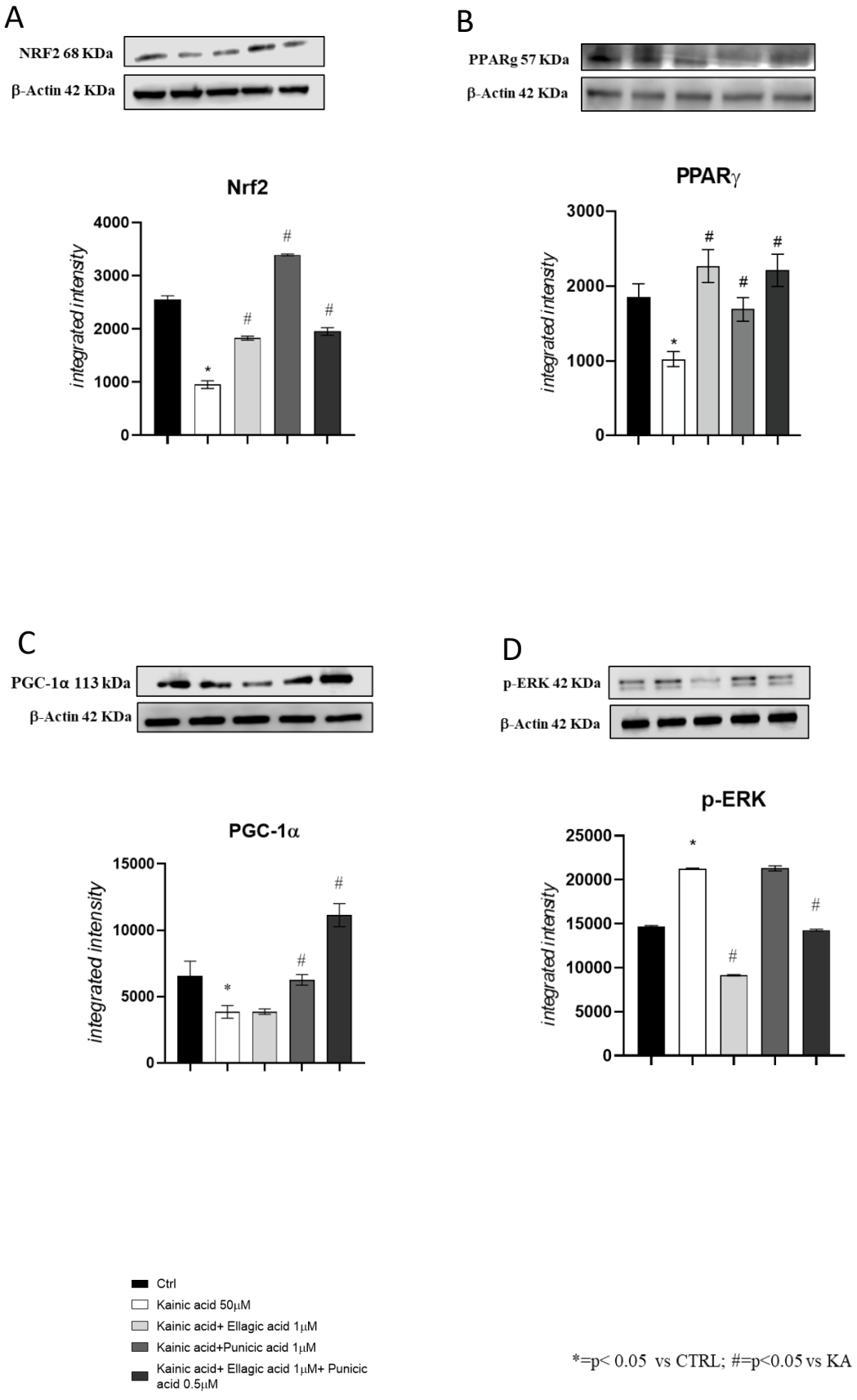

A
NRF2 68 KDa
β-Actin 42 KDa
Nrf2
integrated intensity
4000
3000
2000
1000
0
B
PPARg 57 KDa
β-Actin 42 KDa
PPARγ
integrated intensity
3000
2000
1000
0
C
PGC-1α 113 kDa
β-Actin 42 KDa
PGC-1α
integrated intensity
15000
10000
5000
0
D
p-ERK 42 KDa
β-Actin 42 KDa
p-ERK
integrated intensity
25000
20000
15000
10000
5000
0
Ctrl
Kainic acid 50μM
Kainic acid+ Ellagic acid 1μM
Kainic acid+Punicic acid 1μM
Kainic acid+ Ellagic acid 1μM+ Punicic acid 0.5μM
*=p< 0.05 vs CTRL; #=p<0.05 vs KA

Figure 8

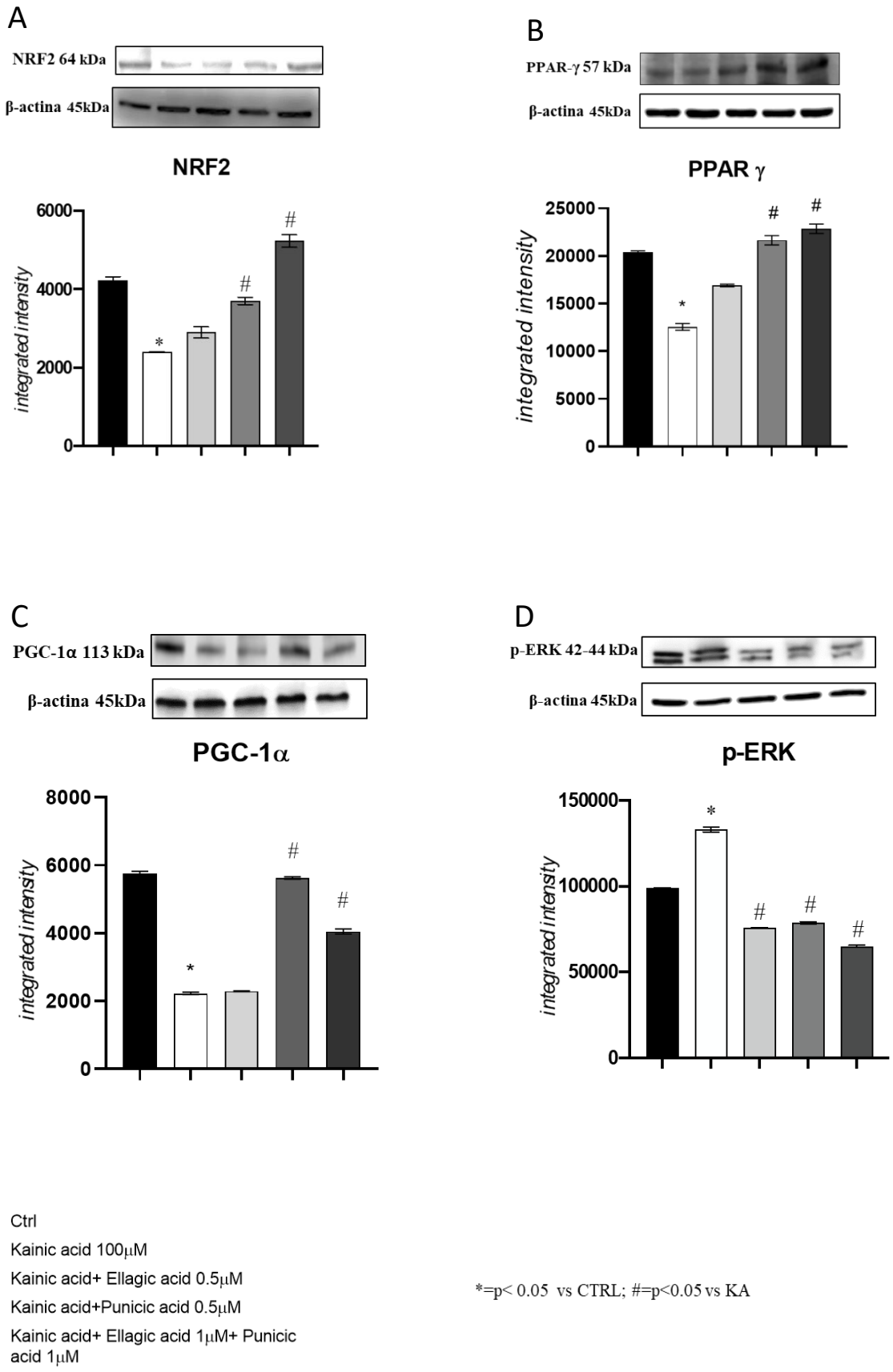

A
NRF2 64 kDa
β-actina 45kDa
NRF2
integrated intensity
6000
4000
2000
0
*
#
#
#
B
PPAR-γ 57 kDa
β-actina 45kDa
PPAR γ
integrated intensity
25000
20000
15000
10000
5000
0
*
#
#
C
PGC-1α 113 kDa
β-actina 45kDa
PGC-1α
integrated intensity
8000
6000
4000
2000
0
*
#
#
D
p-ERK 42-44 kDa
β-actina 45kDa
p-ERK
integrated intensity
150000
100000
50000
0
*
#
#
#
Ctrl
Kainic acid 100μM
Kainic acid+ Ellagic acid 0.5μM
Kainic acid+Punicic acid 0.5μM
Kainic acid+ Ellagic acid 1μM+ Punicic acid 1μM
*=p< 0.05 vs CTRL; #=p<0.05 vs KA

