Marcos André Pinheiro Taveira Monteiro

Lycopersicon esculentum Miller: from by-products towards neuroprotection

Thesis for Doctor Degree in Pharmaceutical Sciences
Phytochemistry and Pharmacognosy Speciality

Work performed under the supervision of
Professor Doctor Paula Cristina Branquinho de Andrade

and co-supervision of
Professor Doctor Patrícia Carla Ribeiro Valentão
Professor Doctor João Paulo Fernandes Teixeira

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To my parents and brothers

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3. Ferreres F, **Taveira M**, Gil-Izquierdo A, Oliveira L, Teixeira T, Valentão P, Simões N, Andrade PB. HPLC-DAD-ESI-MS^n screening of an insect/plant system: the case of *Spodoptera littoralis*/*Lycopersicon esculentum* phenolics and alkaloids. **Rapid Commun Mass Spectrom** 2011, Jul, 25 (14): 1972-80.

4. **Taveira M**, Silva L, Vale-Silva L, Pinto E, Valentão P, Ferreres F, Guedes de Pinho P, Andrade PB. *Lycopersicon esculentum* seeds: an industrial byproduct as an antimicrobial agent. **J Agric Food Chem** 2010, Sep, 58 (17): 9529-36.

5. Ferreres F, **Taveira M**, Pereira DM, Valentão P, Andrade PB. Tomato (*Lycopersicon esculentum*) seeds: new flavonols and cytotoxic effect. **J Agric Food Chem** 2010, Mar, 58(5): 2854-61.

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Oral communications

1. **Taveira M**, Valentão P, Sousa, C, Andrade PB. *Lycopersicon esculentum* Mill. na doença de Alzheimer: que perspetivas? **V Congresso de Análises Clínicas e de Saúde Pública.** 1-3 March 2013 at Escola Superior de Saúde Dr. Lopes Dias do Instituto Politécnico de Castelo Branco, Portugal.

2. **Taveira M**, Ferreres F, Valentão P, Andrade PB. *Lycopersicon esculentum*: potencial químico e biológico. **XI Semana das Ciências Agrárias - As Biociências e o Futuro**. 19-22 April 2010 at Escola Superior Agrária do Instituto Politécnico de Bragança, Portugal.

3. **Taveira M**, Ferreres F, Pereira DM, Valentão P, Andrade PB. Tomato (*Lycopersicon esculentum*) seeds: new flavonoids. **2nd Portuguese Young Chemists Meeting**. 21-23 April 2010 at Universidade de Aveiro, Portugal.

4. **Taveira M**, Pereira MD, Federico F, Valentão P, Andrade PB. Compostos fenólicos no controlo da autenticidade e actividade biológica de sementes de tomate (*Lycopersicon esculentum* Mill.). **Curso de Autenticidade de Produtos Alimentares**. 12-13 March 2010 at Escola Superior Agrária do Instituto Politécnico de Bragança, Portugal.

Poster communications

1. **Taveira M**, Maciejewska M, Costa M, Costa I, Valentão P, Andrade PB. Neuroprotective effect of steroidal alkaloids from *Lycopersicon esculentum* Mill. on glutamate-induced toxicity in SH-SY5Y cells. **4th Workshop Annual BioPlant.** 18 and 19 July 2013, Porto (Portugal).

2. Leitão C, **Taveira M**, Federico F, Gil-Izquierdo A, Valentão P, Andrade PB. *Lycopersicon esculentum* Mill. leaves: from waste to bioactivity. **IJUP13 – 6th Meeting of Young Researchers of U.Porto** 13-15 February 2013, Porto (Portugal).

3. Leitão C, **Taveira M**, Federico F, Gil-Izquierdo A, Valentão P, Andrade PB. HPLC-DAD-ESI/MS^n screening of bioactive compounds in *Lycopersicon esculentum* Mill. leaves. **3rd BioPlant Workshop: Becoming a Plant**. 4 and 5 December 2012, Aveiro (Portugal).

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5. **Taveira M**, Ferreres F, Pereira D, Valentão P, Andrade PB. HPLC/UV-PAD/MS^n-ESI determination of phenolics in *Lycopersicon esculentum* seeds. **XXVth International Conference on Polyphenols**. 24-27 August 2010, Montpellier (France).

6. **Taveira M**, Silva LR, Ferreres F, Vale-Silva L, Pinto E, Valentão P, Andrade PB. *Lycopersicon esculentum* seeds: byproduct valorization. **1ª Workshop Anual BioPlant**. 29 and 30 March 2010, Porto (Portugal).

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AUTHOR'S DECLARATION

The author states to have afforded a major contribution to the conceptual design, technical execution of the work, interpretation of the results and manuscript preparation of the published work included in this dissertation.

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ABSTRACT

ABSTRACT

Tomato (*Lycopersicon esculentum* Miller) processing constitutes one of the major industries worldwide. In Portugal, tomato is one of the main crops, being the second largest exporter of processed tomatoes in Europe. Several tons of residues from its cultivation and processing are produced. Numerous efforts have been made to develop new strategies for an appropriate exploitation of these residues, in order to increase the overall profitability and reduce the environmental impact. In this dissertation the main by-products produced from tomato processing, leaves and seeds, were studied. Two varieties, "bull's heart" and "cherry", were used. In addition, *Spodoptera littoralis* Boisduval is one of the main pests associated to this crop, which can lead to great losses for the producers. This justifies its study as a source of bioactive compounds to take advantage of this infestation and reduce the negative economic impact.

To evaluate tomato seeds health promoting effects, an aqueous extract was analyzed by high-performance liquid chromatography-diode array detection-electrospray ionisation multi-stage mass spectrometry (HPLC-DAD-ESI-MS^n). Fourteen flavonoids were identified, including quercetin, kaempferol and isorhamnetin derivatives, thirteen of them being reported for the first time in tomato seeds. The major identified compounds were quercetin-3-*O*-sophoroside, kaempferol-3-*O*-sophoroside, and isorhamnetin-3-*O*-sophoroside. A concentration-dependent inhibitory effect was observed for acetylcholinesterase (AChE). The same behaviour was noted regarding antioxidant capacity, evaluated against 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$), nitric oxide and superoxide radicals. A weak cell proliferation inhibition, against rat basophile leukemia (RBL-2H3) cell line, was observed with this extract. This vegetable material was further evaluated as a possible antimicrobial agent. The antimicrobial potential of five different tomato seed extracts (methanol, chloroform, ethyl acetate, hexane and sulphuric acid) was checked against Gram-positive (*Staphylococcus aureus*, *Staphylococcus epidermidis*, *Micrococcus luteus*, *Enterococcus faecalis* and *Bacillus cereus*) and Gram-negative (*Proteus mirabilis*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella typhimurium*) bacteria and fungi (*Candida albicans*, *Aspergillus fumigatus* and *Trichophyton rubrum*). The different extracts revealed to be active only against Gram-positive bacteria*, E. faecalis* being the most susceptible one. Concerning antifungal activity, in a general way, *C. albicans* was the most susceptible species. The chemical composition of the extracts was also pursued, concerning organic acids, phenolics and fatty acids and possible relationships with the observed antimicrobial effect were established.

In the materials of *S. littoralis* (larvae, adults, exuviae and excrements) reared in both varieties of *L. esculentum* leaves no phenolics were identified. α-Tomatine was the main steroidal alkaloid in the host plant. The alkaloid composition of the different *S. littoralis* materials was distinct, α-tomatine and dehydrotomatine being the main detected compounds in larvae and excrements. These matrices were the ones with lowest diversity of compounds.

Using HPLC-DAD-ESI-MS^n we were able to characterize fifteen phenolic compounds in *L. esculentum* leaves. Nine of them are reported for the first time. Four steroidal alkaloids were also identified. Some quantitative and qualitative differences were found between leaves of “bull’s heart” and “cherry” varieties, quercetin-3-*O*-rutinoside being the main compound in both. Nevertheless before industrial application, suitable methods to identify and quantify those metabolites should be developed. Solid-phase extraction (SPE) was performed as the purification step before alkaloids analysis. Among the SPE sorbents tested, sulphonic acid bonded silica with H^+ counterion proved to be the most efficient one for removing interfering components. The optimised methods, based on high-performance liquid chromatography coupled to diode array detector (HPLC-DAD), were validated, revealing to be accurate, fast, simple and sensitive. Due to the high content of phenolics, the hydromethanolic extracts of tomato leaves were evaluated concerning their antioxidant potential, showing a concentration-dependent activity against $DPPH^{\bullet}$, nitric oxide and superoxide radicals. Overall, “bull’s heart” variety was more active. The purified extract rich in steroidal alkaloids revealed to have a strong cholinesterase (ChE) inhibition capacity, especially against butyrylcholinesterase (BChE). “Cherry” variety was more active, which can be associated to the presence of high amounts of steroidal alkaloids. Taking into account these results, both purified extracts rich in steroidal alkaloids and their isolated steroidal alkaloids (tomatine and tomatidine) were studied, for the first time, concerning their neuroprotective effect against glutamate-induced toxicity in neuroblastoma cells (SH-SY5Y). The results showed that both purified extracts and isolated compounds, at non-toxic concentrations for gastric (AGS), intestinal (Caco-2) and SH-SY5Y cells, have the capacity to preserve mitochondria membrane potential and to decrease reactive oxygen species levels of SH-SY5Y glutamate-insulted cells. Moreover, the use of specific antagonists of cholinergic receptors allowed observing that tomatine and tomatidine can interact with nicotinic receptors, specifically with the α7 type. No effect on muscarinic receptors was noticed. In addition to the selective cholinesterase’s inhibition revealed by the compounds/extracts, these results provide novel and important insights into their neuroprotective mechanism. This work also demystifies the applicability of these compounds in therapeutics, by demonstrating that their toxicity was overestimated for long time.

Keywords: *Lycopersicon esculentum* Mill.; By-products; Phenolic compounds; Steroidal alkaloids; Alzheimer's disease

RESUMO

RESUMO

A indústria de processamento de tomate (*Lycopersicon esculentum* Miller) é uma das mais importantes a nível mundial. O tomate é um dos principais vegetais produzidos em Portugal, que é o segundo maior exportador de produtos derivados do tomate a nível Europeu. Da indústria do tomate resultam várias toneladas de resíduos. Vários esforços têm sido feitos para tirar partido destes resíduos, de modo a aumentar o lucro das produções agrícolas e reduzir o seu impacto ambiental. Nesta dissertação foram estudados os principais subprodutos do tomate (folhas e sementes) de duas variedades, nomeadamente "coração de boi" e "cereja". Adicionalmente, a *Spodoptera littoralis* Boisduval é uma das principais pragas associadas à produção de tomate. A frequência desta praga justificou a sua caracterização química e avaliação do potencial biológico tendo em vista a sua possível utilização como fonte de compostos bioativos, tirando assim partido da infestação.

Para avaliar os possíveis efeitos benéficos para a saúde das sementes de tomate analisou-se o seu extrato aquoso por HPLC-DAD-ESI-MS^n. Neste extrato foram caracterizados catorze flavonoides, incluindo derivados da quercetina, campferol e isorametina, dos quais treze foram descritos pela primeira vez. Os principais compostos identificados foram a quercetina-3-*O*-soforósido, o campferol-3-*O*-soforósido e a isorametina-3-*O*-soforósido. Relativamente ao seu potencial biológico, foi observada capacidade para sequestrar os radicais $DPPH^{\bullet}$, superóxido e óxido nítrico, assim como para a inibir a enzima acetilcolinesterase, de modo dependente da concentração. Este extrato revelou uma fraca capacidade de inibição da proliferação de basófilos leucémicos de rato (RBL-2H3). Este material foi também avaliado para potencial utilização como agente antimicrobiano. Assim, foram testados diversos extratos de sementes relativamente a bactérias Gram-positivo (*Staphylococcus aureus*, *Staphylococcus epidermidis*, *Micrococcus luteus*, *Enterococcus faecalis* and *Bacillus cereus*) e Gram-negativo (*Proteus mirabilis*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella typhimurium*) e fungos (*Candida albicans*, *Aspergillus fumigatus* and *Trichophyton rubrum*). Os diferentes extratos revelaram-se ativos apenas contra as bactérias Gram-positivo, sendo a *E. faecalis* a espécie mais suscetível. Quanto à atividade antifúngica, os extratos da variedade "coração de boi" foram os mais ativos. De um modo geral, a *C. albicans* foi o fungo mais suscetível. A composição química dos diferentes extratos foi determinada relativamente a ácidos orgânicos, compostos fenólicos e ácidos gordos de e estabeleceu-se uma relação com o efeito antimicrobiano observado.

Nos materiais de *S. littoralis* (larvas, adultos, exúvias e excrementos) alimentados com as duas variedades de *L. esculentum* não foram identificados compostos fenólicos. A α-

tomatina é o principal alcaloide da planta hospedeira. A composição em alcaloides dos diferentes materiais de *S. littoralis* revelou ser distinta, sendo a α-tomatina e desidrotomatina os principais componentes detetados nas larvas e excrementos. Estas matrizes foram as que apresentaram a menor diversidade de compostos.

Nas folhas de *L. esculentum* foram identificados, por HPLC-DAD-ESI-MS^n, quinze compostos fenólicos. Nove deles foram descritos pela primeira vez. Foram igualmente identificados quatro alcaloides esteroídicos. Entre as variedades "coração de boi" e "cereja" foram encontradas algumas diferenças a nível qualitativo e quantitativo, sendo a quercetina-3-*O*-rutinósido o composto principal em ambas. No entanto, para que estes extratos tenham aplicação industrial são necessários métodos adequados para a determinação dos seus constituintes. De modo a viabilizar a análise dos alcaloides esteroídicos procedeu-se à extração em fase sólida (SPE) para purificação dos extratos. Das colunas de SPE testadas, a que tinha como fase estacionária sílica ligada ao ácido sulfónico com o contra-ião H^+ provou ser a mais eficaz na remoção de interferentes. Foram validados métodos baseados na análise por HPLC-DAD, que revelaram ser precisos, rápidos, simples e sensíveis. Face ao seu elevado teor em compostos fenólicos, os extratos hidrometanólicos das duas variedades de folhas de tomateiro foram estudados tendo em conta o seu potencial antioxidante, apresentando uma atividade dependente da concentração contra os radicais $DPPH^{\bullet}$, óxido nítrico e superóxido. De um modo geral, a variedade "coração de boi" foi a mais ativa. O extrato purificado rico em alcaloides esteroídicos revelou ter uma forte capacidade de inibição das colinesterases, especialmente contra a butirilcolinesterase. A variedade "cereja" foi mais ativa, o que pode estar associado à presença de uma quantidade elevada de alcaloides esteroídicos. Atendendo a estes resultados, os dois extratos purificados ricos em alcaloides e os respetivos compostos isolados (tomatina e tomatidina) foram estudados, pela primeira vez, relativamente ao seu efeito neuroprotetor, contra a toxicidade induzida pelo glutamato em células neruonais (SH-SY5Y). Os resultados mostraram que, quer os extratos, quer os compostos isolados, em concentrações não tóxicas para as células gástricas (AGS), do intestino (Caco-2) e SH-SY5Y, têm a capacidade de preservar o potencial da membrana mitocôndrial e para diminuir os níveis de espécie reativas. Adicionalmente, a utilização de antagonistas específicos dos recetores colinérgicos permitiu observar que a tomatina e a tomatidina interagem com os recetores nicotínicos, particularmente com os do tipo α7. Não foi observado qualquer efeito a nível dos recetores muscarínicos. Associando estes resultados à sua capacidade de inibição das colinesterases, podemos estar na presença de novos compostos com potencial para o tratamento da Doença de Alzheimer. Este trabalho desmistifica também a aplicação

destes compostos na terapêutica, demonstrando que a sua toxicidade foi sobrestimada ao longo deste tempo.

Palavras-chave: *Lycopersicon esculentum* Mill.; Subprodutos; Compostos fenólicos; Alcaloides esteroídicos; Doença de Alzheimer

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ABBREVIATIONS AND SYMBOLS

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ACh .....Acetylcholine
AChE.....Acetylcholinesterase
AChR.....Acetylcholine receptors
ACP .....Acyl carrier protein
AD .....Alzheimer's disease
AICD.....Intracellular domain of amyloid precursor protein
APOE .....Apolipoprotein
APP .....Amyloid precursor protein
ATCI .....Acetylthiocholine iodide
Aβ.....Amyloid beta
BChE.....Butyrylcholinesterase
BTCC.....S-Butyrylthiocholine chloride
C4H .....Cinnamate 4-hydroxylase
CFU/ml .....Colony-forming unit per ml
ChE .....Cholinesterase
CHI .....Chalcone isomerase
CHS.....Chalcone synthase
CN .....Ciano
CoA .....Coenzyme A
COMT.....Catechol-*O*-methyltransferase
DAD.....Diode array
DCFH-DA .....2′,7′-Dichlorofluorescein diacetate
DMEM .....Dulbecco's Modified Eagle Medium
DMSO.....Dimethyl sulfoxide
DPPH• .....2,2-Diphenyl-1-picrylhydrazyl radical
DTNB.....5,5'-Dithiobis(2-nitrobenzoic acid)
EC_{50}.....Half maximal effective concentration
ESI .....Electrospray ionisation
FAME .....Fatty acid methyl esters
FBS .....Foetal bovine serum
FGT .....Flavonoid 3-*O*-glucosyltransferase
FLS.....Flavonol synthase
GC-MS .....Gas chromatography coupled with mass spectrometry
HBS.....Hepes buffered saline
HPLC.....High-performance liquid chromatography

HPLC-DAD-ESI-MS^n	High-performance liquid chromatography-diode array detection-electrospray ionisation multi-stage mass spectrometry
IC_{50}	Half maximal inhibitory concentration
IC_{25}	Inhibitory concentration of 25% of reaction
iNOS	Inducible nitric oxide synthase
LC_{50}	Half maximal lethal concentration
LDL	Low-density lipoproteins
LOD	Limit of detection
LOQ	Limit of quantification
mAChR	Muscarinic acetylcholine receptors
MeSA	Methyl salicylate
MHA	Mueller Hinton agar
MHB	Mueller Hinton broth
MIC	Minimum inhibitory concentration
MLA	Methyllycaconitine
MS	Mass spectrometry
MTT	(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
MUFA	Monounsaturated fatty acid
nAChR	Nicotinic acetylcholine receptors
NADH	Nicotinamide adenine dinucleotide reduced form
NBT	Nitroblue tetrazolium
NEAA	Nonessential amino acids
NMR	Nuclear magnetic resonance
$^{\bullet}NO$	Nitric oxide radical
$O_2^{\bullet -}$	Superoxide radical
PAL	Phenylalanine ammonia-lyase
PBS	Phosphate buffer saline
PI3K	Phosphoinositide 3-kinase
PMS	Phenazine methosulfate
PUFA	Polyunsaturated fatty acid
RP-HPLC	Reversed-phase high-performance liquid chromatography
*R*t	Retention time
RSD	Relative standard deviation
SDA	Saboraud dextrose agar
SDB	Sabouraud dextrose broth
SNP	Sodium nitroprusside

SPE .....Solid-phase extraction
SRB .....Sulphorhodamine B
SRSA.....Substance of anaphylaxis
TEAP .....Triethylammonium phosphate
UV .....Ultraviolet

DISSERTATION OUTLINE

DISSERTATION OUTLINE

The present dissertation is divided into five main sections:

Chapter I – Introduction

In chapter I a general overview on the thesis subject and objectives are included. A review on the existing literature about the different topics approached in this dissertation is provided. It affords a basis to understand the objectives and the obtained results.

Chapter II – Experimental section

This section provides detailed information about all the methods and techniques used for the realization of all the works presented in this dissertation.

Chapter III – Results and discussion

This section is divided in four parts. The first three are related to the studies conducted with *L. esculentum* seeds, *S. littoralis* materials and *L. esculentum* leaves, respectively. The last one concerns to the evaluation of the most promising extract for Alzheimer's disease treatment. All of the results are integrated and discussed concerning their relevance, linking them to the existing scientific reports.

Chapter IV – Conclusions and prespectives

The main conclusions that can be taken from the developed work are summarized in this section. In addition, some future prespectives are approched.

Chapter V – References

In this last section are listed all the bibliographic references used in this dissertation.

Chapter I

Introduction

1. *Lycopersicon esculentum* Miller

1.1. General considerations

The plant family Solanaceae contains many taxa, some of them with great relevance in different areas, such as agriculture, medicine and research. The members of this family have a worldwide distribution; nevertheless, the highest diversity of both genera and species is found in the Neotropics. *Solanum* is the largest genus in this family, containing several important crops, namely potato, tobacco and tomato. Species of *Solanum* exhibit an incredible range of morphological forms, ranging from tiny herbs to medium sized forest trees. They are also characterized by the presence of several secondary metabolites, especially alkaloids and phenolics (1).

Among Solanaceae *Lycopersicon esculentum* Miller is highlighted due to its nutritional and economic impact. Its fruits are one of the most consumed and studied matrices in the world, being associated to several health promoting effects on several chronic diseases, including cancer, atherosclerosis, cardiovascular diseases, neurodegenerative diseases, osteoporosis, ageing and male infertility caused by oxidative stress (2).

Linnaeus originally classified tomato as *Solanum lycopersicum* (Linn.). Philip Miller reclassified it to its own genus (1768), naming it *Lycopersicon esculentum* Mill (3). Several years later (1882) Karsten changed it to *Lycopersicum lycopersicum* (Linn.) Karst., retaining the epithet used by Linnaeus, but it was not considered to be correct since the name became a tautonym. Nicolson (1974) suggested an orthographic correction to *Lycopersicon lycopersicum* (Linn.) Karst., indicating that *Lycopersicon* and *lycopersicum* are orthographic variants. Since the name *Lycopersicon lycopersicum* was no longer a tautonym it was accepted as correct. However, as *L. esculentum* Mill. was a more widely known name, a proposal for its conservation was made and accepted in 1983 (4).

Although the botanical classification of this species remains involved in controversy, the plant metabolomics and the leaf structure are the two major reasons for some authors to still consider the genera in separate. For instance, many of the alkaloids common to other *Solanum* species are absent in tomato and their leaves are markedly different from any other *Solanum.* Nevertheless, molecular data from both chloroplast and nuclear genome sequentiation (5, 6) and morphological studies (3, 5) showed that Linnaeus was correct to include tomato in the genus *Solanum*, as they are deeply nested within this

genus, making *Solanum lycopersicum* the correct name. However, both names will probably be found in the literature for some time.

Within the *L. esculentum* species there are several botanical varieties. These varieties can be easily intercrossed with each other to produce viable offspring, creating a huge array of morphological characteristics. However, in spite of the variations between them, they have a common origin (7).

In a general way, *L. esculentum* can be described as a perennial, biennial or annual plant with the following characteristics:

- Branches are usually sprawling or vining, robust to slender and stems are glabrous to pubescent. The trichomes are always simple and usually uniseriate. Sympodial units are di- or trifoliate;
- Leaves are interrupted imparipinnate, sometimes with secondary and tertiary leaflet formation;
- Inflorescences can be simple or have several branches with a peduncle and the flowers are never basal;
- Flowers have a 5-parted calyx with a yellow corolla;
- Fruit is a globose berry and its color can usually range from green to red, yellow or orange. Generally, it has two loci, but several cultivated varieties can be multi-locular. In addition, it has a characteristic accresent calyx;
- Seeds are lenticular, appearing densely hairy due to the elongate testa cell walls (8, 9).

Tomato flowers are hermaphroditic, with both male and female reproductive parts in the same flower. They can be pollinated by bees, which have specialized vibrations of the thoracic flight muscles (10, 11), or by wind (12), the last being mainly responsible for self-pollination.

In this dissertation two different varieties of tomato were studied, namely “cherry” and “bull’s heart”. These varieties were chosen because they are two of the most produced and consumed in Portugal (**Figure 1**).

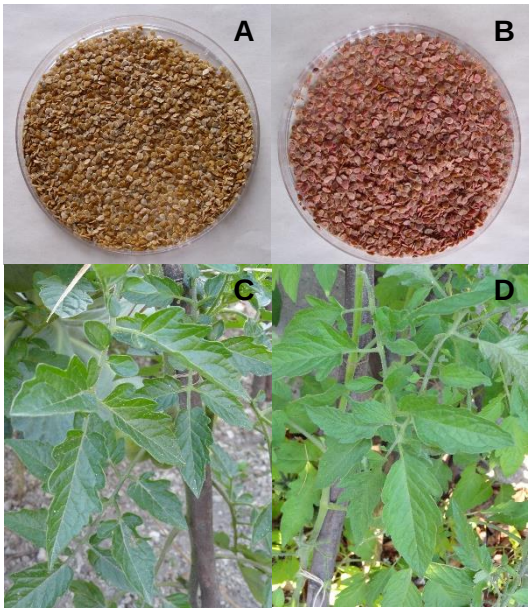


Figure 1. *L. esculentum* materials studied in this dissertation. (**A**) seeds and (**C**) leaves from “cherry” variety; (**B**) seeds and (**D**) leaves from “bull’s heart” variety.

1.2. Economic importance

In terms of agricultural production, tomato and its processing products constitute one of the major industries worldwide. The Asian continent is responsible for about half of the world’s production, followed by the American continent, China and United States being the main producing countries (13).

In Portugal, tomato is one of the main crops (**Figure 2**), being among the 20 greatest producers in 2012 and became the fourth largest exporter of processed tomatoes in the world (second in Europe), having an output of more than 1.2 million tons/year. In addition, our country is the second most productive in the world, with 95 tons/ha, behind California (102 tons/ha) and ahead of Spain (70 tons/ha) (14).

About 95% of domestic production is exported, England, Spain, Holland and Germany being the main markets. Outside Europe, Japan is the largest importer, followed by Kuwait and Australia, with imports of about 7500 tons each. The turnover associated with this activity in 2012 exceeded 250 million Euros (15).

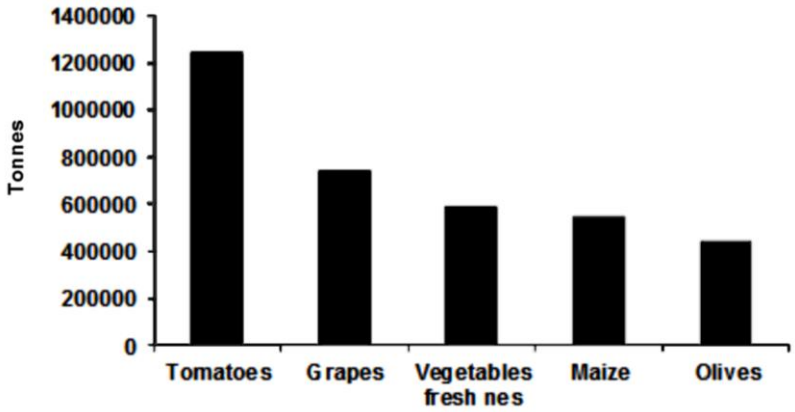


Figure 2. Graphic representation of the most produced commodities in Portugal in 2011; nes: not elsewhere specified (13).

1.3. By-products

Agricultural industry produces several tons of residues derived from the cultivation and processing of different crops. These constitute a pollution, management and economic problem worldwide. Numerous efforts have been made to develop and improve new strategies for the appropriated exploitation of these residues, in order to increase the overall profitability of crops and reduce their environmental impact, using them as a valuable stock material to be applied in a wide range of fields, including bioenergy, biomaterials, compost, fertilizers, food and feed or value-added products (16) (**Figure 3**).

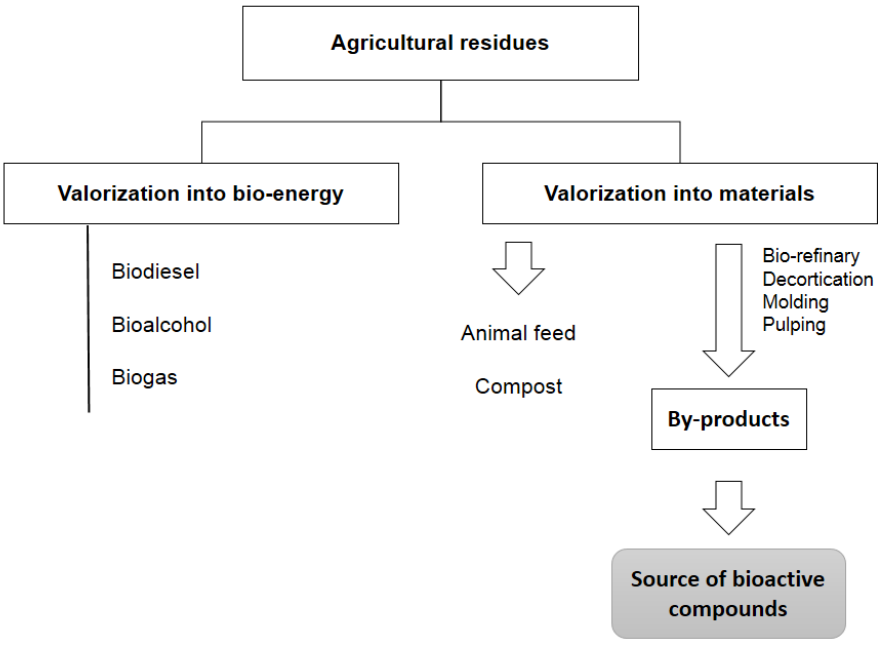


Figure 3. Flow diagram of agricultural biomass exploitation [adapted from (16)].

So, different strategies of utilization of residues from agriculture industry as source for high value-added products can be approached, such as recovery of natural constituents by using conventional or advanced extraction processes.

The main utilization of the agricultural residues is as source of antioxidant compounds, mainly polyphenols, with a potential utilization as dietary supplements and in food fortification, since it is generally assumed that consumers prefer natural compounds to substitute potentially harmful artificial antioxidants (16).

