TOXICITY OF GLYCOALKALOIDS FROM *LYCOPERSICON ESCULENTUM* MILL. – A MECHANISTIC PERSPECTIVE

Daniela Correia da Silva

Dissertação do 2º Ciclo de Estudos conducente

ao **Grau de Mestre em Toxicologia Analítica, Clínica e Forense**
Junho de 2016

Trabalho realizado sob a orientação dos professores:

Prof. Doutora Paula Andrade (Orientadora) e Prof. Doutor David Pereira (Co-orientador)

Acknowledgements

First of all, I would like to express my gratitude to my supervisors, Professor Doctor Paula Andrade and Professor Doctor David Pereira, for their guidance, helpfulness, useful remarks and especially for everything they taught me during the development of this work. I would also like to thank my colleagues for their support along the way, which made the difficulties so much easier to overcome.

Finally, I am grateful to my loved ones, especially my parents, for once again showing so much mindfulness and affection.

Abstract

Glycoalkaloids are steroidal saponins generally occurring in the Solanaceae family, among which we can find tomatine and its aglycon tomatidine, which are mainly synthesised in the tomato plant (*Lycopersicon esculentum* Mill). Several biological activities have been described for these molecules, from anti-cancer to anti-inflammatory and antibacterial.

We have evaluated the toxicity of these molecules in neuronal cells, namely in the neuroblastoma cell line SH-SY5Y. This work aims to clarify the cellular mechanisms underlying the effects of both compounds on these cells. Furthermore, we evaluated the toxicity of these compounds in gastric adenocarcinoma cells (AGS) and macrophages (RAW 264.7).

We have found that tomatine is cytotoxic to neuronal and gastric cells in concentrations starting at 1 µM, while macrophages were only susceptible to this compound at a concentration of 2 µM or superior. The corresponding aglycon, tomatidine, is far less cytotoxic, exerting no toxicity in the tested concentrations (up to 25 µM) in AGS and RAW 264.7 cells, and being safe to SH-SY5Y cells up to 6.25 µM.

In light of these results, we were interested in clarifying the mechanisms underlying cell death. For this purpose, we analysed caspase involvement in the process, and reached the conclusion that tomatine/tomatidine induced-cell death is caspase-independent, a result that was confirmed by the absence of classical traits of apoptosis found upon assessment of cellular morphology. We verified also that these compounds are able to disrupt calcium homeostasis in SH-SY5Y cells. In light of this, we deemed relevant to study the involvement of the endoplasmic reticulum on cell death, and verified the involvement of the PERK/eIF2α branch of the UPR in tomatine/tomatidine-induced neuronal cell death.

This work is important because the tomato is largely consumed by the human population worldwide. It is therefore important to evaluate the toxicity and know the underlying mechanisms of compounds that we often ingest.

Resumo

Os glicoalcaloides são saponinas esteroídicas que geralmente ocorrem na família Solanaceae, entre os quais podemos encontrar a tomatina e a sua aglícona tomatidina, que provêm especialmente do tomateiro (*Lycopersicon esculentum* Mill.). São atribuídas a estas moléculas diversas atividades biológicas, desde anti-cancerígena a anti-inflamatória ou antibacteriana.

Neste trabalho, avaliou-se a toxicidade destas moléculas em células de neuroblastoma humano, nomeadamente na linha cellular SH-SY5Y. Foi avaliada ainda a sua toxicidade em células de adenocarcinoma gástrico humano (AGS) e macrófagos (RAW 264.7).

Concluíu-se que a tomatina é tóxica para as células neuronais e gástricas em concentrações iguais ou superiores a 1 μM, enquanto que os macrófagos só são suscetíveis ao mesmo composto na concentração de 2 μM. Por sua vez, a tomatidina é bastante menos citotóxica, não exercendo toxicidade nas células AGS e RAW 264.7 nas concentrações testadas, e sendo inofensiva para as células SH-SY5Y até uma concentração de 6.25 μM.

À luz destes resultados, considerou-se que seria de interesse clarificar os mecanismos que governam a morte das células nestas condições. Para tal, analisou-se o envolvimento das caspases nesse processo, tendo-se concluído que o mesmo é independente da atividade das caspases, resultado que foi corroborado pela ausência de traços característicos da morfologia apoptótica. Verificou-se ainda que os compostos em estudo têm a capacidade de perturbar a homeostasia do cálcio nas células SH-SY5Y. Tendo isto em consideração, considerou-se importante esclarecer o papel do retículo endoplasmático na morte cellular, constatando-se o envolvimento da via PERK/eIF2α na morte celular.

A importância deste trabalho tem a ver com o facto de que a população humana em todo o mundo inclui o tomate na sua dieta. É portanto importante avaliar a toxicidade e conhecer respetivos mecanismos de compostos que ingerimos com tanta frequência.

General Index

Index of Figures

Index of Tables

Abbreviations

ABC – ATP-binding cassette

AChE – acetylcholinesterase

Akt – protein kinase (B)

AD- Alzheimer's disease

AIF – apoptosis-inducing factor

ATF6 – activating transcription factor 6

BiP/HSPA5/GRP78 – immunoglobulin-heavy-chain-binding-protein

BuChE – butyrylcholinesterase

cIAP – cellular inhibitor of apoptosis

Chk2 – checkpoint kinase 2

CHOP – C/EBP homologous protein

COX-1 – cyclooxygenase-1

COX-2 – cyclooxygenase-2

CP – core particle

DBP - dibutyl phthalate

EndoG – endonuclease G

eNOS – endothelial nitric oxide synthase

ER – endoplasmic reticulum

ERAD – endoplasmic reticulum associated degradation

ERK – extracellular-signal regulated kinase

IL-1β – interleukin-1β

IL-6 – interleukin-6

iNOS – inducible nitric oxide synthase

IRE1α – inositol-requiring 1α

JNK – c-Jun N-terminal kinase

LPS – lipopolysaccharides

MDR – multidrug resistance

MEK-2 – mitogen-activated protein kinase kinase

MMP – matrix metalloproteinases

NF-κB – nuclear factor kappa B

nNOS – neuronal nitric oxide synthase

NO – nitric oxide

p53 – tumour protein p53

PCD – programmed cell death

PD – Parkinson's disease

PERK – double-stranded RNA-dependent protein kinase PKR-like ER kinase

PGE_2 – prostaglandin E_2

P-gp – P-glycoprotein

RIP1 – death domain receptor-associated adaptor kinase RIP

ROS – reactive oxygen species

RP – regulatory particle

SERCA – sarco/endoplasmic reticulum Ca^{2+}-ATPase

TNF-α – tumour necrosis factor-α

u-PA - urinary plasminogen activator

UPR – unfolded protein response

UPS – ubiquitin-proteasome system

XBP – X box binding protein

1. Introduction

1.1. Taxonomy and Ecology of *Lycopersicon esculentum* Mill.

The tomato plant belongs to the Solanaceae family. This family includes a large number of domesticated species, comprising several economically important ones. The tomato is one of the most important, along with potato, pepper, aubergine and tobacco. Furthermore, the Solanaceae family also comprises a number of poisonous plants, which contain substances used as biological pesticides. Thus, this taxon holds a large economic value (1, 2).

The tomato plant originates from South America, in the Andes regions, but it is currently cultivated all around the globe, as its fruit represents a major element in the human diet, whether it is consumed fresh or as processed products. The global production of tomato fruits is currently estimated at 159,000,000 tons (1, 3, 4).

1.2. Secondary metabolism

For millions of years, land plants have been forced to interact with insects. This interaction can be of different natures, and therefore beneficial or not to one or both parties involved. For instance, among the beneficial interactions is pollination. However, most insect-plant interactions associated to herbivory trigger a defence response, since the vast majority of plants are targets to predation (5).

Unlike other organisms, plants are unable to move, and therefore they have come to develop, throughout millions of years of selective pressure, multiple strategies to defend themselves, which spread through genetic mutations and consequent natural selection exerted upon them. These defence strategies can be divided in the development of physical features and the biosynthesis of chemical compounds. These chemical defences arose from mutations in primary metabolic pathways. For the plant to be able to produce this chemical compounds, it is essential that their toxicity to the plant itself or metabolic cost associated to its production do not outweigh their toxic or discouraging action towards their targets, improving the fitness of the plant in a sustainable manner. Furthermore, these defence strategies can be distinguished between constitutive and induced – the first are present at all times and all through the plant organism and are named phytoanticipins, and the latter are specifically induced by an attack, for instance in response to defence signals

which are induced when an insect starts feeding on the plant, and are designated as phytoalexins (2, 5, 6).

In the tomato plant, several classes of secondary metabolites are synthesised, including polyphenols, carotenoids and glycoalkaloids (7). Given its economic value and considerable production for human consumption, tomato plants are bred in order to improve their stress resistance and nutritional value, thus increasing the content of specialized chemical compounds such as steroidal alkaloids (1).

1.2.1. Glycoalkaloids

This class of secondary metabolites is comprised in a larger group, namely alkaloids.

Alkaloids represent a group of about 15,000 natural products which contain nitrogen in their chemical structure, commonly incorporated in a heterocyclic ring, and which can be found in a wide variety of vascular plants (around 20% of all of them) (6).

The class of alkaloids can be further divided in three different groups, namely the true alkaloids, the pseudoalkaloids and the protoalkaloids. True alkaloids are basic, derive from an amino acid, and incorporate their nitrogen atom in a heterocyclic ring. In this group we can find, for instance, nicotine and atropine. Protoalkaloids, such as mescaline, do not incorporate their nitrogen atom in a heterocyclic ring. In turn, pseudoalkaloids differ from the true alkaloids because they do not derive from an amino acid. It is in this group that we can find the glycoalkaloids (8). Examples of such alkaloids are in Figure 1.

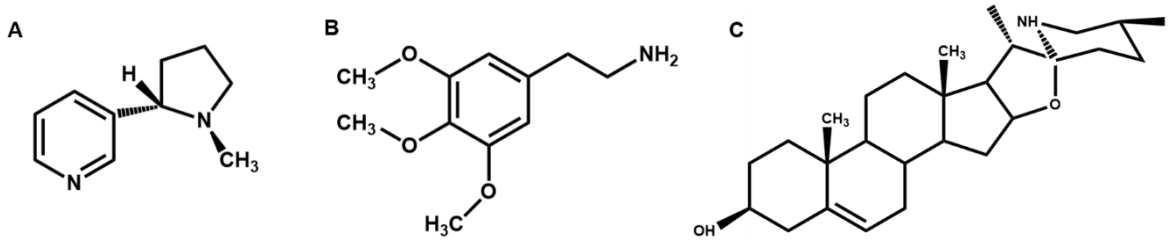

Figure 1 – Chemical structures of nicotine (A), a true alkaloid; mescaline (B), a protoalkaloid; and solasodine (C), a pseudoalkaloid.

Most alkaloids are alkaline at physiological conditions, positively charged and water soluble, when in the form of salts of organic acids. Regarding their biosynthesis, most of these compounds are derived from amino acids, such as lysine, tyrosine, tryptophan and phenylalanine. Nevertheless, that is not the case of some compounds, such as the

glycoalkaloids (6). The components of their carbon skeleton derive from the mevalonic acid pathway, which is the typical biosynthetic pathway of terpenes and of the majority of steroids. Specifically, the precursor for glycoalkaloid biosynthesis is cholesterol (Figure 2), which is itself evanescent in the plant, as it is immediately transformed into other compounds (6, 9).

In general, alkaloids are notorious for their toxicity and pharmacological applications. Regarding their value to the plant itself, they are commonly associated with plant defence against herbivory and pathogen organisms of several taxonomic groups (6).

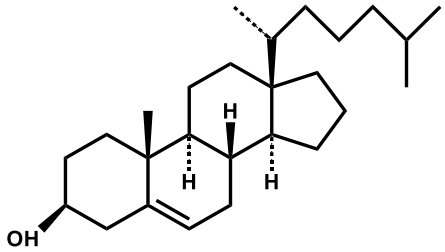

Figure 2 – Chemical structure of cholesterol.

In order to properly introduce the glycoalkaloids, it is also important to discuss the group of saponins. This is a family of steroidal and triterpene aglycons attached to oligosaccharide lateral chains (hydrophilic moieties), which are constitutively produced by several organisms. They provide protection against predators and pathogens, given their antibacterial, antifungal, and insecticidal activities, as well as their capability to prevent the uptake of essential nutrients such as vitamins and minerals and impair protein digestion in the gut (10, 11, 12). These compounds are designated saponins because of their foaming and emulsifying properties, which in turn are due to their amphipathic character (12).

As it was stated before, these compounds might display aglycons of steroidal or triterpene nature. The triterpene type is more often found, and it is typical of the Caryophyllaceae, Primulaceae and Sapotaceae. The steroidal compounds can be found, for instance, in taxons such as Solanaceae, Liliaceae, Agavaceae, Dioscoreaceae. In some particular cases, we can find steroidal and triterpene saponins in the same organism, as is the case of *Avena* sp., although this is not a common occurrence. Examples of crops which produce steroidal and triterpene saponins can be found on Table 1 (13).

Besides their relevance for plant ecology, saponins are known to have multiple pharmacological properties useful to humans, being active principles in traditional folk medicines. Furthermore, other applications can be found in the food and cosmetic fields (10, 11, 12).

Table 1 – Examples of crops which produce steroidal and triterpene saponins (14).

Steroidal Saponins	***Triterpene Saponins***
Oat	Soybean
Pepper	Sunflower
Aubergine	Pea
Potato	Bean
Tomato	Spinach
Yam	Quinoa
Asparagus	Liquorice

Steroidal alkaloids are analogues of steroidal saponins which incorporate nitrogen, and thus they possess sometimes similar bioactivities. These compounds incorporate a nitrogen atom from an amino acid through a transamination reaction. Regarding their bioactivities, they differ from their oxygen analogues essentially for their higher toxicity (15). In ripe tomato fruits, saponins exist in levels significantly higher than lycopene (about 4-fold) (16).

α-tomatine and dehydrotomatine (Figure 3), which are from the **spirosolane type**, are among the most important compounds of the group of glycoalkaloids. Other examples include α-solanine and α-chaconine, from the **solanidane type** (Figure 3), characteristic from *Solanum tuberosum* L., and solasonine and solamargine (spirosolane type), characteristic from *Solanum melongena* L.. Solanidane, spirosolane glycosides have been described in a large number of Solanaceae species, in particular from the genus *Solanum*. Regarding this terminology, the spirosolane type is characterized by the incorporation of the nitrogen atom in an oxa-azaspirodecane structure, like the α-tomatine and dehydrotomatine. The solanidane type is characterized by an indolizidine ring which incorporates the nitrogen atom (2, 17, 18, 19).