Figure 9

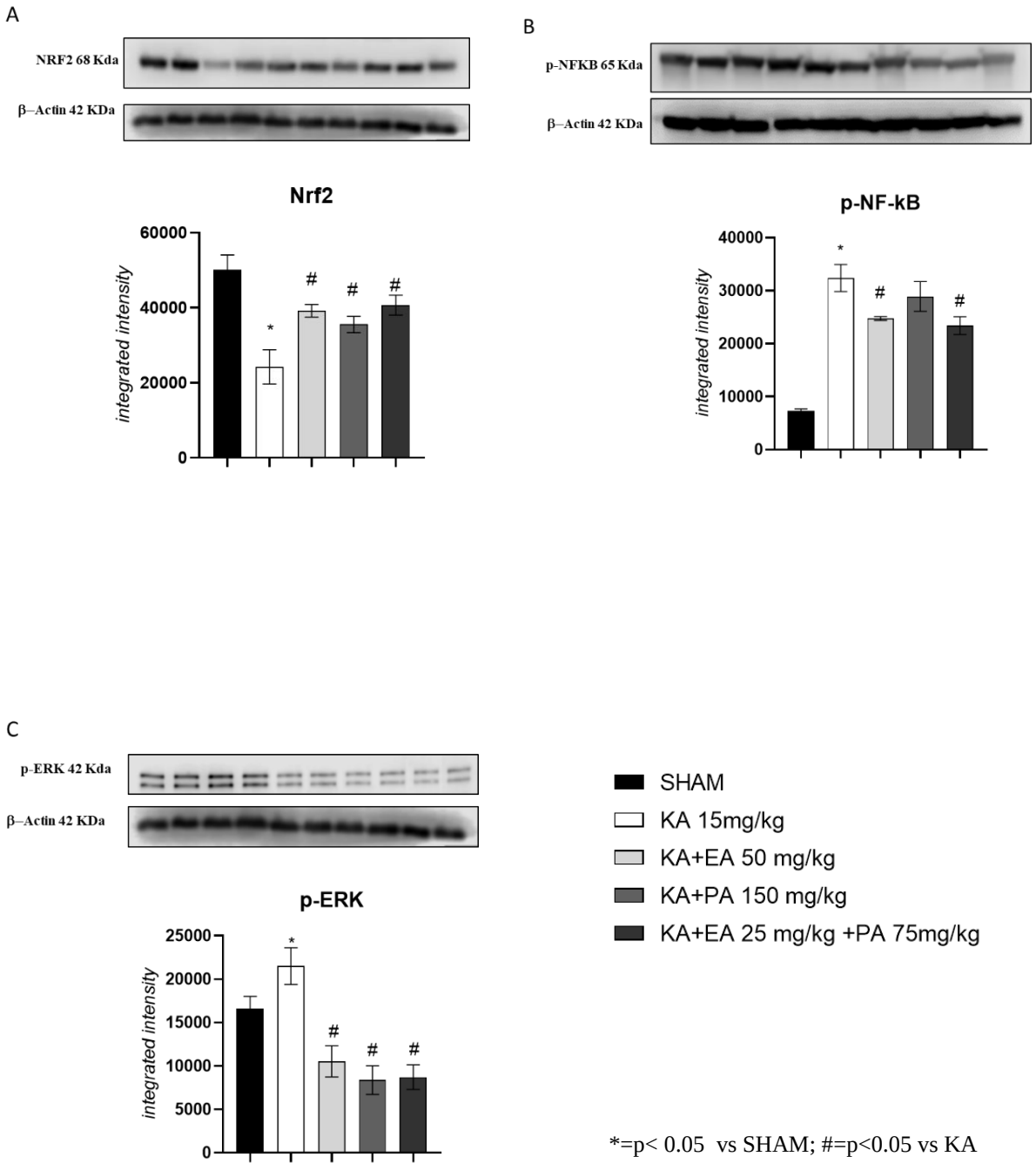

A
NRF2 68 Kda
β–Actin 42 KDa
Nrf2
integrated intensity
60000
40000
20000
0
*
#
#
#
B
p-NFKB 65 Kda
β–Actin 42 KDa
p-NF-kB
integrated intensity
40000
30000
20000
10000
0
*
#
#
C
p-ERK 42 Kda
β–Actin 42 KDa
p-ERK
integrated intensity
25000
20000
15000
10000
5000
0
*
#
#
#
SHAM
KA 15mg/kg
KA+EA 50 mg/kg
KA+PA 150 mg/kg
KA+EA 25 mg/kg +PA 75mg/kg
*=p< 0.05 vs SHAM; #=p<0.05 vs KA

Figure 10

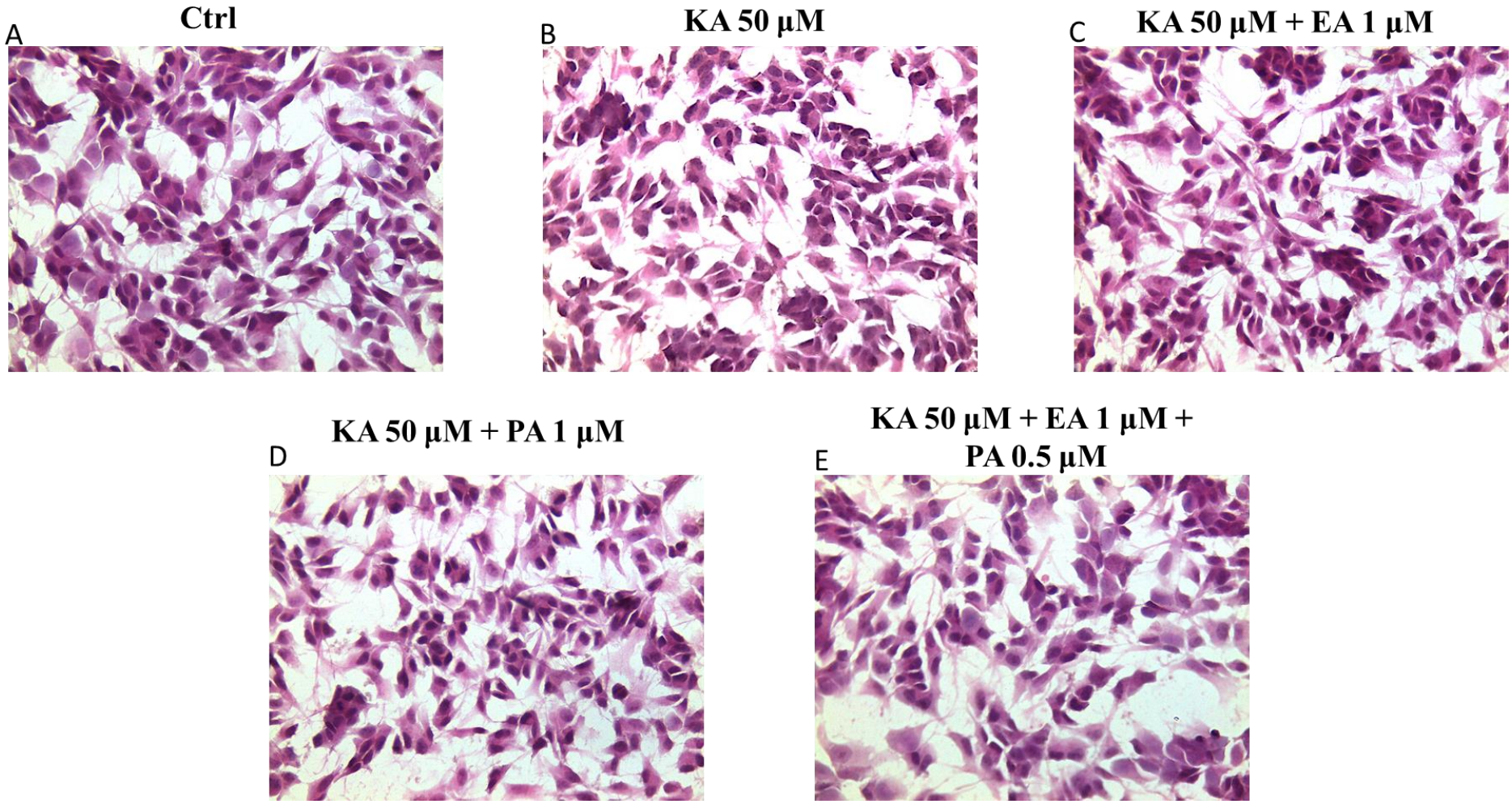

A
Ctrl
B
KA 50 µM
C
KA 50 µM + EA 1 µM
D
KA 50 µM + PA 1 µM
E
KA 50 µM + EA 1 µM +
PA 0.5 µM