Antimicrobial and antiviral properties of numerous plant by-products (16-18) have also been investigated, being mainly attributed to their phenolics content. So, another possibility is their use in biopreservation of food systems, in the pursuit of shelf life extension and enhancement of food safety by using natural antimicrobial compounds (19).

Other properties attributed to bioactive compounds that can be obtained from by-products are anti-inflammatory (20), antidiabetic (21), cholesterol reduction capacity (22, 23), anti-atherogenic (24) and anticancer (16).

Tomato fruit is mostly consumed in the form of processed products, such as tomato juice, paste, puree, ketchup, sauce and salsa. So, the industrial processing of tomato leads to several by-products, namely seeds, peels and leaves (25). However, most of the studies are focused on the fruit and there is little information about its by-products, which account for about 5-13% of tomato production (16).

In fact, in 2012 Portugal produced 1 294×10^3 tons of tomato. Peel and seed by-products are estimated to correspond to a total residue production of 25.9×10^3 tons/year. The two by-products may be available individually, but are usually combined, the mixture being known as pomace. Tomato leaves correspond to a total residue production of 646.9×10^3 tons/year (16).

One use for these by-products is their inclusion in animal feeds or as fertilizer. However, tomato by-products have a high moisture content, which incurs a drying expense if they are to be used in the dry form. In addition, the demand for feed may vary and be dependent on agricultural yields. Thus, the search for an efficient, inexpensive and environmentally sound application for this material is becoming more important (26).

Tomato by-products are rich in bioactive compounds (16, 25) that can be extracted and used with health promoting purposes. In fact, tomato by-products contain increased amounts of lycopene, β-carotene, tocopherols, sterols, phenolics and terpenes (25, 27).

Some of their biological activities were already reported. Pomace has antiplatelet and antidyslipidemic properties (28, 29). Tomato peels present a strong antioxidant activity, especially due to their high content in phenolic compounds (16, 30). Regarding tomato seeds, their antioxidant activity against synthetic radicals was already reported (30, 31), as well as the presence of several classes of metabolites, like fatty acids, carotenoids, saponins (19), phenolic compounds (32), proteins (33), amino acids and vitamins (34).

Leaves are the less studied by-product, especially due to their possible toxicity, which is associated with the presence of high amounts of steroidal alkaloids (35). However, this matrix presents several other bioactive compounds, such as organic acids, sugars, amino acids (36, 37), phenolics (38), terpenes, fatty acids (39, 40), phytosterols (41), among others. For this matrix it was already reported antibacterial and anti-inflammatory activity through the inhibition of prostaglandin E_2 release (16, 42).

2. Insect-plant interaction

2.1. General considerations

In natural ecosystems, plants and insects are living organisms continuously interacting in a complex way. Herbivores, especially insect larvae, represent a major challenge for plants in their natural environment and the study of the relationships between them is of fundamental importance. Insects cannot exist in the absence of green plants, which serve as the primary source of energy-rich compounds. In addition, long-standing exposure to insects increases cross-pollination, which is responsible for the great genetic diversity in plant kingdom. However, plants are unable to avoid hostile visits by herbivorous insects: depending on the intensity of the attack, herbivores might be extremely harmful to plants leading them to death (43).

Due to these complex interactions, the concept of coevolution was developed to describe the results of the relationship between a particular plant species and the herbivorous insects that feed on it. This concept has been applied to describe the evolutionary results of a wide variety of ecological relationships (44).

As a consequence of this coevolution, insects developed several strategies to overcome plants' defence barriers, allowing them to feed, grow and reproduce on their host plants (45). So, adapted phytophagous insects developed mechanisms of protection like feeding only on those plants' parts that contain minimal amounts of defence compounds, development of guts that are not permeable to the allelochemicals and allow a fast excretion of toxins, detoxification of plant metabolites by several metabolic ways, accumulation, modification or concentration of these defence compounds for their own benefit, either actively or passively and often highly selectively. So, insects can also constitute a source of natural chemicals, some of them different from the ones present in their host plants (43, 45).

2.2. *Spodoptera littoralis* Boisduval

Lepidoptera represent a diverse and important group of agricultural insect pests that cause widespread economic damage on different agricultural crops. Among Lepidoptera, the genus *Spodoptera* is largely studied, including more than 25 species. Some species have a cosmopolitan distribution; others are widespread in Africa and South America, where most of the species are concentrated (46).

Spodoptera larvae prefer to feed on young and tender leaves. However, they may also feed on other plant parts, such as fruits, young stalks, bolls or buds. Young larvae start to feed at numerous small feeding points and eventually spread over the entire leaf. If larvae feed on a young plant heavily, the plant's development is retarded and its production will be seriously affected. In addition, the damages caused in the plant constitute an open gate for other plant diseases (47).

Members of the genus *Spodoptera* undergo complete metamorphosis, passing by egg, larva (caterpillar), pupa and adult (moth). Within it we can highlight *Spodoptera littoralis* Boisduval, which is one of the most destructive agricultural Lepidoptera pests and is widely spread through subtropical and tropical regions, with special emphasis in the Mediterranean area. *S. littoralis* can spread to different countries, through imported commodities, such as glasshouse crops, both ornamentals and vegetables from infested areas. In addition, adult moths can also be spread through wind or attachment to other organism (48).

It is a polyphagous species, which can attack plants belonging to more than 44 different families, including grasses, Solanaceae, crucifers and several fruit trees, all containing species of high economic importance. They heavily attack the crops, reducing the production and exploitation profit (46, 47).

Adult moth from *S. littoralis* can reach up to 2 cm long, with a wingspan of approximately 4 cm and they are usually characterized by white v-shaped forewing marking and white, glossy hind wing. Fully developed larvae can reach 35 to 45 mm long, its color varying from grey to reddish or yellowish. Eggs usually have round shape, upright with a small pore on top; scales cover the eggs and there is an outer membrane with about 40 to 50 longitudinal ribs. They are laid in batches and covered with orange-brown hairs (47).

The digestive system of larvae is remarkably adapted to food scarcity: if food is scarce, it retains and processes the nutrients efficiently. This is possible because *S. littoralis* maintains gut conditions that enables it to maximize the extraction of nutrients from plant tissues and minimizes any deleterious effects of pro-oxidant secondary plant metabolites. The lumen characteristics are the main responsible for that: alkaline pH (8.2-8.5) and reducing conditions promote the oxidation and reduction of allelochemicals, particularly phenolics, and participate in the defence against pathogens (48, 49).

In addition, to minimize the deleterious effect of pro-oxidant secondary plant metabolites, *S. littoralis* presents antioxidant enzymes, namely superoxide dismutase, catalase, ascorbate peroxidase and glutathione peroxidase (49).

Digestive enzymes, such as proteases (serine proteinases, trypsin, chymotrypsin and elastase) (50-53) and amylases (54), are also found in *S. littoralis*. These digestive enzymes, which hydrolyse the food into products suitable for uptake and absorption, are mainly secreted in the midgut, where the majority of general digestion occurs.

When food is abundant, food passes quickly and inefficiently and larvae consume far more food than their subsistence requires. In field crops where host material is abundant, larvae are avid consumers and the damage they can cause is extensive (47).

Furthermore, it should be highlighted the *in vitro* angiotensin converting enzyme inhibitory activity of a nonwater soluble protein fraction (Ala-Val-Phe) of *S. littoralis*. After oral administration, a dipeptide (Val-Phe) is released by *in vivo* peptidases, which is an even more potent inhibitor of this enzyme (55).

2.2.1. Life cycle

S. littoralis needs 19 to 144 days to complete its life cycle. Host plant species and environmental conditions may influence the time necessary for development. Adults usually emerge over a brief period in warm weather and immediately mate. In cold areas, adults may wait 2 to 11 days before mating. The female moth lays eggs in batches of 30 to 300, shedding hairs from the abdomen onto the eggs. The eggs are mainly laid on the lower surface of leaves in the upper third of the plant (47, 48) (**Figure 4**).

Incubation of eggs requires from 2 to 26 days. Under natural good conditions the mortality of eggs is very low and this species may have up to eight generations each year. In colder areas, there may be only three generations *per* year (47).

Larvae develop through five or six instars, which can be reached after 12 to 85 days. Unfavourable climatic conditions, such as rain, falling temperatures and short days increase mortality and prolong the larval period. Pupation takes place in cells 2.5 to 5 cm below the soil surface and varies from 5 to 31 days. Females live longer than males and periods can range from 2 days (in summer) to 22 days (in winter) (47).

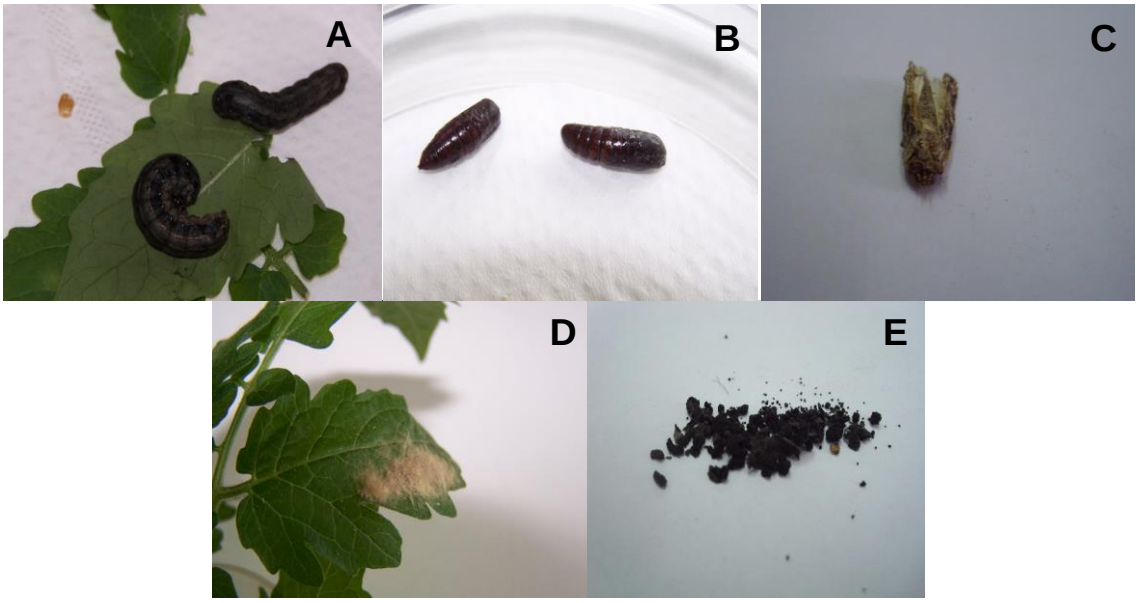


Figure 4. *S. littoralis* materials. (**A**) larvae; (**B**) exuviae; (**C**) adult; (**D**) eggs; (**E**) excrements (photos kindly provided by Doctor Luísa Oliveira from Azores University).

Typically, larvae in the first two instars cluster on the upper third of herbaceous plants, where they feed in small groups on the undersurfaces of leaves. Larvae of the early instars (l^{st}-3^{rd}) feed primarily on leaves during night and during the day they hide in the soil beneath the plants. In the latter instars larvae feed during day and night (47, 48).

They are extremely sensitive to climatic conditions, especially combinations of high temperature and low humidity. Temperatures above 40 °C or below 13 °C may increase mortality (48).

2.2.2. Rearing

S. littoralis can be reared in laboratory under controlled conditions. They can be reared from eggs, larvae, pupae or adults. Eggs should be collected carefully without putting pressure on them, because it will kill the developing larvae, once the chorion is relatively soft. Humidity should be maintained at approximately 70-80%. After hatching most *Spodoptera* larvae are easy to rear on grasses, bean or corn leaves. Alternatively, to reduce mortality rate, artificial diets can be used in the first instars and then larvae are changed to vegetable materials. So far, various artificial diets have been developed and proposed for the maintenance and continuous rearing of insects (56).

Moreover, lepidopteran insects display experimental advantages, such as large body size, accessible genetics and extreme diversity. They show a large spectrum of interactions with plants and with numerous parasites or pathogens (47, 48).

2.3. *S. littoralis vs L. esculentum*

There are already some studies concerning the interaction between *S. littoralis* and different host plants, such as *Arabidopsis thaliana* (L.) Heynh, cotton, maize and lima been plants. These studies mainly focus on the oviposition behaviour, larval development and induction of defences, with special emphasis on the volatile compounds. Nevertheless, there are only few reports about the interaction between *S. littoralis* and *L. esculentum*.

An attack by *S. littoralis* causes an immediat increase of volatile compounds released by tomato plant. Constitutive compounds (mono- and sesquiterpenes) are immediately released after physical damage (caused by the feeding of the herbivores), their rate being correlated with the amount of damage; there is a relatively constant release along time and they rapid decline after feeding ceases (57). In contrast, induced compounds (linalool and indole) show a delay between the time feeding starts and the release of volatiles. In addition, these induced compounds exhibit a diurnal cycling of release, which continues after herbivore's feeding has ceased (57-59).

The content of reducing sugars improves *S. littoralis* leaf consumption. The amount of of ascorbic acid also seems to be important for the development of *S. littoralis*, as there is a negative correlation between this organic acid and insect's mortality (60). In addition, the total phenolic content in tomato leaves is positively correlated with the mortality of *S. littoralis*, this class of compounds being a chemical defence of tomato plant (43, 60).

Besides the chemical defences, it was already reported that tomato leaves present on their surface a sticky exudate produced by the glandular trichomes. This exudate serves as a physical barrier, which can cover *S. littoralis* mouthparts and rear legs. The larvae eventually become entrapped in the exudate and subsequently die. However, this barrier is only efective for larvae at the 1st instar (61).

3. Secondary metabolism

Plants' metabolism leads to the formation of secondary metabolites that are associated with adaptive and physiological functions. Many of these compounds are bioactive and extra nutritional constituents synthesized in small quantities, which have an important biological role. Alkaloids and phenolic compounds are the main classes of compounds characteristic of Solanaceae secondary metabolism (1).

3.1. Alkaloids

Alkaloids are one of the largest classes of secondary metabolites, being widely distributed in the plant and animal kingdoms. In plants, they are most often synthesized in specific sites, namely laticifers (specialized cells) or chloroplasts. Afterwards, they are transported to storage places, mainly located in peripheral tissues like external stem bark and root or seed coat. Due to their basicity and antimetabolite action they have to be compartmentalized in the plant and usually occur as salts of organic or inorganic acids, sometimes exist as tannin complexes and often together with non-alkaloidal compounds (62-64).

Alkaloids play important roles in the organism that produces them, such as nitrogen reserves, antibacterial, antifungal and antiviral agents. They can also be involved in several biological mechanisms, namely chemical defence against herbivores, predators or other plants and attraction of pollinating or seed-dispersing animals (63, 65). This wide range of activities in the nature is only possible due to the enormous variety of structures, which make them also extraordinary difficult to define. Generally, they are defined as natural compounds with a ring structure and a nitrogen atom usually derived from an amino acid. In most of the cases nitrogen is located inside the ring structure. They also have basic characteristics and give positive reactions in precipitation assays, such as those with Dragendorff's, Mayer's or Bertrand's reagents (63, 66).

Alkaloids can have different precursors: ornithine, lysine, phenylalanine, tyrosine, tryptophan, histidine, valine, nicotinic and anthranilic acids. Alkaloids biosynthesis may require the intervention of a single amino acid, two molecules of the same amino acid, rarely two different amino acids or several individual molecules of the same amino acid. In addition, these molecules can suffer different reactions, such as oxidation, esterification, etherification, among others, which justifyes the existence of many structural variations in nature (66).

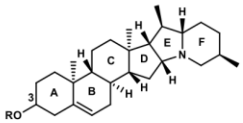
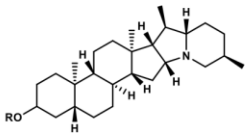
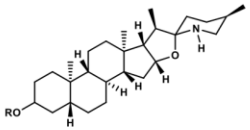
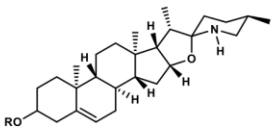
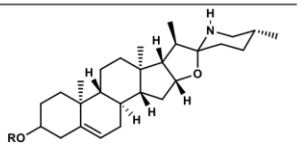
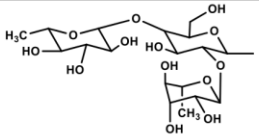
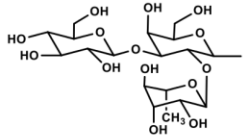
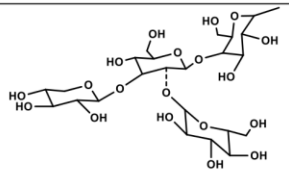
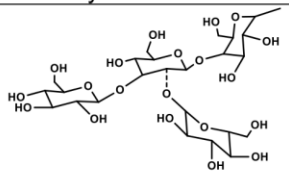
Some alkaloids can also have a mixed biogenetic origin like indolo-monoterpenic alkaloids (tryptophan and secologanoside) or furoquinoleine (anthranilic acid and isopentenyl diphosphate). Nevertheless, there are also other alkaloids that are not derived from the metabolism of an amino acid, namely steroidal, mono-, sesqui-, di- or triterpenic alkaloids, which belatedly incorporate a nitrogen atom, and there are those who classify them as pseudo-alkaloids (66). Among them, the steroidal alkaloids widely present in dietary plants, namely in some Solanaceae species, can be highlighted (35, 67).

3.1.1. Steroidal alkaloids

The structure of these compounds consists on a hydrophobic C_{27} skeleton of cholestane (aglycone) (35), being already described five different aglycone groups: solanidanes (eg. solanidine), spirosolanes (eg. tomatine), epiminocholestanes (eg. solacongestineine), solanocapsines (eg. solanocapsine) and 3-aminospirostanes (eg. jurubine) (68). Steroidal alkaloids usually occur as glycosides in plants. The carbohydrate moiety of the glycoalkaloid is attached to the 3-hydroxyl group of the first ring of the aglycone. Tri- and tetrasaccharides are the most common carbohydrate fraction of glycoalkaloids. The most frequent individual sugars are D-glucose, D-galactose, D-xylose, and L-rhamnose (69).

As so, several structurally different steroidal alkaloids are found in nature (67), some of them sharing the same aglycone (**Table 1**).

Table 1. Chemical structure of some steroidal alkaloids [adapted from (67)].

Glycosidic residue (R at position 3)	Aglycone				
	 Solanidine	 Demissidine	 Tomatidine	 Tomatidenol	 Solasodine
 Chacotriose	**α-Chaconine**	**-**	**-**	**β-Solamarine**	**α-Solamargine**
 Solatriose	**α-Solanine**	**-**	**-**	**α-Solamarine**	**α-Solasonine**
 Lycotetraose	**-**	**Demissine**	**α-Tomatine**	**Dehydrotomatine**	**-**
 Commertetraose	**Dehydrocommersonine**	**Commersonine**	**Sisunine**	**-**	**-**

3.1.1.1. Biosynthetic pathway

The steroidal alkaloids synthesis begins with the germination process (70) and they are firstly synthesized in microsomal organelles or plastids in the cell and then accumulated in the cytoplasm or vacuoles. Unlike other alkaloids, steroidal alkaloids are not transported from one part of the plant into another, remaining at the site of synthesis (62, 70).

Although the biosynthesis of steroidal alkaloids is not exactly known, a summary of the postulated pathway is illustrated in **Figure 5** (35, 67, 71). It has been reported that it starts with the reaction of acetate with acetyl-CoA and then follows through the intermediates of mevalonic acid, squalene, lanosterol and cycloartenol to cholesterol (71, 72). Furthermore, aglycones with double bonds are formed prior to saturate ones (1, 73).

The next step after aglycone formation is glycosylation. Many studies have shown that aglycones are rapid and enzymatically glycosylated to the α-form of glycoalkaloids (74).

As referred before, in this class of alkaloids the nitrogen atom is belatedly incorporated, arginine, glycine and alanine being the most likely source (75). Nevertheless, in tomato leaves steroidal alkaloids biosynthesis is more conditioned by the carbohydrate content than by the nitrogen content (76).

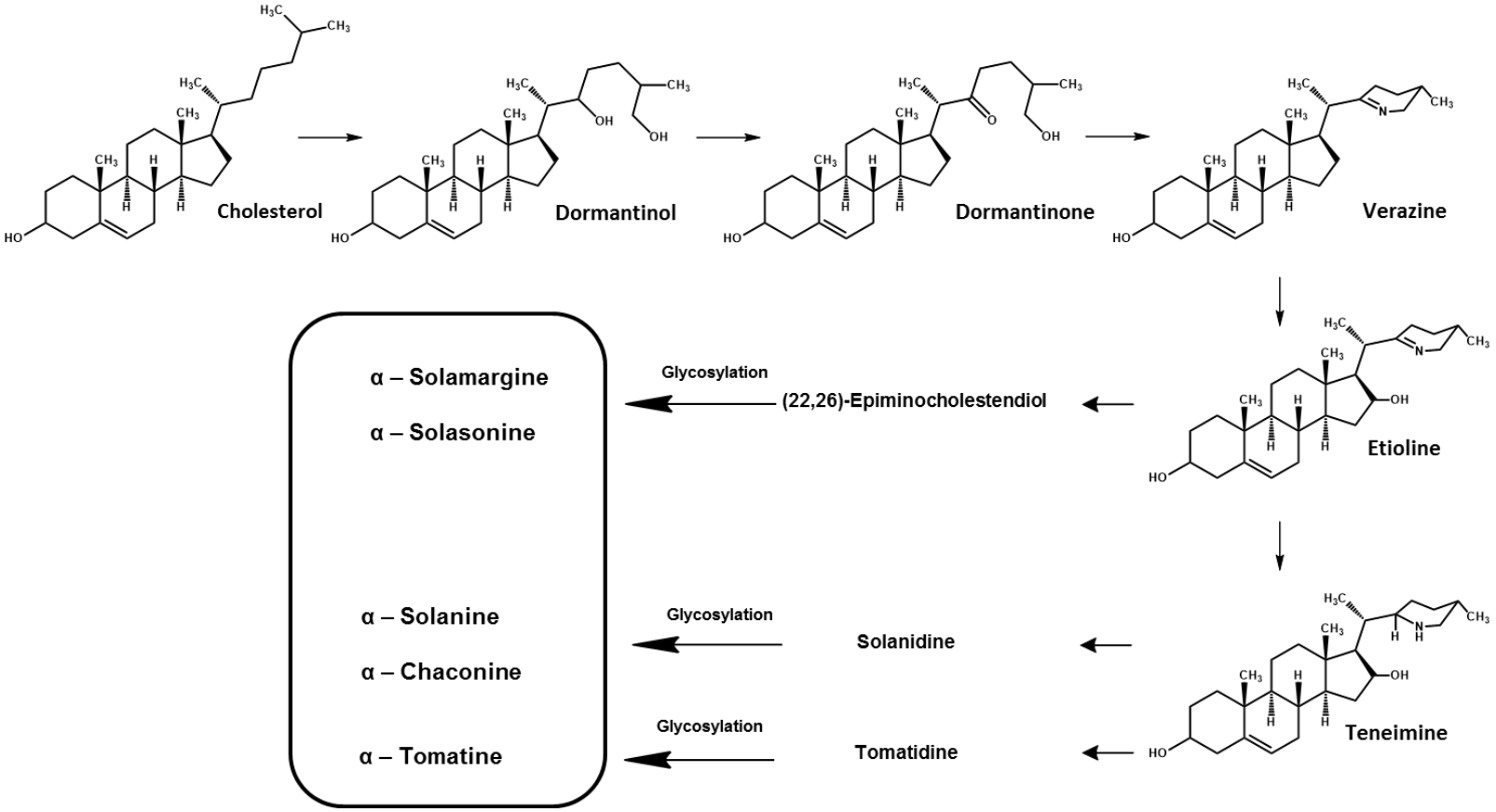


Figure 5. Part of the hypothetical biosynthetic pathway of steroidal alkaloids [adapted from (35, 67)].

3.1.1.2. Extraction, separation and identification

Several solvents have been used in the extraction of steroidal alkaloids, including acetic acid, methanol, methanol with acetic acid, heptane sulfonic acid with acetic acid, methanol with chloroform and tetrahydrofuran/water/acetonitrile/acetic acid mixture (73, 77, 78).

Among them, acetic acid seems to be the most efficient solvent for dried samples. For fresh samples, a mixture of methanol and chloroform (1:1) (79) or tetrahydrofuran/water/acetonitrile/acetic acid (50:30:20:1) (80) is recommended. Freeze-drying is the best method for sample conservation, once it prevents alkaloids' hydrolysis and enzymatic degradation (81).

After extraction, different methods for steroidal alkaloids purification can be used. Traditional precipitation with ammonium hydroxide is not reliable because this process is not quantitative and leads to the loss of several compounds (82). So, to overcome this disadvantage the best method is solid-phase extraction (SPE). Different sorbents can be used, such as C_{18} (83-85), aminopropyl bonded silica (86), octyl and benzenesulfonic acid (SCX)/silica based, SCX (87) and ciano (CN) (88).

For structural determination of aglycones, gas chromatography coupled with mass spectrometry (GC-MS) can be used. In most cases this method requires a pre-hydrolysis and/or derivitization step due to their low volatility (67). Since some steroidal alkaloids share the same aglycone (**Table 1**), this technique can lead to misinterpretations.

For identification and quantification of steroidal alkaloids, high-performance liquid chromatography (HPLC) is the principal method used. Nevertheless, the utilization of ultra-performance liquid chromatography is increasing. This technique allows better resolution and faster separations, mainly due to the small particle size and low flow rates (89, 90).

Different detection procedures can be coupled to HPLC, such as ultraviolet (UV), pulsed amperometry and fluorescence. The most commom detector used is diode array (DAD); however, due to the low specificity of the absorption wavelength (200-210 nm) of steroidal alkaloids, this method may suffer the interference of other compounds. To overcome this disadvantage, a previous sample purification method can be applied (35).

Nevertheless, for structural elucidation, the main methods employed are nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) (63, 91). It should be highlighted that NMR usually requires relatively large amounts of material in comparison to MS and is inefficient in the structural assignment of steroidal alkaloids mixtures (67).

The combination of tandem MS detectors and HPLC constitutes a useful and rapid method for steroidal alkaloids analysis, as it provides information about the nature of the aglycone unit and of the glycosidic linkages. Derivatization or hydrolysis is not required for analysis and limited sample pretreatment is needed. In addition, it gives a highly specific ion signal for each steroidal alkaloid, so it can be used for the analysis of mixtures (63).

Electrospray ionisation (ESI) system can be used to couple HPLC to MS (91, 92). This technique is the most used for alkaloids detection, since it is regarded as a soft ionisation technique, in contrast to fast atom bombardment and matrix-assisted laser desorption/ionisation, because fragmentation of intact quasi-molecular ions is a minor process. In addition, structure-specific fragmentation can be induced in a collision chamber of an MS/MS system, meaning that an ion is formed by ESI, selected in a primary MS experiment and online analysed by a second MS experiment (89). The identification of steroidal alkaloids is usually first based on their molecular weight and further confirmed by comparing the resultant fragmentation pattern with literature or standards (**Table 2**).

To identify aglycone moieties, fragment ions possibly corresponding to aglycones are screened. These ions occur at m/z 410-450 in all steroidal alkaloids. In addition, two distinctive fragment ion pairs are reported to occur by EF-ring cleavage of the steroidal spirosolane, such as m/z 273 and 255 ions in cholestan-3-ol derivatives like tomatidine (89, 93, 94), and ions at m/z 271 and 253 in cholesten-3-ol derivatives, such as tomatidenol (93) (**Table 2**).

Table 2. Most common fragments in steroidal alkaloids HPLC-MS analysis [adapted from (95)].

Ions (m/z)	Structure
253, 271	Cholesten-ol derivative
255, 273	Cholestan-ol derivative
$[M+H]^+$-618	Lycotetraose
$[M+H]^+$-456	Glucose-xylose-glucose
$[M+H]^+$-294	Glucose-xylose
$[M+H]^+$-162	Glucose
$[M+H]^+$-132	Xylose

Fragment ions indicating glycosidic cleavages are also frequent and the composition of the sugar moiety can be elucidated by calculation of neutral loss in MS^n data (95) (**Table 2**).

The use of positive ion mode proved to be advantageous for steroidal alkaloids identification, as it has lower detection limit, is more structurally informative due to the increased amount of fragment ions and increased capabilities for the analysis of crude mixtures (96). In addition, high and low-energy collision-induced disassociation methods can be used (97).

3.1.1.3. Biological activities

Steroidal alkaloids have several physiological functions in plants, such as resistance against bacteria, fungi, nematodes, molluscs and insects. These activities are mainly correlated with the ability to inhibit both acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) and to bind to membrane 3β-hydroxy sterols, causing membrane disruption (67).

The consumption of steroidal alkaloids results in gastrointestinal disturbances, namely vomiting, diarrhoea and usually severe abdominal pain. They can also be potent irritants to the intestinal mucosa (lytic effect), affecting the intestinal permeability and aggravating inflammatory bowel disease. The consumption of high doses can also result in fever, rapid and weak pulse, low blood pressure, rapid respiration and neurological disorders, including drowsiness, apathy, confusion, weakness and visual disturbances. In addition,

as these alkaloids are AChE and BChE inhibitors, they can lead to cholinergic symptoms (35, 67). Moreover, steroidal alkaloids have been found to affect the stability of biomembranes, presenting strong hemolytic properties (67).

Nevertheless, several health promoting effects, such as anticancer, anti-inflammatory, antinociceptive, antipyretic, anticholesterol, antifungal, antimalarial and antibacterial, have been described for these compounds. Some efforts were made to elucidate the structure-activity relationship of glycoalkaloids and aglycones, but only small progresses were made. Generally, it was observed that all these activities can be correlated with both the composition of the carbohydrate side chain and the nature of the aglycone moiety. In addition, it is clear that in most cases the modification of the F ring of any of the aglycone moieties leads to a loss of activity. The saturation between C5 and C6 is more important for solanidane glycoalkaloids than for tomatidine glycoalkaloids. A range of modifications made at the 3-hydroxyl group lead to biological activity variations, both increasing and decreasing depending on the biological system (67).