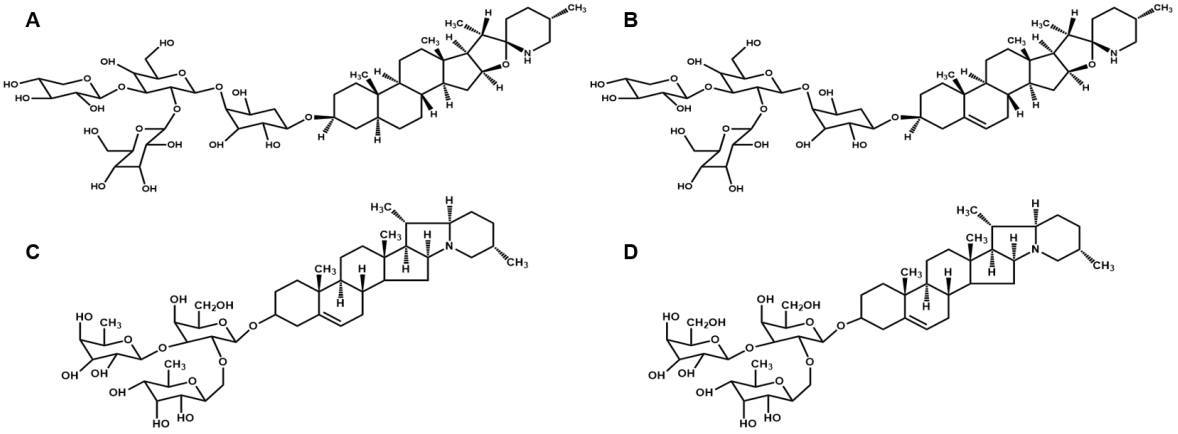

Figure 3 – Chemical structures of the glycoalkaloids α-tomatine (A), dehydrotomatine (B), α-chaconine (C) and α-solanine (D).

Glycoalkaloids, as phytoanticipins, are present all through the body of the plant. Nevertheless, we can pinpoint the areas in which they are more abundant, namely younger tissues such as leaves, sprouts, flowers and unripe fruits. Being saponins, these compounds are constituted by a polar and an apolar moiety, the first consisting of a sugar moiety, and the latter the aglycon – tomatidine (Figure 4), for instance, is the corresponding aglycon of α-tomatine (20). Although they can be very useful to the plant, they are not necessary for the plant to survive and develop normally (18, 21, 22).

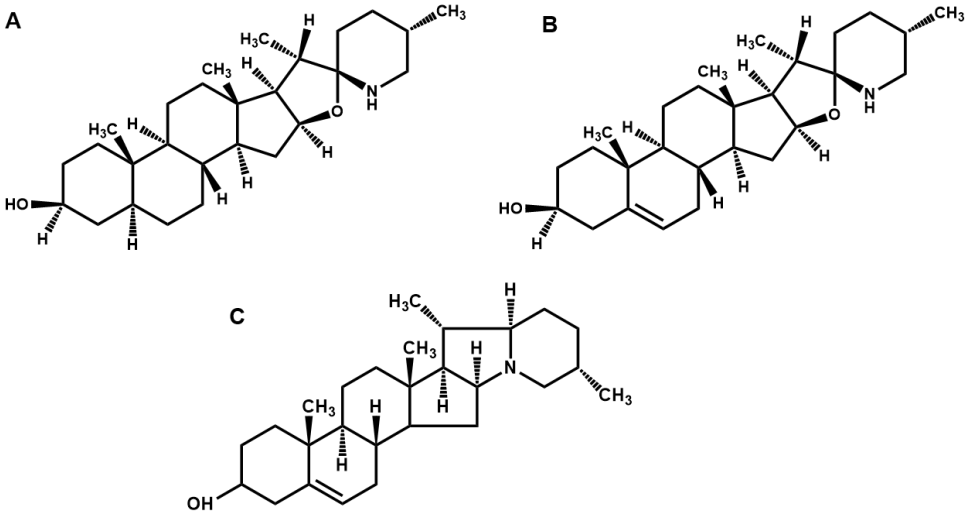

Figure 4 – Chemical structure of tomatidine (A), aglycon of α-tomatine; tomatidenol (B), aglycon of dehydrotomatine; and solanidine (C), aglycon of α-solanine and α-chaconine.

Among the about 100 steroidal alkaloids which are described to the tomato plant (1), we can find esculeoside A, a saponin which can be found only in ripe tomato fruits and which was first isolated in 2003. This compound can be converted to esculeosides B-1 and B-2 (16, 23). Its corresponding aglycon, esculeogenin A, seems to be very similar to the hormone pregnane, given that it can be converted to 3*β*-hydroxy-5*α*-pregn-16-ene-20-one, a pregnane derivative, by refluxing in aqueous pyridine. It is also possible to convert esculeosides B-1 and B-2 to this pregnane derivative through other chemical reactions, by refluxing in a KOH solution, followed by the reaction with HCl/MeOH. Furthermore, a correlation has been established between the ingestion of tomatoes and the presence of androstane derivatives in urine. Along with the fact that a pregnane glycoside has already been found in tomato fruits, this suggests that the intake of steroidal glycosides such as these spirosolane glycosides can promote the synthesis of steroidal hormones like pregnane and progesterone. This steroidal hormone is known to share some of the potential beneficial effects of tomatine, such as anti-tumour or antiosteoporosis activities (16).

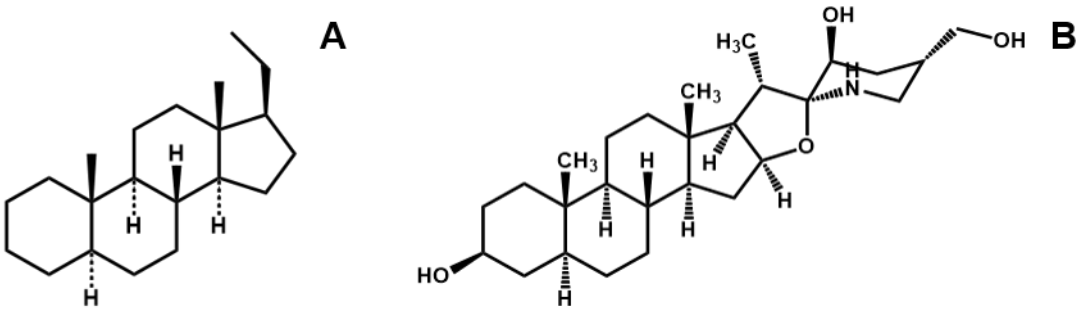

Figure 5 – Chemical structures of pregnane (A) and esculeogenin A (B).

1.3. Bioactivities of glycoalkaloids from *Lycopersicon esculentum* Mill.

1.3.1. Toxicity

As stated before, glycoalkaloids are known to exert toxic effects on several kinds of organisms, from fungi to animals. This toxicity shows to be dose-dependent (21).

The toxicity of steroidal alkaloids was first acknowledged after the observation of gastrointestinal and neurological symptoms, such as vomiting and somnolence, induced by the glycoalkaloids of *Solanum nigrum*. Several decades later, in 1945, tomatine was first isolated and described as an antibiotic agent. Later on, it was found that the designation "tomatine" was attributed to two different compounds, dehydro- and α-tomatine (1).

Essentially, there are two known mechanisms of toxicity exerted by tomatine, the first relying in the disruption of cellular membranes. Tomatine is able to depolarize the cellular membranes by binding to its sterols, leading to the leakage of cellular contents (2, 18, 21). The other mechanism is at the level of the nervous system, where it is known to inhibit acetylcholinesterase (AChE) and butirylcholinesterase (BuChE) activities, like other glycoalkaloids such as α-solanine and α-chaconine (21, 24). Cholinesterase inhibitors currently raise interest as potential drugs for the treatment of neurodegenerative diseases, such as Alzheimer's (AD) or Parkinson's disease (PD), since the inhibition of these enzymes will prolong the action of acetylcholine as a neurotransmitter. In the brain of a patient suffering from AD, BuChE activity is abnormally high, which compromises cholinergic transmission. For this reason, cholinesterase inhibitors are a common approach in the treatment of this illness (24, 25).

1.3.2. Anti-inflammatory activity

Once an organism identifies the presence of an undesired source of infection and/or damage, it may trigger an inflammatory response, a defence process which involves the production of inflammatory mediators by specialized sensors activated by the action of exogenous or endogenous *stimuli*, such as an infection or trauma (26). There are a number of phenomena involved in the inflammatory response, as well as many signalling pathways involved. For instance, in response to lipopolysaccharides (LPS), monocytes migrate

towards the affected tissue, where they suffer differentiation to macrophages. Macrophages play a crucial part in this pathophysiological process, as they are able to produce pro-inflammatory cytokines such as interleukins (IL-1β and IL-6, for instance) and the tumour necrosis factor-α (TNF-α), as well as other inflammatory mediators, as is the case of prostaglandin E_2 (PGE_2) and nitric oxide (NO) (27, 26, 28).

The production of the free radical NO is performed by nitric oxide synthases, a group of enzymes among which we can find iNOS (inducible nitric oxide synthase), eNOS (endothelial nitric oxide synthase) and nNOS (neuronal nitric oxide synthase). The overexpression of iNOS, resulting in increased amounts of nitric oxide, is an important marker of inflammation (29). COX-2 (cyclooxygenase-2) synthesises various molecules which act as inflammatory mediators, such as prostaglandins. This enzyme is the inducible COX isoform, while the constitutive isoform is COX-1. They are both involved in prostaglandin biosynthesis, although the latter is not relevant to the inflammatory response (30, 31, 32, 33).

One of the bioactivities described for tomatine is the anti-inflammatory activity. The mechanisms underlying its anti-inflammatory properties have been previously studied in RAW 264.7 macrophages, where the authors concluded that α-tomatine in concentrations between 0,5 and 2 μM is capable of preventing the secretion of pro-inflammatory cytokines, as well as inhibiting the LPS-induced expression of iNOS and COX-2 enzymes, therefore reducing the levels of PGE_2 and NO, produced by the referred proteins (respectively). There was also evidence of a decrease in the phosphorylation of ERK1/2 (extracellular signal-regulated kinase 1/2) caused by LPS treatment by the action of tomatine (27).

Furthermore, it is known that among the mechanisms underlying the anti-inflammatory activity of tomatine is the inhibition NF-κB (nuclear factor kappa B) and JNK (c-Jun N-terminak kinase) signaling (34).

1.3.3. Muscle hypertrophy

Tomatidine is thought to be a possibly useful compound in the treatment of skeletal muscle atrophy, given that it was able to induce muscle hypertrophy both *in vivo* and *in vitro.* Regarding the *in vitro* studies, an incubation period of 48h with 1 μM tomatidine resulted in increased protein content of the cell, as well as stimulated hypertrophy and increased mitochondrial DNA, in terminally differentiated skeletal myotubes. The former

results were reproducible *in vivo* – it was observed that the effect of tomatidine resulted in a significantly increased muscular mass in young and older mice, which came along with decreased fat mass. The compound shows itself as effective in preventing skeletal muscle atrophy during fasting or limb immobilization, as well as in stimulating the recovery after muscle disuse (35).

1.3.4. Anti-cancer activity

Given that in the past few years many natural products came up as potential anti-cancer drugs, there has been a considerable number of studies regarding the potential anti-cancer activity of tomatine. It was verified that this glycoalkaloid possesses an antiproliferative effect against an array of cancer cell lines, listed on the table below (Table 2) (34).

Table 2 – Human cancer cell lines which have shown to be inhibited by tomatine (36, 37, 38, 39).

Cell Line	***Cancer***	***Concentration***	***Author***
HT29	Colon	≈ 1 μM	Lee et al., 2004
HepG2	Liver	≈ 1 μM	Lee et al., 2004
PC-3	Prostate	1-5 μM	Lee et al., 2011
A549	Lung	2-4 μM	Sheih et al., 2011
MOLT-4	T-lymphoblastic leukemia	1-4 μM	Kúdelová et al., 2013
MCF-7	Breast	≈ 5 μM	Friedman et al., 2013
AGS	Stomach	≈ 0,03 μM	Friedman et al., 2013

Although the aglycon must significantly contribute to the anti-cancer activity of tomatine, it is established that its anti-cancer activity is considerably lower (3).

However, there is still much to clarify regarding the mechanisms of this anti-cancer activity. Studies in the human acute lymphoblastic leukemia cell line MOLT-4 indicate that DNA fragmentation is not involved in the cytostatic action of the compound, although p53 (tumour protein p53) and Chk2 (checkpoint kinase 2) are activated. The phosphorylation of p53 on serine 15 was also induced, as well as the cyclin-dependent kinases inhibitor p21.

As for Chk2, the overall amount was the same, increasing the proportion of Chk2 phosphorylated on threonine 68. These studies indicate that tomatine induces cell cycle arrest, leading to accumulation of cells in G1 phase (34).

Survivin is a protein which is involved in caspase-dependent cell death, once it inhibits caspase-9 and binds to Smac/Diablo, but also in caspase-independent pathways, since it acts inhibiting the AIF (apoptosis-inducing factor). Consequently, survivin is able to promote cell survival. It was reported that tomatine is able to inhibit survivin, as well as induce the release of the AIF as a consequence of causing loss of mitochondrial membrane potential (40).

The anti-cancer activity of tomatine was found to be reproducible *in vivo*, namely in the rainbow trout, by reducing tumour incidence in animals which were subjected to the carcinogenic compound dibutyl phthalate (DBP) in their diet (41).

Another important mechanism of the anti-cancer activity of tomatine is its anti-metastatic effect, which relies on the inactivation of the PI3K/Akt signalling pathway, by inhibiting Akt (protein kinase B) and ERK-1 and -2 (extracellular signal-regulated kinases 1 and 2) phosphorylation, which is the onset of the metastisation process. Tomatine inhibits the binding capacity of the NF-κB, and, as a consequence, inhibits of MMP-2 (matriz metalloproteinase-2), MMP-9 (matrix metalloproteinase-9) and u-PA (urinary plasminogen activator). Processes like angiogenesis are regulated by the matrix metalloproteinases (MMPs), which are themselves upregulated by the NF-κB (36, 42, 43).

Furthermore, it is known that tomatidine is a chemosensitizer, being able to potentiate the cytotoxic effect of chemotherapy drugs in tumours which have developed multidrug resistance (MDR). This effect is related to overexpression of the P-glycoprotein (P-gp) and other transporters from the ATP-binding cassette (ABC) superfamily, which act as efflux pumps to eject the drugs to the extracellular media. In this case, it was verified that tomatidine can potentiate the effects of the anti-cancer molecules adriamicine and vinblastine (44).

1.4. Endoplasmic reticulum (ER) stress and the unfolded protein response (UPR)

The endoplasmic reticulum is constituted by a network of tubules which begins in the nucleus and is present all through the eukaryotic cell (45). This organelle is in charge of multiple functions, primarily synthesis, storage, and folding of proteins into their respective native conformation, as well as post-translational modifications and transport of proteins. It is also the place of biosynthesis of several lipids. The products of this biosynthesis pathways exit the ER through vesicles of its secretory pathway. The ER is also the major intracellular Ca^{2+} reservoir (Figure 6), being able to keep calcium concentrations up to a low milimolar level. The ER also plays a role in the intracellular calcium signalling, involving the exit of calcium ions through several channels, driven by the concentration gradient. The SERCA pump (sarco/endoplasmic reticulum Ca^{2+}-ATPase) is one of the most relevant ATPases in what concerns the control of calcium gradients (46, 45, 47, 48, 49).