Figure 11

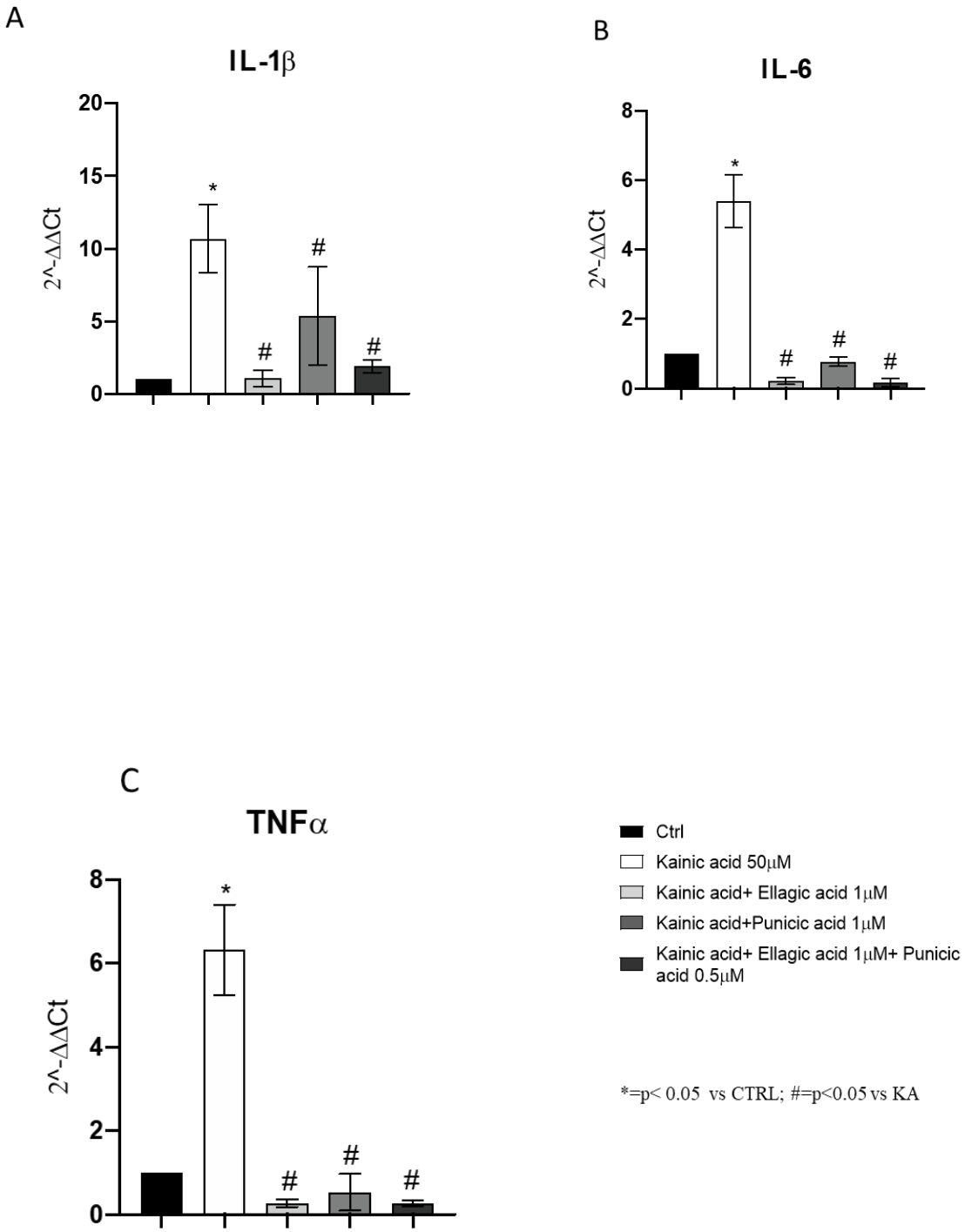

A
IL-1β
2^-ΔΔCt
20
15
10
5
0
*
#
#
#
B
IL-6
2^-ΔΔCt
8
6
4
2
0
*
#
#
#
C
TNFα
2^-ΔΔCt
8
6
4
2
0
*
#
#
#
Ctrl
Kainic acid 50μM
Kainic acid+ Ellagic acid 1μM
Kainic acid+Punicic acid 1μM
Kainic acid+ Ellagic acid 1μM+ Punicic acid 0.5μM
*=p< 0.05 vs CTRL; #=p<0.05 vs KA

Figure 12

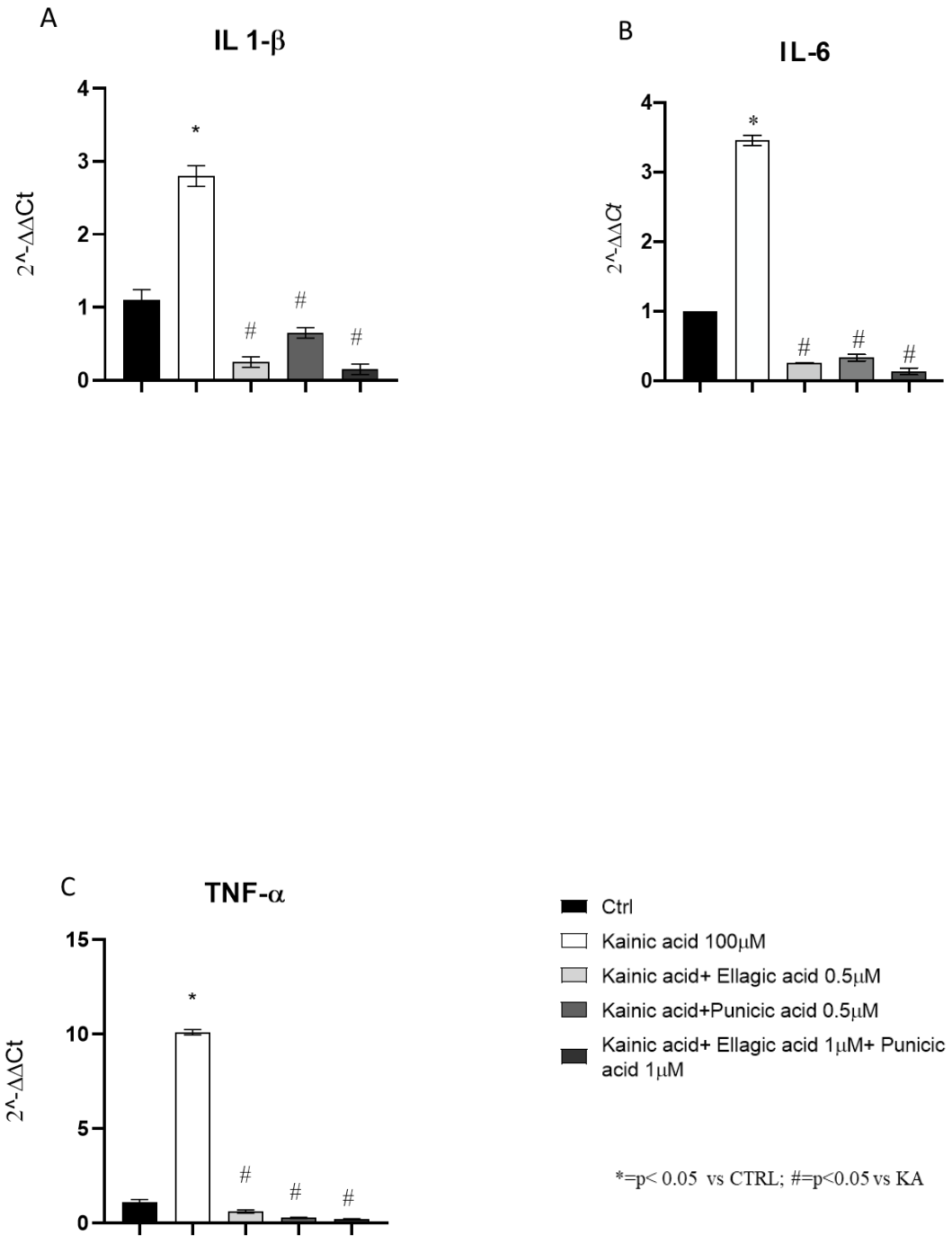

A
IL 1-β
2^-ΔΔCt
B
IL-6
2^-ΔΔCt
C
TNF-α
2^-ΔΔCt
Ctrl
Kainic acid 100μM
Kainic acid+ Ellagic acid 0.5μM
Kainic acid+Punicic acid 0.5μM
Kainic acid+ Ellagic acid 1μM+ Punicic acid 1μM
*=p< 0.05 vs CTRL; #=p<0.05 vs KA