3.1.2. Steroidal alkaloids in *L. esculentum*

The major steroidal alkaloid in tomato, α-tomatine, was reported to be present in all parts of tomato plant, together with dehydrotomatine (74, 98). α-Tomatine structure consists on a C_{27} cholestane skeleton with an oxa-azaspirodecane system (spirosolane), with a 22*S*, 25*S* configuration (tomatidine) and a tetrasaccharide residue (β-lycotetraose) containing D-galactose, two molecules of D-glucose and D-xylose (**Figure 6**). Dehydrotomatine is the unsaturated ∆5 analogue of α-tomatine and is usually present in lower level (~10-fold less). Their respective genines also appear, but in low amounts (35).

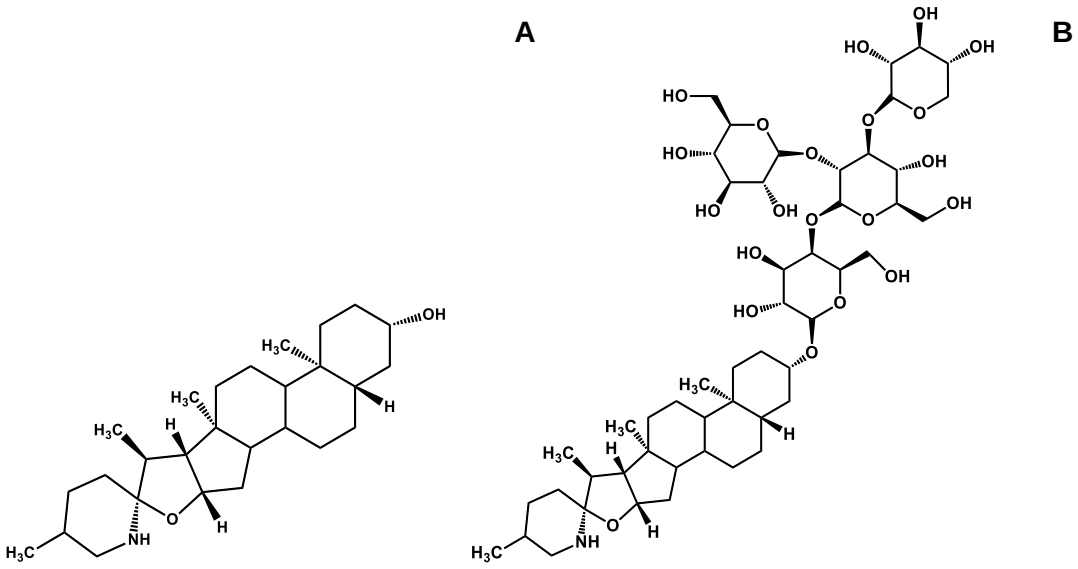


Figure 6. Chemical structures of tomatidine (**A**) and α-tomatine (**B**).

It should be noted that several other steroidal alkaloids have been found in various tomato plant tissues, particularly in the fruit (90, 92, 99-101), and some of them have been associated with the fruit maturation process. The bitter principal factor tomatoside A is found in seeds, dehydrotomatoside, esculeosides A and B in fruit and lycoperosides A-H in leaves and fruits. These steroidal alkaloids differ from α-tomatine in the stereochemistry and substitution of the F-ring (67).

Regarding the quantity of steroidal alkaloids in all parts of tomato plant, leaves and flowers are reported to possess the highest content of these compounds (~1100 mg/kg fresh weight), followed by stems and calyxes (35). Roots and immature fruits are the poorest matrices in terms of these compounds, which are almost absent in mature fruit. Among leaves, senescent ones seem to have higher content of alkaloids. Steroidal alkaloids levels can be increased by several conditions, such as unfavourable climatic conditions, high temperatures, exposure to artificial light, high intensity of sunlight and both mechanical and insect damage (35, 67).

The production of α-tomatine in early tomato fruit serves as a protecting mechanism against predators, which decreases during the maturation process to promote seeds' dispersal (90, 102). This occurs because during the maturation process tomato produces enzymes to metabolize the steroidal alkaloids. In fact, immature green tomatoes contain up to 500 mg of α-tomatine/kg of fresh fruit, reaching levels of ~5 mg/kg of fresh fruit weight at the mature stage (35).

α-Tomatine degradation (103) results from an enzymatic oxidation to give esculeoside A and B, the first being more abundant (104, 105). It was reported that its content increases during fruit ripening in contrast to the decrease of α-tomatine (90, 99, 101). It was also described that ethylene production or signaling during ripening affects these changes, suggesting that esculeoside A is synthesized from α-tomatine as a precursor (94). Furthermore, esculeoside A can be converted into a pregnane glycoside present in the over-ripe fruit (106).

The enzymes responsible for this transformation are activated or synthesized during fruit maturation. Such enzyme activation probably does not occur in mature green fruits of wild lines with high α-tomatine content. These degradation products are possibly channeled into carotenoids and chlorophyll formation (35).

The steroidal alkaloids from *L. esculentum* have revealed several biological activities, such as antibacterial, antifungal, antiviral, anti-inflammatory and immunopotentiation (35, 67, 107-110). Tomatine has protective effects against dibenzo[a,l]pyrene-induced liver and stomach tumours in rainbow trout. It has also exhibited anti-proliferative and apoptotic effects against several cancer cells (colon, liver, breast, stomach, leukemic, lung, mammary and prostate) in *in vitro* and *in vivo* systems (111-120).

In addition, the structure of steroidal alkaloids is quite similar to that of the glucocorticoids, which are precursors of steroidal hormones and anti-inflammatory steroids. Corticoids inhibit the inflammatory response mainly by repressing inducible nitric oxide synthases (iNOS)-mediated nitric oxide production and cyclooxygenase-2 expression. Steroidal alkaloids can act by the same mechanism and modulate inflammatory effects by inhibiting cyclooxygenase-2 pathway and prostaglandin E_2 production decrease (35, 67). It was also reported that tomatine exerts a nonspecific effect in antagonizing the contractions induced by histamine, bradykinin, slow-reacting substance of anaphylaxis (SRS-A), acetylcholine (Ach) and 5-hydroxytryptamine (121), which can open perspectives to its use as anti-allergic drug.

Steroidal alkaloids are also able to form insoluble complexes with cholesterol, which are excreted in the faeces. This leads to a lower level of low-density lipoproteins (LDL) in blood without changing high-density lipoproteins cholesterol levels (35).

3.2. Phenolic compounds

Phenolic compounds comprise a diverse group of molecules in plants with a large range of structures and functions. This class of compounds has been considered one of the most important, numerous and ubiquitous in the plant kingdom (122), being found in all plant organs, including flower, fruit, leaf, stem, seed and root (66).

In plants they act against the attack of different pathogens, herbivores and UV radiation. On non-adapted insects these compounds can have a negative impact, reducing the nutritive value of their food, acting as antifeedants, digestibility reducers, digestion inhibitors, toxic agents, growth inhibitors and prooxidants (43).

In addition, they can act as strong antioxidants, reducing the plant stress. They also participate in plants' colour, mechanical support, growth, nutrient uptake, protein synthesis, regulation of enzyme activity and reproduction, acting as phytoalexins or being involved in pollinators' attraction (43).

These compounds possess an aromatic ring bearing one or more hydroxyl groups and their structures may range from a simple phenolic molecule to a complex high-molecular mass polymer. They are classified according to the number and arrangement of the carbon atoms of the basic structure and can be found in the free form or conjugated with sugar and organic acids moieties (66). In this dissertation the focus will be on flavonoids and phenolic acids, since these are the most relevant groups of phenolics in nature and particularly in Solanaceae.

3.2.1. Phenolic acids

Phenolic acids constitute about one-third of the dietary phenols and can be present in plants as free acids or linked through ester, ether, or acetal bonds either to structural components of the plant (cellulose, proteins, lignin), to larger polyphenols (flavonoids), or to smaller organic molecules (glucose, quinic, malic or tartaric acids). There is a large variability of compounds in this group and they can be found in different plant tissues (66, 123).

Phenolic acids mainly comprise two subgroups: hydroxybenzoic and hydroxycinnamic acids (**Figure 7**). The first include gallic, *p*-hydroxybenzoic, protocatechuic, vanillic and syringic acids, which have in common the C_6-C_1 structure. These compounds may be found in plants in their soluble form conjugated with sugar groups or organic acids and bound to cell wall fractions (lignin) (66).

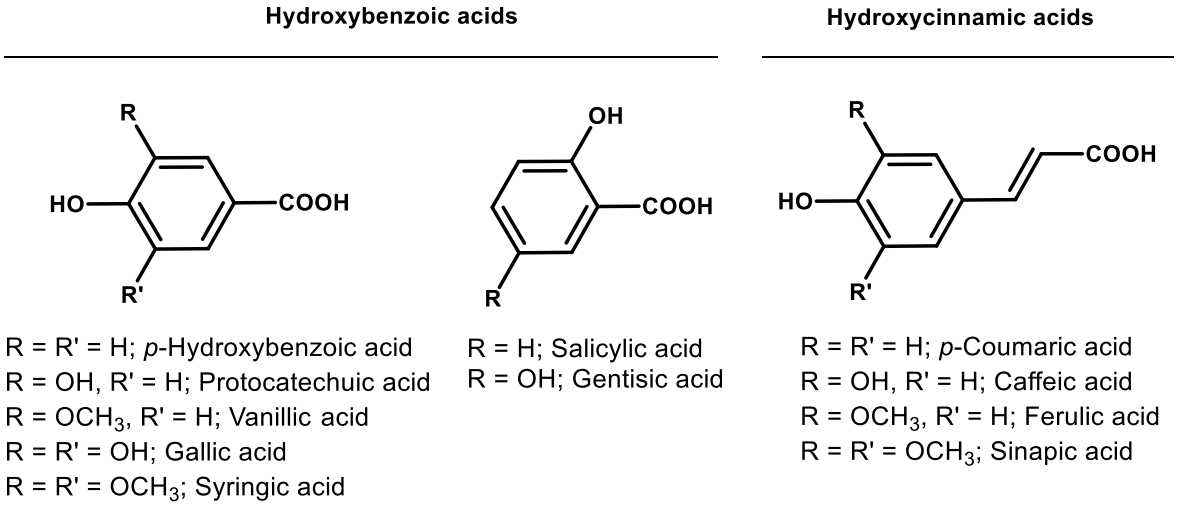


Figure 7. Chemical structure of the main hydroxybenzoic and hydroxycinnamic acids.

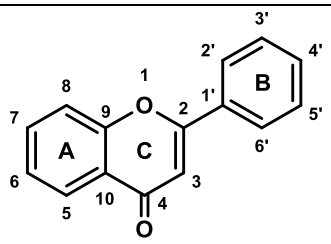
Hydroxycinnamic acids are aromatic compounds with a three-carbon side chain (C_6-C_3). This subgroup presents a much higher quantity and diversity of compounds, caffeic, ferulic, *p*-coumaric and sinapic acids being the most common representatives (27, 66). They usually occur in the conjugated form, as esters of sugars or tartaric, quinic and shikimic acids. The presence of a double bond in the side chain of hydroxycinnamic acids results in the existence of *cis* and *trans* isomers. In nature only the *trans* form occurs, but when in solution the two isomers can be found (66, 124).

3.2.2. Flavonoids

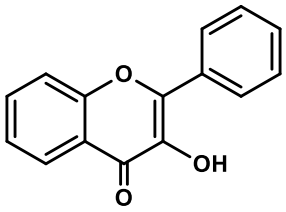
Flavonoids are low molecular weight compounds, consisting of fifteen carbon atoms, arranged in a C_6-C_3-C_6 configuration. The structure essentially consists of two aromatic rings, A and B, joined by a three-carbon bridge, usually in the form of a heterocycle (ring C). This structure is produced by two distinct biosynthetic pathways: the aromatic A ring is derived from the acetate pathway, while B ring is derived from phenylalanine through the shikimate pathway (125).

They are the most abundant group of phenolic compounds and are subdivided into distinct classes differing in the oxidation level of the central heterocyclic ring. These comprise flavones, flavanones, flavonols, dihydroflavonols, chalcones, flavan-3,4-diols flavan-3-ols, isoflavones, aurones and anthocyanidins (66). The chemical structures of the main classes of flavonoids are presented in **Figure 8**.

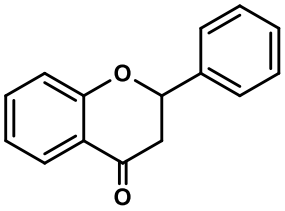
Flavonoids



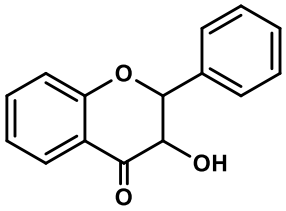
Flavones



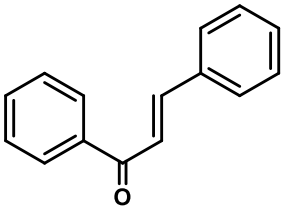
Flavonols



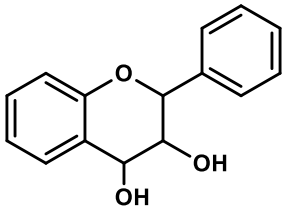
Flavanones



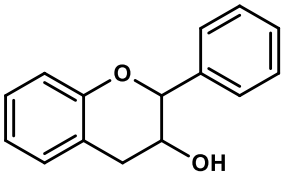
Dihydroflavonols



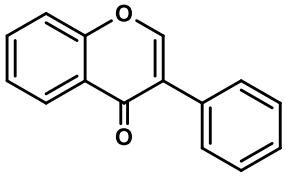
Chalcones



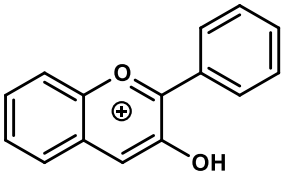
Flavan-3,4-diols



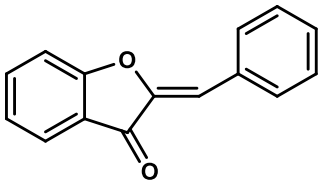
Flavan-3-ols



Isoflavones



Anthocyanidin



Aurones

Figure 8. Basic structure of the main flavonoids' subclasses.

Due to their biosynthetic pathway the most common substitution pattern of A ring is the hydroxylation at positions 5 and 7. For ring B, the hydroxylation may be 4'-OH, 3',4'-OH or 3',4',5'-tri-OH. Nevertheless, the great variety of structures found in this group results from further modifications, glycosylation and methylation of the hydroxyl groups being the most common. Other modifications, such as methoxylation, additional hydroxylation, prenylation, acylation of the hydroxyl groups or sugars moieties and dimerization, are less common (66).

In the nature, flavonoids occur preferably in the heterosidic form, in which one or more hydroxyl groups of the flavonoid are linked to a sugar through a hemiacetalic bound. They are usually found as *O*-heterosides, mainly on the hydroxyl at the 7 position of the aglycone, at the 3 position (flavonols), on the 4'-OH and less frequently on other positions. *C*-glycosyl flavonoids are also found, exhibiting C-C bonds that belong almost exclusively to positions 6 and/or 8. Other group includes *O*-glycosyl-*C*-glycosyl compounds, where *O*-glycosylation may be on the phenolic hydroxyl or on the sugar moiety from *C*-glycosylation, mainly at 2'' position, followed by 6'' position, and more rarely on other positions. These kinds of links mainly occur in flavones. Glycosylation generally turns the molecule more soluble and less reactive in the vacuoles. On the other hand, methyl groups and isopentyl units turn them more lipophilic (66).

Glucose is the most commonly found sugar moiety, followed by galactose, rhamnose, xylose and arabinose. The glycosidic residue may also be acylated with other molecules (hydroxycinnamic acids, acetic acid, malonic acid), establishing an ester bound between them and a sugar hydroxyl group (66, 124).

3.2.3. Biosynthetic pathway

Despite their great structural diversity, these compounds have some structural similarities, once their biosynthetic origin derives from the aromatic amino acids L-phenylalanine and L-tyrosine.

In general, both hydroxybenzoic and hydroxycinnamic acid derivatives have their biosynthetic origin in L-phenylalanine, synthesized itself from chorismate, the final product of the shikimate pathway (66, 123) (**Figure 9**).

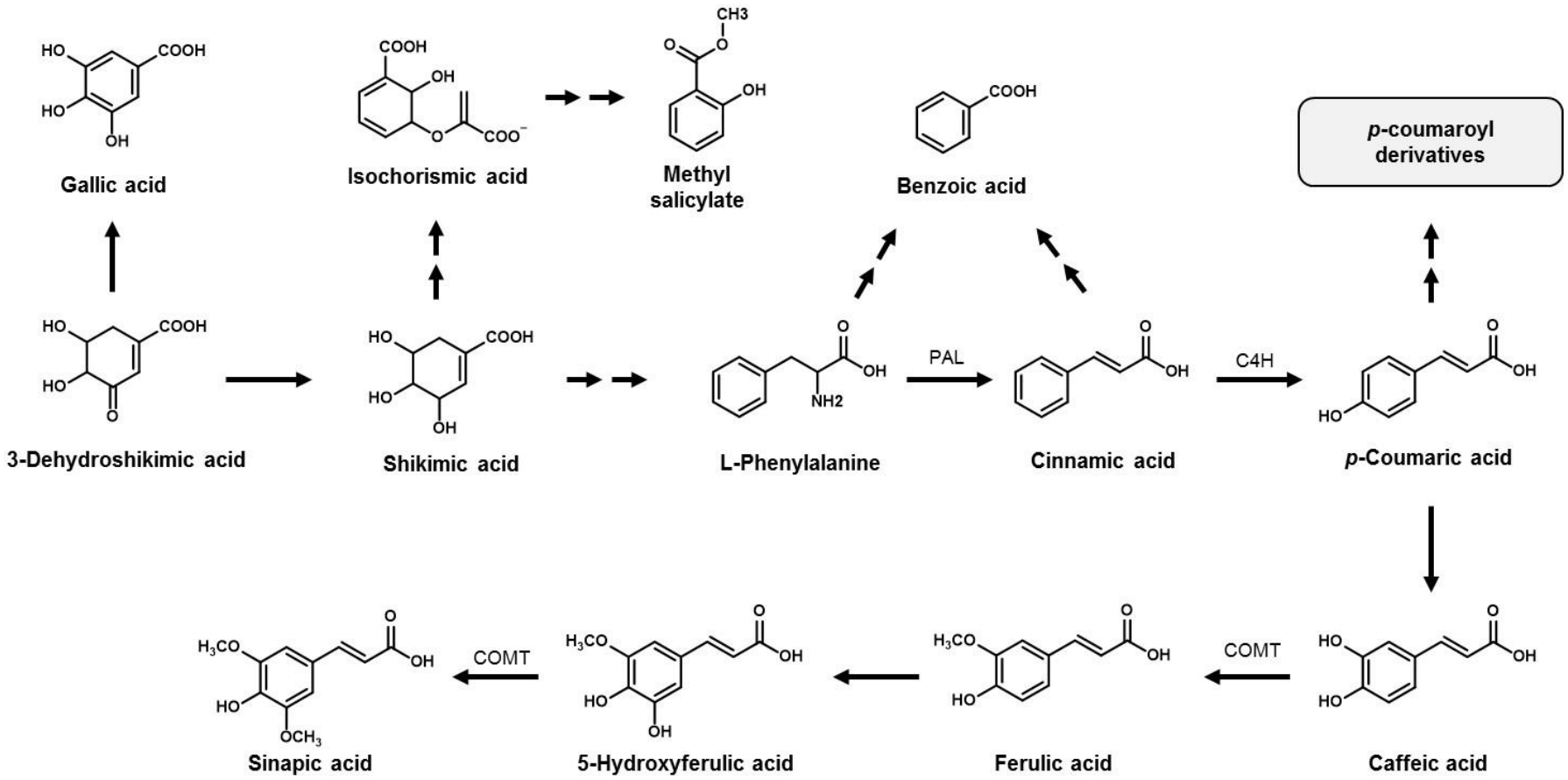


Figure 9. Phenolic acids biosynthetic pathway and key enzymes involved. PAL: phenylalanine ammonia-lyase; C4H: cinnamate 4-hydroxylase; COMT: catechol-*O*-methyltransferase [adapted from (126)].

Gallic acid is obtained from 3-dehydroshikimic acid and can be converted into ellagic acid, which is the basic unit of gallotannins. Several steps lead shikimic acid to originate L-phenylalanine, starting the phenylpropanoid pathway. The deamination of L-phenylalanine results in the formation of cinnamic acid, which can be hydroxylated at position 4 of the aromatic ring to generate *p*-coumaric acid. Subsequently, formation of the *p*-coumaric coenzyme A (CoA) ester (activated form) through enzymatic conversion occurs. Consequent enzyme reactions, involving hydroxylation of the aromatic ring and methylation, originate other derivatives, such as caffeic, ferulic and sinapic acids derivatives. The hydroxybenzoic acid derivatives may result from L-phenylalanine or from a side branch of the general phenylpropanoid pathway, by β-oxydation of *trans*-cinnamic acid into benzoic acid and acetic acid (66, 126).

Alternatively, certain compounds, such as methyl salicylate (MeSA) and methyl benzoate, can directly arise from isochorismate in the shikimate pathway (127) (**Figure 9**). MeSA has been a center of attention, as its release is often induced after herbivores or pathogens attack (128, 129). In addition, it acts as a long-distance signal compound within the plant, leading to systemic acquired resistance (129). In fact, this compounds was reported to be increased in tomato plant after *S. littoralis* attack (58).

As referred above, flavonoids have a mixed biosynthetic origin (shikimate and acetate pathways). They are synthesized from phenylpropanoid derivatives by condensation of *p*-coumaroyl-CoA with three malonyl-CoA units (C_3). Chalcone synthase (CHS) catalyses this reaction, producing naringenin chalcone. The intramolecular cyclization of the chalcone is mediated by chalcone isomerase (CHI) to obtain naringenin, which is the key precursor for the biosynthesis of all the other classes of flavonoids (66) (**Figure 10**).

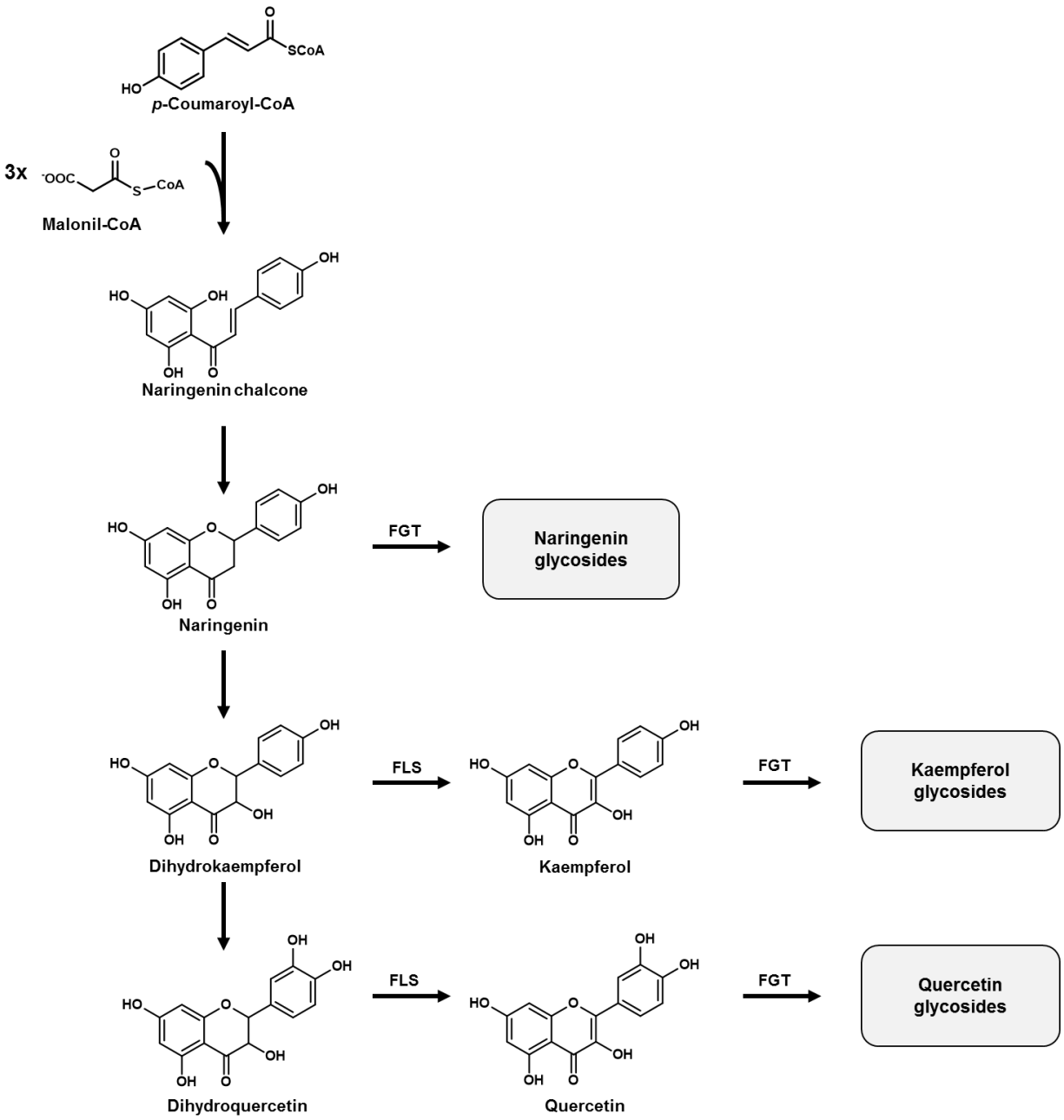


Figure 10. Flavonoids biossynthetic pathway [adpated from (126)]. FGT: flavonoid 3-*O*-glucosyltransferase; FLS: flavonol synthase.

3.2.4. Extraction, separation and identification

Several extraction methods for phenolic compounds are described in literature. Nevertheless, the most commonly used is solvent extraction. Several solvents and conditions have been reported, but acidified methanol, ethanol, acetone and ethyl acetate or the combination of these solvents with water are the most frequent (130, 131).

The process can be enhanced by associating it to ultrasounds, thus reducing the extraction time. In addition, ultrasounds cause the disruption of cell walls, reduction in particle size and enhancement of the mass transfer of cell contents to the solvent, caused by the collapse of the bubbles produced by cavitation, which leads to the membrane destruction (130, 132-134).

One of the disadvantages of solvent extraction is the coextraction of other compounds, such as chlorophylls, peptides, proteins, sugars and organic acids, among others. To remove these interferences different techniques can be performed, the use of C_{18} Sep-Pak cartridges being the most common (27).

Several techniques have been reported for the separation of phenolics, namely thin-layer chromatography, gas chromatography, capillary electrophoresis, high-speed counter current chromatography, supercritical fluid chromatography, among others. Nevertheless, reversed-phase HPLC is the main method used for the separation of phenolic compounds in plant-food material, in which the stationary phase is less polar than the mobile phase. So, the retention time of phenolic compounds is higher for substances that are less polar (flavonoids), while polar molecules (phenolic acids) are eluted more easily. However, if the compound contains apolar substituents, such as methoxyl groups, retention time increases; if it includes sugars or quinic acid residues it tends to elute before the corresponding free compound. So, retention time can be used as an indication of polarity, allowing predicting the class of metabolite according to the region in which each family elutes in the chromatogram (135).

Regarding the stationary phase, silica-bonded C_{18} columns are the most used (130). Taking into account the chemical complexity and similarity of phenolic compounds, the use of gradient elution instead of isocratic mode is required. Usually, a binary system composed by high quality ultrapure acidified water (phosphoric, acetic or formic acids) as the polar solvent and by a less polar solvent, such as acetonitrile and methanol, is used (136). Solvent acidification is required to suppress the ionisation of phenolic and

carboxylic groups, which will improve parameters like retention time and resolution. Formic and acetic acids are the most used. While the addition of formic acid only affects resolution, acetic acid also substantially reduces the retention time, which can be explained by its low ability to form ionic pairs (130, 137, 138).

The chromatographic conditions should always be optimized and several gradients, flow rates and temperatures should be tested to choose the best conditions, which result from the compromise between resolution and retention time, leading to an acceptable and an adequate separation of the different classes of metabolites, including several isomeric forms (130).

HPLC systems can be equipped with a wide range of detectors (refractive index, fluorescence, electrochemical, DAD, MS and UV/Vis), which can be used to detect and quantify phenolics. DAD detection is one of the most widely used in the analysis of phenolic compounds in different matrices. Its main advantage is that several information can be obtained from a single run, as each class has a characteristic UV spectrum (136, 139). In addition, it is possible to determine the correct wavelength to quantify the compounds and evaluate peak purity (130).

However, in spite of UV/Vis information being a very important analytical tool, it may be not enough for the complete characterization of a complex mixture. For phenolic compounds' structure elucidation some techniques can be used, such as NMR, near infrared and MS, the last one being the most frequent (27).

Regarding phenolic compounds analysis, several methods can be used to couple HPLC with MS (140). ESI ionisation is better suited for the analysis of thermally unstable compounds with higher molecular weights and polarity. This technique has a good sensitivity for flavonoid glycosides present in plant extracts, being one of the most widely used (27).

In the positive mode, the background noise is relatively high, preventing the detection of some compounds, being the negative mode more sensitive and less affected by impurities or interfering background noise. In addition, phenolics and their carboxylic acid derivatives ionise better in negative mode (27).

From the MS analysis we can know the compound's molecular weight, the aglycone's structure (hydroxylation profile and point of connection between rings B and C) and if there is any methylation, sulphatation or glycosylation (27).