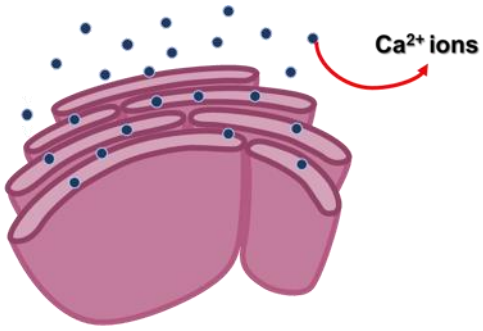

Figure 6 – Schematic representation of calcium ions exiting the lumen of the ER.

Concerning protein folding, cells can generally keep its normality by the action of foldases, lectines, and molecular chaperones. However, when this process is compromised, aberrant proteins are targeted for ERAD (endoplasmic reticulum associated degradation), which implies their ubiquitination and proteolysis in the proteasome. If this response is compromised or if it reveals itself insufficient, misfolded proteins accumulate and the eukaryotic cells triggers a chain of events known as the UPR (unfolded protein response) (50, 51, 52).

The UPR, as it was previously mentioned, generally follows the accumulation of un- or misfolded proteins in the ER lumen, products of upregulated synthesis or deficiencies in protein processing. It can, however, be induced by other kinds of *stimuli*, external to the organelle, as is the case of high glucose concentrations. For these reasons, we can recognise the importance of the UPR regarding metabolic or physiological stress factors (Figure 7) (53).

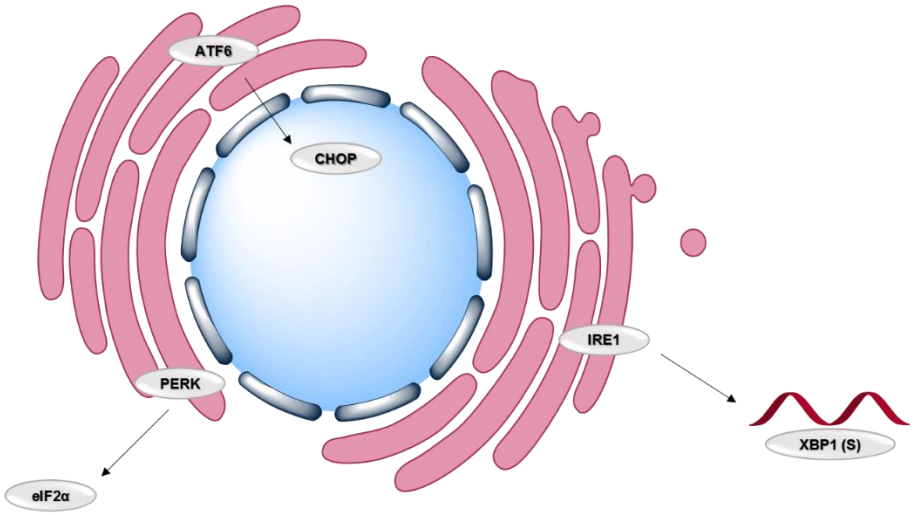

Figure 7 – Major endoplasmic reticulum stress sensors.

The endoplasmic reticulum possesses proteins which act as stress sensors; for this purpose, each one of them possesses a luminal, a transmembrane and a cytosolic domain. The luminal domain senses the accumulation of unfolded proteins, whereas the cytosolic domain is in charge of transducing the signal. From this group of proteins, we can highlight three as the major known stress sensors – PERK (double-stranded RNA-dependent protein kinase PKR-like ER kinase), IRE1α (inositol-requiring 1α) and ATF6 (activating transcription factor 6). While the cell keeps its homeostasis, these proteins remain in their inactive form through association with ER chaperones, as, for instance, BiP/HSPA5/GRP78 (immunoglobulin-heavy-chain-binding-protein). The table below (Table 3) lists the functions of these proteins whilst in their active forms (45, 54, 55, 56).

Table 3 – Modifications and mechanisms of action of ER stress sensor proteins after chaperone dissociation.

Protein	***Modifications***	***Effect***
PERK	Homodimerization, autophosphorylation	Protein-kinase activity; phosphorylates the eIF2α.
IRE1-α	Homodimerization, autophosphorylation	Protein-kinase and RNAse activity, splices the mRNA encoding the XBP.
ATF6	Translocation to Golgi apparatus, cleavage to p50ATF6	Activates transcription of UPR genes, CHOP upregulation.

The activated form of PERK phosphorylates eIF2α at S51. In turn, eIF2α blocks protein synthesis by stopping translation initiation. This allows the relief of the folding load of the ER, given the reduction of the amount of new unfolded proteins. Furthermore, the accumulated proteins are eliminated by ERAD and pro-survival proteins increase, as is the case of cIAP (cellular inhibitor of apoptosis). One of the genes which translation is not blocked by eIF2α is ATF4, which will, similarly to ATF6, upregulate the expression of CHOP (C/EBP homologous protein). CHOP is an inducer of apoptosis, mainly by reducing the expression of Bcl2 and by sensitizing cells to agents which induce ER stress. The activated form of IRE1α possesses endoribonuclease activity (RNAse), removing a 26-nucleotide from the XBP (X box binding protein) mRNA, originating sXBP1. This spliced form of XBP constitutes a transcription factor, activating the UPR target genes (45, 50).

The UPR comprises a phase of adaptation, a phase of alarm and, finally, cell death, generally by apoptosis. So, when the UPR is not sufficient for the ER to recover its homeostasis, it may culminate in programmed cell death, as well as inflammation, cell cycle arrest or even autophagy (45, 50).

1.5. Proteasome

The ubiquitin-proteasome system (UPS) is present in the nucleus and cytoplasm of every eukaryotic cell, where it plays a determinant part in regulating a wide array of essential cellular processes, such as the cell cycle, signal transduction, apoptosis or even inflammation (57, 58, 59).

Structurally, the proteasome (Figure 10) can be divided in two distinct parts: a core particle (CP) or the 20S proteasome, and a regulatory particle (RP) or the 19S proteasome. Together, they form the proteolytic machine known as the 26S proteasome (57).

The CP is composed of four rings, two α and two β. In turn, each one of this rings is composed by seven units. The four rings are joined together in a way that the α rings face the outside of the CP and the β rings are on the inside. The catalytic sites of the proteasome can be found in the β rings. We can identify three of them, according to the substrates they cleave. The β1 or trypsin-like cleaves basic residues, the β2 or caspase-like cleaves acidic residues, and finally the and finally the β5 or chymotrypsin-like cleaves proteins after hydrophobic residues. So, the CP is in charge of the proteolytic activity, whereas the RP is in charge of the translocation of the ubiquitinated proteins to the catalytic site (57, 58).

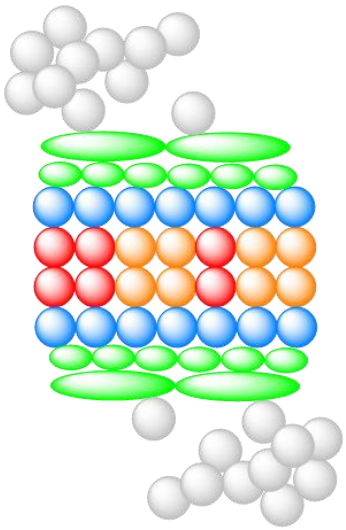

Figure 8 – Illustration of the structure of the proteasome.

Given that the UPS is a main regulator of cellular proteostasis, along with the lysosomes, disturbances of its homeostasis are connected to several diseases. It is therefore important to know how to modulate this system, in order to respond to this sort of pathologies, among which neurodegenerative diseases such as AD or PD (59, 60). Furthermore, recently, the proteasome has risen as a new target for cancer chemotherapy, since its inhibition has shown to effectively induce apoptosis through ER stress in a wide array of cancer cell lines, which naturally hold a very active protein synthesis rate (57).

1.6. Cell death mechanisms

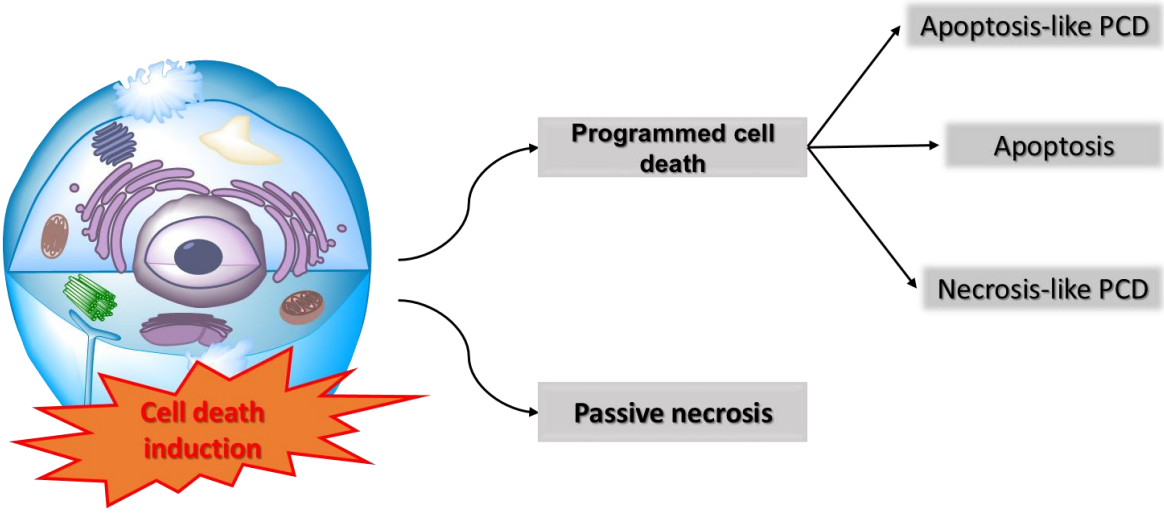

Figure 9 – Representation of the different cell death pathways in which the cell can engage.

There are several aspects to take into account in the classification of cell death mechanisms, for instance, whether they are programmed (genetically determined and energy dependent) or passive, caspase-dependent or independent, or whether the morphology is, for example, apoptotic or necrotic (61, 62).

In order for a cell to be considered irreversibly dead, which implies that the cell death process is complete, it has to be fully disintegrated into apoptotic bodies, its cellular membrane must have been disrupted, or it was phagocytosed by the surrounding cells (61).

1.6.1. Apoptosis

Apoptosis is the term used to describe the most common form of programmed cell death, since it is the mechanism of cell self-destruction which occurs during the normal development of an organism. Furthermore, it is also triggered by a wide array of exogenous stimuli. As it was previously mentioned, apoptosis is a genetically regulated process. Its underlying genes have shown to be highly conserved (62, 63).

Morphologically, its main hallmarks include chromatin condensation (resulting in pyknosis), cellular membrane blebbing and fragmentation into apoptotic bodies, formed

after the cellular membrane blebs and engulfs whole organelles and nucleus and endoplasmic reticulum fragments, and shrinkage of the cell, leading to a separation from the surrounding cells (62, 64).

It is generally accepted that apoptosis might follow the death receptor (or extrinsic) pathway or the mitochondrial (or intrinsic) pathway, although there may be some overlap between the two (62). Recently, new cell death mechanisms which result in the morphological hallmarks of apoptosis were described. This mechanism are related to grazyme A and/or B, however, granzyme A induces cell death in a caspase-independent manner (65).

Although the molecular events underlying apoptosis are diverse, the most part of them involve the mitochondria. ROS (reactive oxygen species) have an importance on the phosphorylation of some proteins which exert regulatory roles on apoptosis - that is the case, for instance, of tyrosine. The activation of caspases is also a key event in apoptosis. Caspases are a group of cysteine-aspartic acid proteases which may act as initiators, activating other caspases (as is the case of caspase-8 and -9) or as executioners (as are caspase-3, -6 and -7). However, the mitochondrial pathway of apoptosis can occur in a caspase-independent manner, as long as the apoptosis inducing factor (AIF) and the endonuclease G (EndoG) migrate from the mitochondria to the nucleus. This also results in DNA fragmentation, yet in larger fragments (66, 67, 68).

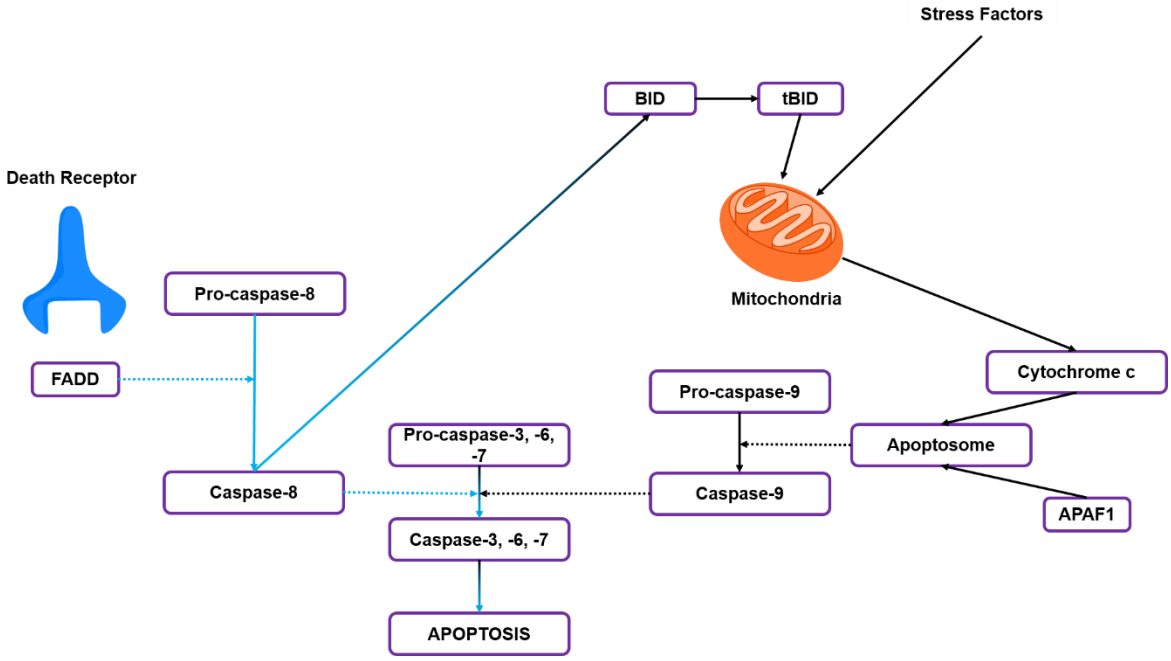

Figure 10 – Representation of the death receptor and mitochondrial pathways of apoptosis.

1.6.2. Necrosis

Necrosis is the designation given to passive, energy-independent cell death, which occurs when the cell is physically injured (69).