Figure 13

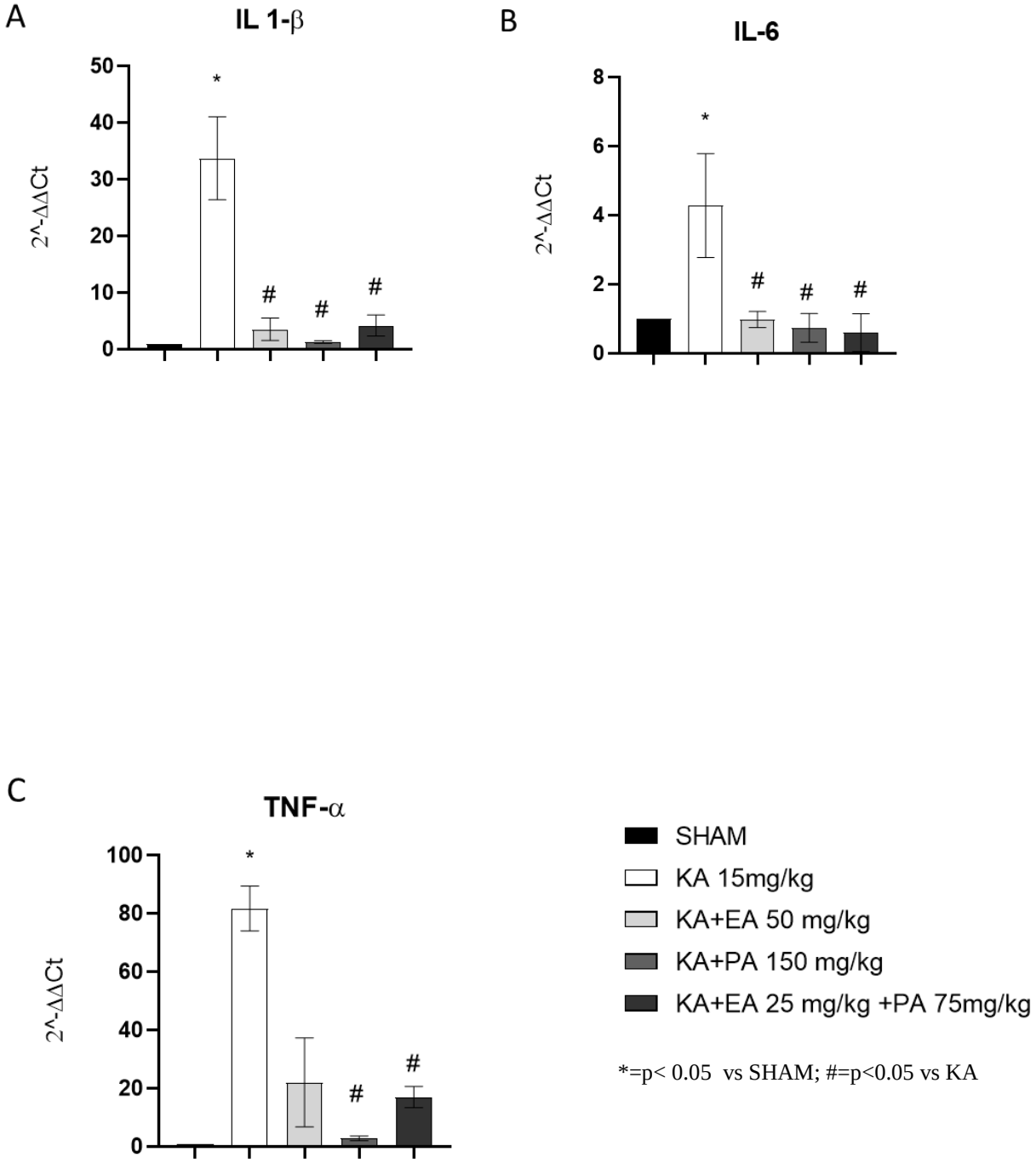

A
IL 1-β
2^-ΔΔCt
50
40
30
20
10
0
*
#
#
#
B
IL-6
2^-ΔΔCt
8
6
4
2
0
*
#
#
#
C
TNF-α
2^-ΔΔCt
100
80
60
40
20
0
*
#
#
SHAM
KA 15mg/kg
KA+EA 50 mg/kg
KA+PA 150 mg/kg
KA+EA 25 mg/kg +PA 75mg/kg
*=p< 0.05 vs SHAM; #=p<0.05 vs KA

Figure 14

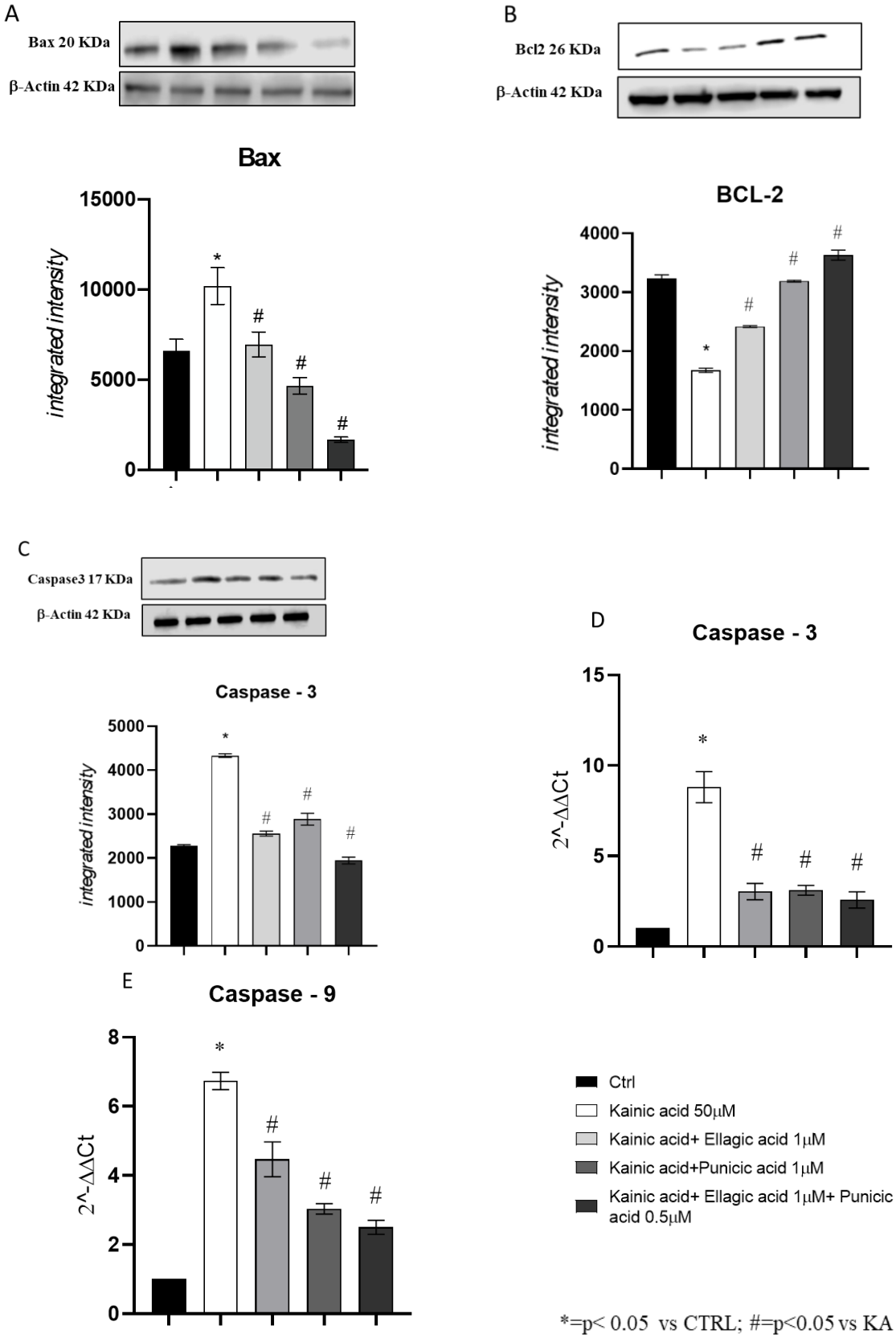

A
Bax 20 KDa
β-Actin 42 KDa
B
Bcl2 26 KDa
β-Actin 42 KDa
Bax
integrated intensity
15000
10000
5000
0
BCL-2
integrated intensity
4000
3000
2000
1000
0
C
Caspase3 17 KDa
β-Actin 42 KDa
Caspase - 3
integrated intensity
5000
4000
3000
2000
1000
0
D
Caspase - 3
2^-ΔΔCt
15
10
5
0
E
Caspase - 9
2^-ΔΔCt
8
6
4
2
0
Ctrl
Kainic acid 50μM
Kainic acid+ Ellagic acid 1μM
Kainic acid+Punicic acid 1μM
Kainic acid+ Ellagic acid 1μM+ Punicic acid 0.5μM
*=p< 0.05 vs CTRL; #=p<0.05 vs KA