Regarding the glycosidic fraction, it is possible to know the number of residues (mono, di, tri and tetrasaccharides) and type of sugar (hexoses, pentoses or deoxyoses). It is also possible to obtain information about the terminal monosaccharide stereochemistry, the glycosidic sequence, the type of inter-glycosidic connections, the localization of the glycosidic fraction on the aglycone and information about the sugar possible acylation (141).

For example, between heterosides with the same type of sugar in hydroxyl at positions 3 and 7, fragmentation preferentially occurs at carbon 7, which means that at the MS conditions used the glycosidic linkage at carbon 7 is more labile than that of carbon 3. The type of sugar is established by the difference between the mass of the glycoside and of the aglycone: 132 u for pentose, 146 u for deoxyhexose, 162 u for hexose and 308 u for deoxyhexosylhexose (137).

The presence of diglycosidic compounds is very usual in nature, those with sophorose [glucose-(1 → 2)-glucose] and gentiobiose [glucose-(1 → 6)-glucose] being the most common. Both lead to the loss of 324 u (2 glucoses: 162 + 162); nevertheless, for sophorose the intermediary fragments of 162 u are observed, while they are almost absent in gentiobiose fragmentation (142).

MS fragmentation also allows distinguishing between *O*- and *C*-heterosides. For *O*-heterosides the route of fragmentation involves the cleavage of the glycosidic linkages and the elimination of sugars, being observed mainly fragments from aglycone and sugar moieties. In the case of *C*-glycosides internal sugar fragmentation ions are mainly noticed (137, 141).

Taking into to account the molecular weight resulting from the glycosidic fraction remotion, it is possible to tentatively identify the remaining aglycones; for example, 300/301 u correspond to quercetin, 285 u to kaempferol and 315 u to isorhamnetin (141).

To study acyl flavonoids alkaline hydrolysis may be performed, followed by MS analysis of the deacylated derivatives. This hydrolysis procedure may be necessary since, for example, losses of 146 u for *p*-coumaroyl moieties and of 162 u for caffeoyl residues

coincide with those of rhamnosyl and hexosyl residues, respectively. Otherwise, a misassignment of the mass spectrometric data might occur (137).

Hydroxycinnamic acids derivatives originate the precursor ion, which allows knowing from which hydroxycinnamic acid is derived (caffeic: 179 u; ferulic: 193 u; *p*-coumaric: 163 u). Concerning derivatives with quinic acid, the MS^2 fragmentation is characteristic for each of the three possible isomers (hydroxycinnamic acid binding to the hydroxyl group of carbons 3, 4 or 5 of quinic acid), allowing the full compound identification by the fragments obtained and their relative intensity (143).

3.2.5. Biological activities

The potential positive impact of phenolic compounds on human health has been widely reported. One of the most important properties is their antioxidant capacity against reactive species, which are involved in ageing, chronic, degenerative, autoimmune, inflammatory and coronary diseases (144-147).

The antioxidant capacity of phenolic compounds is dependent on the chemical structure, nucleus rearrangement and the presence of functional groups (148). In the presence of free radicals phenolic compounds have the capacity to stabilize the unpaired electron by donating a hydrogen atom of their hydroxyl groups and stabilize the phenoxyl radical so formed (149).

Quercetin (**Figure 10**) is the compound that has the highest antioxidant activity because of the presence of two hydroxyl groups in ring B. Comparatively, kaempferol (**Figure 10**) that only contains one hydroxyl group at C4', has a lower capacity. However, the presence of a higher number of OH groups is no requirement for a higher antioxidant activity (149).

Moreover, it is also known that the presence of a 3-OH group increases the antioxidant activity, since it turns planar the conformation of the B ring, allowing conjugation to occur, and an electronic stability displacement reaction. Also the double bond between C2 and C3 influences electronic displacements and maintains the planar conformation. For this reason, flavonols and flavan-3-ols are classes of flavonoids generally with stronger antioxidant capacity (149).

Hydroxycinnamic acids have some structural features similar to those of flavonoids that may be important for their antioxidant capacity. For example, caffeic acid (**Figure 7**) has a catechol group, a double bond in the side chain conjugated with the phenolic ring and a carbonyl group that allow the phenoxyl radical stabilization (150).

Furthermore, phenolic compounds are also known to increase cells levels of antioxidant enzymes, like superoxide dismutase, glutathione peroxidase and catalase, and to inhibit the pro-oxidant ones, such as xanthine oxidase and lipoxygenases (151).

Besides the antioxidant capacity, phenolic compounds are known as antiviral, antibacterial, antimutagenic, fungicidal and reducing agents and metal chelators (152-154). These compounds also have neuroprotective effects, since they interfere in the regulation of transcription proteins factors and cell signalling, which lead to neurodegeneration (155).

In addition, these compounds, especially flavonoids, have demonstrated therapeutic potential in metabolic disorders, such as diabetes, obesity and dyslipidemia, by regulating the activity of nuclear receptors and increasing the tolerance to insulin (156). Furthermore, they have proved to have a role in the anticancer activity and inhibition of metastases progression (157, 158).

In addition to the potential effects on human health, phenolics have been reported to have excellent properties as food preservatives. Moreover, polyphenols have many industrial applications: for example, they may be used as natural colourings and in the production of paints, paper and cosmetics (159).

3.2.6. Phenolic compounds in *L. esculentum*

L. esculentum is rich in phenolic compounds. The expression of phenolic compounds can vary between the plant tissues (160-162) and the profile may be influenced by the cultivar, cultivation conditions and storage methods (148, 163-167). More than 100 phenolic compounds were already described in tomato fruit. The main flavonoids and phenolic acids identified are displayed in **Table 3**.

Many phenolic acids have been reported to occur both in tomato skin and pulp. Within them, hydroxycinnamic acid derivatives are in larger quantity, esters of quinic acid being the most common, namely 3, 4 or 5-*O*-caffeoylquinic acids (161).

Depending on the ripening stage of the fruit, different compounds can be found: immature tomato fruits mainly present sinapic acid, while vanillic and salicylic acids are abundant on ripe tomato (148). These compounds are found at higher concentrations in pulp than in pericarp tissue (168).

The highest concentration of flavonoids is found in epidermal and placental tissues, followed by seeds and pulp. More specifically, derivatives of quercetin, kaempferol, naringenin and chalconaringenin are mainly present in the epidermis, while some others, such as the aglycones chalconaringenin and naringenin and the trisaccharides of kaempferol and quercetin, are found in both epidermis and the vascular attachment region (160).

Table 3. Main flavonoids and phenolic acids identified in tomato fruit of non-transgenic cultivars [adapted from (148, 160, 161)].

Phenolic compound class	Main compounds
Flavonoids	
Chalcones	Chalconaringenin; chalconaringenin-hexose derivatives; chalcoeriodictyol; chalcoeriodictyol-hexoside
Dihydrochalcones	Phloretin-3',5'-di -*C*- glucoside
Flavanones	Naringenin; naringenin-7-*O*-rutinoside; naringenin-hexose derivatives; eriodictyol; eriodictyol-hexose derivatives
Flavones	Apigenin acetylhexoside
Flavonols	Kaempferol; kaempferol glycosides; quercetin; quercetin glycosides; quercetin acyl glycosides[a]; myricetin; myricitrin
Dihydroflavonols	Dihydrokaempferol-7-*O*-hexoside
Anthocyanidins	Peonidin-3-*O*-(acyl)rutinoside-5-*O*-glucoside[b]; delphinidin-3-*O*-rutinoside-5-*O*-glucoside; delphinidin-3-*O*-(acyl)rutinoside-5-*O*-glucoside[b]; petunidin-3-*O*-rutinoside-5-*O*-glucoside; malvidin-3-*O*-rutinoside-5-*O*-glucoside; malvidin-3-*O*-(acyl)rutinoside-5-*O*-glucoside[b]
Phenolic acids	
Hydroxybenzoic acids	Benzoic acid; *p*-hydroxybenzoic acid; *p*-hydroxybenzoic acid-hexoside; *p*-hydroxybenzoic acid-pentoside; salicylic acid; gentisic acid; vanillic acid; homovanillic acid
Hydroxycinnamic acids	Cinnamic acid; *m*-coumaric acid; *p*-coumaric acid; *p*-coumaric acid hexose derivatives; *p*-coumaroylquinic acid; caffeic acid; caffeic acid hexose derivative; caffeoylquinic acid derivatives; ferulic acid; isoferulic acid; ferulic acid hexose derivatives; sinapic acid; sinapic acid hexose derivatives

[a] Acyation: *p*-coumaroyl or feruloyl. [b] Acylation: *p*-coumaroyl or caffeoyl.

Only few works focused their attention on tomato plant roots, seeds and leaves. *p*-Hydroxybenzoic, vanillic, *p*-coumaric, *o*-coumaric, ferulic, sinapic, gentisic, salicylic, caffeic, 3,5-dimethoxycinnamic and syringic acids were described in the roots (169, 170).

Some studies reported the phenolic composition of the seeds' cavity (including columella, placenta tissue and seeds) (32) and that of a mixture of seeds and skin. Rutin, rutin-apioside, naringenin, chalconaringenin and 5-*O*-caffeoylquinic acid were described (171). However, no work dealt with the molecular speciation of tomato seeds. As far as we know, only 5-*O*-caffeoylquinic acid, naringenin, rutin, and myricetin were specifically described in hydroethanolic seeds' extract (162).

Regarding the leaves, some phenolic acids, including caffeic acid derivatives, *trans p*-coumaric, *cis p*-coumaric, caffeic, ferulic, sinapic, *p*-hydroxybenzoic, protocatechuic and vanillic acids, were detected (38, 172).

4. Primary metabolism

Primary metabolites are compounds that possess fundamental roles in plant development steps, such as growth, respiration, photosynthesis and hormone and protein synthesis. Among them fatty and organic acids are highlighted, since they are the most represented in Solanaceae and because of their biological activities (173).

4.1. Organic acids

Organic acids are a heterogeneous group of compounds. Their nature and concentration influence the organoleptic characteristics of fruits and vegetables. They are weak acids that can be found in the plant cellular vacuoles. The great accumulation of these metabolites is probably due to their role in the photosynthetic process. Nevertheless, they can have several other functions in plants, such as energy production, formation of amino acids, osmotic adjustment and interaction with microorganisms (174). In addition, they also have a role in the internal mechanisms of heavy metals detoxification. As so, they are essential for the adaptation of plants to environmental changes (175).

Various organic acids are produced during plant growth, being present in the whole plant. Malic, citric and tartaric acids are mostly found in fruits, whereas oxalic acid is more common in leaves (176). Generally, citric and malic acids are those present in higher quantities in plants (177).

4.1.1. Biosynthetic pathway

Organic acids have low molecular weight (178) and their synthesis can occur mostly in the mitochondria *via* the Krebs cycle or from glucose direct oxidation (175). Citric, isocitric, α-ketoglutaric, succinic, fumaric, malic, *cis*-aconitic and oxaloacetic acids are the ones resulting from the Krebs cycle (179) (**Figure 11**).

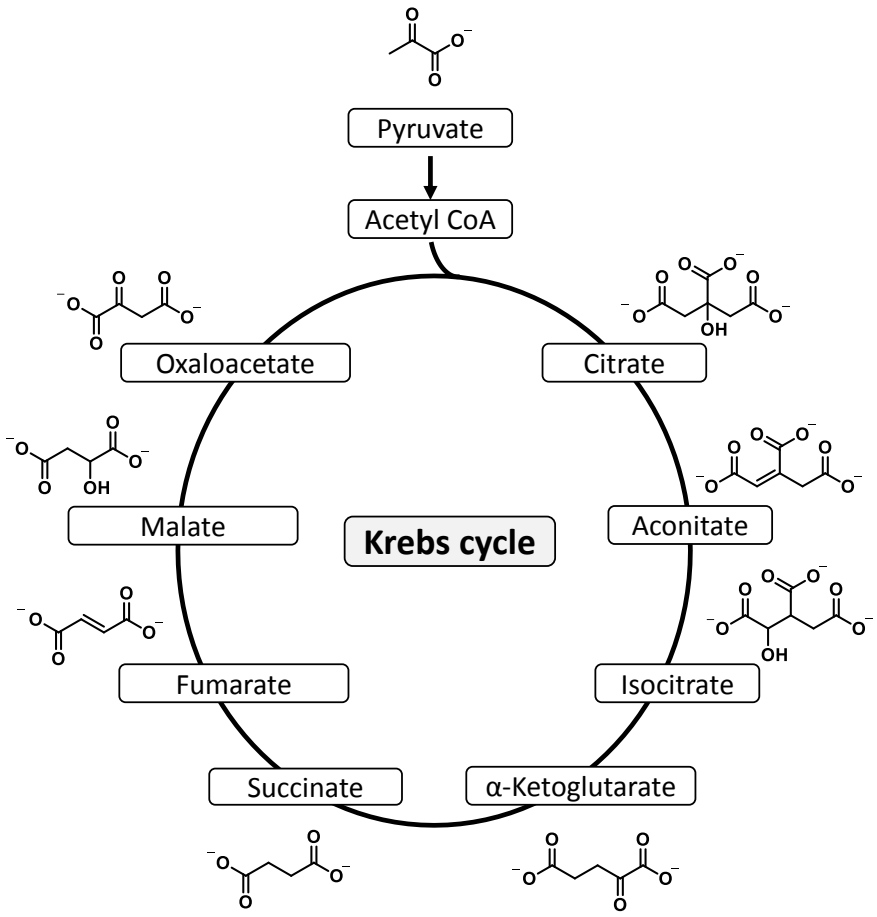


Figure 11. Krebs cycle.

4.1.2. Extraction, separation and identification

Organic acids are soluble in water and hydroalchoolic solvent mixtures. Solvent acidification contributes to increase their extraction and for further purification of these extracts SPE can be used (180-183).

The separation by HPLC is particularly useful for the analysis of polar or moderately polar compounds, such as organic acids (184). This can be achieved with an ion exclusion column, consisting on an ion-exchange resin, which splits ionised and neutral species. The ionised compounds are rejected by the resin and eluted through the column, while the neutral or slightly ionised molecules are retained. As so, it is possible to conveniently separate acid molecules from highly ionised substances (185). Elution can be isocratic or in gradient mode, the first being the most used for the determination of organic acids (139, 186).

There are several techniques for detection of these metabolites, such as UV, MS and fluorescence (184), the first being the most common. In this case external standards for compound's identification and quantification is required (181).

4.1.3. Biological activities

Organic acids are recognized by their ability to scavenge free radicals and chelate metal ions; therefore, they can be used in the prevention of several diseases, including cancer and atherosclerosis (187). At industrial level they can be used as flavourings, preservatives and antioxidants (188).

Citric acid is one of the most used antioxidants in industry (189), as it has the ability to chelate metals (187), forming inactive complexes (190). It is also used to improve flavour and aroma of several products (191). The same happens with malic acid (187). Their antimicrobial and antifungal properties, probably due to variations in pH, are also important (179, 191).

Ascorbic acid cannot be synthetized by the human body, so it should be obtained from the diet. This organic acid is important for the production of certain hormones and neurotransmitters and in the metabolism of certain amino acids and vitamins. Its antioxidant activity is well known, being important for the protection against oxidative damage (192).

4.1.4. Organic acids in *L. esculentum*

Aspartic, citric, malic, succinic, ascorbic, aconitic, oxalic acetic and glyceric acids are reported in tomato fruits and leaves (35, 193). Pyroglutamic, pyruvic, fumaric, succinic, citric and malic acids were found in the seeds. Citric, malic and succinic acids were reported as the main organic acids in seeds and seedling exudates (194).

Citric acid is described as the major organic acid in all stages of plant growth. The percentage of malic acid drastically decreases and that of succinic acid strongly increases when seedlings become plants (194).

4.2. Fatty acids

Fatty acids are involved in many plants' physiological processes, such as adaptation, survival, ion channel modulation, endo and exocytosis, pollen formation, defence and development of chloroplasts (195), being structurally important for them (196).

They are characterized by a carbon chain with a terminal carboxyl group. Generally, they can be classified as saturated or unsaturated (**Figure 12**). The first are molecules with a carbon chain containing no double bonds, which gives them a greater rigidity. They are primarily involved in the production of triglycerides and cholesterol, being associated with the prevalence of obesity, cardiovascular and other diseases (197).

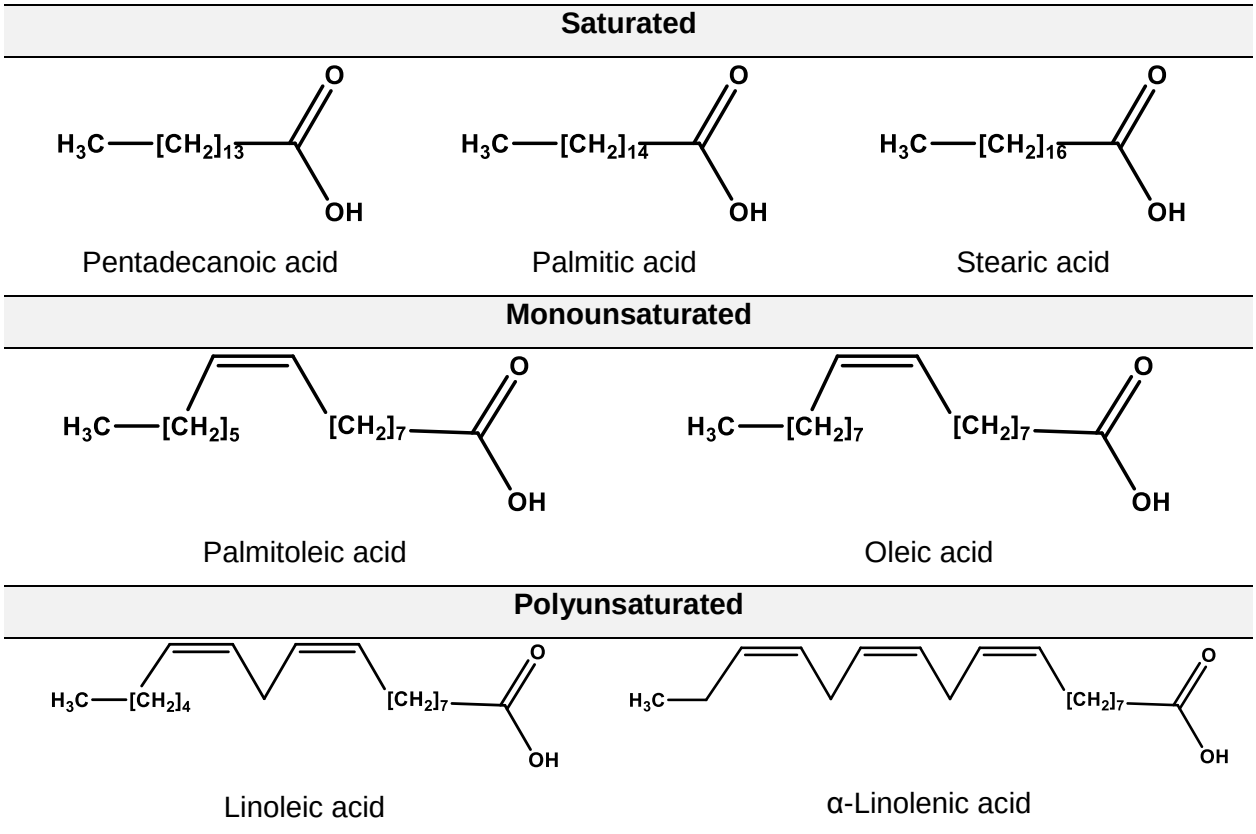


Figure 12. Chemical structure of the main fatty acids described in *L. esculentum*.

Unsaturated fatty acids may be monounsaturated (MUFA) or polyunsaturated (PUFA), depending on the number of double bonds. Due to the presence of double bonds they can appear as *cis* or *trans* isomers. In contrast to saturated fatty acids, they provide flexibility and fluidity to cell membrane, playing an important role in its permeability (195).

4.2.1. Biosynthetic pathway

The biosynthesis of fatty acids occurs in the cytosol and begins with the oxydation of a molecule of acetyl-CoA to malonyl-CoA. Malonyl-CoA reacts with another acetyl-CoA molecule and the resulting compound suffers further condensation, reduction and dehydration reactions to originate palmitic (16:0) or stearic acid (18:0), which are later desaturated to MUFA and PUFA (198).

The occurrence of double bonds in unsaturated fatty acids is a microsomal process and requires the presence of nicotinamide adenine dinucleotide reduced form (NADH) and oxygen as co-substrates and cytochrome b5. CoA esters, as well as polar lipids like phosphatidylcholine, are substrates for the introduction of the double bonds (199).

4.2.2. Extraction, separation and identification

The solvents used for fatty acids extraction should offer a good polarity range to extract both the polar lipids from cellular membranes and the non-polar lipids, a mixture of chloroform and methanol (2:1) being the most used (200).

The separation of fatty acids is mainly performed by GC-MS. In this context, the samples must be derivatized to give rise to less polar and sufficiently volatile compounds, which could be eluted at reasonable temperatures without undergoing thermal decomposition or molecular rearrangements (201).

For the derivatization process different methods can be used, such as esterification, silylation and acetylation. Esterification is the most commonly used and can be done with an appropriate alcohol, such as methanol, and an inorganic acid to catalyze the reaction. Boron trifluoride or boron trichloride are very useful for forming ester derivatives, the reactions catalyzed by the first being faster (201).

4.2.3. Biological activities

The effects of fatty acids are complex and greatly vary according to the dose and the nature of the molecule. Saturated fatty acids are negatively correlated with the development of caridiovascular diseases. On the other hand, unsaturated fatty acids (MUFA and PUFA) have cardioprotective effects. Within these, PUFAs have a more prominent effect (200).

In addition, PUFAs have also revelead to be active at neuronal level: low concentrations of these acids are associated with a worse visual and cognitive development in children and an increased risk of dementia in the elderly (202). The literature also refers the fatty acids importance in the bone matrix, since they interfere with signaling pathways of osteoclasts and osteoblasts, favouring bone growth (203).

Among PUFAs, the ones belonging to omega-3 (ω-3) and omega-6 (ω-6) families are highlighted. The human organism is not able to synthesize them due to the absence of specific enzymes. So, it is necessary to obtain them from the diet (204, 205). These fatty acids are recognized by their ability to lower serum cholesterol levels, leading to the reduction of blood pressure, thus decreasing the risk of cardiovascular disease (206).

4.2.4. Fatty acids in *L. esculentum*

Some fatty acids have been described in *L. esculentum* fruit: pentadecanoic, palmitic and stearic acids (saturated), and linoleic, oleic, palmitoleic and α-linolenic acids (unsaturated). In quantitative terms, linoleic acid is the most abundant, followed by palmitic and oleic acids (207).

In tomato fruit fatty acids are mostly found in the seeds and cuticle. Myristic, palmitic, heptadecanoic, stearic, arachidic, myristoleic, palmitoleic, oleic, linoleic, α-linolenic and gadoleic acids are described in the seeds, which are considered to be a good source of edible oil (161, 208). The cuticle is a thin lipophilic layer that covers the aerial organs of all plants and provides protection against diverse stress conditions. It is composed mainly by C_{16} and C_{18} hydroxy fatty acids and waxes (90, 161).

The studies performed with leaves showed the presence of palmitic, stearic, oleic, linoleic and α-linolenic acids, the latter being the major compound (207).

5. Alzheimer's disease (AD)

AD is one of the most common forms of dementia. It is a progressive neurodegenerative disorder with a mean duration of around 8.5 years between the first clinical symptoms and death. The criteria for the diagnosis of AD include established dementia by clinical examination and neuropsychological tests (209).

The development of AD is characterised by progressive impairments of cognitive function, including memory loss, particularly of recent events during the initial phases, and impairments in other cognitive domains that interfere with mood, reason, judgment and language. It is often accompanied by behavioural disturbances, such as aggression, depression and wandering (210). Extracellular deposits of amyloid plaques, intracellular formation of neurofibrillary tangles and loss of neuronal synapses and pyramidal neurons from the cholinergic system are observed in AD patient's brain (211).

AD aetiology is still unknown and several aetiological/pathogenetic hypotheses have been advanced, such as genetic defect, slow or latent virus disorder, energy metabolism deficit, altered amyloid precursor protein (APP) processing, deficiency of neurotrophic factors, glutamate toxicity (excitotoxicity), mitochondrial defect, trace elements neurotoxicity and free radical-induced neuron degeneration or oxidative stress. It is possible that all of them are involved and interact between them (212) (**Figure 13**).

There is no cure for AD and the current therapeutics focuses only on ameliorating the symptons or slowing its progression. The treatment strategies include the use of cholinesterases (ChE) inhibitors, such as galantamine, and glutamate receptors antagonists like memantine (210). By blocking the enzyme responsible for ACh breakdown, ChE inhibitors increase the levels of ACh in the brain and indirectly lead to acetylcholine receptors (AChR) activation. The combination of both ChE inhibitors and AChR agonists has revealed promising results both in *in vivo* (213) and *in vitro* (214) models of AD.

The selectivity of enzyme inhibition is crucial for ChE inhibitors profile. They can act as inhibitors of AChE, which is found primarily in neural tissue, but may also inhibit BChE, which acts mainly in the periphery. For example, donepezil and galantamine are AChE-selective inhibitors and rivastigmine a dual AChE and BChE inhibitor (215).

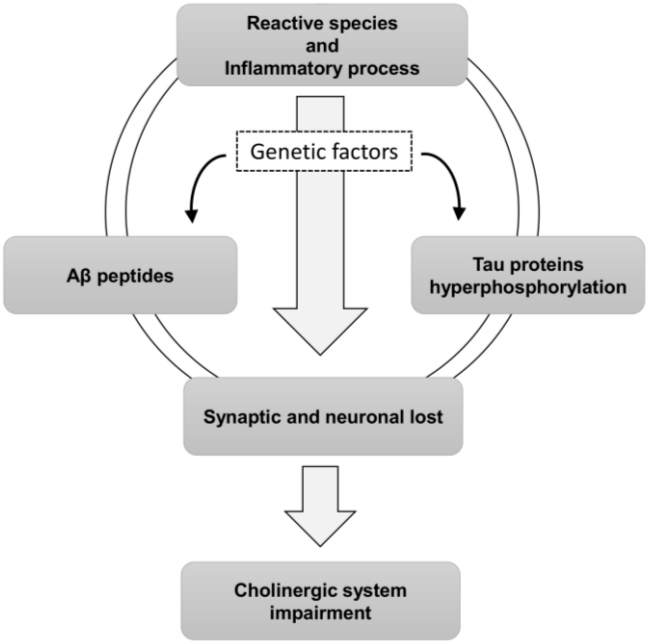


Figure 13. Main hypotheses for AD aetiology.

BChE represents only 10% of total ChE activity in the temporal cortex of the healthy human's brain, but it is increased in AD patients' brain: during the disease progression, AChE activity decreases, while that of BChE increases (216, 217). In addition, the ratio BChE:AChE also changes from 0.6 to 11.0, showing that BChE may have a primordial role in AD pathology (216).

Another possible therapeutic target could be amyloid-β petide (Aβ). Aβ is a fragment from the larger protein APP, which is critical to neuron growth, survival and post-injury repair (218).

APP can be processed by secretases and cleaved in three different fragments by two pathways: non-amyloidogenic (α-secretase) or amyloidogenic (β-secretase). If α-secretase acts on APP, a non-pathogenic fragment called P3 will be formed. On the other hand, sequential cleavage by β-secretase and γ or ε-secretase produces an Aβ peptide (219) (**Figure 14**). Aβ monomers self-assemble into amyloid fibrils by nucleation-dependent polymerization creating amyloid plaques. These plaques are dense, insoluble and can be found outside and around neurons (220).

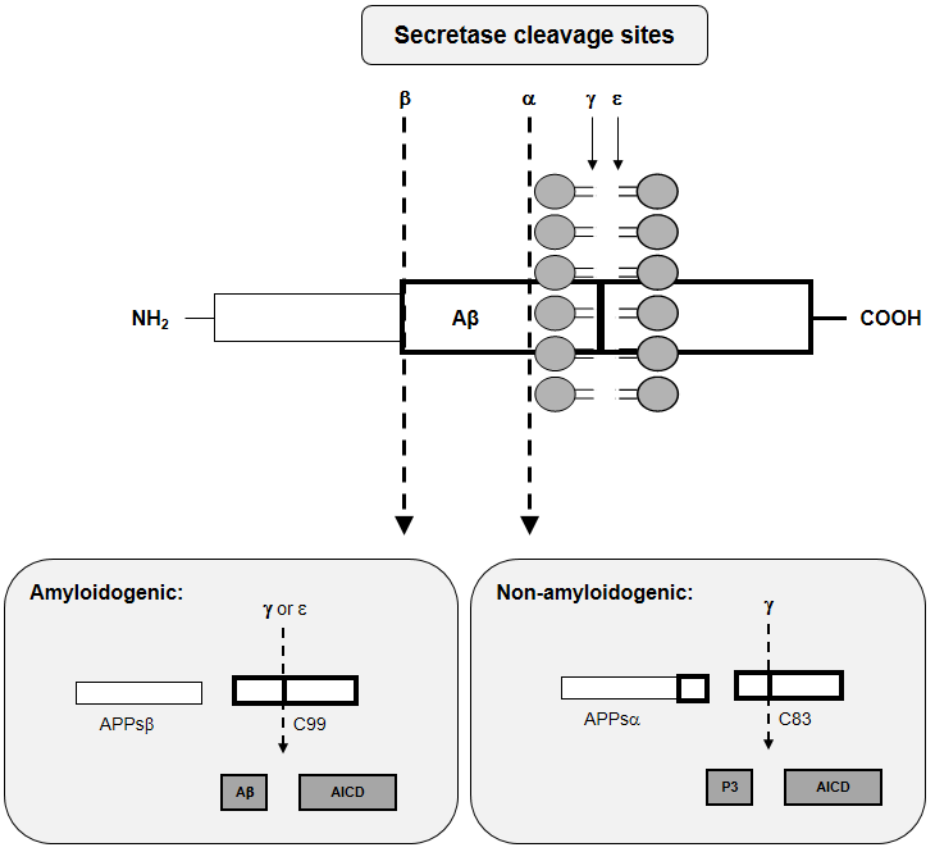


Figure 14. Aβ formation pathway [adapted from (219)].