Regarding the hallmarks of necrosis, prior to the disruption of the cell membrane itself we can find organelles such as mitochondria and lysosomes with disrupted membranes or swollen, distended endoplasmic reticulum with disaggregated ribosomes, and cytoplasmic vacuolization. Furthermore, necrosis is commonly associated with inflammation, since the leakage of the intracellular compounds signals the danger to the surrounding area (62, 70).

Unlike what occurs with the apoptotic bodies, which are completely phagocyted by the neighbour cells, only a part of the remains of the necrotic cell is engulfed by macropinocytosis. This the process by which large endocytic vacuoles named macropinosomes are formed, in an actin-dependent manner (71, 72).

1.6.3. Autophagy

As it is implied by the designation itself, autophagy is the process by which a cell feeds on itself, ultimately obtaining its energy when the uptake of nutrients is not sufficient for it to keep its homeostasis. Generally, it occurs when the cell stops receiving normal signaling, nutrients or oxygen, but it may also play a protective role in eliminating damaged or prejudicial cell components. For instance, autophagy plays a very important part in eliminating excess ER resulting from the UPR. The cells undergoing autophagy commonly engage in programmed cell death without caspase activation (69, 73).

Mechanistically, autophagy consists in extensive formation of multimembrane autophagic vesicles containing cell components to be digested. These vesicles are ultimately engulfed by the lysosomes (69).

1.6.4. Paraptosis

Paraptosis is a form of apoptosis-like PCD which was recently described and that does not meet the criteria to be considered apoptosis, both from a mechanistic and morphological perspective. It may also be referred to as non-lysosomal vacuolated degeneration. Morphologically, paraptosis does not result in apoptotic morphology - the major hallmark of paraptosis is vacuolization of the cytoplasm, derived from the endoplasmic reticulum. There is also swelling of the mitochondria. Furthermore, this type of PCD is caspase-3-independent, and so caspase inhibitors are unable to prevent the cells from dying. However, caspase-9 is known to be involved. Although paraptosis might still be underexplored from a mechanistic point of view, some of its mediators have already been identified. It is known that the mitogen-activated protein kinase kinase (MEK-2) is involved in the process, as well as Jun N-terminal kinase (JNK) and the calcium-binding protein ALG-2 (74, 75, 76, 77).

This phenomenon is associated to the cell death which occurs during the normal neural development, as well as to some neurodegenerative diseases (75, 77).

1.6.5. Necroptosis

Most of the stimuli which induce apoptosis can also result in necrotic cell death, as long as the duration or intensity of said stimuli is overpowering to the cell. The term necroptosis, or necrosis-like PCD, emerged out of need to distinguish this cell death mechanism from the classic necrosis previously described, given that this is a genetically regulated process which might only be activated when the cell, for some reason, cannot undergo apoptosis. Like apoptosis, necroptosis is a regulated process which occurs during development and in healthy tissues. This process relies on the activity of the serine/threonine kinase RIP1. Although it was clarified that RIP1 is an upstream intervenient in necroptosis, there are several models for its mechanism of action. Some of the described downstream events in this signaling cascade include increased permeability of mitochondrial membranes and build-up of ceramides with pro-necrotic activity (68, 72, 78).

Necroptosis is connected to increases in Ca^{2+} levels arising from the ER, and consequently it may occur along with apoptosis. The increase in calcium levels may end up disrupting lysosomal membranes, and therefore activating its resident proteases, for

instance, calpains. Since there is calpain activation, this cell death mechanism may overlap with autophagy (78).

1.7. Objectives

This work aims to clarify the cellular mechanisms underlying the toxicity exerted by Solanaceae glycoalkaloids, namely tomatine and tomatidine, in neuroblastoma (SH-SY5Y) and gastric adenocarcinoma (AGS) cell lines.

For this reason, the main objectives were:

- Assess the effect of the compounds in cell viability and membrane integrity;
- Observe the morphological modifications induced by the compounds;
- Determine the involvement of caspases in cell death;
- Analyse their effect on calcium homeostasis;
- Determine the importance of the UPR and the ER stress sensors involved in the process;
- Define whether the compounds are able to inhibit 20S proteasome activity.

Furthermore, another goal of this work is to verify the anti-inflammatory activity of tomatine and tomatidine in a cell-free system and in RAW 264.7 macrophages, by:

- Analysing if there is any inhibitory activity over phospholipase A_2;
- Determining whether they are able to diminish NO production by LPS-stimulated macrophages.

2. Materials and Methods

2.1. Standards and Reagents

Tomatine and tomatidine were obtained from Extrasynthese (Genay, France). The Fura-2 AM fluorescent probe was purchased from Abcam Biochemicals. 3-(4,5-dimethylthiazolyl- 2)-2,5-diphenyltetrazolium bromide (MTT), trypan blue, propano-2-ol, dimethyl sulfoxide (DMSO), β-nicotinamide adenine dinucleotide (NADH), sodium pyruvate, Triton™ X–100, sodium deoxycholate, Trizma® hydrochloride, soybean lipooxygenase (LOX) from *Glycine max* (L.) Merr. (Type V-S; EC 1.13.11.12), phospholipase A_2 from honey bee venom *(Apis mellifera)*, 1,2-dilinoleoyl-sn-glycero-3-phosphocholine, *N*-(1-naphthyl)ethylenediamine, sulphanilamide, lipopolysaccharide (LPS) from *Salmonella enterica*, Giemsa dye, DPX mountant, and thapsigargin were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM), Hank's balanced salt solution (HBSS), penicillin-streptomycin solution (penicillin 5000 units/mL and streptomycin 5000 µg/mL), foetal bovine serum (FBS) and 0,05% trypsin-EDTA were acquired from GIBCO, Invitrogen™ (Grand Island, NY, USA). Staurosporin, carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (Z-VAD.fmk) and salubrinal were purchased from Santa Cruz Biotechnology, Inc. Phosphoric acid (H_3PO_4) was acquired through Scharlau. Methanol and potassium dihydrogen phosphate were purchased from Merck (Darmstadt, Germany). Caspase-Glo® 3/7 luminescent kit was obtained from Promega Corporation. Lactacystin, 20S proteasome and Suc-Leu-Leu-Val-Tyr-AMC (20S proteasome fluorogenic substrate) were purchased from Enzo Life Sciences, Inc (Farmingdale, NY, USA).

2.2. Cell culture conditions

Human neuroblastoma cell line SH-SY5Y, human gastric adenocarcinoma cell line AGS and RAW 264.7 mouse macrophages were maintained in DMEM culture medium with 1% penicillin/streptomycin and 10% FBS at a temperature of 37 ºC, with 5% CO_2.

2.3. MTT assay

This assay is widely used to determine the proportion of viable cells. The assay comprises incubation of cells with MTT, which is reduced to formazan in mitochondria by cells with active metabolism. These cells are considered to be viable, as opposed to non-viable cells, which cannot reduce MTT (79).

SH-SY5Y and AGS cells were washed twice with HBSS, trypsinized, centrifuged and plated at a density of 3 x 10^4 and 1,5 x 10^4 cells/well, respectively, followed by a period of incubation of 24h at the previously described conditions. In the case of RAW 264.7 macrophages, they were washed, scraped and plated at a density of 2,5 10^4 x cells/well.

After a new period of incubation of 24h, every well was filled with 100 µL of a MTT 0.5 mg/mL solution and incubated for 2 hours. The MTT solution was then removed from the wells, and the formazan in each one of them was dissolved in 200 µL of a solution of 3:1 DMSO:isopropanol. The absorbance at 560 nm was read in a Thermo Scientific ™ Multiskan ™ GO microplate reader, in order to determine the amount of formazan. As the product of MTT reduction, the amount formazan is presumably proportional to the population of viable cells (79).

2.4. LDH assay

LDH leakage was evaluated, in order to assess membrane integrity. In the case of cellular death by necrosis, the cells will swell and their membranes will eventually disrupt, causing cytosolic enzymes such as the LDH, to leak to the extracellular media. To determine the LDH leakage, a kinetic NADH oxidation assay was conducted, using sodium pyruvate as substrate. The cells were incubated in the presence of each compound for 8 or 24 hours. After the incubation period, 20 µL of culture media was removed from each well. To these aliquots, NADH at 210,7 µM and sodium pyruvate at 1,363 µM (final concentrations) were added. The absorbance was then read at 340 nm in a Thermo Scientific ™ Multiskan ™ GO microplate reader. As a positive control for maximum LDH leakage to the extracellular media, 1% Triton X-100 was used.

2.5. Cellular density assay

This assay was executed as described before (80) (80). SH-SY5Y cells were plated in 96-well plates at a density of 3 x 10^4 cells/well and incubated overnight at the previously described conditions. The cells were then incubated in the presence of the compounds for 24 hours. When this period was finished, the cells were fixed with previously chilled 40% trichloroacetic acid for 60 min at 4 ºC. Ended this hour, the wells were carefully washed with water and left to dry. A 30 min incubation with 0,4% SRB in 1% acetic acid followed. The solution was then removed, and the wells were washed several times with 1% acetic acid. Finally, an incubation with tris-base for 10 min was performed and the absorbance was read at 492 nm in a Thermo Scientific ™ Multiskan ™ GO microplate reader.

2.6. Cell morphology assessment

The assay was performed as described before (81). Cells were plated in 24-well plates, at a density of 7 x 10^4 cells/well, and incubated at 37ºC for 24h. Cover slips had been previously placed on the wells. In the following day, the medium was removed, and the cells were incubated with the compounds of interest for another 24h. Ended this period, the fixation with methanol was carried out. The medium was removed, the wells were washed with HBSS and 600 µL of previously chilled methanol or 4% formaldehyde were added. The plate was then placed on ice for 30 min. After removal of the methanol, the wells were washed with HBSS one more time, and incubated with the Giemsa colouration (1:10 dilution with distilled water, filtrated) for 25 min. Finally, the cover slips were washed three times with water, dried and fixed with DPX mountant.

2.7. Intracellular Ca^{2+} quantification

SH-SY5Y cells were plated in black bottom 96-well plates at a density of 3 x 10^4 cells/well, followed by a period of incubation of 24 hours to allow the cells to adhere to the surface of the wells. Ended this period, the cells were exposed to the fluorescent calcium probe Fura-2/AM, at a concentration of 5 µM in HBSS, and then the probe was removed, the cells were washed with HBSS and the cells were incubated with the compounds for

another hour, in HBSS supplemented with 10% FBS. Thapsigargin at 5 µM was used as a positive control to assess the maximum cytosolic calcium levels which we could obtain. Finally, the calcium levels were read in a Cytation™ 3 (BioTek) multifunctional microplate reader. The relative amount of cytosolic calcium was determined by calculating F_{340}/F_{380}.

2.8. Determination of the involvement of the PERK/eIF2α branch of the UPR

Cells were plated at a density of 3 x 10^4 cells/well in 96-well plates. The cells were then subjected to the action of the compounds for 8 hours, both in the presence or absence of salubrinal at 40 µM. The latter is known to prevent ER stress through inhibition of the eIF2α phosphorylation. The differences is cell viability between group subjected to the glycoalkaloids alone or co-incubated with the compounds and salubrinal were then evaluated by MTT assay.

2.9. Caspase inhibition assay

Cells were seeded in 96-well plates at a density of 3 x 10^4 cells/well. After this time period, the culture medium was replaced by new medium or new medium with Z-VAD-FMK at 50 µM, followed by 1 hour of incubation at 37ºC, at the end of which 2 µL of a solution fifty-fold concentrated of each compound was added to the respective wells. The cells were subjected to the action of tomatine and tomatidine for 8h, both in the presence and absence of Z-VAD-FMK. The pan-caspase inhibitor shown to be non-toxic at the referred concentration and incubation period. The positive control group was subjected to staurosporin at 500 nM for the same time period. After this 8 hour incubation, the cell viability was determined performing an MTT assay as described before.

2.10. Caspase-3/7 activity assay

Caspase-3 activity was assessed using the Caspase-3/7-Glo (Promega Corporation) kit. Cells were plated at a density of 3 x 10^4 cells/well and subjected to the presence of tomatine of tomatidine for 8 hours. Staurosporin at 500 nM was used as a positive control for maximum caspase-3 activity. Ended the 8 hours of incubation, 60 µL of culture medium were removed from each well, followed by the addition of 40 µL of the luminescent kit (in order to achieve a 1:2 dilution).

A period of incubation of 10 minutes at 22ºC followed, at the end of which luminescence was read in a Cytation™ 3 (BioTek) multifunctional microplate reader.

2.11. 20S proteasome inhibition assay

The assay was carried out in black-bottom 96-well plates. The first thing to be added to the respective wells was the compounds, in a solution of 50 µL (and therefore twofold concentrated). To the positive control group we resorted to lactacystin, known to be a 20S proteasome inhibitor, here used in final concentration of 10 and 20 µM. The previous step was followed by the addition of 70 ng of 20S proteasome in 25 µL of assay buffer. Finally 25 µL Suc-LLVY-AMC were added, so that the final substrate concentration on the well was of 40 µM. In each well, the final volume was 100 µL. The plate was left to incubate in the dark at 37ºC for two hours. Ended this time, the absorbance was measured at 340 nm absorption and 460 nm emission in a Cytation™ 3 (BioTek) microplate reader.

2.12. Determination of the activity of the honey bee phospholipase-A_2 (PLA_2) activity

For this assay PLA_2 was used at 0.25 µg/mL and 5-LOX was used at 0.23 µg/mL. The enzymes and the compounds were dissoved in sodium deoxycholate at 3 mM in tris-HCl at 50 mM and pH 8.5. PLA_2 substrate (1,2-dilinoleoyl-sn-glycero-3-phosphocholine) was used at 65 µM, dissolved in sodium deoxycholate at 10 mM in tris-HCl at 50 mM and pH 8.5. The assay was performed in 96-well plates. To each well, 50 µL of sample, 20 µL of each

enzyme and 20 µL of substrate were added, plus 30 µL of buffer (the enzymes and substrate were prepared 7-fold concentrated, and the samples 2.8-fold concentrated). After na incubation period of 35 min at 37 ºC, the absorbance was read at 234 nM in a Thermo Scientific ™ Multiskan ™ GO microplate reader.

2.13. Determination of nitric oxide levels

RAW 264.7 cells were plated in 96-well plates at a density of 3,5 x 10^4 cells/well. In the following day, they were incubated with the compounds under analysis. 2 hours after the incubation, 2 µL of a fifty-fold concentrated LPS solution were added, in order to reach a final LPS concentration in the well of 0,05 mg/mL, and so that the cells were co-exposed to the compounds and LPS for 22 hours. Ended this 22 hours, 75 µL of the supernatant were collected from each well. To each of these aliquots, 75 µL of Griess reagent were added, and the plate was incubated in the dark for 10 minutes. Finally, the absorbance at 562 nm was read in a Thermo Scientific ™ Multiskan ™ GO microplate reader.

2.14. Statistical analysis

For the statistical analysis, GraphPad Prism software was employed. T-test were performed with a level of significance of $p<0,05$.

3. Results and discussion

3.1. SH-SY5Y and AGS cells

3.1.1. Glycoalkaloid cytotoxicity

The first step was to evaluate the concentrations at which tomatine and tomatidine exerted their toxic effect upon the cell lines used.