Figure 15

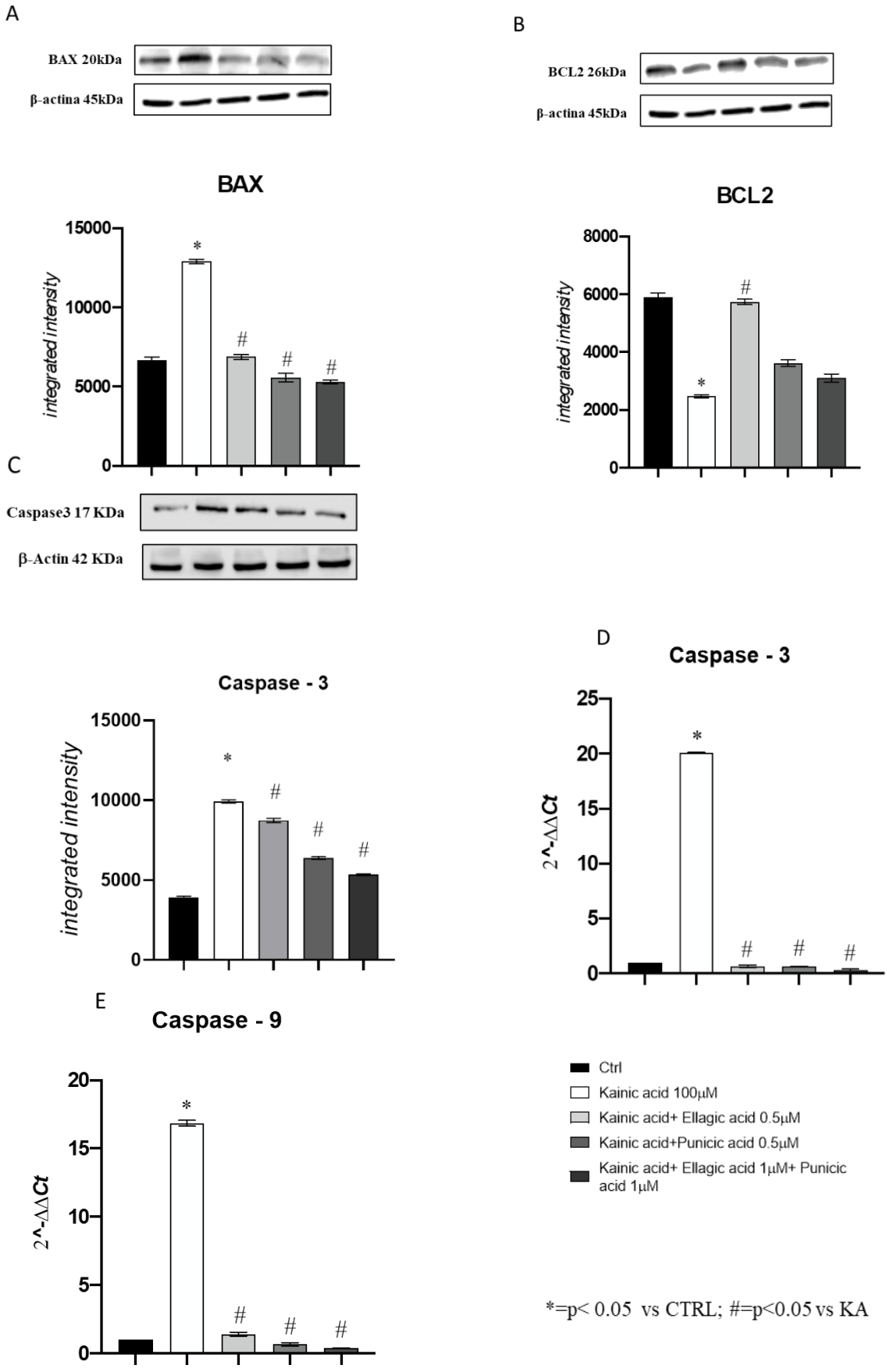
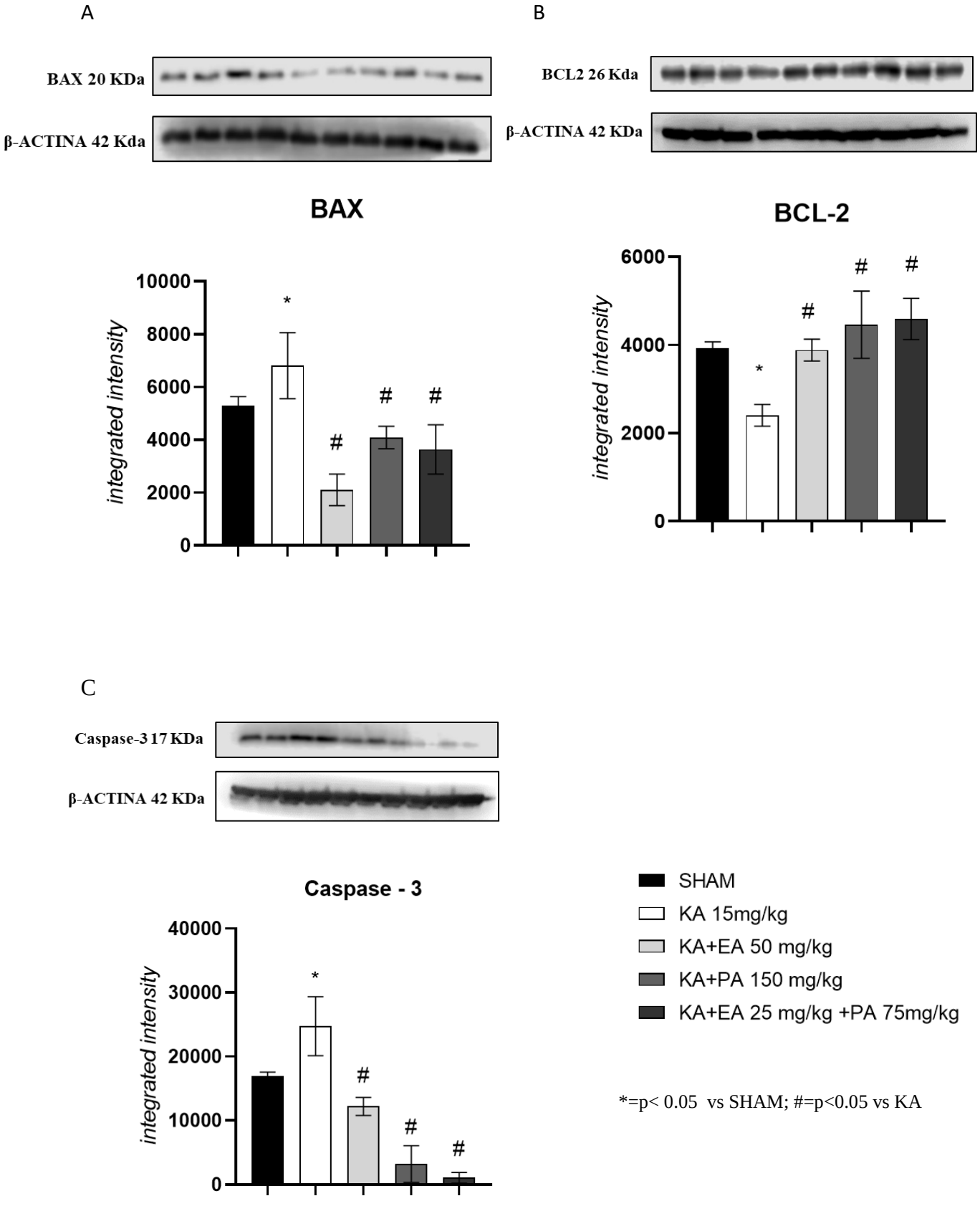
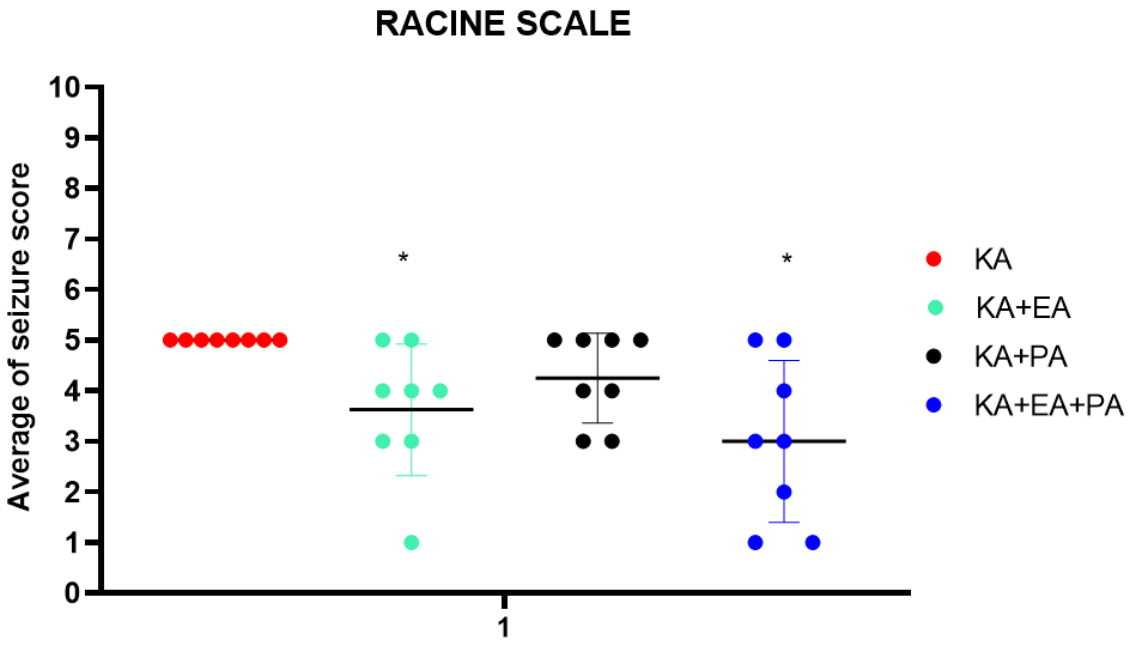

A
BAX 20kDa
β-actina 45kDa
B
BCL2 26kDa
β-actina 45kDa
BAX
integrated intensity
15000
10000
5000
0
BCL2
integrated intensity
8000
6000
4000
2000
0
C
Caspase3 17 KDa
β-Actin 42 KDa
Caspase - 3
integrated intensity
15000
10000
5000
0
D
Caspase - 3
2^-ΔΔCt
25
20
15
10
5
0
E
Caspase - 9
2^-ΔΔCt
20
15
10
5
0
Ctrl
Kainic acid 100μM
Kainic acid+ Ellagic acid 0.5μM
Kainic acid+Punicic acid 0.5μM
Kainic acid+ Ellagic acid 1μM+ Punicic acid 1μM
*=p< 0.05 vs CTRL; #=p<0.05 vs KA