The precise mechanism by which Aβ exerts its toxic effects on neurons remains unclear, but it seems to induce neuronal apoptosis, most likely through the p53-Bax cell death pathway (221).

Aβ can also directly or indirectly induce free radicals generation in neurons. It can also interfere with different cell metabolic processes, like calcium ion (Ca^{2+}) homeostasis or glucose uptake (221).

AD is also considered a tauopathy due to abnormal aggregation of tau protein. Every neuron has a cytoskeleton, an internal support structure composed by microtubules. These microtubules act like pathways, guiding nutrients and molecules from the body of the cell to the ends of the axon and back. Tau protein stabilises the microtubules when phosphorylated and is, therefore, called a microtubule associated protein. In AD, tau protein suffers chemical changes, becoming hyperphosphorylated, which leads to the formation of neurofibrillary tangles that accumulate inside the cells themselves, with consequent disintegration of the neuron's transport system (219, 222).

Several factors can lead to tau hyperphosphorylation, such as Aβ oligomers, oxygen free radicals, iron overload, cholesterol levels in neuronal rafts, LDL and high levels of homocysteine. They can activate microglial cells, with the consequent release of proinflammatory cytokines that modify neuronal behaviour through anomalous signalling cascades, finally promoting tau hyperphosphorylation (219).

As referred above, oxidative stress and inflammation can also have a role in AD progression. Inflammation is a general marker of tissue damage in any disease and may be either secondary to tissue damage in AD or a marker of an immunological response. So, several inflammatory processes and different cytokines (tumor necrosis factor, interleukins 1 and 6) may also contribute to the development of AD (223).

The central nervous system is especially vulnerable to free radical damage, as a result of the brain's high oxygen consumption rate, its abundant lipid content and the relative scarcity of antioxidant enzymes when compared to other organs. In addition, antioxidant defences decrease in brain and plasma with ageing, rendering the brain even more susceptible to reactive species. According to the oxidative stress hypothesis, the cumulative oxidative damage over time could account for the late life onset and the slow progressive nature of this neurodegenerative disorder (224, 225). A consequence of oxidative stress is the formation of advanced glycation end products (AGEs), which are proteins or lipids that become glycated after exposure to sugars. Accumulation of AGEs in cells and tissues is a normal feature of ageing, but is accelerated by the oxidative action of reactive species. They can be detected in pathological deposits, such as amyloid plaques and neurofibrillary tangles. AGEs explain many of the neuropathological and biochemical features of AD, like extensive protein crosslinking, glial induction of oxidative stress and neuronal cell death. Activation of the receptor for advanced glycation end products (RAGE) by AGEs causes upregulation of the transcription factor nuclear factor-κB and its target genes. AGEs block nitric oxide activity in the endothelium and cause the production of reactive oxygen species, leading to neuronal death (223, 225, 226).

AD seems to be a sporadic disease with no genetic heredity, although some genes may act as risk factors. Most of autosomal dominant family AD can be attributed to mutations of the genes of amyloid precursor protein (chromosome 21), presenilin-1 (chromosome 14) or presenilin-2 (chromosome 1). These mutations increase the production of Aβ42, which is the main component of senile plaques, leading to an increased accumulation of amyloid plaques in the brain (227).

A specific isoform of apolipoprotein (APOE), namely APOE4, is also correlated with a higher probability of AD development. Apolipoproteins enhance the breakdown of Aβ, but some isoforms, like APOE4, are not very effective in this task, leading to excessive amyloid buildup in the brain (228, 229).

Mutations in the triggering receptor expressed on myeloid cells 2 gene have also been associated with a higher risk of AD. This mutation can change white blood cells in the brain, reducing their capacity to remove Aβ proteins. Endothelial nitric oxide synthase-3 gene (230) and α2-macroglobulin gene (231) have also been been implicated in this disease.

5.1. *In vitro* models

The human neuroblastoma cell line SH-SY5Y has been extensively used as AD model (232-234). Differentiated or undifferentiated cells can be used. Nevertheless, when undifferentiated, they also express functional nicotinic (nAChR) and muscarinic (mAChR) acetylcholine receptors (235, 236). In addition, Cheung *et al*. (237) reported that undifferentiated SH-SY5Y cells are more appropriate for studying neurotoxicity or neuroprotection effect, because differentiation increases the tolerance to the aggression agents (237). Different aggression agents can be used to evaluate neuroprotective capacity, glutamate, Aβ peptides, okadaic acid and H_2O_2 being the most common (232, 233, 238).

Glutamate is an endogenous neurotransmitter and has distinct roles in the central nervous system, including potentiation and plasticity of the brain *via N*-methyl-D-aspartate (NMDA) receptors (239). However, glutamate is neurotoxic at high concentrations due to a process called excitotoxicity, which is also mediated by NMDA receptors (240, 241), as the over activation of these receptors triggers Ca^{2+} fluxes and enhances cellular oxidative stress (242). Thus, glutamate-induced neuronal death, especially by pro-oxidant and mitochondrial dysfunction effects, is associated with the development of several neurodegenerative diseases and is thought to be a key factor in the pathogenesis of AD (239, 243, 244). Concerning mitochondrial dysfunction, it leads to the appearance of all of the histological modifications of AD, including tau hyperphosphorylation, processing of APP to β-amyloid, tangle formation and neurodegeneration (245).

Okadaic acid is also used in AD studies. It is a toxin produced by marine algae that blocks protein phosphatases inducing hyperphosphorylation of tau protein and formation of neurofibrillary tangles. Okadaic acid-induced toxicity has been considered a good model for the neuronal death occurring in AD, linked to tau hyperphosphorylation (246).

Oxidative stress results in different types of neuronal death, including necrosis and apoptosis, which are known to occur in the course of AD, as previously referred (247, 248). Thus, direct therapeutic efforts towards oxidative events in the pathway of neuron degeneration and death are important for AD treatment (239). To study the antioxidant effect on neuronal cells, different oxidant agents can be used, H_2O_2 being the most common (249-251).

6. Objectives

The main objectives of this dissertation were:

1- To evaluate the chemical composition of *L. esculentum* by-products, namely seeds and leaves;

2- To evaluate the health promoting effects of *L. esculentum* seeds;

3- To assess the possible industrial application of *L. esculentum* seeds and leaves;

4- To evaluate *S. littoralis* fed with *L. esculentum* leaves as a possible source of bioactive compounds;

5- To evaluate the ChE inhibition capacity of non-toxic concentrations of steroidal alkaloids (tomatine and tomatidine) and tomato leaves purified extracts, as well as their neuroprotective potential against glutamate-induced toxicity in SH-SY5Y cells;

6- To establish possible relationships between chemical composition and biological activity of the different matrices.

Chapter II

Experimental section

1. Standards and reagents

Tomatine, tomatidine, quercetin-3-*O*-rutinoside, kaempferol-3-*O*-rutinoside, isorhamnetin-3-*O*-rutinoside and 5-*O*-caffeoylquinic, *p*-coumaric, ferulic and sinapic acids were purchased from Extrasynthése (Genay, France).

Oxalic, citric, fumaric, malic, acetic, aconitic and pyruvic acids, pentadecanoic, palmitic, palmitoleic, heptadecanoic, stearic, oleic and linoleic acids methyl esters, boron trifluoride 10% methanolic solution, 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$), NADH, phenazine methosulfate (PMS), bovine serum albumin, nitroblue tetrazolium (NBT) chloride, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), sulphanilamide, AChE (EC 232-559-3) from electric eel (type VI-s, lyophilized powder), BChE (EC 3.1.1.8) from equine serum, sulphorhodamine B (SRB), acetylthiocholine iodide (ATCI), S-butyrylthiocholine chloride (BTCC), galantamine hydrobromide, dimethyl sulfoxide (DMSO), L-glutamic acid, triton X-100, (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), rhodamine 123, methyllycaconitine (MLA) citrate salt, mecamylamine, 2′,7′-dichlorofluorescein diacetate (DCFH-DA), Folin-Ciocalteu's reagent and Tris-HCl were purchased from Sigma-Aldrich (St. Louis, MO, USA). *N*-(1-Naphthyl)-ethylene-diamine dihydrochloride, sodium nitroprusside (SNP) dehydrate, ciprofloxacin, fluconazole, ammonia 25%, acetonitrile, methanol and chloridric, trichloroacetic, sulphuric and acetic acids were obtained from Merck (Darmstadt, Germany). Sodium chloride was purchased from José M. Vaz Pereira, S.A. (Sintra, Portugal). Magnesium chloride hexahydrate and triethylammonium phosphate (TEAP 1.0 M) buffer were from Fluka (Buchs, Switzerland). Dulbecco's Modified Eagle Medium (DMEM), phosphate-buffered saline (PBS), hepes buffered saline (HBS), nonessential amino acids (NEAA), foetal bovine serum (FBS), antibiotic (10,000 U/ml penicillin, 10,000 µg/ml streptomycin), fungizone (250 µg/ml amphotericin B), human transferrin (4 mg/ml) and trypsin-EDTA were from Invitrogen (Gibco Laboratories; Lenexa, KS). Scopolamine was obtained from Tocris bioscience (Bristol, UK). Mueller Hinton Broth (MHB) and Mueller Hinton Agar (MHA) media were purchased from Liofilchem (Teramo, Italy) and Saboraud dextrose agar (SDA) and Sabouraud dextrose broth (SDB) from Bio-Mèrieux (Marcy L'Etoile, France).

All SPE columns were obtained from Supelco (Bellefonte, USA).

Water was treated in a Milli-Q (Millipore, Bedford, MA, USA) water purification system.

2. Samples

2.1. Plant materials

Seeds of *L. esculentum* from “bull’s heart” (RJS, Portugal) and “cherry” (Galassi Sementi, Italy) varieties were purchased from local commerce. No antibacterial or antifungal agents were added by the producer. Seeds were washed with water, then surface sterilized with 10% sodium hypochlorite solution, rinsed with sterile distilled water and were air dried at room temperature. The samples were ground into a fine powder and kept in a desiccator in the dark until analysis.

L. esculentum leaves from “cherry” and “bull’s heart” varieties were cultivated in a greenhouse without pesticide applications. Authenticity of the plant material was assured by Doctor Luísa Oliveira (CBA, CIRN, Department of Biology of the University of Azores). Leaves were freeze-dried, powdered (mean particle size lower than 910 µm) and kept in a desiccator in the dark until analysis.

Voucher specimens were deposited at the Laboratory of Pharmacognosy of the Faculty of Pharmacy of Porto University under the following identification: LESBH-092010 and LESCH-092010 for seeds from “bull’s heart” and “cherry” varieties, respectively; LEBH-112010 and LECH-112010 for leaves from “bull’s heart” and “cherry” varieties, respectively.

2.2. *S. littoralis* materials

Larvae and adults of *S. littoralis* were collected in S. Miguel Island (Azores archipelago, Portugal). They were kept in the laboratory with artificial diet (252), at 25±2 ºC, 70-80% relative humidity and photoperiod of 14:10 h (light:dark). The insects used came from the 3rd and 4th laboratory generations. After emergence, larvae were fed separately *ad libitum* with medium leaves of *L. esculentum* from “cherry” and “bull’s heart” varieties, cultivated in a green house without pesticide applications and disinfected with formalin 5%. Fourth instar larvae and their excrements were collected for analysis. Larvae were kept without food for 12 h before freezing. Other larvae were allowed to reach the adult stage, being collected less than 24 h after emergence, together with exuviae. *S. littoralis* and plants’ materials were freeze-dried and kept in a desiccator in the dark until analysis.

3. Extraction procedures

3.1. *L. esculentum* seeds

For phenolics characterization, 0.5 g of seeds were extracted with 2 ml water:methanol (1:1) mixture, by sonication for 1 h, followed by 15 h maceration and another sonication period (1 h). The resulting extract was centrifuged and filtered through 0.45 μm membrane before analysis.

For phenolics quantification and evaluation of cytotoxic effect on RBL-2H3 cells, antioxidant proprieties and acetylcholinesterase (electric eel) inhibition capacity an aqueous extract was prepared, by boiling ca. 6 g of powdered seeds in 600 ml water, for 30 min. The resulting extract was filtered through a Büchner funnel, frozen and lyophilized. A yield of 770 mg was obtained. The lyophilized aqueous extract was kept in a desiccator, in the dark, until analysis. For phenolics determination this extract was redissolved in water, while the bioactivity assays were performed after redissolving it in water or buffer.

For the antimicrobial assays, seeds were extracted using five different solvents: methanol, chloroform, ethyl acetate, hexane and sulphuric acid 0.005 M. Powdered seeds (8 g) were extracted with 200 ml of each solvent with sonication (5 min), followed by 24 h of extraction at room temperature, with mechanic stirring (200 rpm). The resulting extracts were filtered through Buchner funnel and evaporated to dryness under reduced pressure using a rotary evaporator, at 40 ºC. The extracts were redissolved in 4 ml of methanol and stored at -20 ºC until analysis.

3.2. *L. esculentum* leaves

3.2.1. Phenolic compounds

Phenolic compounds were extracted using a mixture of methanol:water (1:1), which allows the recovery of a wide range of polyphenols with diverse structures (253, 254).

For the study of the ecological duo *S. littoralis*/*L. esculentum*, 80 mg of “cherry” variety lyophilized leaves were thoroughly mixed at room temperature with 0.5 ml of solvent, sonicated (1 h), followed by 15 h maceration and another sonication period (1 h). The resulting extract was centrifuged (12000 rpm, 5 min). The same procedure was followed

for “bull’s heart” variety, but starting with a distinct amount of sample (100 mg). The obtained extracts were filtered through a 0.20 µm size pore membrane before HPLC-DAD-ESI-MS^n analysis.

For quantitative analysis and evaluation of antioxidant capacity against $DPPH^{\bullet}$, $^{\bullet}NO$ and $O_2^{\bullet-}$ radicals, dried *L. esculentum* leaves (160 mg) were sonicated (30 min) with 2 ml of solvent, followed by 2 h mechanical agitation (Stuart Scientific, Staffordshire) at 300 rpm and another sonication period (30 min). During this process the temperature was controlled to keep it under 40 ºC, to avoid compounds degradation. The resulting extract was centrifuged (12000 rpm, 5 min). The supernatant was collected and evaporated to dryness under reduced pressure (40 ºC). The obtained residue was redissolved in methanol and filtered through a 0.45 µm size pore membrane previous to HPLC-DAD analysis (**Figure 15**).

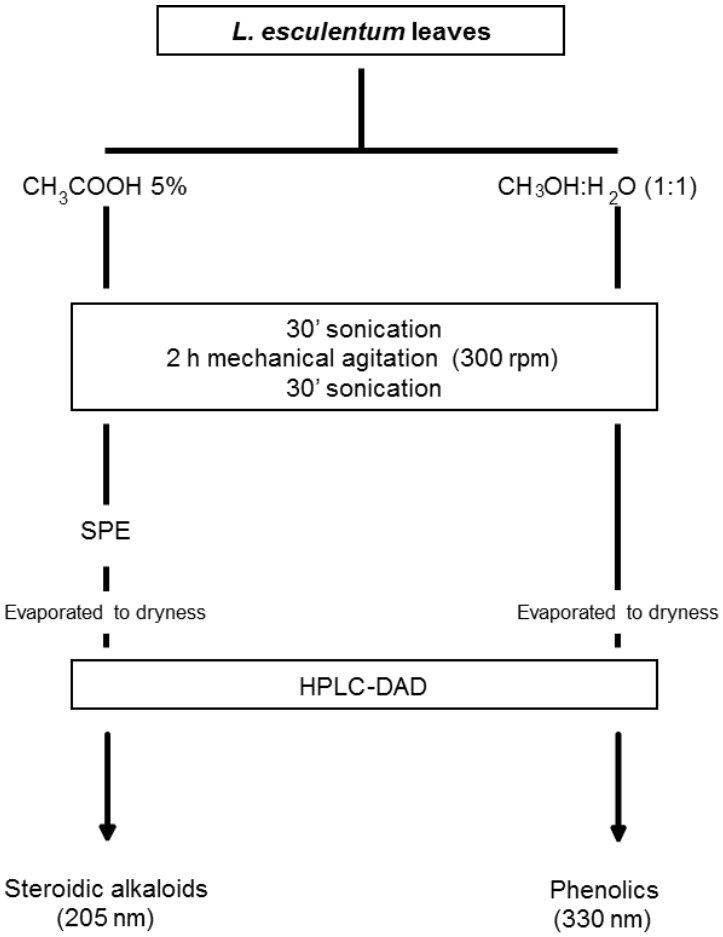


Figure 15. Overview of the experimental procedure for quantification of phenolic compounds and steroidal alkaloids.

3.2.2. Steroidal alkaloids

The extraction of steroidal alkaloids was carried out with acetic acid 5%, since it is a nontoxic and inexpensive solvent, being most commonly used for the extraction of these compounds (35, 80). The extraction procedure was similar to the one referred above for phenolic compounds quantification, excepting that the supernatant containing the alkaloids was subjected to a purification step before analysis (**Figure 15**).

3.2.2.1. SPE columns

Several commercially available sorbents were compared: ion-exchange (SCX), reversed-phase (octadecyl bonded silica (C_{18})), normal phase (cyanopropyl bonded silica (CN), NH_2 and combined packings (CN/SiOH; NH_2/C_{18}) (**Table 4**). Preconditioning and elution conditions were established according to supplier's information and bibliography (86, 87).

Table 4. SPE conditions for the different sorbents[a].

Sorbent	Preconditioning	Washing	Elution
SCX (1000 mg)	10 ml MeOH + 10 ml 5% AcOH	20 ml 5% MeOH	20 ml 2.5% NH_3 in MeOH
C_{18} (1000 mg)	10 ml MeOH + 10 ml 5% AcOH	10 ml 5% MeOH	20 ml 2.5% NH_3 in MeOH
CN (1000 mg)	10 ml MeOH + 10 ml 5% AcOH	20 ml 5% MeOH	20 ml 25% AcOH in MeOH
NH_2 (1000 mg)	10 ml MeOH + 10 ml 5% AcOH	20 ml 5% MeOH	20 ml 25% AcOH in MeOH
CN/SiOH (1500 mg)	10 ml MeOH + 10 ml 5% AcOH	20 ml 5% MeOH	20 ml 25% AcOH in MeOH
NH_2/C_{18} (1000 mg)	10 ml MeOH + 10 ml 5% AcOH	20 ml 5% MeOH	20 ml 25% AcOH in MeOH

[a] MeOH: methanol; AcOH: acetic acid.

3.2.2.2. Purification

The supernatant containing the alkaloids was directly applied in the preconditioned SPE columns and purification followed the conditions indicated in **Table 4**. All the resulting purified extracts were evaporated to dryness under reduced pressure, at 40 ºC. The residues were dissolved in methanol, filtered through a 0.45 µm size pore membrane and analysed by HPLC-DAD. SCX columns were used in the purification of the extracts for quantitative analysis.

3.3. *S. littoralis* materials

In addition to the very small size of the insect's organism itself, the mortality of *S. littoralis* reared on *L. esculentum* leaves from both varieties was high (data not shown), which conditioned the amounts of samples used in the ecological duo study.

Larvae (40 mg/0.3 ml), excrements (20 mg/0.3 ml), exuviae (20 mg/0.3 ml) and adults (80 mg/0.5 ml) of *S. littoralis* reared on leaves from "cherry" variety were thoroughly mixed at room temperature with methanol/water (1:1), sonicated (1 h), followed by 15 h maceration and another sonication period (1 h). This methodology was also applied to materials of *S. littoralis* fed with leaves of "bull's heart" variety, but using distinct sample/solvent ratios: 130 mg/0.5 ml for larvae; 500 mg/ml for excrements; 30 mg/0.3 ml for exuviae; 130 mg/0.5 ml for adults. The resulting extracts were centrifuged (12000 rpm, 5 min) and filtered through a 0.20 µm size pore membrane.

4. HPLC-DAD-ESI-MS^n phenolics qualitative analysis of *L. esculentum* seeds

Chromatographic separation was carried out with a LiChroCART column (250 x 4 mm, RP-18, 5 µm particle size, LiChrospher®100 stationary phase; Merck, Darmstadt, Germany), protected with a LiChroCART guard column (4 x 4mm, RP-18, 5 µm particle size, Merck, Darmstadt, Germany). The mobile phase consisted of two solvents: water-acetic acid (1%) (A) and methanol (B), starting with 5% B and using a gradient to obtain 50% at 30 min and 80% at 37 min.

The flow rate was 1 ml/min and the injection volume 30 µl. Spectral data from all peaks were accumulated in the range 240-400 nm and chromatograms were recorded at 350 nm. Analyses were carried out with an Agilent HPLC 1100 series equipped with a photo-diode array detector and mass spectrometer in series (Agilent Technologies, Waldbronn, Germany). The HPLC consisted of a binary pump (model G1312A), an autosampler (model G1313A), a degasser (model G1322A) and a photo-diode array detector (model G1315B). The HPLC system was controlled by a ChemStation software (Agilent, v. 08.03). The mass spectrometer was an ion trap mass analyzer (model G2445A) equipped with an electrospray ionisation interface and was controlled by LCMSD software (Agilent, v. 4.1).

The ionisation conditions were adjusted at 350 °C and 4 kV for capillary temperature and voltage, respectively. The nebulizer pressure and flow rate of nitrogen were 65.0 psi and 11 l/min, respectively. The full scan mass covered the range from m/z 100 up to m/z 1200. Collision-induced fragmentation experiments were performed in the ion trap using helium as the collision gas, with voltage ramping cycles from 0.3 up to 2 V. Mass spectrometry data were acquired in the negative ionisation mode. MS^n was carried out on the most abundant fragment ion observed in the first-generation mass spectrum. For the compounds present in trace amounts MS^n was carried in the Multiple Reaction Monitoring mode.

5. HPLC-DAD-ESI-MS^n phenolics and alkaloids qualitative analysis in *L. esculentum* leaves and *S. littoralis* materials

Chromatographic separation was carried out with an ACE 3 C-18-AR column (150 x 0.5 mm, 3 µm particle size; Advanced Chromatography Technologies, Aberdeen, Scotland). The mobile phase consisted of water (1% acetic acid) (A) and methanol (B). Elution started with 20% B and a gradient was used to obtain 60% at 20 min and 90% at 35 min. The flow rate was 10 µl/ml and the injection volume was 2 µl for phenolic compounds and 0.3 µl for glycoalkaloids. Spectral data from all peaks were accumulated in the 195-400 nm range and chromatograms were recorded at 202, 205 and 335 nm. The HPLC-DAD-ESI-MS^n analyses were carried out in an Agilent HPLC 1200 series, equipped with a diode array detector and mass detector in series (Agilent Technologies, Waldbronn, Germany). The HPLC consisted of a binary pump (model G1376A), an autosampler (model G1377A) refrigerated at 4 ºC (G1330B), a degasser (model G1379B) and a diode array detector (model G1315D). The HPLC system was controlled by ChemStation

software (Agilent, v. B.01.03-SR2). The mass detector was a Bruker ion trap spectrometer (model HCT Ultra), equipped with an electrospray ionisation interface, and was controlled by LCMSD software (Agilent, v. 6.1). The ionisation conditions were adjusted to 300 °C and 4.0 kV for capillary temperature and voltage, respectively. The nebulizer pressure and nitrogen flow rate were 5.0 psi and 3 l/min, respectively. The full scan mass covered the range from m/z 100 up to m/z 1200. Target mass of 400 u for phenolic compounds and of 750 u for alkaloids was used. Collision-induced fragmentation experiments were performed in the ion trap using helium as collision gas, with voltage ramping cycles from 0.3 up to 2 V. Mass spectrometry data were acquired in the negative and positive ionisation modes for phenolic compounds and alkaloids, respectively. Automatic MS^n was carried out in Selected Ion Monitoring mode.

6. Quantitative analysis of *L. esculentum* seeds

Phenolic compounds, organic acids and fatty acids in *L. esculentum* seeds extracts were quantified by distinct methodologies, as described below.

6.1. HPLC-DAD phenolic compounds analysis

For quantification of phenolic compounds, 20 µl of redissolved aqueous lyophilized extract (80 mg/ml) were analyzed using a HPLC-DAD unit (Gilson) and a Spherisorb ODS2 (25.0 x 0.46 cm, 5 µm particle size; Waters, Milford, USA) column. Elution was performed under the conditions described above for phenolics identification (Chapter II, item 4). Detection was achieved with a Gilson diode array detector. Spectral data from all peaks were accumulated in the range of 200-400 nm and chromatograms were recorded at 350 nm. The data were processed on Unipoint system Software (Gilson Medical Electronics, Villiers le Bel, France).

Phenolic compounds quantification was achieved by the absorbance recorded in the chromatograms relative to external standards. Since standards of all identified compounds were not commercially available, kaempferol derivatives were quantified as kaempferol-3-*O*-rutinoside, quercetin derivatives as quercetin-3-*O*-rutinoside and isorhamnetin derivatives as isorhamnetin-3-*O*-rutinoside. Kaempferol-3-*O*-sophoroside and isorhamnetin-3-*O*-sophoroside were quantified together as kaempferol-3-*O*-rutinoside.

6.2. HPLC-UV organic acids analysis

The extracts used to assess the antimicrobial activity (Chapter II, item 3.1.) were analysed on an analytical HPLC unit (Gilson), using an ion exclusion column Nucleogel® Ion 300 OA (300 x 7.7 mm; Macherey-Nagel, Düren, Germany), in conjunction with a column heating device set at 30 ºC. Elution (70 min) was carried out at a solvent flow rate of 0.2 ml/min, isocratically, with sulphuric acid 0.005 M as the mobile phase. Detection was performed with a Gilson UV detector at 214 nm. Organic acids quantification was achieved by the absorbance recorded in the chromatograms relative to external standards.

6.3. GC-MS fatty acids analysis

6.3.1. Derivatization

Sterified fatty acids in the extracts prepapred with chloroform, methanol, ethyl acetate, hexane and sulphuric acid (Chapter II, item 3.1.) were hydrolysed with potassium hydroxide methanolic solution (11 mg/ml), at 90 ºC for 10 min. The free fatty acids originally present and those resulting from the alkaline hydrolysis were derivatised to their methyl ester forms with BF_3 methanolic solution (10%), at 90 ºC for 10 min. The methyl esters derivatives were extracted with isooctane and anhydrous sodium sulphate was added to assure the total absence of water. The resulting extract was evaporated to dryness under a stream of nitrogen and redissolved in isooctane.

6.3.2. GC-MS conditions

The standard mixture/sample extracts (1 µl) were analysed using a Varian CP-3800 gas chromatographer (USA) equipped with a VARIAN Saturn 4000 mass selective detector (USA) and a Saturn GC/MS workstation, software version 6.8. A VF-5 ms (30m x 0.25mm x 0.25 µm) column (VARIAN) was used. The injector port was heated to 250 °C. Injections were performed in split mode, with a ratio of 1/40. The carrier gas was Helium C-60 (Gasin, Portugal), at a constant flow of 1ml/min. The oven temperature was set at 40 °C for 1 min, then increasing 5 °C/min to 250 °C, 3 °C/min to 300 °C and held for 15 min. All mass spectra were acquired in electron impact mode. Ionisation was maintained off during the first 4 min, to avoid solvent overloading. The ion trap detector was set as follows: transfer line, manifold and trap temperatures were 280, 50 and 180 °C,

respectively. The mass ranged from m/z 50 to 600, with a scan rate of 6 scan/s. The emission current was 50 µA and the electron multiplier was set in relative mode to auto tune procedure. The maximum ionisation time was 25,000 µs, with an ionisation storage level of 35 m/z. The analysis was performed in Full Scan mode. Identification of compounds was achieved by comparison of their retention time and mass spectra with those from pure standards injected under the same conditions and from NIST 05 MS Library Database. The amount of fatty acids methyl esters (FAME) present in the extracts was achieved from the calibration curve of the respective FAME standard. The FAME values were then converted into their respective fatty acid contents.

7. Quantitative analysis of *L. esculentum* leaves

Steroidal alkaloids and phenolics, the main bioactive compounds in leaves, were quantified by HPLC-DAD. The methods were optimized and fully validated.

The HPLC-DAD system used comprised a liquid chromatographer equipped with models 302 and 305 pumps (Gilson Medical Electronics, Villiers le Bel, France), a 20 µl loop and a diode array detector (170, Agilent, Villiers le Bel, France), controlled by Unipoint system software (Gilson Medical Electronics, Villiers le Bel, France). Separation of both kinds of analytes was performed using a reversed-phase ACE 3 C-18-AR column (150 x 4.6 mm, 3 µm particle size; Advanced Chromatography Technologies, Aberdeen, Scotland). Data were processed on Unipoint system.

7.1. Phenolic compounds

The mobile phase was a mixture of two solvents: acetic acid (1%) and methanol. Two gradient conditions were used (**Table 5**) to establish the most suitable concerning chromatographic separation and run time. Elution was performed at different flow rates (0.5-1 ml/min) in order to obtain the optimal conditions. Spectral data from all peaks were accumulated in the range of 200-400 nm and chromatograms were recorded at 330 nm. The different phenolic compounds were identified by comparing their chromatographic behaviour and UV-vis spectra in the 200-400 nm range with authentic standards and with data from our previous work using HPLC-DAD-ESI-MS^n.

Table 5. Gradient elution programs tested for HPLC-DAD quantification of phenolic compounds and steroidal alkaloids in *L. esculentum* leaves.