We verify that the impact of tomatine and tomatidine on cell viability is dose-dependent for both compounds (Figure 11).

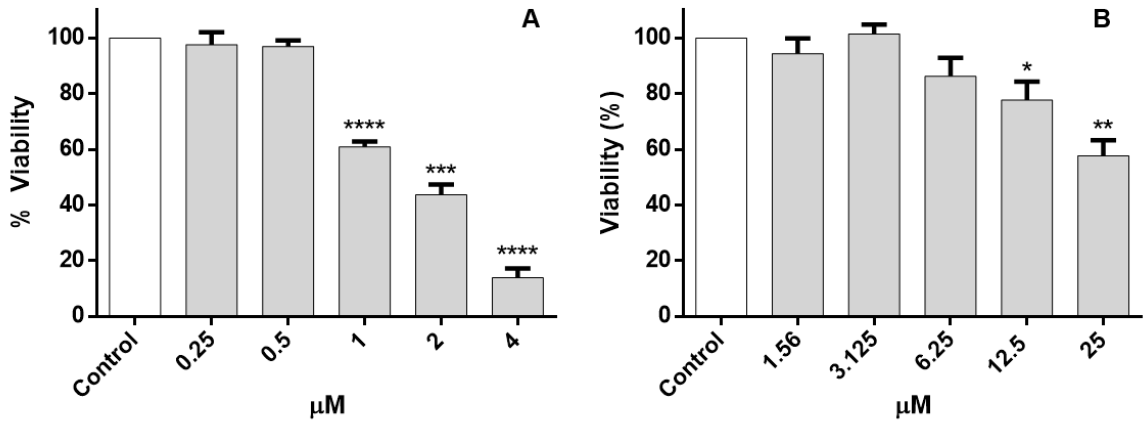

Figure 11 – Effect of tomatine (A) and tomatidine (B) on SH-SY5Y cell viability after 24 hours, evaluated by MTT reduction assay. Results presented as mean ± SEM.

However, although the behavior of dose-response curve is similar, the IC_{50} is quite different: while it is only 1.63 μM for tomatine, it is considerably higher for tomatidine (above tested concentrations), indicating that tomatine is more cytotoxic to SH-SY5Y cells than tomatidine. This agrees with previous literature, which reports the higher toxicity of the glycoalkaloid when compared to the respective aglycon, since the glycoalkaloid is an amphipatic molecule and therefore able to interact with the membranes in a more efficient manner, as the glycosidic side chain is determinant to the toxicity of glycoalkaloids.

In order to elucidate the type of cell death involved, we evaluated the activity of the cytosolic enzyme LDH in the extracellular media, in order to determine whether the cell death is related or not to the occurrence of necrosis.

The action of tomatidine in the concentrations tested showed no increase in the leakage of the LDH to the extracellular media when compared to the control group, and therefore the hypothesis of cell death by necrosis can be excluded (Figure 12). However, tomatine has shown to increase the activity of this enzyme in the extracellular media, although only in the highest concentration tested (4 µM). This result agrees with the literature, which describes the ability of the glycoalkaloids to disrupt cellular membranes. In addition, we conclude that concentrations up to 2 µM may be used without the interference of this property of the saponins (Figure 12).

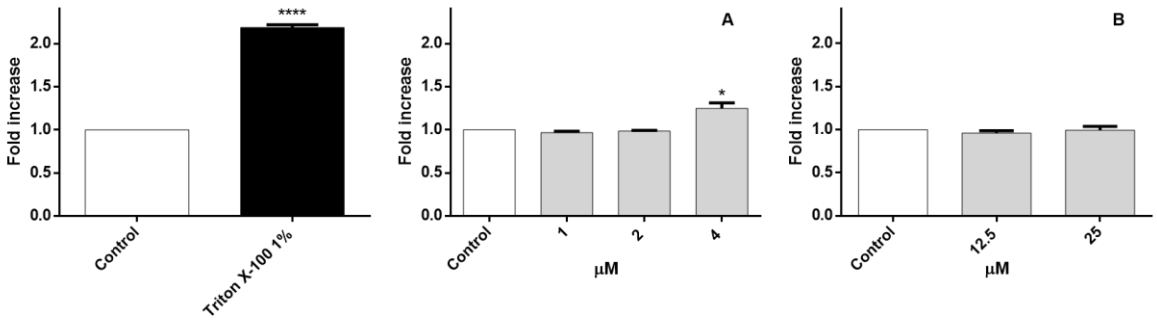

Figure 12 – Influence of tomatine (A) and tomatidine (B) on membrane integrity in SH-SY5Y cells, assessed by cytosolic LDH leakage. Triton X-100 at 1% was used as a positive control for maximum extracellular LDH activity. Results presented as mean ± standard deviation of the mean.

Considering that the glycoalkaloids are known to interact with the cholesterol molecules in the cell membranes, leading to their disruption, the determination of the levels of activity of cytosolic lactate dehydrogenase in the extracellular media allows us to assess if and to what extent the cell membranes are rupturing by action of our compounds of interest. Here we determined that, in the tested concentrations, tomatidine does not cause membrane disruption. As for tomatine, we verify an increase of about 30% in the LDH activity in the highest concentration tested (4 µM). In light of these results, we can assume

that observed decreases in cell viability are not completely due to the occurrence of necrosis, but to cell death by mechanisms which remain to be clarified.

In order to evaluate if the toxicity found is neuron-specific, we have tested the two molecules in the stomach adenocarcinoma cell line AGS. We verify that tomatidine does not exert any toxicity to AGS cells up to 25 µM. However, tomatine is toxic to these cells in concentrations as low as 1 µM (Figure 13) (which are, however, higher than previously reported), behaviour which is similar to that found in SH-SY5Y cells. The glycosidic side chain of tomatine confers it some hydrophilicity, resulting in a greater readiness for interacting with the cell.

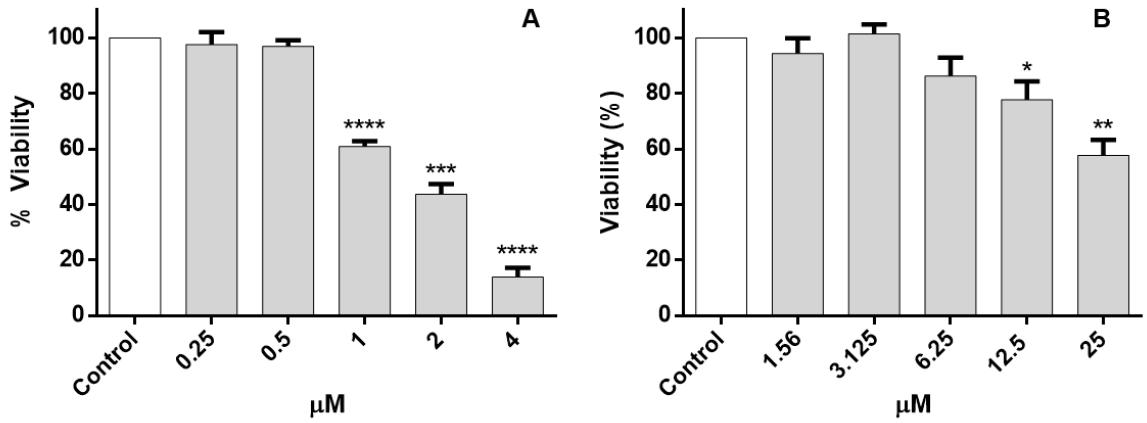

Figure 13 – Effect of tomatine (A) and tomatidine (B) on AGS cell viability after 24 hours, evaluated by MTT reduction assay. Results presented as mean ± SEM.

In what regards membrane integrity, it was observed that AGS cells were more susceptible for the occurrence of cytosolic LDH leakage than SH-SY5Y cells (Figure 14). In fact, 1 µM tomatine lead to membrane disruption, while the same concentration elicited no such effect in the neuronal cell line. This concurs with the literature which, as previously mentioned, describes the disruption of cell membranes along the gastrointestinal tract as one of the main mechanisms of glycoalkaloid toxicity.

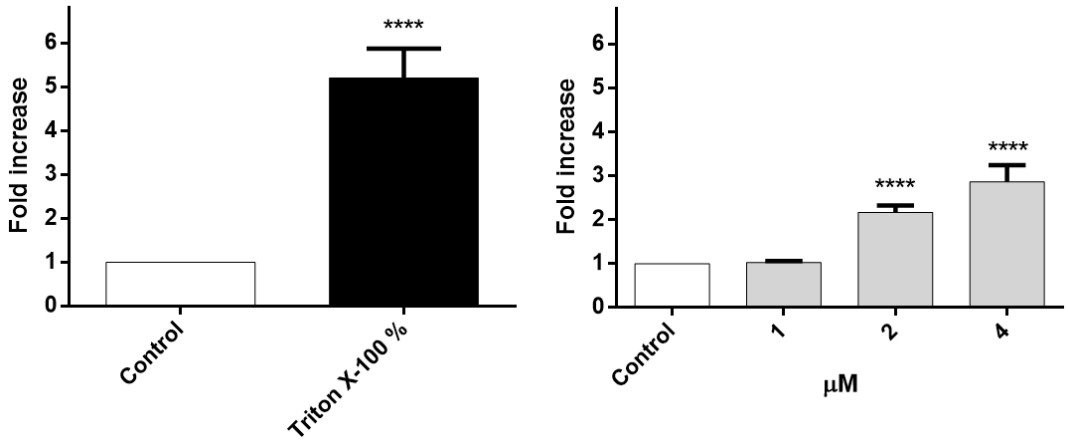

Figure 14 – Influence of tomatine on membrane integrity in AGS cells assessed by cytosolic LDH leakage. Triton X-100 at 1% was used as a positive control for maximum extracellular LDH activity. Results presented as mean ± SEM.

After interpreting the results from both the MTT and LDH assays, we were interested in knowing if the molecules under study could be exerting an anti-proliferative effect. As it can be seen in Figure 15, incubation with tomatine results in minor anti-proliferative effect.

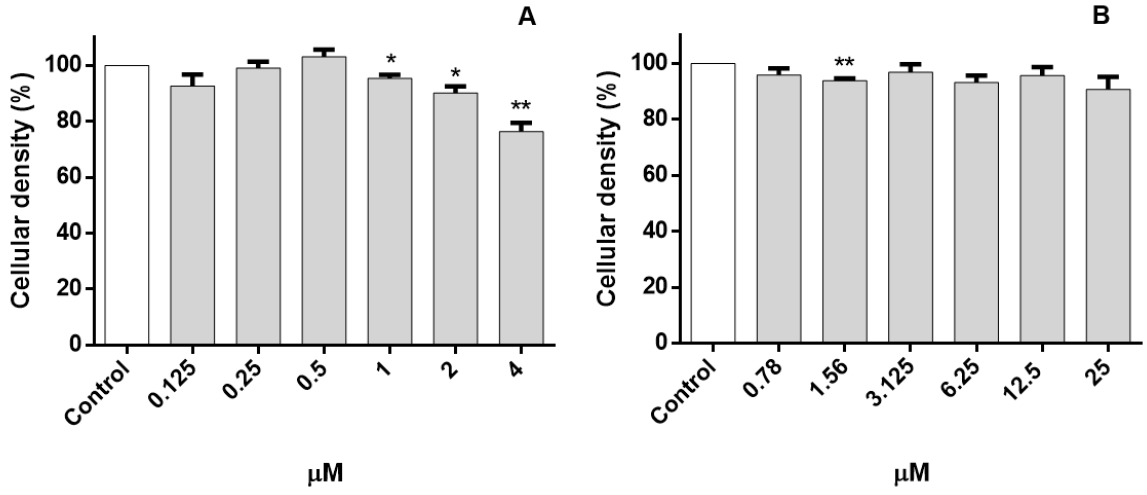

Figure 15 – Influence of tomatine (A) and tomatidine (B) on cellular density in SH-SY5Y cells. Results presented as mean ± standard deviation of the mean.

3.1.2. Cell morphology

Given the effect upon cellular viability indicated by the MTT assay, we were interested in shedding some light on the mechanism of cell death which is taking place. For this reason, we have conducted experiments for cell morphology assessment.

As it can be in Figure 16, despite the decrease in cell viability, no classical traits of apoptotic nor necrotic morphologies can be found. We can highlight that the cells are still extending their neurites to their surrounding area, which constitutes an important hallmark of a healthy phenotype in SH-SY5Y cells.

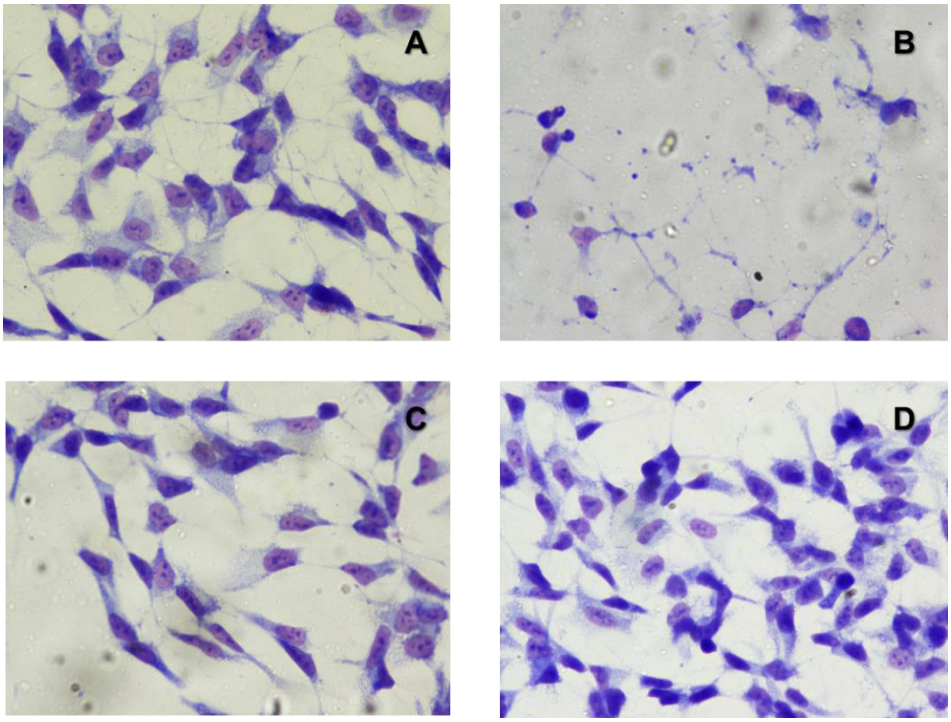

Figure 16 - Influence of 2 µM tomatine (C) and 25 µM tomatidine (D) on the morphology of SH-SY5Y cells after 24 hours, when compared to a control group (A). Staurosporine at 500 nM (B) was used as a positive control for cell death. Images obtained by Giemsa coloration.

Necrosis, opposed to programmed cell death, involves the swelling of the cells and membrane disruption (69). Such necrotic traits cannot be found in these images.