Figure 16



*=p< 0.05 vs SHAM; #=p<0.05 vs KA

Figure 17



* =p<0.05 vs KA

Figure 18

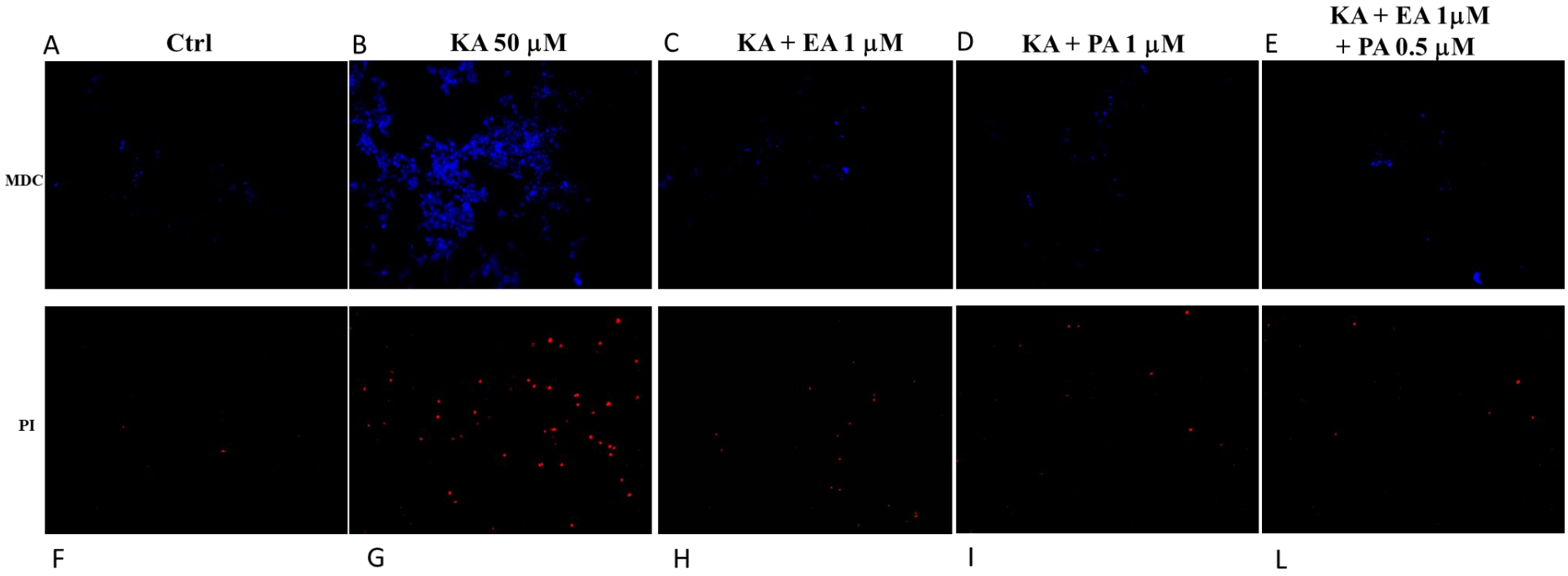

A
Ctrl
B
KA 50 µM
C
KA + EA 1 µM
D
KA + PA 1 µM
E
KA + EA 1µM
+ PA 0.5 µM
MDC
PI
F
G
H
I
L

Figure 19

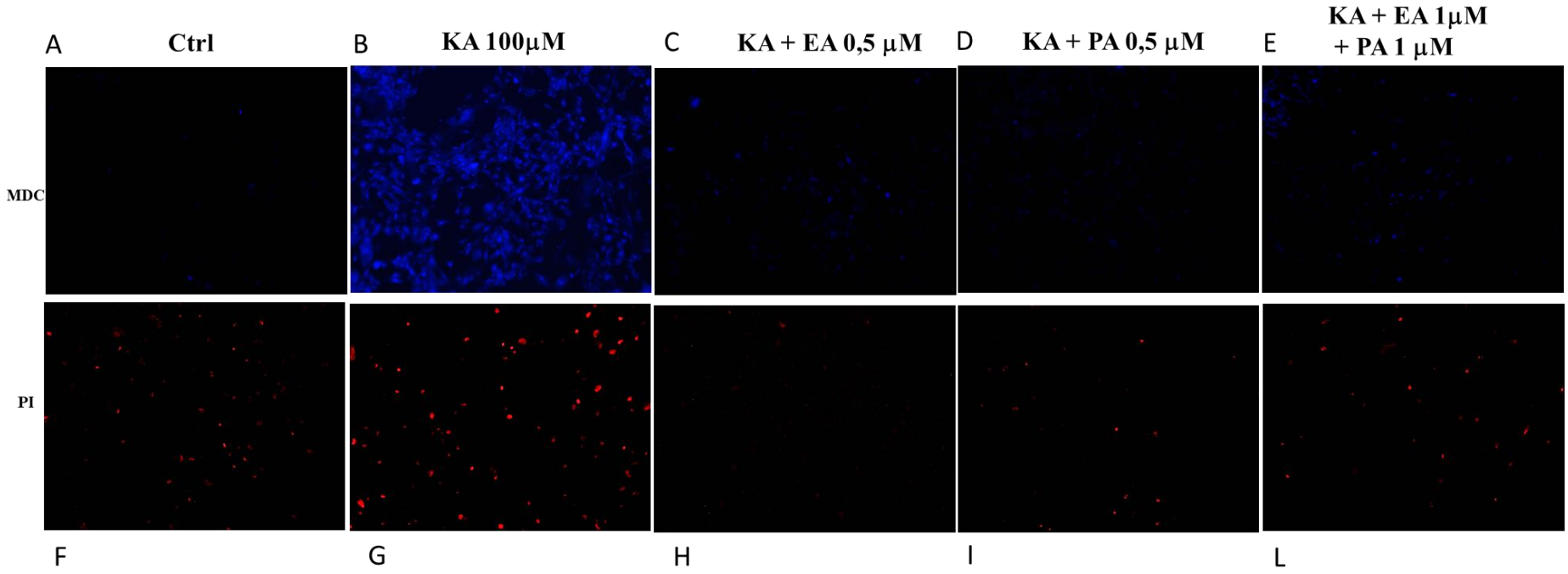

A
Ctrl
B
KA 100µM
C
KA + EA 0,5 µM
D
KA + PA 0,5 µM
E
KA + EA 1µM
+ PA 1 µM
MDC
PI
F
G
H
I
L

Figure 20

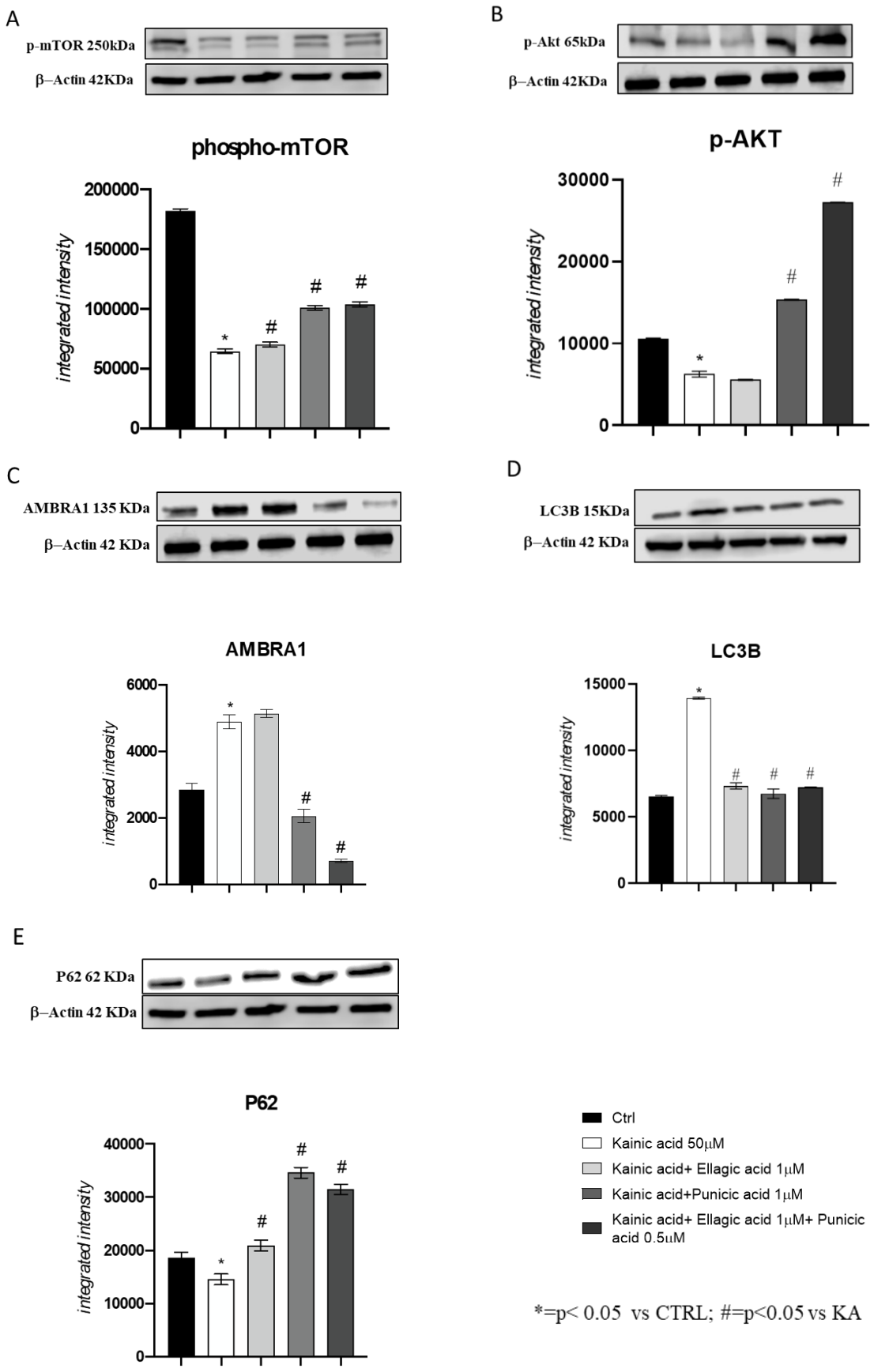

A
p-mTOR 250kDa
β–Actin 42KDa
B
p-Akt 65kDa
β–Actin 42KDa
phospho-mTOR
integrated intensity
200000
150000
100000
50000
0
p-AKT
30000
20000
10000
0
C
AMBRA1 135 KDa
β–Actin 42 KDa
D
LC3B 15KDa
β–Actin 42 KDa
AMBRA1
6000
4000
2000
0
LC3B
15000
10000
5000
0
E
P62 62 KDa
β–Actin 42 KDa
P62
40000
30000
20000
10000
0
Ctrl
Kainic acid 50μM
Kainic acid+ Ellagic acid 1μM
Kainic acid+Punicic acid 1μM
Kainic acid+ Ellagic acid 1μM+ Punicic acid 0.5μM
*=p< 0.05 vs CTRL; #=p<0.05 vs KA