Gradient	Phenolics			Alkaloids		
	Time (min)	Eluent system[a]		Time (min)	Eluent system[a]	
		MeOH (%)	AcOH (%)		ACN (%)	TEAP (%)
A	0	15	85	0	20	80
	30	40	60	12	25	75
	35	60	40	15	35	65
	37	80	20	17	45	55
	45	90	10	25	65	35
B	0	20	80	0	20	80
	20	60	40	12	45	55
	35	90	10	17	55	45
				20	57	43

[a] MeOH: methanol; AcOH: acetic acid 1%; ACN: acetonitrile; TEAP: triethylammonium phosphate buffer.

Phenolic compounds quantification was achieved by the absorbance recorded in the chromatograms relative to external standards. Because standards of all identified compounds were not commercially available, kaempferol derivatives were quantified as kaempferol-3-*O*-rutinoside, quercetin derivatives as quercetin-3-*O*-rutinoside and *p*-coumaric, sinapic and ferulic acids derivatives as *p*-coumaric, sinapic and ferulic acids, respectively. Caffeoyl-hexoside and *p*-coumaroyl-hexoside acids were quantified together as 5-*O*-caffeoylquinic acid and the pair feruloyl-hexoside acid plus sinapoyl-hexoside acid isomer was determined as ferulic acid.

7.2. Steroidal alkaloids

The mobile phase consisted of acetonitrile and TEAP buffer (25 mM) at pH 3.0. As for phenolic compounds, the elution conditions were optimized to establish the most suitable ones concerning run duration and chromatographic separation (**Table 5**). Different flow rates (0.5-1 ml/min) were assayed in order to achieve the optimal conditions. Spectral data from all peaks were accumulated in the range of 200-400 nm and chromatograms were recorded at 205 nm. The different steroidal alkaloids were identified by comparison with authentic standards and with data from our previous study using HPLC-DAD-ESI-MS^n.

Quantification was achieved by the absorbance recorded in the chromatograms relative to external standards. As the commercial standards of tomatine and tomatidine correspond to the mixtures of α-tomatine plus dehydrotomatine and of tomatidine plus tomatidenol, respectively, α-tomatine and dehydrotomatine were quantified together as tomatine and tomatidine plus tomatidenol were quantified together as tomatidine.

7.3. Methods validation

The linearity range, limit of detection (LOD), limit of quantification (LOQ), precision and recovery were studied for the developed methods. The linearity of the calibration curve was assessed by the analysis of individual reference compounds. Calibration curves were obtained by plotting the peak areas against analytes concentrations. Five solutions of 5-*O*-caffeoylquinic acid (0.200, 0.100, 0.050, 0.025 and 0.003 mg/ml), quercetin-3-*O*-rutinoside (0.160, 0.080, 0.040, 0.020 and 0.003 mg/ml), tomatine (3.100, 1.550, 0.775, 0.388 and 0.097 mg/ml) and tomatidine (3.500, 1.750, 0.875, 0.438 and 0.109 mg/ml) were analysed, each one of them for four times. LOD and LOQ were determined by signal-to-noise ratios (S_0/b, where S_0 is the standard deviation of signal-to-noise ratio and b is the slope of the calibration plot) of 3:1 and 10:1, respectively.

Intra-day and inter-day variations were chosen to determine the precision. For the intra-day precision, the leaves' extracts were analysed four times within one day, while for the inter-day precision they were examined in quadruplicate in three consecutive days. The relative standard deviation (RSD) of the peak area was calculated as measure of precision.

The recoveries were determined by adding known amounts of quercetin-3-*O*-rutinoside (0.69, 0.34 and 0.016 mg/ml), 5-*O*-caffeoylquinic acid (0.45, 0.37 and 0.01 mg/ml), tomatine (1.93, 0.58 and 0.5 mg/ml) and tomatidine (0.23, 0.12 and 0.05 mg/ml). As most of the identified phenolic compounds are not commercially available, quercetin-3-*O*-rutinoside and 5-*O*-caffeoylquinic acid were chosen to assess phenolic compounds recovery, as they represent the two main classes of phenolics found in this matrix (flavonoids and hydroxycinnamic acids derivatives, respectively).

8. Antioxidant potential

8.1. DPPH• scavenging assay

DPPH• is a stable free radical with a strong absorbance at 515 nm, able to accept an electron or hydrogen atom, becoming a non-radical and hardly oxidizable species (255).

The antiradical activity was determined spectrophotometrically in a plate reader (Multiskan Ascent, Thermo Electron Corporation). For each extract, a dilution series (five different concentrations) was prepared in a 96-well plate. The reaction mixtures in the sample wells consisted of 25 μl of extract (redissolved in methanol) and 200 μl of 150 mM methanolic DPPH•. The plate was incubated for 30 min at room temperature after addition of DPPH• solution and the absorvance was determined at 515 nm (256). Three experiments were performed in triplicate.

8.2. Superoxide ($O_2^{\bullet -}$) scavenging assay

Superoxide was generated by the NADH/PMS system, according to a described procedure: PMS reduced by NADH reacts with oxygen to produce $O_2^{\bullet -}$; this radical then reduces NBT to a formazan blue dye, which has an absorption maximum at 560 nm (256).

The reaction mixtures in the wells consisted on 50 µl of sample dissolved in buffer (KH_2PO_4 19 mM, pH 7.4), 50 µl NADH (166 μM), 150 µl NBT (43 μM) and 50 µl of PMS (2.7 μM). The rate of the reaction was assessed at 560 nm, during 2 min after PMS addition (256, 257). Three experiments were performed in triplicate.

8.3. •NO scavenging assay

•NO was spontaneously generated by a SNP solution at pH 7.4 and subsequentially reacted with oxygen, producing nitrite that was determined by Griess reagent, as reported before (256, 258).

Antiradical activity was determined spectrophotometrically in a 96-well plate reader. The reaction mixtures in each well consisted on 100 µl of sample dissolved in buffer

(KH_2PO_4 100 mM, pH 7.4) and 100 µl of SNP (20 mM). The plates were incubated at room temperature for 60 min, under light. Griess reagent (1% sulphanilamide and 0.1% naphthylethylenediamine in 2% H_3PO_4) (100 µl) was then added and 10 min later the absorbance of the chromophore formed during the diazotisation of nitrite with sulphanilamide and subsequent coupling with naphthylethylenediamine was determined at 560 nm. Three experiments were performed in triplicate.

9. Antimicrobial potential

9.1. Microorganisms

Nine bacterial species were used for the experiments: *Staphylococcus aureus* (ATCC 20231), *Staphylococcus epidermidis* (ATCC 20044), *Salmonella typhimurium* (ATCC 43971), *Proteus mirabilis* (ATCC 4479), *Escherichia coli* (ATCC 30083), *Pseudomonas aeruginosa* (ATCC 50071), *Bacillus cereus* (ATCC 31000), *Enterococcus faecalis* (ATCC 20477) and *Micrococcus luteus* (ATCC 20030). Cultures were obtained from the Laboratory of Microbiology, Faculty of Pharmacy, Porto University. Stock cultures were maintained on MHA at 4 ºC.

Antifungal activity was checked against *Candida albicans* (ATCC 10231), *Aspergillus fumigatus* (ATCC 46645) and *Trichophyton rubrum* (CECT 2794). *Candida parapsilosis* (ATCC 22019) and *Candida krusei* (ATCC 6258) were used for quality control. All strains were stored in SDB with 20% glycerol at -70 ºC and subcultured in SDA before each test, to ensure optimal growth conditions and purity.

9.2. Antibacterial activity

Bacterial inocula were prepared by growing cells in MHB for 24 h, at 37 ºC. Cell suspensions were diluted in sterile MHB to provide initial cell counts of about 10^6 colony-forming units *per* ml (CFU/ml). The minimum inhibitory concentration (MIC) of seeds extracts, organic acids and quercetin-3-*O*-rutinoside were determined by two-fold serial dilution method, in 96-well plates. All tests were performed in MHB.

The initial concentration was 20 mg/ml for the seeds extracts (Chapter II, item 3.1.) and all organic acids, with the exceptions of aconitic (10 mg/ml) and fumaric (2.5 mg/ml) acids. Quercetin-3-*O*-rutinoside was tested at a maximum concentration of 69 µg/ml. The final

concentration of methanol did not exceed 1% (v/v). Briefly, 90 µl of MHB and 10 µl of suspension containing 10^6 CFU/ml were added in each well, which contained 100 µl of extract/compound. The reference antibacterial drug ciprofloxacin was also tested. Negative controls in MBH alone and with 1% methanol (v/v) and sterility and growth controls were included. Plates were incubated for 24 h, at 37 ºC, and then examined by a binocular microscope.

The MIC was determined as the lowest concentration of seeds extracts or standard compounds (organic acids or quercetin-3-*O*-rutinoside) inhibiting the visual growth of the test culture on the microplate. The experiments were performed in duplicate and repeated independently three times, yielding essentially the same results (a range of values is presented when different results were obtained).

9.3. Antifungal activity

Broth microdilution methods based on the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) reference documents M27-A3, and M38-A2, for yeast and filamentous fungi, respectively, with minor modifications, were used to determine MICs. Briefly, inocula suspensions were prepared from SDA cultures at the final required density in RPMI-1640 medium (with glutamine, without bicarbonate, and with phenol red as pH indicator).

Seeds extracts were dissolved in 50% methanol (v/v) and organic acids and quercetin-3-*O*-rutinoside were dissolved in RPMI-1640 broth. Two-fold serial dilutions were prepared in RPMI-1640 broth, starting from 20 mg/ml for seeds extracts and organic acids, with the exceptions of aconitic (10 mg/ml) and fumaric (2.5 mg/ml) acids and quercetin-3-*O*-rutinoside (0.21 mg/ml). The solutions and cell suspensions in the test medium were then distributed into sterile 96-well plates. Maximum methanol concentrations were kept at 1% (v/v). The plates were incubated at 35 ºC in a humid atmosphere, without agitation, for 48 h for *C. albicans* and *A. fumigatus*, and for four days for *T. rubrum*. The reference antifungal drugs fluconazole (*C. albicans*) and voriconazole (*A. fumigatus* and *T. rubrum*) were also assyed. MICs correspond to the lowest concentrations resulting in 100% growth inhibition.

Fluconazole MICs for *C. parapsilosis* (ATCC 22019) and *C. krusei* (ATCC 6258) were determined as quality controls and the results were within the recommended limits.

Sterility and growth controls in RPMI-1640 medium alone and with 1% of methanol (v/v) were included. The experiments were performed in duplicate and repeated independently three times, yielding essentially the same results (a range of values is presented when different results were obtained).

10. Non-human cholinesterases inhibition

Several tests allow evaluating this property, the most widely used being the spectrophotometric determination based on the method of Ellman. This consists on monitoring the hydrolysis of acetylthiocholine, followed by reaction with Ellman's reagent to produce 5-thio-2-nitrobenzoate. Several enzymes from different sources are commercially available, although the results obtained can be different depending on the enzyme source (259).

Acetylcholinesterase inhibitory activity was determined spectrophotometrically in a plate reader (Multiskan Ascent, Thermo Electron Corporation). In each well the mixture consisted on 25 μl ATCI (15 mM), 125 μl DTNB (3 mM), 50 μl buffer (50 mM Tris-HCl, pH 8, containing 0.1% BSA) and 25 μl of sample dissolved in buffer with 10% of methanol. The absorbance was read at 405 nm during 2 min. After this step, 25 μl of AChE (0.44 U/ml) was added and the absorbance was read again. The rates of reactions were calculated by Ascent Software version 2.6 (Thermo Labsystems Oy). The rate of the reaction before adding the enzyme was subtracted from that obtained after adding the enzyme in order to correct eventual spontaneous hydrolysis of substrate (260, 261).

BChE inhibition assay was performed in a similar way, but using 25 μl of 15 mM BTCC as substrate and 25 μl of enzyme (0.1 U/ml of BChE) (260, 261).

For each enzyme, three experiments were performed in triplicate.

11. Cell system assays

11.1. *L. esculentum* seeds aqueous extract

11.1.1. Culture conditions and treatments

Rat basophile leukemia (RBL-2H3) cell line from American Type Culture Collection (LGC Standards S.L.U., Spain) was maintained in DMEM with 15% FBS and 2% penicillin, in an incubator with 5% CO_2. Cells were plated with a density of $1x10^4$ cells/well and allowed to attach for 24 h at 37 °C, with 5% CO_2. On the following day the medium was removed and cells were gently washed with warm PBS. Tomato seeds aqueous extract (Chapter II, item 3.1.) redissolved in media was added to the wells at final concentrations ranging from 4 to 10 mg/ml and incubated for 48 h at 37 °C, with 5% CO_2. The antiproliferative capacity was assessed by SRB assay. Media alone was used as negative control.

11.1.2. SRB assay

The SRB assay relies on the uptake of the negatively charged pink aminoxanthine dye, sulphorhodamine B, by basic amino acids in the cells. The greater the number of cells, the higher amount of dye is taken up and, after fixing, when the cells are lysed, the released dye will give a more intense color and higher absorbance (262).

The method described by Houghton *et al.* (262) was followed, with modifications. After the incubation period, media was removed, 100 µl of cold 40% trichloroacetic acid was added and plates were maintained at 4 °C for 60 min. Plates were then washed 5 times with tap water and allowed to dry. Afterwards, 50 µl of 0.4% SRB in 1% acetic acid was added and plates were incubated for 30 min at 37 °C, with 5% CO_2.

After the incubation period, plates were quickly washed with 1% acetic acid, in order to remove unbound dye, allowed to dry and then 100 µl of tris-base were added. Plates were shaken after 10 min.

Absorbance was read in a multi-plate reader, at 492 nm.

11.2. *L. esculentum* leaves purified alkaloids extract and steroidal alkaloids

11.2.1. Culture conditions and treatments

Human colorectal adenocarcinoma (Caco-2) cell line from the American Type Culture Collection was routinely cultured using DMEM supplemented with 10% FBS, 1% NEAA, 1% antibiotic, 1% fungizone and 6 µg/ml transferrin. Human gastric adenocarcinoma (AGS) cells and human neuroblastoma (SH-SY5Y) cells from the American Type Culture Collection were grown in DMEM media supplemented with 10% FBS, 2% penicillin/streptomycin and 1% NEAA. All cell lines were cultured at 37 ºC in a 5% CO_2 incubator. Cells were washed with HBS, trypsinized and sub-cultured in 96-well plates at a density of 25000 cells/cm^2.

The purified leaf extracts (Chapter II, item 3.2.2.) (6.17-790 µg/ml), tomatine (0.79-101.53 µM) and tomatidine (3.95-505 µM) were dissolved in medium containing 0.1% (v/v) DMSO. The final concentration of DMSO did not affect cellular viability. To determine the effect of the compounds/extracts on cells, viability was assessed 24 h after exposure.

The potential protective effect against glutamate toxicity was assessed by co-exposition with compounds/extracts. The toxicity induced by glutamate (12.5-200 mM) was also evaluated by MTT reduction after 24 h, as described by Silva *et al.* (263). To evaluate the possible involvement of nAChEr and nBChEr in the protective effect, the incubations were performed in the presence of mecamylamine (1 µM) and scopolamine (1 µM), respectively. The specific α7-nAChR inhibitor MLA (1 µM) was also tested. To measure the reactive species and mitochondrial membrane potential cells were exposed to 25 mM glutamate, with or without compounds/extracts, and incubated for 20 min and 4 h, respectively, at 37 ºC in a 5% CO_2 incubator.

11.2.2. AChE and BChE inhibitory activity

ChE inhibition capacity was determined by Ellman's method, as previously reported (264). As undifferentiated SH-SY5Y cells present low ChE activity (265), to evaluate both AChE and BChE inhibition a SH-SY5Y protein extract was used. Briefly, SH-SY5Y cells were washed with PBS, then lysis buffer (1% triton-X 100 in PBS) was added and cells were scraped. The suspension was centrifuged (13000 rpm during 20 min, at 4 ºC) and the supernatant was collected. The protein concentration of the supernatant was adjusted

to provide a control absorbance between 0.50 and 2.00 after 30 min of incubation at 32 ºC, in the presence of the substrate ATCI or BTCC. Absorbance was measured at 405 nm before and after incubation with the enzyme and the initial absorbance was subtracted in order to correct eventual spontaneous hydrolysis of substrate.

11.2.3. MTT reduction

The MTT assay allows evaluating cells' mitochondrial activity and to determine the number of viable cells. MTT is reduced to formazan by mitochondrial dehydrogenase succinate, this enzyme being only active in cells with intact metabolism and respiratory chain (266).

After cells treatment, the medium was removed and the cells were incubated for 30 min, at 37 °C, with culture medium containing 0.5 mg/ml MTT. Afterwards, the solution was removed and formazan crystals were solubilized in 200 µL DMSO. The resulting purple solution was measured spectrophotometrically at 570 nm (267). Data are presented as the percentage of MTT reduction of treated cells relative to control.

11.2.4. Lactate dehydrogenase (LDH) leakage

LDH is rapidly released from damaged cells. The consumption of NADH is kinetically measured in the supernatant and is correlated with the amount of extracellular LDH. Thus, the cell viability is inversely proportional to the amount of released LDH (267).

Under the conditions of the test, LDH catalyses the conversion of pyruvate to lactate as NADH is oxidized to NAD^+. The catalytic activity is determined from the rate of disappearance of NADH at 340 nm during 4 min (background correction at 620 nm). Thus the decrease in absorbance is proportional to LDH activity in the sample (233).

After 24 h treatment, an aliquot of culture medium was taken to determine the activity of LDH leaked through cell membranes. The cell monolayer was lysed in the remaining medium with lysis buffer (1% triton-X 100 in PBS) to determine total LDH activity. Results are expressed as LDH activity in media relative to total activity (medium plus cell lysate).

11.2.5. Measurement of reactive species with DCFH-DA

DCFH-DA is the most widely used probe for detecting intracellular reactive species. DCFH-DA diffuses through the cell membrane of viable cells, being deacetylated to 2',7'-dichlorofluorescein (DCFH), which is not fluorescent. This compound reacts quantitatively with reactive species (of oxygen and of nitrogen) inside the cell to produce the fluorescent dichlorofluorescein (DCF), which remains trapped within the cells and can be measured to provide an index of intracellular oxidative species (268).

Intracellular reactive species production was measured using DCFH-DA, as previously described (269), with some modifications. After 20 min of incubation with 12.5 µM of DCFH-DA, the excess of the probe was washed out and 200 µl of 1% solution of triton X-100 was thoroughly mixed. Cells were incubated for 5 min at room temperature and the fluorescence of the lysate at 485 nm excitation and 520 nm emission was recorded in a microplate spectrofluorimeter (Synergy^{TM} HT, Biotek Instruments Winooski, USA) operated by Gen5 Software.

11.2.6. Measurement of mitochondrial membrane potential

Mitochondrial membrane potential was monitored using the fluorescent dye rhodamine 123, which preferentially distributes into active mitochondria based on the highly negative mitochondrial membrane potential. Depolarization of mitochondrial membrane results in the loss of rhodamine 123 from the mitochondria and in a decrease of intracellular fluorescence (270).

Cells were incubated with rhodamine 123 (5 µg/ml) during 30 min at 37 °C, then washed twice with PBS (270). Fluorescence was recorded at 485 nm excitation and 520 nm emission in a microplate spectrofluorimeter (Synergy^{TM} HT, Biotek Instruments Winooski, USA) operated by Gen5 Software.

11.2.7. Protein content

Protein content was measured by Lowry's method as previously described (271), using bovine serum albumin as standard. Briefly, proteins react with copper [II] ions under alkaline conditions and the subsequent reduction of the Folin-Ciocalteu's reagent to heteropolymolybdenum blue by the copper-catalysed oxidation of aromatic amino acids was measured at 650 nm in a microplate reader.

12. Statistical analysis

All statistical calculations were made using GraphPad Prism version 6.00 for Windows (GraphPad Software, San Diego California, USA). For all the cell system assays, the statistical comparison between groups was estimated using the parametric method of one-way ANOVA on ranks followed by the Bonferroni's post hoc test. For the evaluation of neuroprotective effect against glutamate-induced toxicity and of acetylcholine receptors envolvement in the neuroprotective effect, the concentration-response curves were fitted using the least squares as the fitting method and the comparisons between curves (bottom, top and log EC_{50}) were made using the extra sum-of-squares *F* test. In all cases, *p* values lower than 0.05 were considered statistically significant.

For the antioxidant potential ($DPPH^{\bullet}$, $^{\bullet}NO$ and $O_2^{\bullet-}$ radicals) and cholinesterase inhibition (human and non-human enzymes) evaluation the IC values were estimated by interpolation of the nonlinear regression curve built using the mean values of three experiments.

Chapter III

Results and discussion

1. *L. esculentum* seeds

1.1. Aqueous extract

Tomato seeds are often considered as waste of the production of tomato derivatives, and some consumers remove them from the fruit before use. From a nutritional point of view, different compounds with health promoting effects can be present in the aqueous extract obtained after boiling for 30 min, which resembles the cooking procedure.

So, this work aimed to improve the knowledge on the phenolic profile of tomato seeds and to evaluate some of their health promoting capacities. For these purposes, phenolics were screened for the first time, by HPLC-DAD-ESI-MS^n. The antioxidant capacity was assessed against $DPPH^{\bullet}$, $O_2^{\bullet -}$ and $^{\bullet}NO$ radicals and AChE inhibitory capacity was checked. Cytotoxicity was evaluated by SRB assay in RBL-2H3 cells.

1.1.1. HPLC-DAD-ESI-MS^n phenolic compounds qualitative analysis

The HPLC-DAD-ESI-MS^n screening of phenolic compounds in tomato seeds hydromethanolic extract showed two major peaks (compounds **7** and **10**+**11**), besides others present in small amounts (**Figure 16**). The structures of the compounds were characterized, excepting those of compounds **A** to **F**. The UV spectra of these latter pointed to hydroxycinnamic acids, although this could not be confirmed by their mass spectra.

The mass spectrum of compounds **1**-**14** indicated that they were flavonoid-*O*-glycosides. In the MS^2 fragmentation of compounds **7**-**14** or in the MS^3 of compounds **1**-**6** the aglycone-related ion ($[Aglc-H/2H]^-$) (300/301 for quercetin, 285 for kaempferol and 315 for isorhamnetin) (**Table 6**) was the base peak or an abundant one.

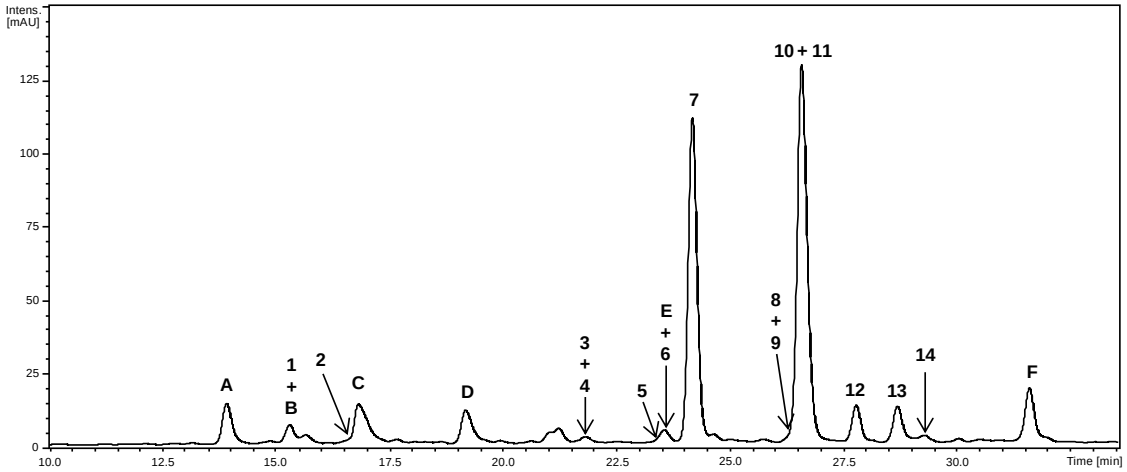


Figure 16. HPLC phenolic profile of tomato seeds. Detection at 350 nm. Peaks: (**1**) quercetin-3-*O*-sophoroside-7-*O*-glucoside; (**2**) kaempferol-3-*O*-sophoroside-7-*O*-glucoside; (**3**) quercetin-3-*O*-gentiobioside-7-*O*-glucoside; (**4**) quercetin-3-*O*-sophoroside-7-*O*-rhamnoside; (**5**) kaempferol-3-*O*-sophoroside-7-*O*-rhamnoside; (**6**) isorhamnetin-3-*O*-sophoroside-7-*O*-rhamnoside; (**7**) quercetin-3-*O*-sophoroside; (**8**) kaempferol-3-*O*-(2-sophorosyl)glucoside or kaempferol-3-*O*-glucosyl-(1→2´´´)-glucosyl-(1→2´´)-glucoside; (**9**) quercetin-3-*O*-(2-pentosyl)rutinoside or quercetin-3-*O*-(2-pentosyl,6-rhamnosyl)glucoside; (**10**) kaempferol-3-*O*-sophoroside; (**11**) isorhamnetin-3-*O*-sophoroside; (**12**) isorhamnetin-3-*O*-gentiobioside; (**13**) quercetin-3-*O*-rutinoside; (**14**) kaempferol-3-*O*-(2-pentosyl)glucoside; (**A**-**F**) non identified compounds.

According to the MS fragmentation profile, these compounds were divided into three different groups. The MS^2 fragmentation of the deprotonated molecule ($[M-H]^-$) from the first group (compounds **1**-**6**) showed the base peak, almost the unique ion, corresponding to the loss of a hexose (162 u, **1**-**3**) or a deoxyhexose residue (146 u, **4**-**6**). This MS^2 fragmentation results from the loss of glycosylation in position 7, which is characteristic of flavonol-3-*O*-glycoside-7-*O*-glycosides (272) and also agrees with the fragmentations observed in the majority of the Brassicaceae glycosides, published before by our group (273-276).

Additionally, it was already reported the presence of several compounds with substitution in 3 and 7 positions and never in the B ring. Thus, biosynthetically these compounds were considered to have the same substitution pattern.

Moreover, the MS fragmentation was similar to that of the compounds with this kind of substitution. The resulting ion corresponded to the aglycone with the glycosidic fraction in the 3 position (di-hexosides). In the MS^3 fragmentation of these ions ($MS^3[(M-H)\rightarrow(M-H-162/146)]^-$) it could be observed, besides the ion $[(M-H)-120)]^-$ resulting from the loss of a

hexose residue in positions 0-3, ions that involved the cleavage of the interglycosidic bond, with the loss of a hexose residue (162 u) and/or a hexose (180 u) (**Table 6**).

This behaviour is typical of a 1→2 bond (compounds **1**, **2**, **4**-**6**) *vs* 1→6 one (compound **3**), in which these ions are absent or present in low abundance (272). Considering that the hexoses detected in different parts of the fruit from diverse tomato cultivars are glucosides (148), we tentatively assigned the compounds to quercetin-3-*O*-sophoroside-7-*O*-glucoside (**1**), kaempferol-3-*O*-sophoroside-7-*O*-glucoside (**2**), quercetin-3-*O*-gentiobioside-7-*O*-glucoside (**3**), quercetin-3-*O*-sophoroside-7-*O*-rhamnoside (**4**), kaempferol-3-*O*-sophoroside-7-*O*-rhamnoside (**5**) and isorhamnetin-3-*O*-sophoroside-7-*O*-rhamnoside (**6**).

Table 6. *R*t, UV, MS: $[M-H]^-$, $-MS^2\,[M-H]^-$ and $-MS^3\,[(M-H)\rightarrow(M-H-162/146)]^-$ data of flavonoid-*O*-glycosides from tomato seeds.[a]

	Compounds[b]	*R*t (min)	UV (nm)	$[M-H]^-$	$-MS^2[M-H]^-$, m/z (%)	$-MS^3[(M-H)\rightarrow(M-H-162/146)]^-$, m/z (%)				
						-120	-162	-180		$[Aglc-H/2H]^-$
					Flavonol-3-*O*-diglucoside-7-*O*-glucosides					
					-162					
1	Q-3-Soph-7-Glc	15.2	----[c]	787	625(100)	505(5)	463(8)	445(25)		300(100)
2	K-3-Soph-7-Glc	16.6	----[c]	771	609(100)	489(13)	447(5)	429(37)		285(100)
3	Q-3-Gtb-7-Glc	21.8	----[c]	787	625(100)					300(100)
					Flavonol-3-*O*-sophoroside-7-*O*-rhamnosides					
					-146					
4	Q-3-Soph-7-Rh	21.8	----[c]	771	625(100)	505(30)	463(7)	445(30)		300(100)
5	K-3-Soph-7-Rh	23.3	----[c]	755	609(100)		447(27)	429(75)		285(100)
6	I-3-Soph-7-Rh	23.5	----[c]	785	639(100)			459(28)		315(100)
					Flavonol-3-*O*-triglycosides					
					$-MS^2[M-H]^-$					
					-120	-132	-150	-162	-342	
8	K-3-triGlc	26.5	----[c]	771	651(21)			609(100)	429(48)	285(50)
9	Q-3-Pent-Rut	26.5	----[c]	741		609(56)	591(22)			300(100)
					Flavonol-*O*-diglycosides					
					$-MS^2[M-H]^-$					
					-120	-132	-150	-162	-180	
7	Q-3-Soph	24.1	256, 266sh, 298ssh, 354	625	505(10)			463(18)	445(28)	300(100)
10	K-3-Soph	26.6	266, 298sh, 350[d]	609	489(6)				429(43)	285(100)
11	I-3-Soph	26.8	----[d]	639	519(11)				459(45)	315(100)
12	I-3-Gtb	27.8	256, 266sh, 304sh, 354	639					459(7)	315(100)
13	Q-3-Rut	28.7	256, 266sh, 299sh, 354	609						301(100
14	K-3-Pent-Glc	29.3	----[c]	579		447(32)	429(37)			284(100)

[a] Main observed fragments. Other ions were found but they have not been included.
[b] Q: quercetin; K: kaempferol; I: isorhamnetin; Soph: sophoroside (glucosyl(1→2)glucoside); Gtb: gentiobioside (glucosyl(1→6)glucoside); Rut: rutinoside (rhamnosy(1→6)glucoside); Glc: glucoside; Rh: rhamnoside; Pent: pentoside.
[c] Compounds hidden by others or in traces. Their UV spectra have not been properly observed.
[d] UV spectrum of **10** predominates over the spectrum of **11**.