On the other hand, the apoptotic morphology is characterized by blebbing of the cell membranes, presence of apoptotic bodies, shrunken cells and chromatin condensation and posterior fragmentation (82). Neither of this phenomena can be detected in the pictures above, which suggests that the decreased cell viability we obtain in MTT assays might be due to other form of programmed cell death, rather than classical apoptosis.

In the case of AGS cells (Figure 17), we verify that after 24 hours in the presence of 2 µM tomatine, the cells start to display some characteristics that could be compatible to necrosis/necroptosis, namely the presence of swollen nuclei. However, in order to definitely confirm this hypothesis, more data would be necessary. In addition, the cytosolic structures found in treated cells could be compatible with autophagosomes.

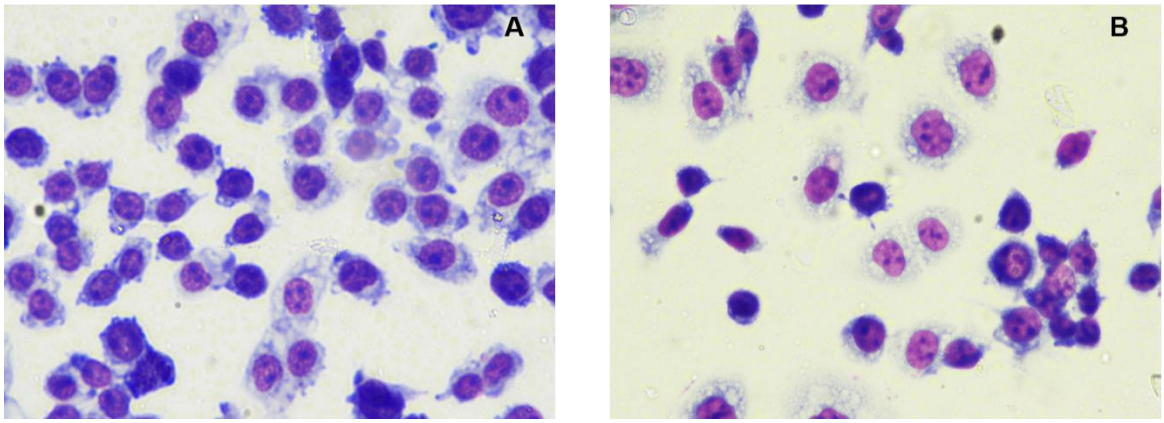

Figure 17 - Influence of 2 µM tomatine (B) on the morphology of AGS cells after 24 hours, when compared to a control group (A). Images obtained by Giemsa colouration.

3.1.3. Tomatine and tomatidine interfere with calcium homeostasis

In order to produce further information regarding the mechanism of action of these molecules, we have evaluated their ability to interfere with calcium homeostasis. Ca^{2+} levels must be tightly regulated since their rise in the cytosol is toxic and can potentially lead to cell death. Cytosolic concentrations of free ions are generally kept at a nanomolar range (83).

Thapsigargin, used here as a positive control, is known to deeply disturb Ca^{2+} homeostasis, given that it irreversibly inhibits the sarco/endoplasmic reticulum pump Ca^{2+}-ATPase (SERCA), which is localized in the ER membrane and exerts its function pumping free calcium ions into the ER lumen (45, 84). Thus, the SERCA pump is crucial for maintaining Ca^{2+} levels within a normal range, and inherently for keeping ER homeostasis, and therefore it is essential to the survival of the cell (85).

Our results show that glycoalkaloids disrupt calcium homeostasis as we verify that the incubation of cells for 1 hour with tomatine or tomatidine induces a substantial increase of the amounts of calcium ions in the cytosol in the same range of the positive control (Figure 18).

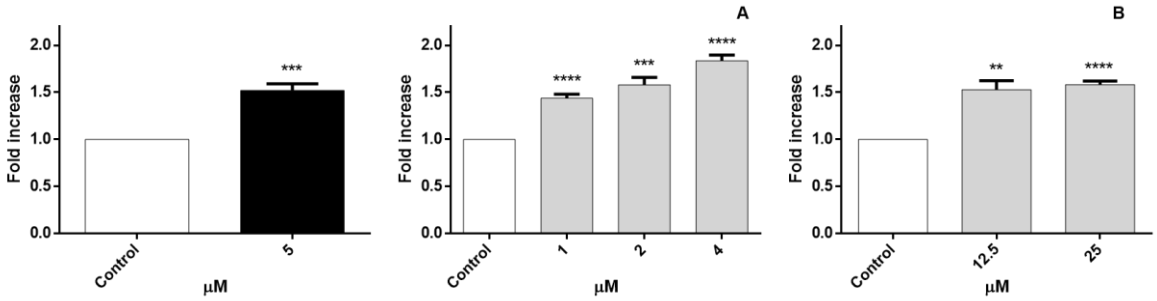

Figure 18 – Effect of glycoalkaloids on calcium levels, evaluated by relative amounts of cytosolic calcium, in SH-SY5Y cells. 5 µM thapsigargin was used as a positive control for the calcium efflux from the endoplasmic reticulum. A: tomatine; B: tomatidine. Results presented as mean ± standard deviation of the mean.

In light of these results, we can conclude that the molecules under study disturb calcium homeostasis. However, the precise target in which they exert their effect remains to be determined.

3.1.4. eIF2α phosphorylation is involved in glycoalkaloid toxicity

Given its impact on Ca^{2+} levels, we hypothesised that the glycoalkaloid toxicity could be a consequence of its effect upon molecular targets in the ER, the major calcium storage organelle in cells.

In this regard, we determined the involvement of the PERK/eIF2α branch of the UPR in the toxicity exerted by these molecules. For this purpose, we incubated cells with the compounds in the presence of salubrinal, a selective inhibitor of eIF2α phosphorylation (86, 87). As it can be seen in Figure 19, co-incubation of tomatine and tomatidine with salubrinal rescues cells from the damage caused by these molecules, which indicates that the glycoalkaloid toxicity in these cells involves activation of the PERK/eIF2α pathway. In light of these results, we can infer the tomatine and tomatidine trigger ER stress and may cause the onset of the UPR.

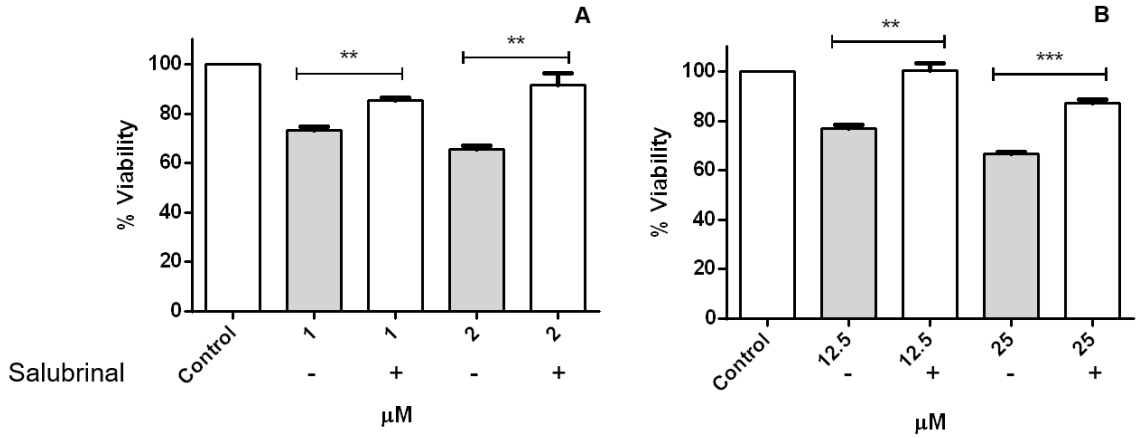

Figure 19 – Differences on cell viability of SH-SY5Y cells when exposed to tomatine (A) or tomatidine (B) alone or with co-incubation with the eIF2α phosphorylation inhibitor salubrinal. Results presented with mean ± standard deviation of the mean.

Salubrinal acts on the phosphatases which act on eIF2α, as is the case of PERK. From this inhibitory action results an increase on eIF2α phosphorylation, to which is associate an impairment of protein synthesis. The consequential decrease in the load of proteins to fold in the ER is crucial to cell recovery from situations of endoplasmic reticulum stress (88, 89).

In order to elucidate if the effect upon the ER stress status was neuron-specific, AGS cells were subject to the same experience. As it can be seen in Figure 20, co-exposition with salubrinal failed to produce significant differences when compared to the groups treated with tomatine alone. Our results indicate that glycoalkaloids toxicity at the level of the ER may be exerted selectively on neuronal cells.

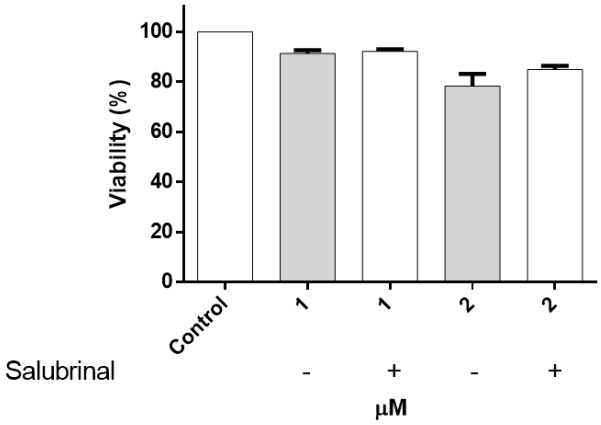

Figure 20 – Differences on cell viability of AGS cells when exposed to tomatine alone or with co-incubation with the eIF2α phosphorylation inhibitor salubrinal. Results presented with mean ± standard deviation of the mean.

3.1.5. Glycoalkaloid toxicity in neurons is caspase-independent

In order to evaluate the possible contribution of caspases to glycoalkaloid-induced cell death, we incubated the cells with the compounds in the presence of Z-VAD.fmk, a pan-caspase inhibitor. This experiment did not result in any significant differences in toxicity (Figure 21), which strongly suggests that the cell death induced by tomatine and tomatidine relies on caspase-independent mechanisms.

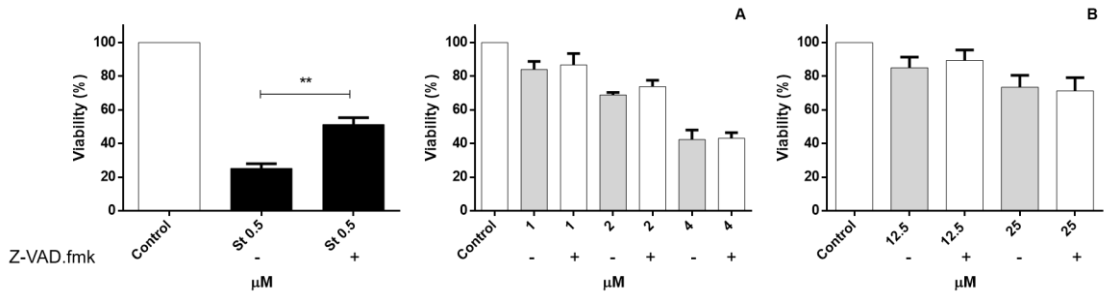

Figure 21 – Effect of co-exposition of glycoalkaloids with Z-VAD.fmk, a pan-caspase inhibitor, on SH-SY5Y cells. Staurosporine at 0.5 µM was used as a positive control for the

effect of caspase inhibition on cell viability. Results presented with mean ± standard deviation of the mean.

This result was confirmed by evaluating caspase 3/7 activity following incubation with the compounds, which did not elicit any activation (Figure 22). This results confirm the occurrence of a cell death mechanism other than classical apoptosis through the mitochondrial or death receptor pathways.

These results agree with previous literature, which reports the same results on different cell lines and in *in vivo* experiments regarding the anti-cancer potential of tomatine (90).

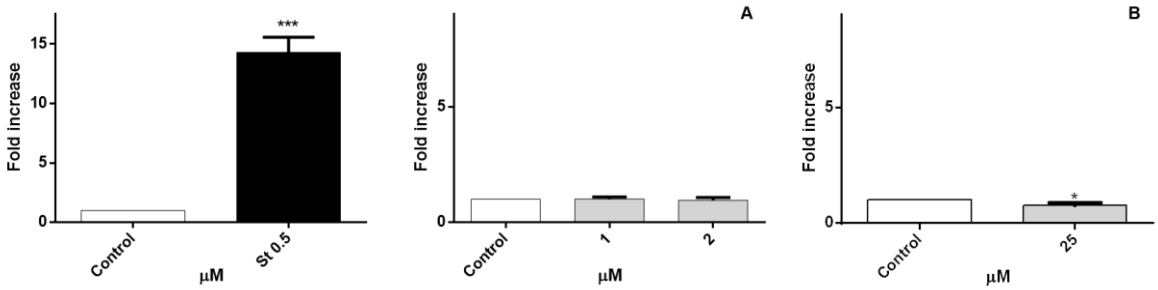

Figure 22 – Effect of glycoalkaloids in caspase 3/7 activities in SH-SY5Y cells. Staurosporine at 0.5 µM was employed as a positive control for maximum caspase activity. Results presented as mean ± standard deviation of the mean.

Differently, in the case of AGS cells there is an involvement of caspases in glycoalkaloid toxicity, as shown in Figure 23. Along with the results obtained concerning cytosolic LDH leakage and cell morphology, these results raise the possibility that AGS cell death induced by glycoalkaloids occurs through necroptosis.

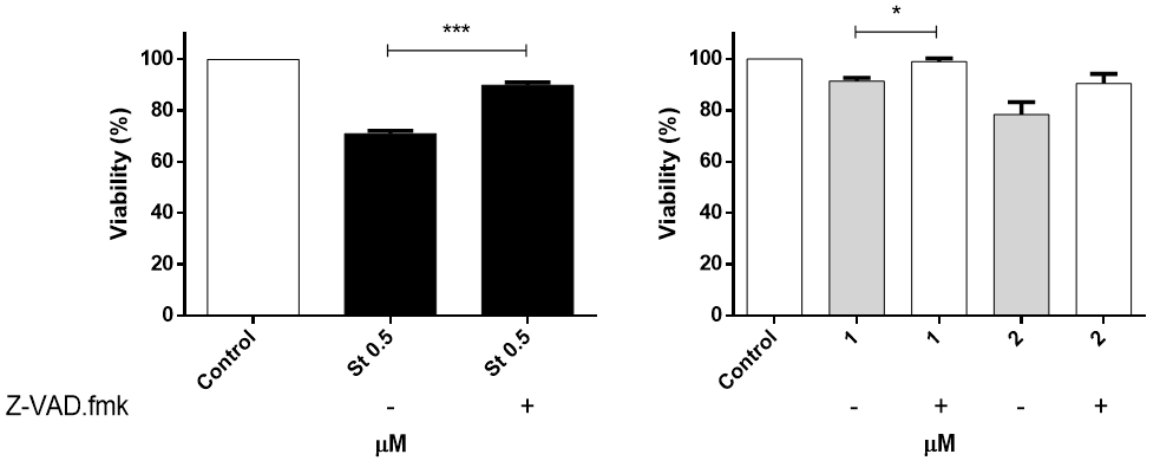

Figure 23 – Effect of co-exposition of glycoalkaloids with Z-VAD.fmk, a pan-caspase inhibitor, on AGS cells. Staurosporine at 0.5 µM was used as a positive control for the effect of caspase inhibition on cell viability. Results presented with mean ± standard deviation of the mean.