Figure 21

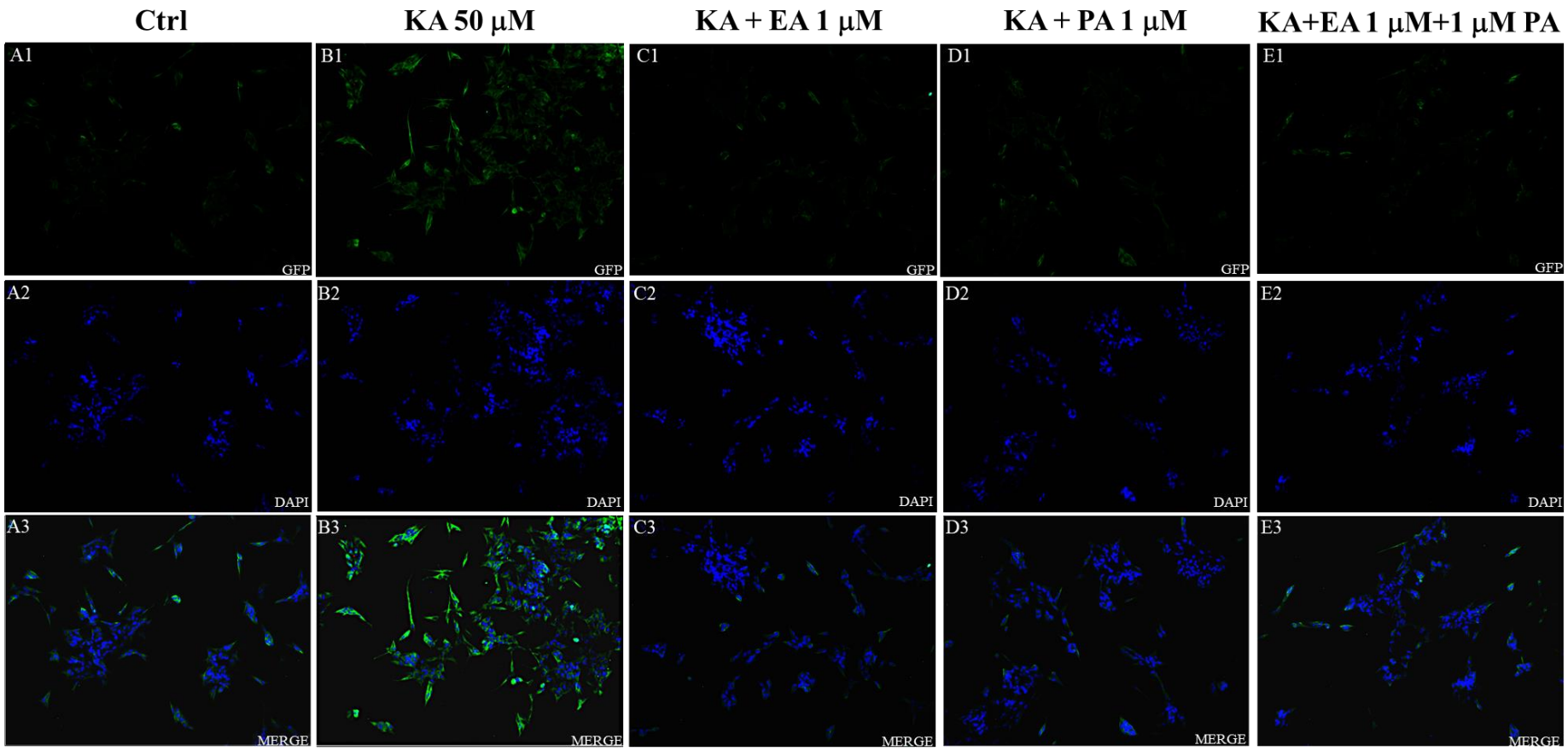

Ctrl
KA 50 µM
KA + EA 1 µM
KA + PA 1 µM
KA+EA 1 µM+1 µM PA
A1
B1
C1
D1
E1
GFP
A2
B2
C2
D2
E2
DAPI
A3
B3
C3
D3
E3
MERGE

Figure 22

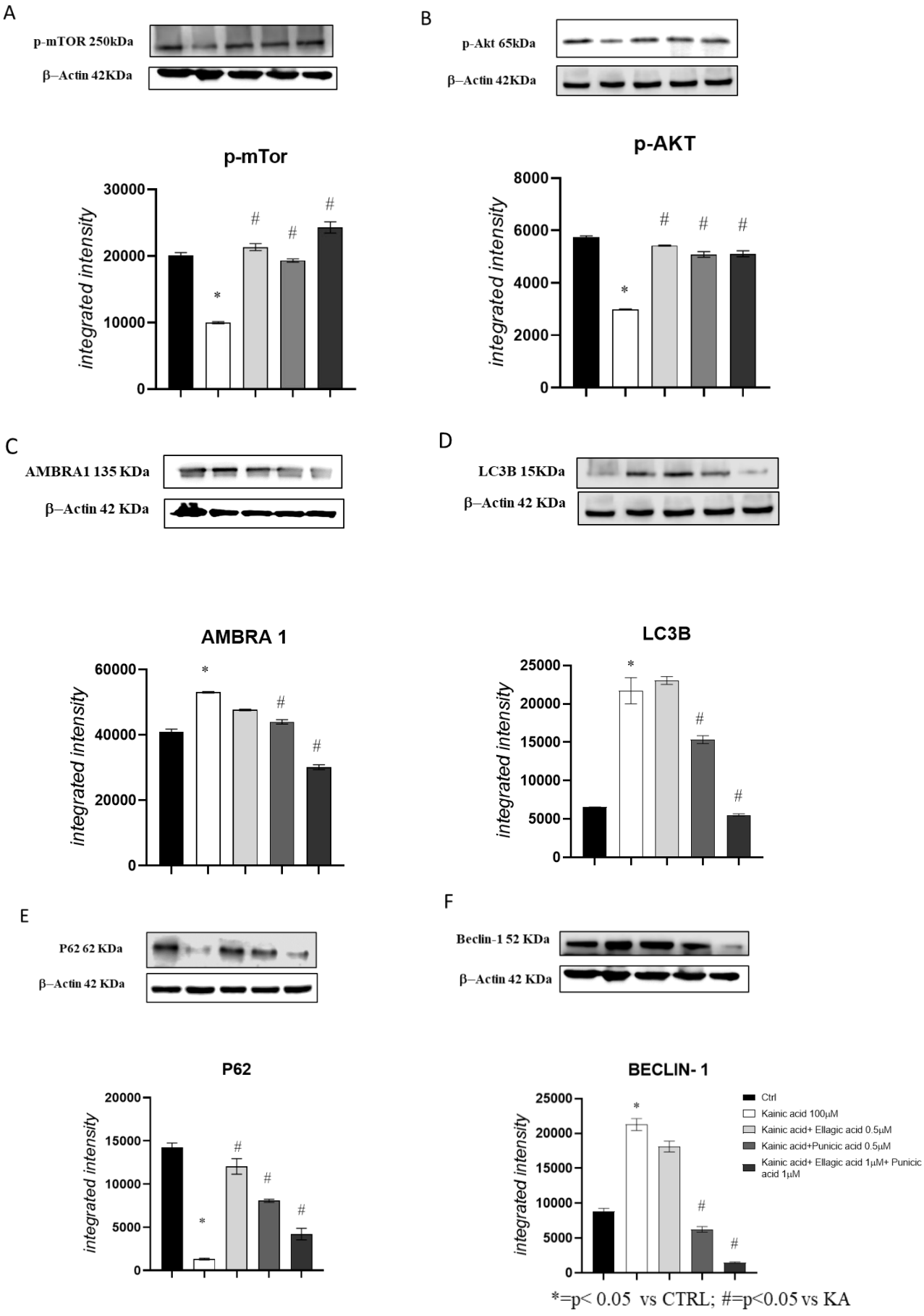
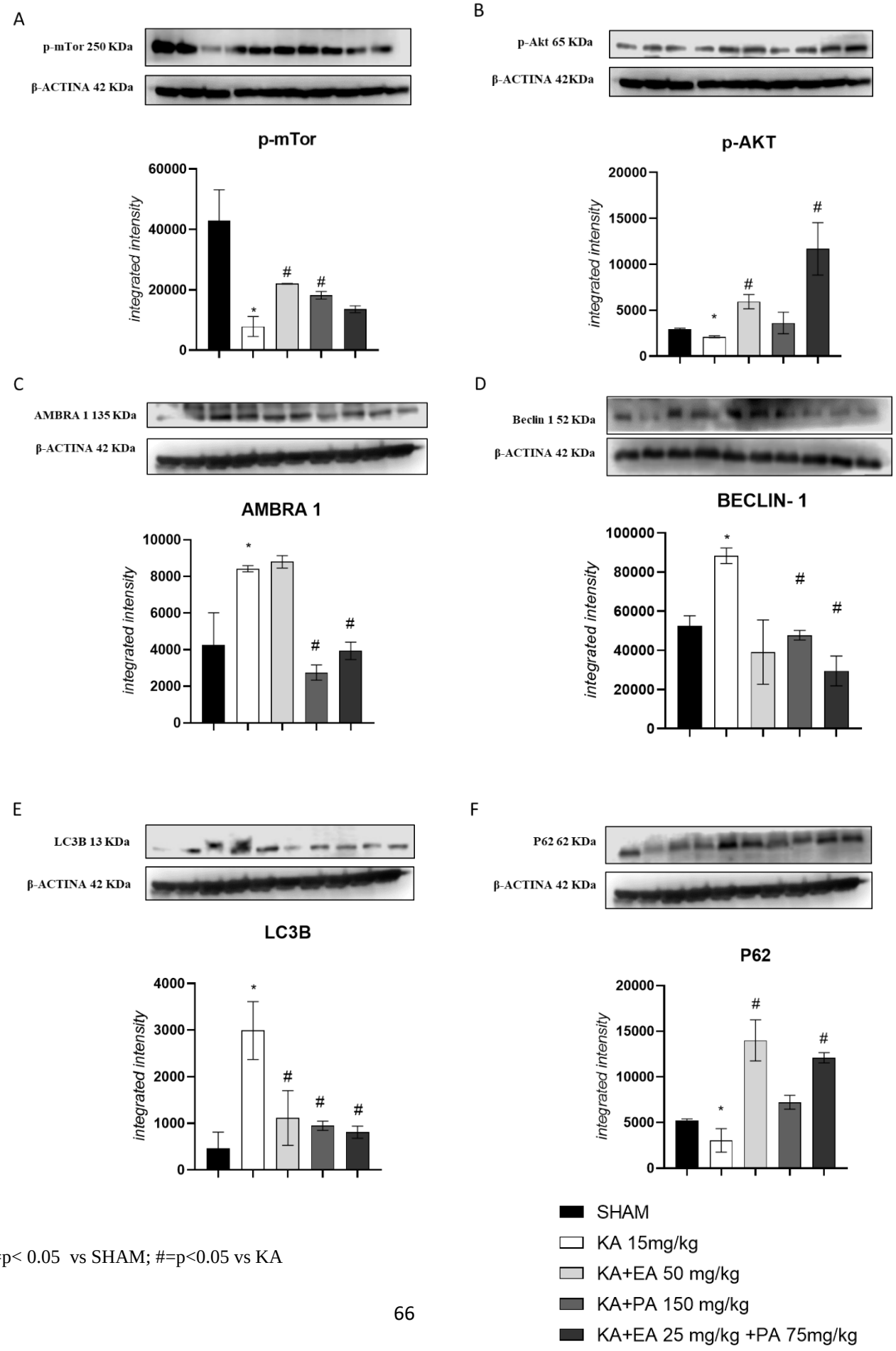