The MS^2 $[M-H]^-$ fragmentation of another group of compounds, also triglycosides (compounds **8** and **9**), was different from the one previously discussed. In their fragmentation, besides the aglycone-related ions, other ions resulting from the rupture of the interglycosidic bond of the triglycoside were observed. Thus, in compound **8** (kaempferol-tri-hexoside) it was noticed the presence of the ion at m/z 429, which resulted from the combined loss of a hexose residue (162 u) and a hexose plus water (180 u) (**Figure 17**).

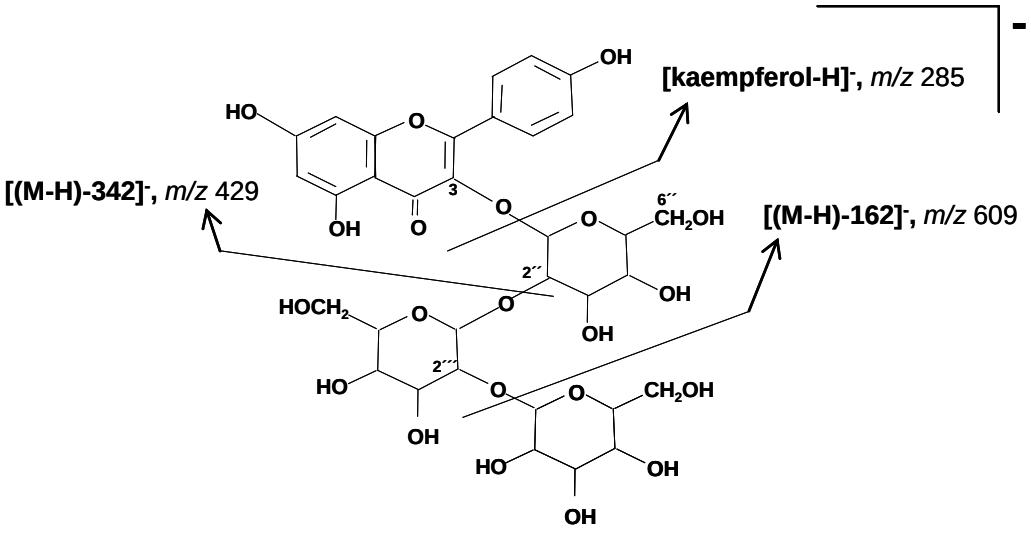
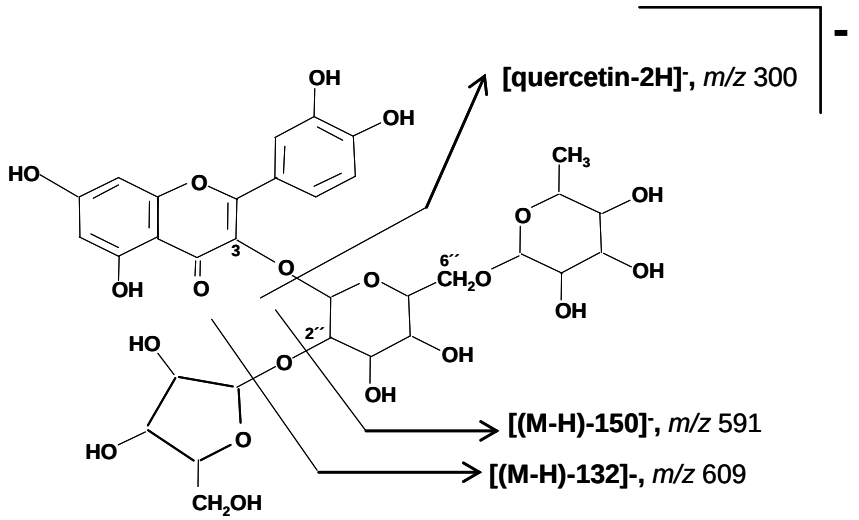


Figure 17. Fragmentation pattern of compound **8**.

This water loss indicated that the di-hexosyl was linked to another sugar, instead of a phenolic hydroxyl (277). This behaviour suggested that the di-hexosyl fraction should not be linked to the hydroxyl in 6 position of the hexose, because comparing 1→2 *vs* 1→6 bonds, the latter is hard to be broken. Thus, it should be in other position, probably 2''. On the other hand, the presence of ions at m/z 609 (base peak), resulting from the loss of 162 u from the $[M-H]^-$, indicated that the di-hexosyl group should also have a 1→2 interglycosidic linkage (**Figure 17**). Therefore, compound **8** was tentatively identified as kaempferol-3-*O*-(2-sophorosyl)glucoside or kaempferol-3-*O*-glucosyl-(1→2´´´)-glucosyl-(1→2´´)glucoside.

Compound **9** exhibited a $[M-H]^-$ ion at m/z 741 and in its MS^2 fragmentation the base peak corresponded to the quercetin aglycone anion (m/z 300 $[quercetin–2H]^{–}$). Thus, it corresponds to a quercetin triglycoside (quercetin+pentose+rhamnose+hexose, 741=301+132+146+162), substituted only at one phenolic hydroxyl group (272). Other important ions resulted from the loss of a pentose residue (132 u) or a pentose (150 u), indicating that the pentose was linked to the hydroxyl from a sugar moiety different from the 6''position of the hexose (**Figure 18**).



$[M-H]^-$, *m/z* 741

Figure 18. Fragmentation pattern of compound **9**.

In the MS^3 $[(M-H)\rightarrow(M-H-132)]^-$ event (results not shown in **Table 6**) no ions involving the cleavage of the interglycosidic bond in the rhamnohexosyl part were noticed. Only the aglycone-related ion and the ion at m/z 343 were detected, which corresponded to an internal cleavage of the hexose in the positions 0-3. These data indicated that compound **9** could be a quercetin rutinoside substituted with a pentose. Thus, compound **9** was tentatively identified as quercetin-3-*O*-(2-pentosyl)rutinoside or quercetin-3-*O*-(2-pentosyl, 6-rhamnosyl)glucoside. In fact, it agrees with quercetin-3-*O*-(2''-*O*-β-apiofuranosyl-6''-*O*-α-rhamnopyranosyl-β-glucopyranoside), reported before in tomato (167).

The other compounds (**7** and **10**-**14**) are diglycosides of flavonoids whose MS^2 fragmentations presented, as base peak, the aglycone-related ion with only one substitution in the flavonol skeleton (**Table 6**).

For compounds **7**, **10, 11** and **14** the characteristic MS^2 fragmentation from diglycosides with interglycosidic linkage 1→2 was observed, exhibiting several ions corresponding to the loss of a hexose residue (162 u) and/or a hexose plus water (180 u) (**7**+**10** and **11**) and of 132 and 150 u corresponding to pentosyl or pentosyl plus water, respectively (**14**), in considerable abundance. Thus, these compounds were identified as quercetin-3-*O*-sophoroside (**7**), kaempferol-3-*O*-sophoroside (**10**), isorhamnetin-3-*O*-sophoroside (**11**) and kaempferol-3-*O*-(2-pentosyl)glucoside (**14**). These ions were not observed for compound **13**, while for compound **12** (isomer of **11**) the ion at m/z 459 ($[M-H-180)]^-$ had a relative abundance of 7% *vs* 45% for compound **11**, indicating that the interglycosidic bonds of these compounds were 1→6. So, these compounds were identified as isorhamnetin-3-*O*-gentiobioside (**12**) and quercetin-3-*O*-rutinoside (**13**).

From these compounds, only quercetin-3-*O*-rutinoside (**13**) was described in tomato seeds (162). Moreover, quercetin-3-*O*-sophoroside-7-*O*-rhamnoside (**4**) and kaempferol-3-*O*-sophoroside-7-*O*-rhamnoside (**5**) were already reported in tomato fruit (148, 160).

1.1.2. HPLC-DAD phenolic compounds quantitative analysis

The phenolic composition of *L. esculentum* seeds aqueous lyophilized extract from both varieties (“bull’s heart” and “cherry”) was similar to that of the hydromethanolic one. In order to get a better characterization of the extract used in the evaluation of cytotoxic effect on RBL-2H3 cells, radical scavenging and AChE inhibition capacity, phenolic compounds were quantified by HPLC-DAD.

Both varieties presented a similar profile, “bull’s heart” and “cherry” containing ca. 2066 and 1974 mg/kg phenolic compounds, respectively (**Table 7**). The compounds in highest amounts in both varieties were quercetin-3-*O*-sophoroside (**7**) and the pair kaempferol-3-*O*-sophoroside (**10**) plus isorhamnetin-3-*O*-sophoroside (**11**), representing ca. 30 and 57% of total phenolics, respectively. All the other compounds were found in much smaller amounts (**Table 7**).

Phenolic compounds are present in most plant seeds and grains (160), playing vital roles in defence against pathogens and predators and contributing to physiological functions, such as seed maturation and dormancy (278). As referred above, at the subcellular level, they may be accumulated in vacuoles or in the cell walls. All of the

flavonoids present in tomato seeds are diglycosides or triglycosides, being known that they are mainly confined to hydrophilic regions, such as vacuoles and apoplasts (278).

Table 7. Phenolic composition of tomato seeds aqueous lyophilized extracts (mg/kg, dry basis)[a].

	Compounds[b]	"Cherry"	"Bull's heart"
1+B	Q-3-Soph-7-Glc+B	nq	32.8 (0.1)
2	K-3-Soph-7-Glc	nq	nq
3+4	Q-3-Gtb-7-Glc + Q-3-Soph-7-Rh	58.1 (2.8)	35.4 (1.1)
5	K-3-Soph-7-Rh	nq	nq
6+E	I-3-Soph-7-Rh +E	53.7 (3.1)	15.1 (0.6)
7	Q-3-Soph	655.6 (12.3)	603.3 (15.6)
8+9	K-3-triGlc + Q-3-Pent-Rut	nq	nq
10+11	K-3-Soph + I-3-Soph	1078.6 (46.4)	1221.2 (51.8)
12	I-3-Gtb	41.7 (1.2)	73.8 (2.5)
13	Q-3-Rut	86.4 (1.0)	84.0 (7.6)
14	K-3-Pent-Glc	nq	nq
	∑	1974.1	2065.7

[a] Results are expressed as mean (standard deviation) of three determinations. nq: not quantified. ∑: sum of the determined flavonoids.
[b] Q: quercetin; K: kaempferol; I: isorhamnetin; Soph: sophoroside (glucosyl(1→2)glucoside); Gtb: gentiobioside (glucosyl(1→6)glucoside); Rut: rutinoside (rhamnosy(1→6)glucoside); Glc: glucoside; Rh: rhamnoside; Pent: pentoside.

1.1.3. AChE inhibitory activity

The effect of tomato seeds aqueous extracts on AChE activity was assessed for the first time. Tomato seeds exhibited a concentration-dependent inhibitory capacity (**Figure 19**). This activity can be partially explained by the presence of flavonoids, especially quercetin derivatives, which have been described as AChE inhibitors (279).

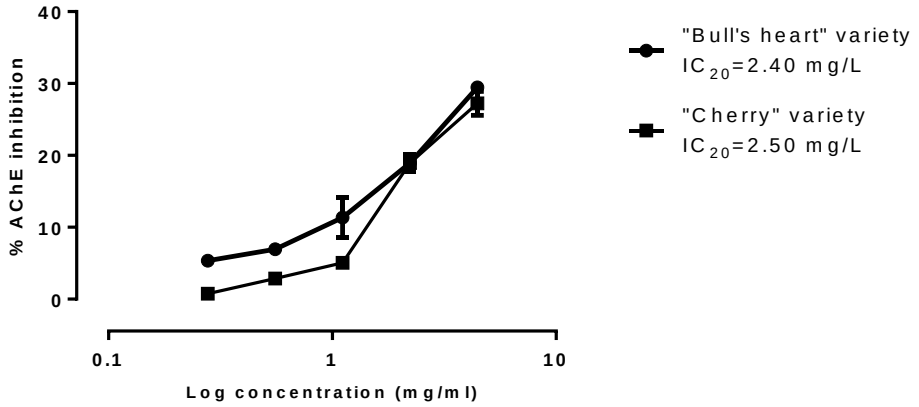


Figure 19. AChE inhibitory effect of tomato seeds aqueous extract. Values show mean ± SE from three experiments performed in triplicate.

1.1.4. Antioxidant capacity

The DPPH• assay provides basic information on the antiradical activity of extracts. In this assay, tomato seeds exhibited a concentration-dependent antioxidant potential (**Figure 20**).

Tomato seeds also provided protection against •NO, in a concentration-dependent way. The same was observed against superoxide anion, being particularly effective against this reactive species (**Figure 20**). The scavenging of these two radicals can be of major importance due to their role in the formation of other reactive species, such as peroxynitrite, which can be extremely deleterious to cells (280).

In addition, comparing both varieties, “bull’s heart” one seemed to be more active, possibly due to its higher content of phenolic compounds (**Table 7** and **Figure 20**). In fact, the total antioxidant activity of extracts results from the interaction between their several constituents, which may include synergistic or additive effects. Phenolic compounds like those present in the extract, namely quercetin, kaempferol and isorhamnetin glycosides, were already described as having antioxidant proprieties in several systems (281, 282).

The antioxidant potential exhibited by seeds can be explained by the need to protect their storage lipids from oxidation and to ensure their viability, which is especially important during germination, when oxygen demand is high (283).

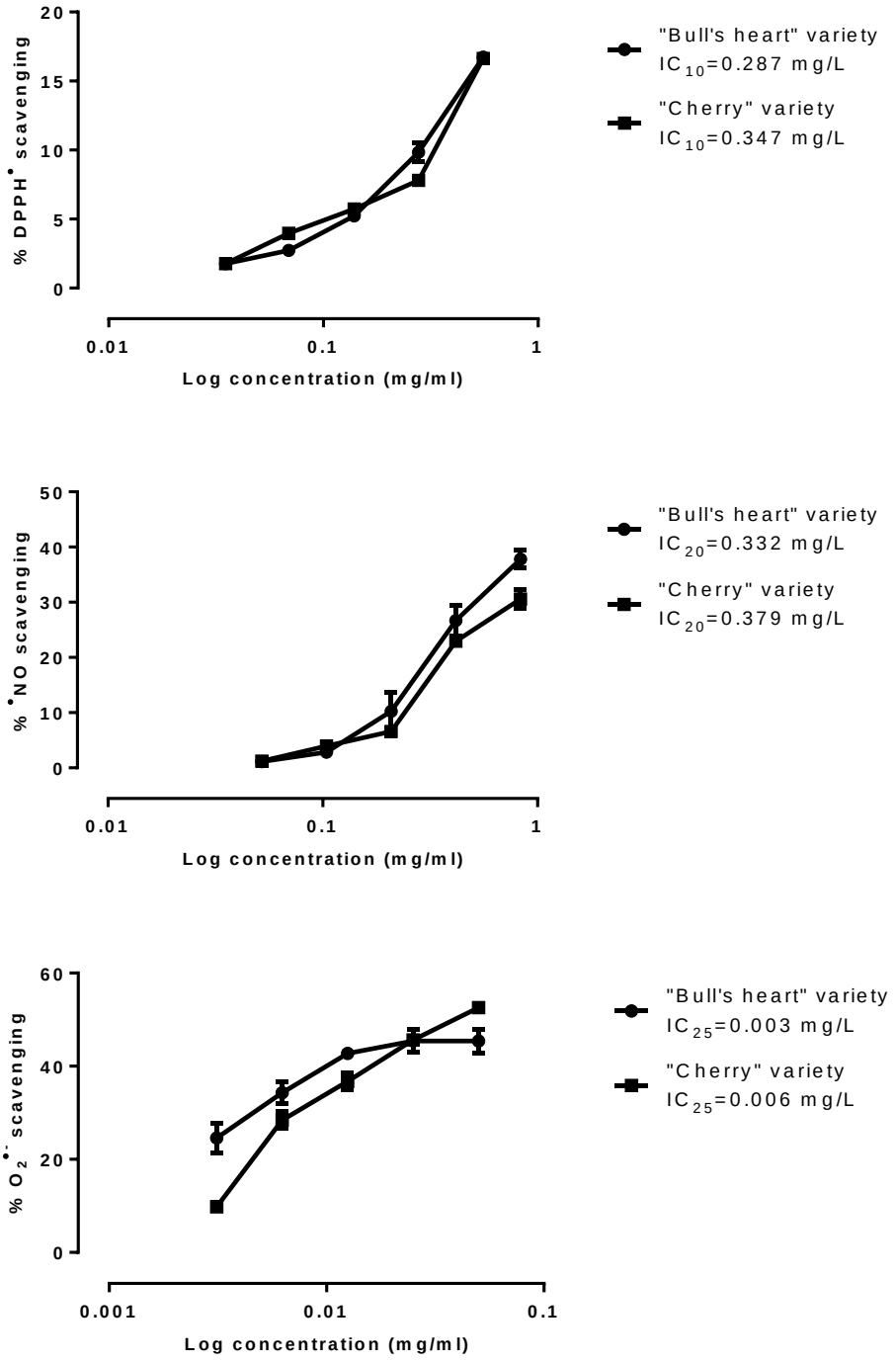


Figure 20. Effect of tomato seeds aqueous lyophilized extracts against DPPH•, •NO and $O_2^{\bullet-}$. Values show mean ± SE from three experiments performed in triplicate.

1.1.5. Cytotoxicity

Tomato seeds aqueous extract from “cherry” variety displayed a concentration-dependent effect against RBL-2H3 proliferation in the SRB assay (IC_{50} = 5.98 mg/ml). At the highest tested concentration (8 mg/ml), the inhibition reached 80%; due to solubility issues, no concentrations above this could be tested (**Figure 21**).

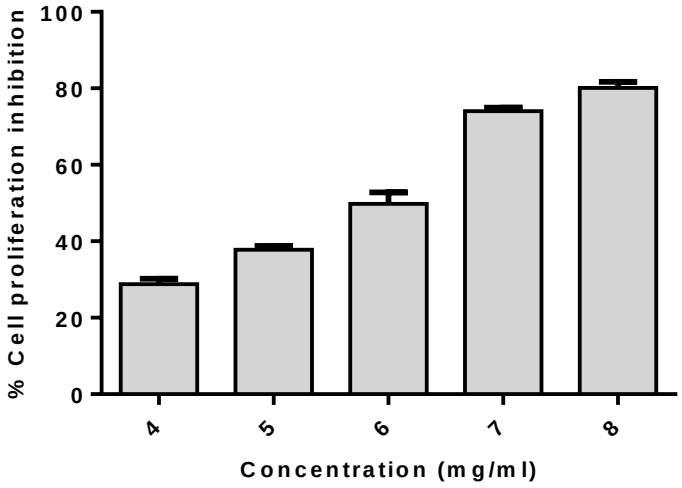


Figure 21. RBL-2H3 proliferation inhibitory capacity of the aqueous extract from tomato seeds of “cherry” variety. Values show mean ± SE from three experiments performed in triplicate.

Due to the chemical complexity of tomato seeds aqueous extract, it is not easy to establish which compounds are responsible for the displayed activity. Quercetin derivatives account for ca. 37% of the total amount of flavonoids. The aglycone has been implicated in growth inhibition of several malignant tumour cell lines *in vitro*. These include NK/Ly ascites tumour cells, gastric cancer cells (HGC-27, NUGC-2, NKN-7 and MKN-28), colon cancer cells (COLON 320 DM), human breast cancer cells, human squamous and gliosarcoma cells, ovarian cancer cells and Ehrlich ascites cells and also the cell lines L1210 and P-388, which are leukemia cells like RBL-2H3 (153).

Although several molecules and pathways have been proposed as targets of flavonoids, the precise mechanism by which these compounds exert their cancer-protective effects are still poorly understood. Most studies concerning cytotoxic effects of flavonoids use the free aglycone. However, in natural extracts the predominant compounds usually are glycosylated derivatives. It could be assumed that sterically hindered molecules, such as di and triglycosylated flavonoids present in this extract, do not manage to pass through the cell membrane in high amounts. Nevertheless, some

simpler molecules, like quercetin-3-*O*-sophoroside, kaempferol-3-*O*-sophoroside or isorhamnetin-3-*O*-sophoroside, could be able to cross the membrane. In fact, it is already described that quercetin-3-*O*-glucoside has potent antiproliferative activity against MCF-7 cells (284).

In addition to the identified metabolites, the role of the unidentified compounds **A**-**F** cannot be ignored. Moreover, the presence of the glycoalkaloids found in tomato plant (285), that could also contribute to this activity, was discarded by the general alkaloids precipitation tests, adding Dragendorff's (solution of potassium bismuth iodide), Mayer's (potassiomercuric iodide solution) and Bertrand's (silicotungstic acid solution) reagents to the extract, purified according to Friedman and co-worker (285).

1.2. Exploitation as antimicrobial agent

In the pursuit of the valorization of this by-product by finding an industrial application, and taking into account that the antioxidant capacity was the most promising activity showed by the seeds aqueous extracts, the possible utilization of this vegetal material as food preservative was further checked by assessing its antimicrobial capacity.

Several solvents were used to obtain an extract with higher antimicrobial activity. Additionally, in order to try to establish a possible relationship between this capacity and the chemical composition of the extracts, phenolic compounds, organic acids and fatty acids were determined by HPLC-DAD, HPLC-UV and GC-MS, respectively. The antibacterial and antifungal activity of both *L. esculentum* seeds extracts and the determined chemical components were evaluated by broth dilution assays.

1.2.1. Extracts chemical characterization

1.2.1.1. Phenolic compounds

The analysis by HPLC-DAD of the different *L. esculentum* seeds extracts revealed the presence of phenolic compounds only in the methanolic extracts (**Figure 22**), which were poorer in terms of diversity of compounds relatively to the aqueous extracts (**Figure 23**).

Both varieties exhibited the same profile: eight flavonoid glycosides were identified, which included quercetin, kaempferol and isorhamnetin derivatives already found in aqueous extract (**Table 8**).

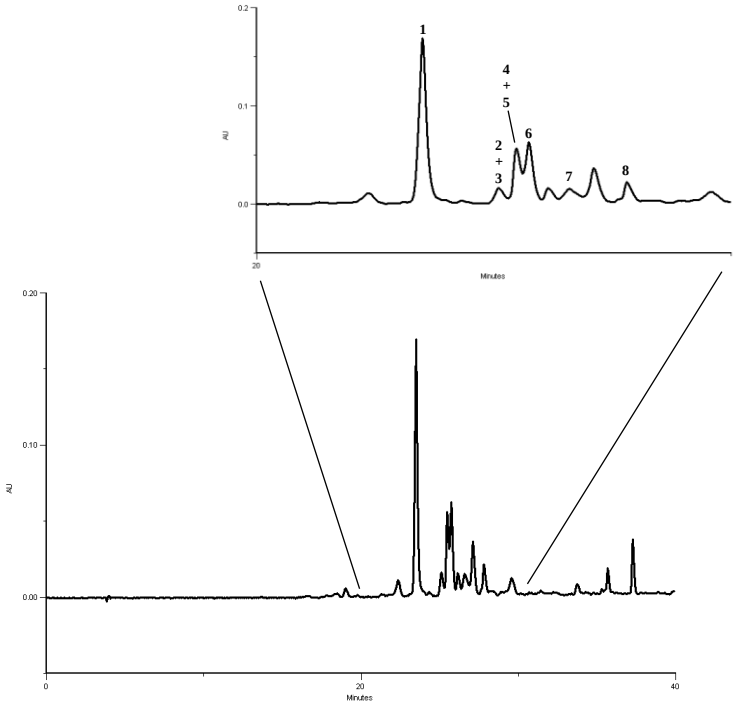


Figure 22. HPLC-DAD phenolic profile of *L. esculentum* seeds ("bull's heart" variety). Detection at 350 nm. Peaks: (**1**) quercetin-3-*O*-sophoroside; (**2**) kaempferol-3-*O*-(2-sophorosyl)glucoside; (**3**) quercetin-3-*O*-(2-pentosyl)rutinoside; (**4**) kaempferol-3-*O*-sophoroside; (**5**) isorhamnetin-3-*O*-sophoroside; (**6**) isorhamnetin-3-*O*-gentiobioside; (**7**) quercetin-3-*O*-rutinoside; (**8**) kaempferol-3-*O*-(2-pentosyl)glucoside.

As observed for the aqueous extract (**Table 7**), "bull's heart" variety exhibited a higher amount of phenolics than "cherry" variety. In both varieties quercetin-3-*O*-sophoroside (compound **1**) was the major component, representing ca. 45% of the identified compounds (**Table 8**).

Table 8. Phenolic composition of *L. esculentum* seeds methanolic extracts (mg/kg of seeds)[a].

	Compounds[b]	**"Bull's heart"**	**"Cherry"**
1	Q-3-Soph	20.30 (1.80)	14.44 (0.12)
2+3	K-3-(Soph)Glc + Q-3-pent-Rut	2.40 (0.28)	nq
4+5	K-3-Soph + I-3-Soph	6.16 (0.32)	10.76 (0.21)
6	I-3-Gtb	13.04 (0.40)	2.92 (0.23)
7	Q-3-Rut	2.88 (0.20)	0.72 (0.04)
8	K-3-Pent-Glc	5.28 (0.04)	0.44 (0.08)
	∑	50.42	29.28

[a] Results are expressed as mean (standard deviation) of three determinations. nq: not quantified. ∑: sum of the determined flavonoids.

[b] Q: quercetin; K: kaempferol; I: isorhamnetin; Soph: sophoroside (glucosyl(1→2)glucoside); Gtb: gentiobioside (glucosyl(1→6)glucoside); Rut: rutinoside (rhamnosy(1→6)glucoside); Glc: glucoside; Pent: pentoside.

Overall, seeds methanolic extracts contained less phenolic compounds, which can be related to the high degree of glycosylation of the compounds found in this vegetable material having more affinity to water (**Figure 23**).

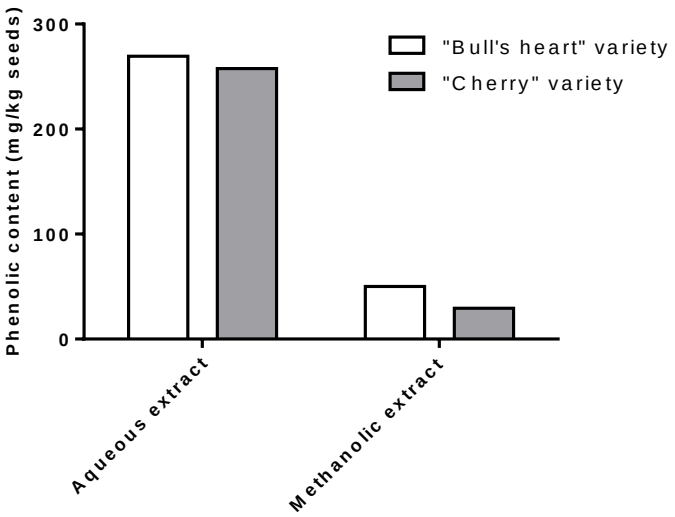


Figure 23. Phenolic content of tomato seeds extracts.

1.2.1.2. Organic acids

Organic acids were found in both methanolic and sulphuric acid tomato seeds extracts (**Figure 24** and **Table 9**).

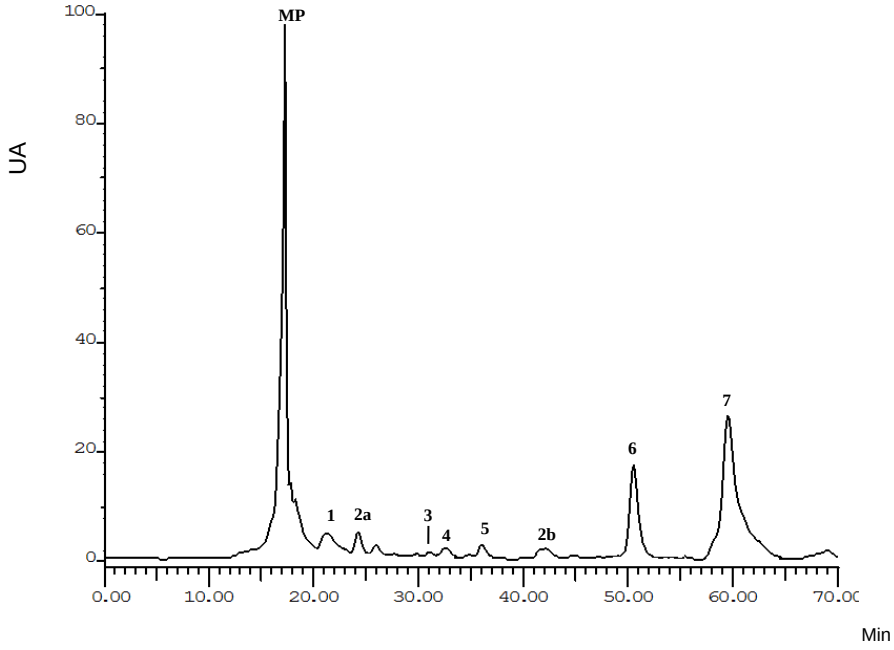


Figure 24. HPLC-UV organic acids profile of *L. esculentum* seeds ("cherry" variety) sulphuric acid extract. Detection at 214 nm. Peaks: (**MP**) Mobile phase; (**1**) oxalic acid; (**2a+b**) aconitic acid; (**3**) citric acid; (**4**) pyruvic acid; (**5**) malic acid; (**6**) acetic acid; (**7**) fumaric acid.