3.1.6. Effect of glycoalkaloids on 20S proteasome activity

In light of the involvement of ER stress to the activity displayed by these tomato plant molecules, we assessed the capacity of these compounds to inhibit the proteasome, given its importance to protein homeostasis.

Results reveal that tomatine can inhibit 20S proteasome activity in very low concentrations (2 µM). Once again the glycoalkaloid displays a stronger activity, when compared to its aglycon, which does not seem to share the potential of tomatine for proteasome inhibition (Figure 24).

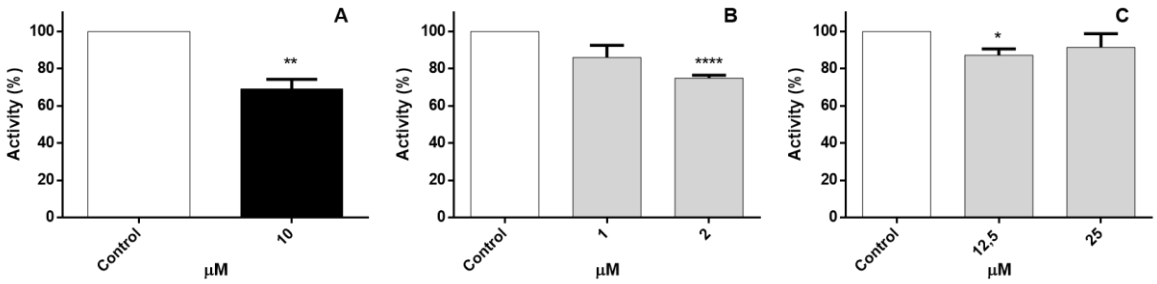

Figure 24 – Influence of tomatine (B) and tomatidine (C) in the 20S proteasome activity. Lactacystin (A) was employed as a positive control for proteasome inhibition. Results presented with mean ± standard deviation of the mean.

Given the capacity of tomatine to inhibit the proteasome, we cannot rule out the hypothesis that the ER stress triggered by this molecule is a consequence of an overload of misfolded/unfolded proteins that would normally by destroyed by this catalytic complex.

20S proteasome inhibitors compose a relatively new approach in the development of anti-cancer drugs. These molecules are able to inhibit cancer cells through several distinct mechanisms – they can induce apoptosis, block the cell cycle, and prevent tumour angiogenesis, among several other processes (57, 91). Considering our results, it can be useful to further investigate the potential of tomatine for this purpose.

3.2. Effect of glycoalkaloids on RAW 264.7 macrophages

Given the fact that natural products frequently exert several biological activities, we were interested in pursuing other biological targets. For this reason, the macrophage cell line RAW 264.7 was used.

3.2.1. Glycoalkaloid cytotoxicity

As usual, the first experiments were used to determine the potential toxicity of the molecules under study in the cell line to be used. Similarly to what we observed with AGS cells, tomatidine did not exert any toxicity on RAW 264.7 macrophages. Interestingly, this cells demonstrate to be more resistant to tomatine toxicity than both AGS and SH-SY5Y cell lines (Figure 25).

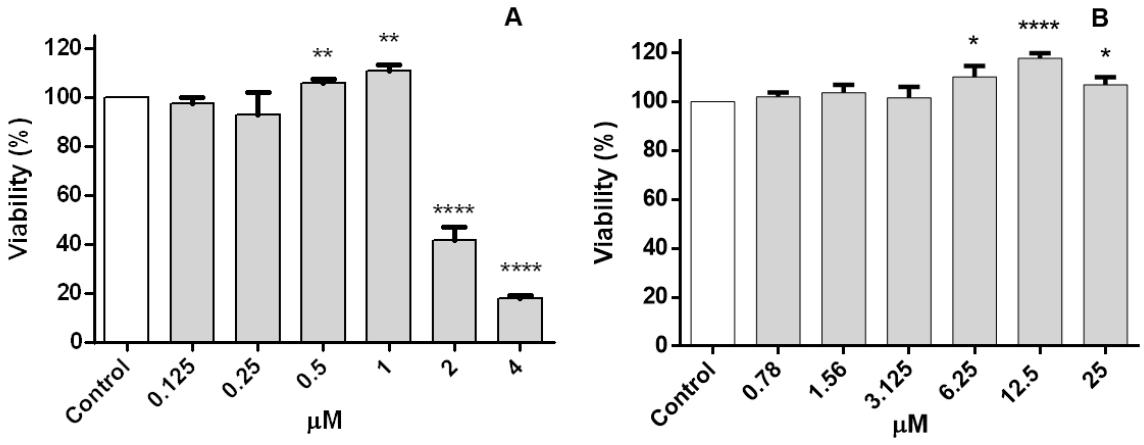

Figure 25 – Effect of tomatine (A) and tomatidine (B) on RAW 264.7 cell viability after 24 hours, evaluated by MTT reduction assay. Results presented as mean ± standard deviation of the mean.

3.2.2. Influence of glycoalkaloids in the production of NO by LPS-stimulated macrophages

Production of NO is one of the hallmarks of inflammation and, for this reason, we have studied the potential anti-inflammatory activity of tomatine and tomatidine by monitoring the levels of this mediator in LPS-challenged macrophages.

Our results show that tomatine is not very effective as an anti-inflammatory agent by inhibition of NO production, as the tested concentrations, 0,125-1 μM, could not lower the amounts of NO in the collected supernatant more than 10% (Figure 27). Concentrations above 1 μM were cytotoxic to RAW 264.7 macrophages and so they could not be applied to this purpose. Tomatidine, less cytotoxic, could be tested up to 25 μM, concentration which was capable of lowering NO production in about 20%.

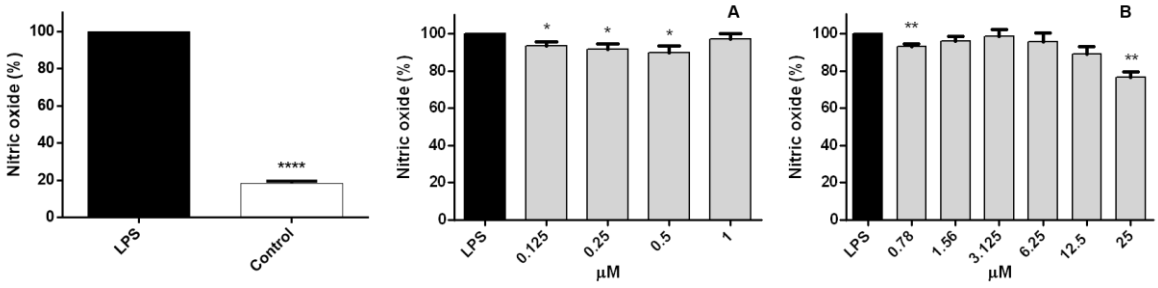

Figure 26 – Influence of tomatine (A) and tomatidine (B) in production of NO by LPS-stimulated RAW 264.7 macrophages. Results presented as mean ± standard deviation of the mean.

3.2.3. Influence of glycoalkaloids in the honey bee PLA_2 activity

Phospholipase A_2 is an enzyme which acts in the arachidonic acid pathway and is able to hydrolyze phospholipids of cellular membranes (92). We determined that glycoalkaloids are not able to inhibit this enzyme (Figure 28), and so their previously described anti-inflammatory activity does not rely in the arachidonic acid pathway inhibition.

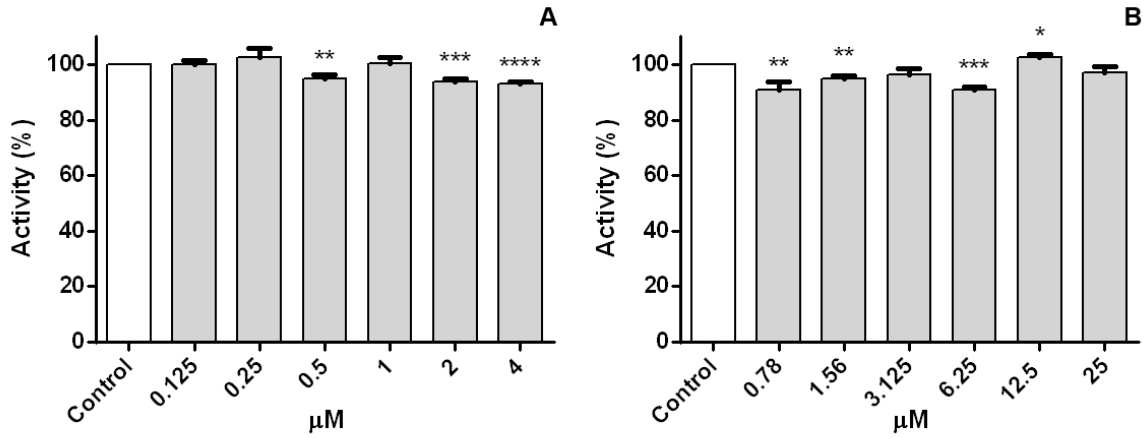

Figure 27 – Influence of tomatine (A) and tomatidine (B) in the activity of the honey bee PLA_2. Results presented as mean ± standard deviation of the mean.

4. Conclusions and future perspectives

In order to clarify the mechanisms underlying glycoalkaloid-induced cell death, we found that glycoalkaloid toxicity is largely caspase-independent. We determined that these compounds exert their effect through the endoplasmic reticulum, since they disrupt calcium homeostasis and promote the UPR by inducing eIF2α phosphorylation.

In order to accomplish our goal, it would be interesting to evaluate the changes in the mitochondrial membrane potential. It could also be relevant to determine if the death domain receptor-associated adaptor kinase RIP (RIP1) is involved in the cell death process.

Furthermore, to the purpose of examining further the involvement of the UPR in glycoalkaloid toxicity, it could be useful to study the remaining branches of this mechanism and to assess the involved genes by RT-PCR.

The tomato is one of the most important foodstuffs worldwide, which justifies the research on its properties. To the best of our knowledge, the effects of tomatine on the ER were still unknown. The obtained results are useful in that they go towards the objectives of this work, which was to unveil new mechanisms underlying glycoalkaloid toxicity.

5. References

1. Cárdenas PD, Sonawane PD, Heinig U, Bocobza SE, Burdman S, Aharoni A. The bitter side of the nightshades: Genomics drives discovery in Solanaceae steroidal alkaloid metabolism. Phytochemistry. 2015;113:24-32.

2. Chowański S, Adamski Z, Marciniak P, Rosiński G, Büyükgüzel E, Büyükgüzel K, et al. A Review of Bioinsecticidal Activity of Solanaceae Alkaloids. Toxins. 2016;8(3):60.

3. Friedman M. Anticarcinogenic, Cardioprotective, and Other Health Benefits of Tomato Compounds Lycopene, α-Tomatine, and Tomatidine in Pure Form and in Fresh and Processed Tomatoes. Journal of Agricultural and Food Chemistry. 2013;61(40):9534-50.

4. Ferreres F, Taveira M, Pereira DM, Valentão P, B. Andrade P. Tomato (Lycopersicon esculentum) Seeds: New Flavonols and Cytotoxic Effect. Journal of Agricultural and Food Chemistry. 2010;58(5):2854-61.

5. Fürstenberg-Hägg J, Zagrobelny M, Bak S. Plant Defense against Insect Herbivores. Int J Mol Sci. 2013;14(5):10242-97.

6. Taiz L, Zeiger E. Plant Physiology: Sinauer Associates Inc., Sunderland, MA U.S.A; 2010.

7. Tohge T, Alseekh S, Fernie AR. On the regulation and function of secondary metabolism during fruit development and ripening. Journal of Experimental Botany. 2014;65(16):4599-611.

8. Waterman PG. Alkaloids: Biochemistry, Ecology, and Medicinal Applications. 2013.

9. Friedman M. Tomato Glycoalkaloids: Role in the Plant and in the Diet. Journal of Agricultural and Food Chemistry. 2002;50(21):5751-80.

10. Simons V, Morissey JP, Latijnhowrs M, Csukai M, Cleaver A, Yarrow C, et al. Dual effects of plant steroidal alkaloids on *Saccharomyces cerevisiae*. Antimicrobial Agents And Chemotherapy. 2006;50(8):2732-40.

11. Hoagland RE. Toxicity of tomatine and tomatidine on weeds, crops and phytopathogens fungi. Allelopathy Journal. 2009;23(2):425-36.

12. Moses T, Papadopoulou KK, Osbourn A. Metabolic and functional diversity of saponins, biosynthetic intermediates and semi-synthetic derivatives. Critical Reviews in Biochemistry and Molecular Biology. 2014;49(6):439-62.

13. Podolak I, Galanty A, Sobolewska D. Saponins as cytotoxic agents: a review. Phytochemistry Reviews. 2010;9(3):425-74.

14. Francis G, Kerem Z, Makkar HPS, Becker K. The biological action of saponins in animal systems: a review. British Journal of Nutrition. 2002;88(06):587-605.

15. Dewick PM. Medicinal Natural Products: A Biosynthetic Approach. 3rd ed: Wiley; 2009.

16. Nohara T, Manabe H, Fujiwara Y, Ikeda T, Ono M, Murakami K, et al. Conversion of tomato saponins to pregnane derivatives. Chem Pharm Bull 2014;62(5):483-7.

17. Nohara T, Ikeda T, Fujiwara Y, Matsushita S, Noguchi E, Yoshimitsu H, et al. Physiological functions of solanaceous and tomato steroidal glycosides. J Nat Med 2007(61):1-13.

18. Itkin M, Rogachev I, Alkan N, Rosenberg T, Malitsky S, Masini L, et al. GLYCOALKALOID METABOLISM1 Is Required for Steroidal Alkaloid Glycosylation and Prevention of Phytotoxicity in Tomato. The Plant Cell. 2011;23(12):4507-25.

19. Bruneton J. Pharmacognosie: Phytochimie, Plantes Médicinales, 4éme ed.2009.

20. Ventrella E, Marciniak P, Adamski Z, Rosinski G, Chowanski S, Falabella P, et al. Cardioactive properties of Solanaceae plant extracts and pure glycoalkaloids on *Zophobas atratus*. Insect Science. 2015;22:251-62.

21. Milner SE, Brunton NP, Jones PW, O Brien NM, Collins SG, Maguire AR. Bioactivities of glycoalkaloids and their aglycones from solanum species. Journal of Agricultural and Food Chemistry. 2011;59(8):3454-84.

22. Sandrock RW, VanEtten HD. Fungal Sensitivity to and Enzymatic Degradation of the Phytoanticipin α-Tomatine. Phytopathology. 1998;88(2):137-43.

23. Nohara T, Fujiwara Y, Zhou JR, Urata J, Ikeda T, Murakami K, et al. Saponins, esculeosides B-1 and B-2, in tomato juice and sapogenol, esculeogenin B1. Chemical and Pharmaceutical Bulletin. 2015;63(10):848-50.