A
p-mTOR 250kDa
β–Actin 42KDa
B
p-Akt 65kDa
β–Actin 42KDa
p-mTor
integrated intensity
30000
20000
10000
0
p-AKT
integrated intensity
8000
6000
4000
2000
0
C
AMBRA1 135 KDa
β–Actin 42 KDa
D
LC3B 15KDa
β–Actin 42 KDa
AMBRA 1
integrated intensity
60000
40000
20000
0
LC3B
integrated intensity
25000
20000
15000
10000
5000
0
E
P62 62 KDa
β–Actin 42 KDa
F
Beclin-1 52 KDa
β–Actin 42 KDa
P62
integrated intensity
20000
15000
10000
5000
0
BECLIN- 1
integrated intensity
25000
20000
15000
10000
5000
0
Ctrl
Kainic acid 100μM
Kainic acid+ Ellagic acid 0.5μM
Kainic acid+Punicic acid 0.5μM
Kainic acid+ Ellagic acid 1μM+ Punicic acid 1μM
*=p< 0.05 vs CTRL; #=p<0.05 vs KA

Figure 23



*=p< 0.05 vs SHAM; #=p<0.05 vs KA

Figure 24

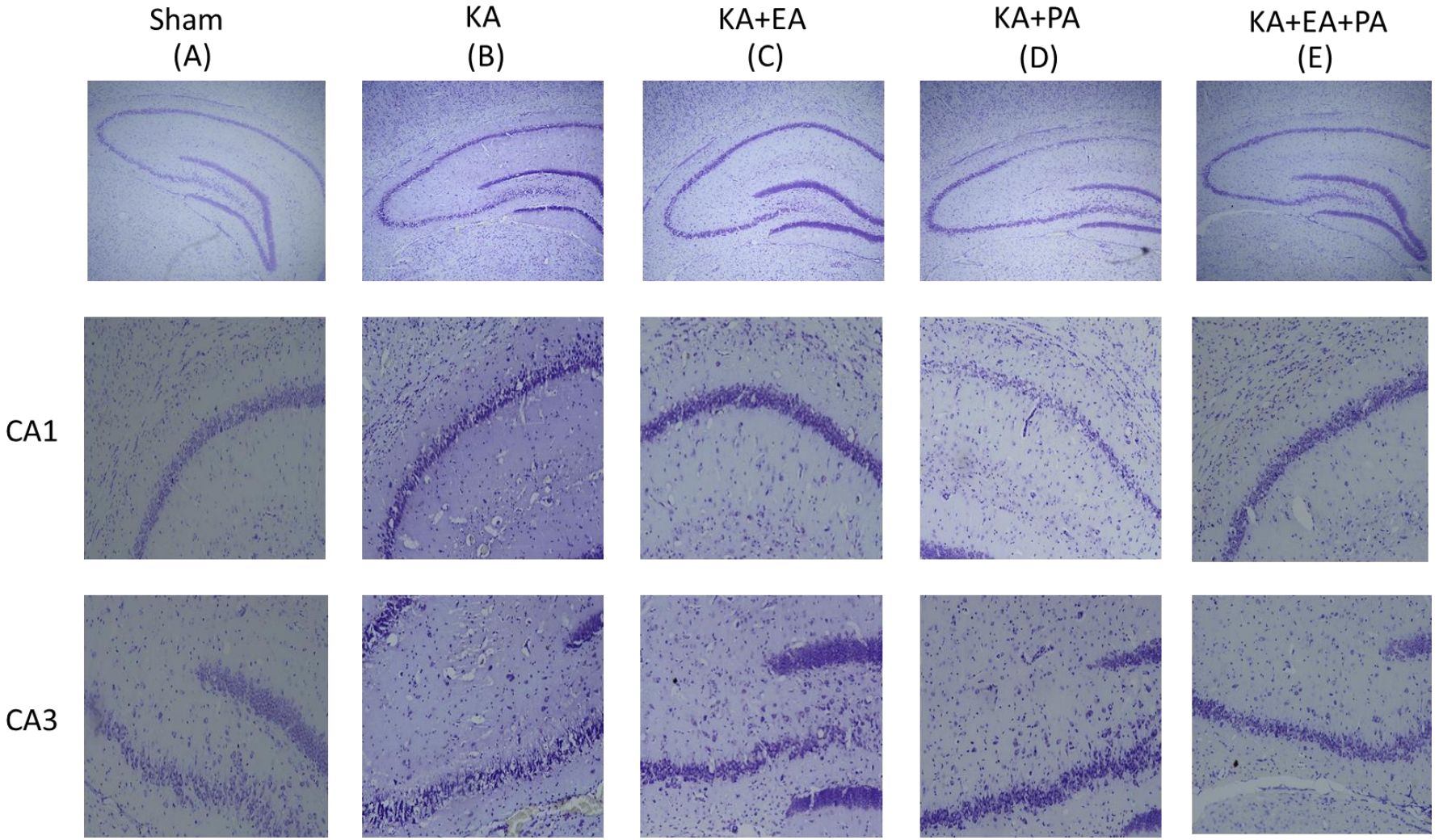


Table1

GENE NAME	**SEQUENCE**
GADPH HUMAN	Primer F GAG TCA ACG GAT TTG GTC GT Primer R TTG ATT TTG GAG GGA TCT CG
IL-1β HUMAN	Primer F GGG CCT CAA GGA AAA GAA TC Primer R TTC TGC TTG AGAGGT GCT GA
TNF-α HUMAN	Primer F TCCTTC AGA CAC CCT CAA CC Primer R AGG CCC CAG TTT GAA TTC TT
IL-6 HUMAN	Primer F TTT TCT GCC AGT GCC TCT TT Primer R TAC CCC CAG GAG AAG ATT CC
CASPASE-3	Primer F GCA GAG TCA AAG GCT GGA AC Primer R GAG TCA ACC AAG TTC GCA CA

CASPASE 9	Primer F GAT GCT GTC CCC TAT CAG GA Primer R GGG ACT GCA GGT CTT CAG AG

Table 2

GENE NAME	**SEQUENCE**
GADPH RAT	Primer F CTC ATG ACC ACA GTC CAT GC Primer R TTC AGC TCT GGG ATG ACC TT
IL-1β RAT	Primer F AGG CTT CCT TGT GCA AGT GT Primer R TGA GTGACA CTG CCT TCC TG
TNF-α RAT	Primer F AGT CCG GGC AGG TCT ACT TT Primer R GGC CAC TAC TTC AGC GTC TC

IL-6 RAT	Primer F CCG GAG AGG AGA CTT CAC AG Primer R ACA GTG CAT CAT CGC TGT TC