24. Pohanka M. Inhibitors of Acetylcholinesterase and Butyrylcholinesterase Meet Immunity. International Journal of Molecular Sciences. 2014;15(6):9809-25.

25. Ahmed F, Ghalib RM, Sasikala P, Ahmed KKM. Cholinesterase inhibitors from botanicals. Pharmacognosy Reviews. 2013;7(14):121-30.

26. Oliveira A, Lobo-da-Cunha A, Taveira M, Ferreira M, Valentão P, Andrade P. Digestive Gland from Aplysia depilans Gmelin: Leads for Inflammation Treatment. Molecules. 2015;20(9):15766.

27. Zhao B, Zhou B, Bao L, Yang Y, Guo K. Alpha-Tomatine Exhibits Anti-inflammatory Activity in Lipopolysaccharide-Activated Macrophages. Inflammation. 2015;38(5):1769-76.

28. Faix JD. Biomarkers of sepsis. Critical Reviews in Clinical Laboratory Sciences. 2013;50(1):23-36.

29. Aktan F. iNOS-mediated nitric oxide production and its regulation. Life Sciences. 2004;75(6):639-53.

30. Abdel-Aziz AAM, Abou-Zeid LA, ElTahir KEH, Ayyad RR, El-Sayed MAA, El-Azab AS. Synthesis, anti-inflammatory, analgesic, COX-1/2 inhibitory activities and molecular docking studies of substituted 2-mercapto-4(3H)-quinazolinones. European Journal of Medicinal Chemistry. 2016;121:410-21.

31. Patrono C. Cardiovascular Effects of Cyclooxygenase-2 Inhibitors: A Mechanistic and Clinical Perspective. British Journal of Clinical Pharmacology. 2016.

32. Rouzer CA, Marnett LJ. Cyclooxygenases: structural and functional insights. Journal of Lipid Research. 2009;50(Suppl):S29-S34.

33. Smith WL, Langenbach R. Why there are two cyclooxygenase isozymes. Journal of Clinical Investigation. 2001;107(12):1491-5.

34. Kúdelová J, Seifrtová M, Suchá L, Tomšík P, Havelek R, Řezáčová M. Alpha-tomatine activates cell cycle checkpoints in the absence of DNA damage in human leukemic MOLT-4 cells. Journal of Applied Biomedicine. 2013;11(2):93-103.

35. Dyle MC, Ebert SM, Cook DP, Kunkel SD, Fox DK, Bongers KS, et al. Systems-based discovery of tomatidine as a natural small molecule inhibitor of skeletal muscle atrophy. Journal of Biological Chemistry. 2014;289(21):14913-24.

36. Shieh JM, Cheng TH, Shi MD, Wu PF, Chen Y, Ko SC, et al. α-Tomatine Suppresses Invasion and Migration of Human Non-Small Cell Lung Cancer NCI-H460 Cells Through Inactivating FAK/PI3K/Akt Signaling Pathway and Reducing Binding Activity of NF-κB. Cell Biochemistry and Biophysics. 2011;60(3):297-310.

37. Friedman M, Levin CE, Lee S-U, Kim H-J, Lee I-S, Byun J-O, et al. Tomatine-Containing Green Tomato Extracts Inhibit Growth of Human Breast, Colon, Liver, and Stomach Cancer Cells. Journal of Agricultural and Food Chemistry. 2009;57(13):5727-33.

38. Lee S-T, Wong P-F, Cheah S-C, Mustafa MR. Alpha-Tomatine Induces Apoptosis and Inhibits Nuclear Factor-Kappa B Activation on Human Prostatic Adenocarcinoma PC-3 Cells. PLoS ONE. 2011;6(4):e18915.

39. Lee K-R, Kozukue N, Han J-S, Park J-H, Chang E-y, Baek E-J, et al. Glycoalkaloids and Metabolites Inhibit the Growth of Human Colon (HT29) and Liver (HepG2) Cancer Cells. Journal of Agricultural and Food Chemistry. 2004;52(10):2832-9.

40. Chao M-W, Chen C-H, Chang Y-L, Teng C-M, Pan S-L. α-Tomatine-Mediated Anti-Cancer Activity <italic>In Vitro</italic> and <italic>In Vivo</italic> through Cell Cycle- and Caspase-Independent Pathways. PLoS ONE. 2012;7(9):e44093.

41. Friedman M, McQuistan T, Hendricks JD, Pereira C, Bailey GS. Protective effect of dietary tomatine against dibenzo[a,l]pyrene (DBP)-induced liver and stomach tumors in rainbow trout. Molecular Nutrition & Food Research. 2007;51(12):1485-91.

42. Shih Y-W, Shieh J-M, Wu P-F, Lee Y-C, Chen Y-Z, Chiang T-A. α-Tomatine inactivates PI3K/Akt and ERK signaling pathways in human lung adenocarcinoma A549 cells: Effect on metastasis. Food and Chemical Toxicology. 2009;47(8):1985-95.

43. Chiu C-T, Chen J-H, Chou F-P, Lin H-H. Hibiscus sabdariffa Leaf Extract Inhibits Human Prostate Cancer Cell Invasion via Down-Regulation of Akt/NF-κB/MMP-9 Pathway. Nutrients. 2015;7(7):5065-87.

44. Lavie Y, Harel-Orbital T, Gaffield W, Liscovitch M. Inhibitory effect of steroidal alkaloids on drug transport and multidrug resistance in human cancer cells. Anticancer Research. 2001; 21(2A):1189-94.

45. Pereira DM, Valentao P, Correia-da-Silva G, Teixeira N, Andrade PB. Translating endoplasmic reticulum biology into the clinic: a role for ER-targeted natural products? Natural Product Reports. 2015;32(5):705-22.

46. Stutzmann GE, Mattson MP. Endoplasmic reticulum Ca^{2+} handling in excitable cells in health and disease. Pharmacol Rev. 2011;63:700-27.

47. English AR, Voeltz GK. Endoplasmic Reticulum Structure and Interconnections with Other Organelles. Cold Spring Harbor Perspectives in Biology. 2013;5(4):a013227.

48. Mekahli D, Bultynck G, Parys JB, De Smedt H, Missiae L. Endoplasmic-Reticulum Calcium Depletion and Disease. Cold Spring Harb Perspect Biol. 2011;3.

49. Solovyova N, Veselovsky N, Toescu EC, Verkhratsky A. Ca(2+) dynamics in the lumen of the endoplasmic reticulum in sensory neurons: direct visualization of Ca(2+)-induced Ca(2+) release triggered by physiological Ca(2+) entry. The EMBO Journal. 2002;21(4):622-30.

50. Chakrabarti A, Chen AW, Varner JD. A review of the mammalian unfolded protein response. Biotechnol Bioeng 2011;108(12):2777-93.

51. Guerriero CJ, Brodsky JL. The delicate balance between secreted protein folding and endoplasmic reticulum-associated degradation in human physiology. Physiol Rev. 2012;92(2):537-76.

52. Minamino T, Komuro I, Kitakaze M. Endoplasmic Reticulum Stress As a Therapeutic Target in Cardiovascular Disease. Circulation Research. 2010;107(9):1071-82.

53. Bravo-Sagua R, Rodriguez AE, Kuzmicic J, Gutierrez T, Lopez-Crisosto C, Quiroga C, et al. Cell death and survival through the endoplasmic reticulum mitochondrial axis. Curr Mol Med. 2013;13(2):317-29.

54. Tsujii S, Ishisaka M, Hara H. Modulation of endoplasmic reticulum stress in Parkinson's disease. European Journal of Pharmacology.EJP41675.

55. Logue S, Cleary P, Saveljeva S, Samali A. New directions in ER stress-induced cell death. Apoptosis. 2013;18(5):537-46.

56. Schönthal AH. Endoplasmic reticulum stress: Its role in disease and novel prospects for therapy. Scientifica. 2012.

57. Frankland-Searby S, Bhaumik SR. The 26S Proteasome Complex: An Attractive Target for Cancer Therapy. Biochimica et biophysica acta. 2012;1825(1):64-76.

58. Jacobson AD, MacFadden A, Wu Z, Peng J, Liu C-W. Autoregulation of the 26S proteasome by in situ ubiquitination. Molecular Biology of the Cell. 2014;25(12):1824-35.

59. Schmidt M, Finley D. Regulation of proteasome activity in health and disease. Biochimica et biophysica acta. 2014;1843(1):10.1016/j.bbamcr.2013.08.012.

60. Dahlmann B. Role of proteasomes in disease. BMC Biochemistry. 2007;8(1):1-12.

61. Kroemer G, Galluzzi L, Vandenabeele P, Abrams J, Alnemri ES, Baehrecke EH, et al. Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. Cell death and differentiation. 2009;16(1):3-11.

62. Elmore S. Apoptosis: A Review of Programmed Cell Death. Toxicologic pathology. 2007;35(4):495-516.

63. Chang HY, Yang X. Proteases for Cell Suicide: Functions and Regulation of Caspases. Microbiology and Molecular Biology Reviews. 2000;64(4):821-46.

64. Ly JD, Grubb DR, Lawen AL. The mitochondrial membrane potential (ΔΨm) in apoptosis; an update. Apoptosis. 2003;8:115–28.

65. Martinvalet D, Zhu P, Lieberman J. Granzyme A Induces Caspase-Independent Mitochondrial Damage, a Required First Step for Apoptosis. Immunity. 2005;22(3):355-70.

66. Simon H-U, Haj-Yehia A, Levi-Schaffer F. Role of reactive oxygen species (ROS) in apoptosis induction. Apoptosis. 2000;5:415-8.

67. McIlwain DR, Berger T, Mak TW. Caspase Functions in Cell Death and Disease. Cold Spring Harbor Perspectives in Biology. 2013;5(4):a008656.

68. Zhivotovsky B, Orrenius S. Calcium and cell death mechanisms: A perspective from the cell death community. Cell Calcium. 2011;50(3):211-21.

69. Bröker LE, Kruyt FAE, Giaccone G. Cell Death Independent of Caspases: A Review. Clinical Cancer Research. 2005;11(9):3155-62.

70. Rock KL, Kono H. The inflammatory response to cell death. Annual review of pathology. 2008;3:99-126.

71. Lim JP, Gleeson PA. Macropinocytosis: an endocytic pathway for internalising large gulps. Immunol Cell Biol. 2011;89(8):836-43.

72. Golstein P, Kroemer G. Cell death by necrosis: towards a molecular definition. Trends in Biochemical Sciences. 2007;32(1):37-43.

73. Altman BJ, Rathmell JC. Metabolic Stress in Autophagy and Cell Death Pathways. Cold Spring Harbor perspectives in biology. 2012;4(9):a008763-a.

74. Sperandio S, de Belle I, Bredesen DE. An alternative, nonapoptotic form of programmed cell death. Proceedings of the National Academy of Sciences of the United States of America. 2000;97(26):14376-81.

75. Lee D, Kim IY, Saha S, Choi KS. Paraptosis in the anti-cancer arsenal of natural products. Pharmacology and Therapeutics. 2016;162:120-33.

76. Bury M, Girault A, Megalizzi V, Spiegl-Kreinecker S, Mathieu V, Berger W, et al. Ophiobolin A induces paraptosis-like cell death in human glioblastoma cells by decreasing BKCa channel activity. Cell Death Dis. 2013;4:e561.

77. Jia DP, Wang S, Zhang BC, Fang F. Paraptosis triggers mitochondrial pathway-mediated apoptosis in Alzheimer's disease. Experimental and Therapeutic Medicine. 2015;10(2):804-8.

78. Portt L, Norman G, Clapp C, Greenwood M, Greenwood MT. Anti-apoptosis and cell survival: A review. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research. 2011;1813(1):238-59.

79. Riss TL, Moravec RA, Niles AL, Benink HA, Worzella TJ, Minor L. Cell Viability Assays. Assay Guidance Manual: Eli Lilly & Company and the National Center for Advancing Translational Sciences, Bethesda (MD); 2004.

80. Pereira DM, Cheel J, Areche C, San-Martin A, Rovirosa J, Silva LR, et al. Anti-Proliferative Activity of Meroditerpenoids Isolated from the Brown Alga Stypopodium flabelliforme against Several Cancer Cell Lines. Marine Drugs. 2011;9(5):852-62.

81. Pereira MD, Correia-da-Silva G, Valentão P, Teixeira N, Andrade BP. Palmitic Acid and Ergosta-7,22-dien-3-ol Contribute to the Apoptotic Effect and Cell Cycle Arrest of an Extract from Marthasterias glacialis L. in Neuroblastoma Cells. Marine Drugs. 2014;12(1).

82. Kögel D, JHM P. Caspase-Independent Cell Death Mechanisms. Madame Curie Bioscience Database [Internet]: Austin (TX): Landes Bioscience.

83. Dang D, Rao R. Calcium-ATPases: Gene disorders and dysregulation in cancer. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research. 2016;1863(6, Part B):1344-50.

84. Bittremieux M, Parys JB, Pinton P, Bultynck G. ER functions of oncogenes and tumor suppressors: Modulators of intracellular Ca2+ signaling. Biochimica et Biophysica Acta - Molecular Cell Research. 2016;1863(6):1364-78.

85. Dubois C, Prevarskaya N, Vanden Abeele F. The calcium-signaling toolkit: Updates needed. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research. 2016;1863(6, Part B):1337-43.

86. Goswami P, Gupta S, Biswas J, Joshi N, Swarnkar S, Nath C, et al. Endoplasmic Reticulum Stress Plays a Key Role in Rotenone-Induced Apoptotic Death of Neurons. Molecular Neurobiology. 2016;53(1):285-98.

87. Nakka VP, Prakash-babu P, Vemuganti R. Crosstalk Between Endoplasmic Reticulum Stress, Oxidative Stress, and Autophagy: Potential Therapeutic Targets for Acute CNS Injuries. Molecular Neurobiology. 2016;53(1):532-44.

88. Kessel D. Protection of Bcl-2 by salubrinal. Biochemical and Biophysical Research Communications. 2006;346(4):1320-3.

89. Harding HP, Zhang Y, Bertolotti A, Zeng H, Ron D. Perk Is Essential for Translational Regulation and Cell Survival during the Unfolded Protein Response. Molecular Cell. 2000;5(5):897-904.

90. Kim SP, Nam SH, Friedman M. The tomato glycoalkaloid α-tomatine induces caspase-independent cell death in mouse colon cancer CT-26 cells and transplanted tumors in mice. Journal of Agricultural and Food Chemistry. 2015;63:1142-50.

91. Adams J, Palombella VJ, Sausville EA, Johnson J, Destree A, Lazarus DD, et al. Proteasome inhibitors: a novel class of potent and effective antitumor agent. Cancer Res. 1999;59.

92. Rivel M, Solano D, Herrera M, Vargas M, Villalta M, Segura Á, et al. Pathogenesis of dermonecrosis induced by venom of the spitting cobra, Naja nigricollis: An experimental study in mice. Toxicon. 2016;119:171-9.