Sulphuric acid extracts presented aconitic, citric, pyruvic, malic, fumaric, oxalic and acetic acids (**Figure 24**). In the methanolic extracts only the last two were detected. All these compounds were already described in tomato plant (35, 193, 194).

The total organic acids content in sulphuric acid extracts was nearly twenty times higher than that found in the methanolic ones (**Table 9**). "Bull's heart" variety exhibited higher levels of these compounds. Malic acid was the main compound in sulphuric acid extracts (ca. 53%), while acetic acid predominated in the methanolic ones (ca. 73%) (**Table 9**).

Table 9. Organic acids composition of *L. esculentum* seeds sulphuric acid and methanolic extracts (mg/kg of seeds)[a].

Compounds		**"Bull's heart"**		**"Cherry"**	
		Sulphuric acid	**Methanol**	**Sulphuric acid**	**Methanol**
1	Oxalic	98.80 (0.70)	7.70 (0.60)	38.60 (0.40)	6.30 (0.90)
2	Aconitic	5.00 (0.00)	-	4.20 (0.50)	-
3	Citric	75.70 (0.10)	-	66.30 (0.20)	-
4	Pyruvic	25.80 (0.90)	-	22.80 (0.10)	-
5	Malic	234.10 (2.00)	-	262.60 (1.30)	-
6	Acetic	56.20 (0.20)	19.10 (0.00)	39.50 (0.30)	18.20 (0.20)
7	Fumaric	7.90 (0.00)	-	8.20 (0.10)	-
	∑	503.50	26.80	442.20	24.50

[a] Results are expressed as mean (standard deviation) of three determinations. ∑: sum of the determined organic acids.

1.2.1.3. Fatty acids

Fatty acids were present in chloroform, methanol, ethyl acetate and hexane extracts of the two seeds varieties (**Figure 25** and **Table 10**).

Chloroform provided the highest fatty acids amounts (**Table 10**), which is not surprising considering the low polarity of this solvent. Seven compounds were identified, distributed by saturated fatty acids (pentadecanoic, palmitic, heptadecanoic and stearic acids), monounsaturated fatty acids (palmitoleic and oleic acids) and polyunsaturated fatty acids (linoleic acid). All these compounds were already described in tomato plant (35, 208, 286-288).

Palmitic acid was the major compound in all analysed extracts, excepting in the chloroformic extract from the “cherry” variety, in which linoleic acid was present in highest amounts (**Table 10**).

The presence of glycoalkaloids in tomato seeds, which could also contribute to the antimicrobial activity (67), was also discarded in these extracts, by using the general alkaloid precipitation tests refered above for the aqueous extracts (Chapter III, item 1.1.5.).

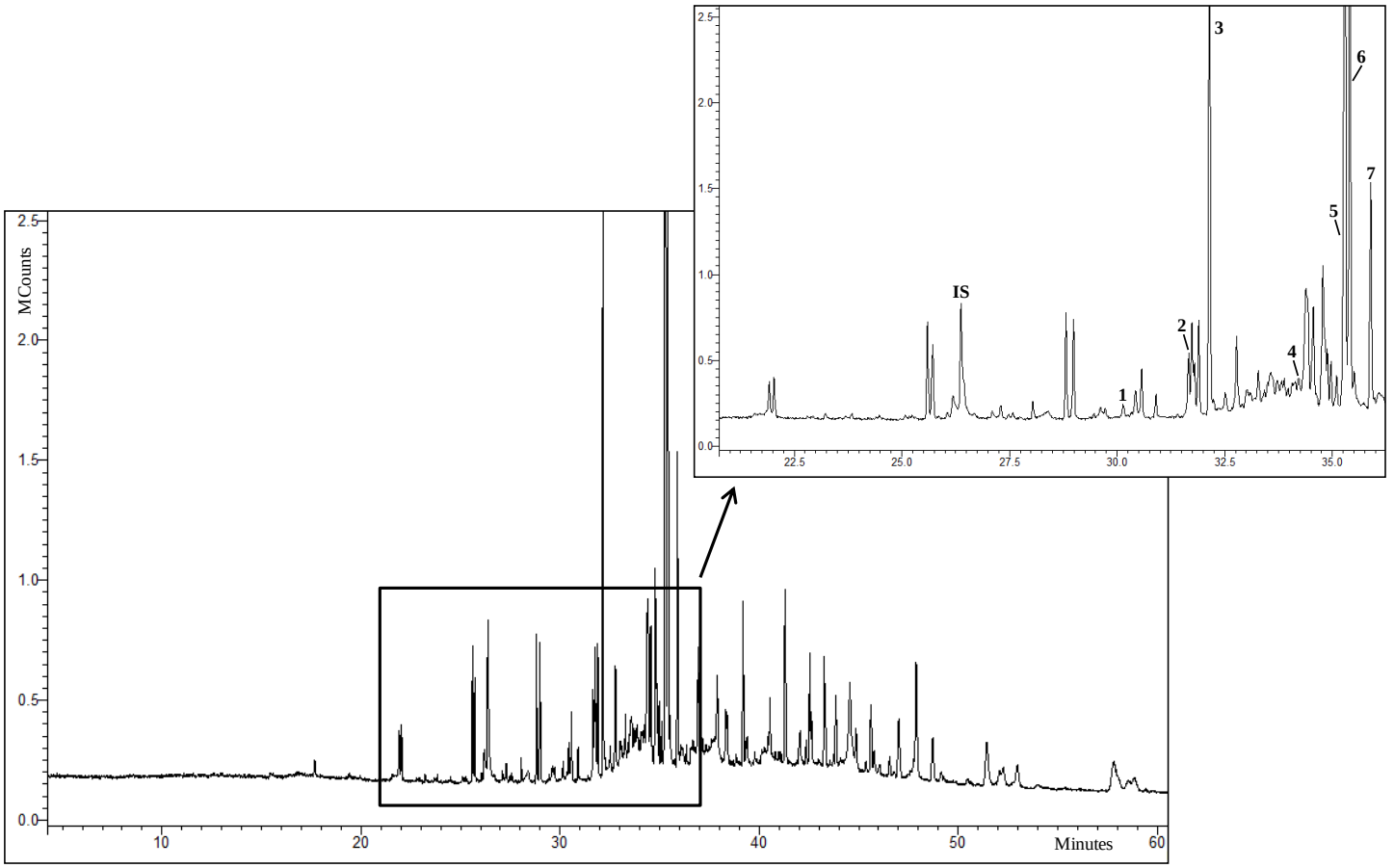


Figure 25. GC-MS fatty acids profile of chloroformic *L. esculentum* seeds extract (“bull’s heart” variety). Full scan mode. Peaks: (**IS**) Internal standard; (**1**) pentadecanoic acid; (**2**) *cis*-9-hexadecenoic acid; (**3**) hexadecanoic acid; (**4**) heptadecanoic acid; (**5**) *cis*-9,12-octadecadienoic acid; (**6**) *cis*-9-octadecenoic acid; (**7**) octadecanoic acid.

Table 10. Fatty acids composition of *L. esculentum* seeds extracts (mg/kg of seeds) [a].

	Compound			"Bull's heart"				"Cherry"			
				Chlor	**Met**	**EtAc**	**Hex**	**Chlor**	**Met**	**EtAc**	**Hex**
1	Pentadecanoic acid	C15:0		0.93 (0.17)	-	-	-	-	-	-	-
2	*cis*-9-Hexadecenoic acid (Palmitoleic acid)	C16:1		2.42 (0.16)	-	-	-	-	-	-	-
3	Hexadecanoic acid (Palmitic acid)	C16:0		12354.54 (1522.12)	1.50 (0.01)	4750.46 (99.51)	1.94 (0.07))	12334.39 (949.68)	0.73 (0.01)	2648.18 (37.38)	2.45 (0.27)
4	Heptadecanoic acid	C17:0		nq	nq	nq	nq	nq	nq	nq	nq
5	*cis*-9,12-Octadecadienoic acid (Linoleic acid)	C18:2		764.75 (29.41)	nq	0.04 (0.00)	nq	28634.22 (361.04)	0.62 (0.01)	949.28 (52.54)	nq
6	*cis*-9-Octadecenoic acid (Oleic acid)	C18:1		0.51 (0.01)	nq	-	-	-	nq	0.06 (0.00)	-
7	Octadecanoic acid (Stearic acid)	C18:0		1.10 (0.12)	0.13 (0.01)	0.25 (0.01)	-	0.24 (0.01)	0.07 (0.02)	0.05 (0.01)	-
			SFA	12356.57	1.63	4750.71	1.94	12334.63	0.80	2648.23	2.45
			MUFA	2.93	nq	-	-	-	nq	0.06	-
			PUFA	764.75	nq	0.04	nq	28634.22	0.62	949.28	nq
			∑	13124.25	1.63	4750.75	1.94	40968.85	1.42	3597.57	2.45

[a] Results are expressed as mean (standard deviation) of three determinations. nq: not quantified. ∑: sum of the determined fatty acids. SFA: saturated fatty acids. MUFA: monounsaturated fatty acids. PUFA: polyunsaturated fatty acids. Chlor: Chloroform; Met: Methanol; EtAc: Ethyl acetate; Hex: Hexane.

1.2.2. Antimicrobial potential

In order to evaluate the antimicrobial potential of *L. esculentum* seeds, the chloroform, methanol, ethyl acetate, hexane and sulphuric acid extracts were screened against Gram-positive and Gram-negative bacteria and fungi. Additionally, in order to try to establish a relationship with the chemical composition, the effect of all identified organic acids and of quercetin-3-*O*-rutinoside was also assessed. Quercetin-3-*O*-rutinoside was the only phenolic compound tested because it was the only one commercially available among the identified phenolics. Fatty acids standards were not assayed because they are insoluble in water and, consequently, in both RPMI and MHB media.

1.2.2.1. Antibacterial activity

Concerning the effect against Gram-positive bacteria, it was possible to determine some MIC values, which are indicative of the antimicrobial potential of tomato seeds (**Table 11**). Regarding the different solvents used, we observed that hexane, sulphuric acid and ethyl acetate seemed to be the most promising (**Table 11**), although none of the tested extracts could be clearly highlighted.

Comparing the extracts of “bull’s heart” and “cherry” varieties, no relevant differences were found, *E. faecalis* being the most susceptible species (**Table 11**). *E. faecalis*, present in the gastrointestinal tract of humans, can cause several infections, especially in nosocomial environments (213). The hexane extract from “cherry” variety and the ethyl acetate extract from both varieties were the most active against this bacterial species (MIC = 2.5 mg/ml) (**Table 11**).

The “bull’s heart” ethyl acetate extract and the hexane extracts from both varieties were the most active against *S. aureus* (**Table 11**), which constitutes an important pathogenic agent in both nosocomial and community environments, being also frequently involved in cases of food poisoning (289).

Table 11. MIC values (mg/ml) obtained with *L. esculentum* seeds extracts against selected bacteria[a].

Bacteria	"Bull's heart"					"Cherry"					Ciprofloxacin (µg/ml)
	Chlor	Met	EtAc	Hex	Sulph	Chlor	Met	EtAc	Hex	Sulph	
Gram-positive											
S. aureus	20	20	5	5	> 20	20	> 20	10	5	20	1.25
S. epidermidis	> 20	> 20	> 20	10	10	20	> 20	10	10	> 20	0.6
M. luteus	> 20	> 20	> 20	20	10	20	20	>20	10	5	1.25
E. faecalis	10	5	2.5	5	10	5	10	2.5	2.5	5	5
B. cereus	20	20	20	20	10	10	>10	20	> 20	5	10
Gram-negative											
P. mirabillis	> 20	> 20	> 20	> 20	> 20	> 20	> 20	> 20	> 20	> 20	1.25
E. coli	> 20	> 20	> 20	> 20	> 20	> 20	> 20	> 20	> 20	> 20	1.25
P. aeruginosa	> 20	> 20	> 20	> 20	> 20	> 20	> 20	> 20	> 20	> 20	2.5
S. typhimurium	> 20	> 20	> 20	> 20	> 20	> 20	> 20	> 20	> 20	> 20	0.6

[a] Chlor: chloroform; Met: methanol; EtAc: ethyl acetate; Hex: hexane; Sulph: sulphuric acid.

Considering the protection against *S. epidermidis*, “bull’s heart” sulphuric acid, “cherry” ethyl acetate and hexane extracts from both varieties were the most effective (MIC = 10 mg/ml) (**Table 11**). Sulphuric acid extract from “cherry” variety was the most active against *M. luteus* and *B. cereus* (**Table 11**). *S. epidermidis* and *M. luteus* are important pathogens in immunocompromised patients (290). *B. cereus* is responsible for food borne illness (291, 292).

Under the tested concentrations all Gram-negative bacteria were found to be resistant to the different extracts (**Table 11**). Previous studies indicated that Gram-negative bacteria appear to be less sensitive to the action of many natural extracts (293), which is in agreement with our results. These results can probably be related with the physical differences between the cell walls of Gram-positive and Gram-negative bacteria. The presence of the external membrane with high content of lipopolysaccharide surrounding the cell wall in Gram-negative bacteria is considered to be the major contributor to its ability to resist to chemical stress (294, 295).

To check whether the observed antibacterial activity could be due to the presence of the identified organic acids and phenolics, the same assays were performed with the available standards. All organic acids presented antibacterial activity at the tested concentrations, with the exception of pyruvic acid. Fumaric acid was generally the most active compound against all tested bacteria (**Table 12**). Gram-negative bacteria were more resistant to organic acids than Gram-positive bacteria (**Table 12**), which agrees with the results obtained with the seeds extracts. Thus, the activity found for tomato seeds can, in part, be due to the presence of this class of compounds.

Organic acids have commonly been used as food preservatives and have been reported to exhibit general antimicrobial activities. Wu et al. (296) demonstrated that the antimicrobial effect of organic acids is linked to pH: they affect bacteria by lowering both environmental and intracellular pH (297).

To check if the antibacterial effect of organic acids was correlated with their capacity to decrease the medium pH, three different solutions with pH 2.2, 3.0 and 4.0 were tested. The smaller value corresponds to that of the organic acids solution with the lowest pH. All bacteria were able to grow only at pH 4.0 (data not shown), which confirms that the antibacterial activity may be linked to the decrease of pH values.

Table 12. MIC values (mg/ml) obtained with organic acids and quercetin-3-*O*-rutinoside against selected bacteria and fungi[a].

Microorganism	Organic acids							Quercetin-3-*O*-rutinoside (µg/ml)
	Pyruvic	Aconitic	Citric	Malic	Acetic	Oxalic	Fumaric	
Gram-positive Bacteria								
S. aureus	> 10	1.25	1.25	1.25	1.25	1.25	0.625	>69
S. epidermidis	> 10	1.25	1.25	1.25	1.25	1.25	0.625	69
M. luteus	> 10	1.25	1.25	1.25	1.25	1.25	1.25	69
E. faecalis	> 10	1.25	1.25	1.25	1.25	1.25	1.25	69
B. cereus	> 10	1.25	1.25	1.25	1.25	1.25	0.625	69
Gram-negative Bacteria								
P. mirabillis	> 10	1.25	2.5	1.25	1.25	1.25	1.25	>69
E. coli	> 10	2.5	2.5	2.5	1.25	1.25	2.5	69
P. aeruginosa	> 10	1.25	1.25	1.25	1.25	1.25	1.25	>69
S. typhimurium	> 10	2.5	2.5	2.5	1.25	2.5	2.5	69
Fungi								
C. albicans	>20	>10	>20	>20	10	10	>2.5	>69
A. fumigatus	>20	>10	>20	>20	10	10	>2.5	>69
T. rubrum	>20	>10	20	20	5	10	>2.5	>69

[a] MIC (µg/ml) for fluconazole was 0.25 (*C. albicans* ATCC 10231), for voriconazole 0.25 (*A. fumigatus* ATCC 46645) and 0.03 (*T. rubrum* CECT 2794). MICs for ciprofloxacin against bacteria were similar to those shown in Table 11.

Quercetin-3-*O*-rutinoside was also tested, presenting activity against all bacteria, excepting *S. aureus*, *P. mirabilis* and *P. aeruginosa* (**Table 12**). Phenolics in seeds are related to their defence against predators and pathogens, including bacteria and fungi (278). Since they are known to be synthesized by plants in response to microbial infection (298), it is not surprising that they have been found to be effective antimicrobial substances against a wide array of microorganisms *in vitro*. The antibacterial activity of quercetin-3-*O*-rutinoside was already noticed in previous works (299). Additionally, other extracts, also characterized by the presence of quercetin, kaempferol and isorhamnetin glycosides, have been shown to possess antimicrobial capacity (300).

Thus, phenolic compounds also seem to give some contribution to the antibacterial properties of tomato seeds. The antimicrobial activity of phenolic compounds can be concentration dependent. At low concentration they affect enzyme activity, particularly those associated with energy production, while at high concentration they cause protein denaturation (295).

Phenolic compounds may also alter the microbial cell permeability, thereby allowing the loss of macromolecules from the interior, interfering with membrane function and interacting with membrane proteins, causing deformation of structure and functionality (295). All these processes are pH-independent (300).

Also fatty acids may participate in the observed effects, as their bactericidal potential is well known (301). Fatty acids and derivatives are most effective against Gram-positive bacteria. Gram-negative organisms are generally not affected, since they are less sensitive to lipophilic agents (302). This natural resistance can be due to the complexity of their cell walls (302). The mechanism of bactericidal action of long chain fatty acids and derivatives is ascribed to a balance between the hydrophilic and hydrophobic parts of the molecule. Regardless of the polarity of the hydrophilic portion, optimum chain length is between C_{12} and C_{16} (302). In addition, palmitoleic, palmitic, linoleic, oleic and stearic acids, present in the tested extracts, are known for their antibacterial activity (301).

1.2.2.2. Antifungal activity

Tomato seeds extracts displayed antifungal properties, although they seemed to be less effective in comparison to Gram-positive bacteria. In a general way, the extracts from "bull's heart" variety were more active (**Table 13**). Under the tested concentrations *A. fumigatus* was resistant to the action of all extracts (**Table 13**).

Candida species, especially *C. albicans*, is a causal agent of opportunistic oral and genital infections in humans. Candidoses have emerged as important causes of morbidity and mortality in immunocompromised patients (303). All extracts from "bull's heart" variety were active against *C. albicans*, excepting the ethyl acetate one. The chloroformic extract was the most active (**Table 13**).

T. rubrum is the commonest causative agent of dermatophytoses worldwide and inhabits soil, humans or animals (302). The methanolic extract from "bull's heart" variety revealed to be the most active against this fungal species (**Table 13**).

Thus, tomato seeds exhibited inhibitory activity against some microorganisms implicated in the pathogenesis of skin diseases, such as *C. albicans* and *T. rubrum*, providing some scientific basis for the possible treatment of skin pathologies.

In addition, quercetin-3-*O*-rutinoside displayed no activity against *C. albicans*, *A. fumigatus* or *T. rubrum* at the tested concentrations (**Table 12**). Regarding the antifungal activity of organic acids, best results were obtained for acetic and oxalic acids (**Table 12**). Although, as referred above, pH is an important factor for antibacterial activity, fungi are more resistant to pH variation, being able to grow under lower values. To verify the effect of pH in fungi growth, solutions with different pH values were also assayed, as indicated above for bacteria. All fungi were able to grow at pH 2.2 (data not shown). These results seemed to indicate that the antifungal activity was not due solely to low pH, but also to the specific organic acid molecule. Antifungal activity of fatty acids has already been reported, being related with their lipophilicity, as it happened against bacteria (301).

Table 13. MIC values (mg/ml) obtained with *L. esculentum* seeds extracts against selected fungi[a].

Microorganism	"Bull's heart"					"Cherry"				
	Chlor	**Met**	**EtAc**	**Hex**	**Sulph**	**Chlor**	**Met**	**EtAc**	**Hex**	**Sulph**
C. albicans	5	5-10	>20	10	5-10	>20	>20	10	>20	>20
A. fumigatus	>20	>20	>20	>20	> 20	>20	> 20	>20	>20	>20
T. rubrum	10	5-10	> 20	20	20	>20	> 20	10	>20	>20

[a] Chlor: chloroform; Met: methanol; EtAc: ethyl acetate; Hex: hexane; Sulph: sulphuric acid. MIC (µg/ml) for fluconazole was 0.25 (*C. albicans* ATCC 10231), for voriconazole 0.25 (*A. fumigatus* ATCC 46645) and 0.03 (*T. rubrum* CECT 2794).

Although organic acids and quercetin-3-*O*-rutinoside alone have been shown to be active, synergic and/or antagonic interactions between these and other components were not evaluated and cannot be excluded. In fact, the precise mechanism of antimicrobial action of the extracts is difficult to elucidate, due to complex interactions between the different constituents (304). As observed with other matrices, the inherent activity of an extract can also be expected to relate with the chemical configuration of its components and the proportion in which they are present (305). Other extractive methods, such as supercritical fluid extraction or sequential extraction, could be used in order to accumulate more active components and improve the antibacterial and antifungal activity.

2. *S. littoralis*/*L. esculentum* system chemical interaction

As far as we are aware, there are no studies involving sequestration and metabolism of phenolic compounds and steroidal alkaloids by *S. littoralis*. Attending to the importance of these compounds in the interactions occurring within an ecological duo and their biological potential, an integrated approach, consisting of HPLC-DAD-ESI-MS^n analysis, was developed to assess their profiles' evolution through the life cycle of *S. littoralis* (larvae, adults, exuviae and excrements) reared on *L. esculentum* leaves. Comparions were made with the host plant.

2.1. HPLC-DAD-ESI-MS^n phenolic compounds analysis

The HPLC-DAD-ESI-MS^n screening of the host *L. esculentum* leaves hydrometanolic extract (Chapter II, item 3.2.1.), with detection at 335 nm (**Figure 26**) and MS^n in negative ionisation mode, revealed the presence of several phenolic compounds (**Figure 26** and **Table 14**).

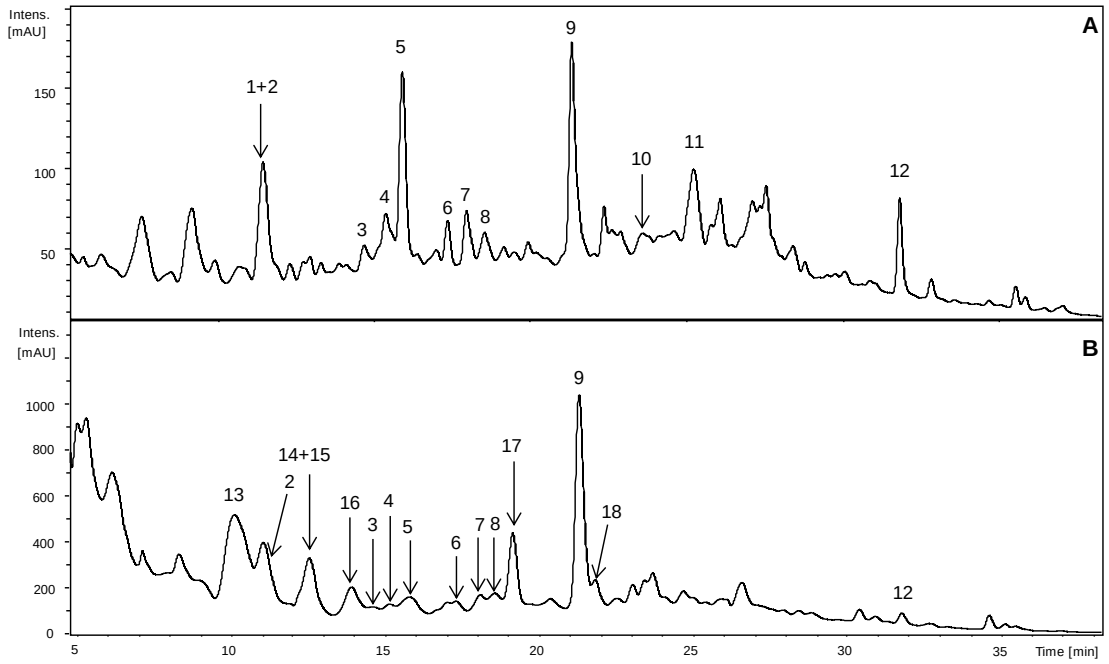


Figure 26. HPLC phenolic profile of *L. esculentum* leaves from "cherry" (**A**) and "bull's heart" (**B**) varieties. Detection at 335 nm. Identity of compounds as in Table 14.

In “cherry” variety (**Figure 26**) we found quercetin-3-*O*-rutinoside (**9**), which was further confirmed by comparison with the authentic standard. Compound **10** eluted 2.3 min later and was present in trace amounts. Its MS spectrum pointed to a tetrahydroxyflavone with the same substitution pattern of compound **9**, being tentatively assigned as kaempferol-3-*O*-rutinoside (**10**).

As for the remaining compounds, some of them were present in trace amounts or coeluting with others. Thus, their UV spectra were not properly observed (**1, 2** and **4**). Compound **1** presented a deprotonated molecular ion at m/z 341 and its MS^2 fragmentation originated an ion at m/z 179 by loss of 162 u. This compound was tentatively identified as caffeoyl-hexoside acid. In fact, this compound may correspond to caffeic acid-4-*O*-β-D-glucoside reported before in *L. esculentum* leaves (306). Compound **1** coeluted with another one, differing in 16 u and exhibiting a similar fragmentation (**Table 14**).

Thus, we believe that compound **2** corresponds to *p*-coumaroyl-hexoside acid (**2**). Another compound with a similar fragmentation pattern, that is, loss of 162 u to obtain an ion at m/z 223 $[sinapic\text{-}H]^-$, was characterized as sinapoyl-hexoside acid (**4**).

In the MS fragmentation of compounds **3**, **5** and **6** it was observed a base peak corresponding to the deprotonated molecular ion of quinic acid (m/z 191). The UV spectra of compounds **5** and **6** corresponded to feruloyl and *p*-coumaroyl derivatives, respectively (**Table 15**). Compounds **3** and **6** could be identified as *p*-coumaroylquinic acid isomers and compound **5** as 5-*O*-feruloylquinic acid (**Figure 26**) (143). Compound **8** exhibited a deprotonated molecular ion at m/z 367, as it happened with compound **5**. However, although its MS^2 fragmentation also showed the ion at m/z 191 as base peak, its MS and UV spectra were different from those of feruloylquinic acid (**Table 14**).

In the MS^2 fragmentation of compound **7** it could be noticed the loss of 44 u (CO_2, carboxyl). For compound **11** the base peak corresponded to the ion at m/z 191 (deprotonated quinic acid) (**Table 14**). However, the obtained data did not allowed suggesting any structure. The same happened regarding compound **12**.

Table 14. *R*t, UV, MS: $[M-H]^-$ and - MS^2 $[M-H]^-$ data of phenolic compounds from host *L. esculentum* leaves[a].

	Compound	*R*t (min)	UV max (nm)	$[M-H]^-$, m/z	$MS^2[M-H]^-$, m/z (%)
13	3-*O*-Caffeoylquinic acid	10.1	296sh, 324	353	191(100), 179(5)
1	Caffeoyl-hexoside acid	11.5	**a**[b]	341	179(100)
2	*p*-Coumaroyl-hexoside acid	11.6	**a**[b]	325	163(100)
14	Feruloyl-hexoside acid	12.5	294sh, 324[c]	355	193(100)
15	Sinapoyl-hexoside acid isomer	12.7	294sh, 324[c]	385	223(100)
16	5-*O*-Caffeoylquinic acid	13.9	**a**[b]	353	191(100), 179(3)
3	*p*-Coumaroylquinic acid isomer	14.7	**a**[b]	337	191(100)
4	Sinapoyl-hexoside acid	15.4	**a**[b]	385	223(100)
5	5-*O*-Feruloylquinic acid	15.9	298sh, 324	367	191(100)
6	*p*-Coumaroylquinic acid isomer	17.3	286sh, 310	337	191(100), 163(7)
7	Unknown	18.0	268, 344sh	471	427(100)
8	Feruloylquinic acid isomer	18.5	284sh, 310	367	365(60), 191(100)
17	Quercetin-3-*O*-pentosyl-rutinoside	19.1	255, 266sh 298sh, 350	741	723(11), 609(72), 591(30), 300(100)
9	Quercetin-3-*O*-rutinoside	21.3	256, 266sh 295sh, 352	609	301(100)
18	Sinapoyl derivative	21.8	**a**[b]	629	223(100)
10	Kaempferol-3-*O*-rutinoside	23.6	**a**[b]	593	285(100)
11	Unknown	25.2	294, 328sh	499	481(20), 467(30), 191(100)
12	Unknown	31.8	328	327	309(60), 201(15), 171(70)

[a] Main observed fragments. Other ions were found but they have not been included.
[b] **a**: Compounds hidden by others or in traces. Their UV spectra have not been properly observed.
[c] UV spectra of **14**+**15**.

The HPLC-DAD-ESI-MS^n screening of *L. esculentum* leaves from "bull's heart" varieties revealed a composition different from that of the "cherry" one (**Figure 26B**). Compounds not observed in this last were found (**Figure 26B**, **Table 14**): feruloyl-hexoside acid (**14**), sinapoyl-hexoside acid isomer (**15**) and two caffeoylquinic acids (**13** and **16**). According to their retention time, these last may correspond to 3- and 5-*O*-caffeoylquinic acids, respectively, although in the MS^2 fragmentation of the 3- isomer the ion at m/z 179 did not showed a great abundance, as Clifford et al. described (143). These caffeoylquinic acids were already identified in *L esculentum* leaves (38). A sinapoyl derivative (**18**) was also found, which MS fragmentation was characterized by the presence of an ion at m/z 223.

In addition, a quercetin-3-*O*-derivative (**17**) was noticed. This compound presented a deprotonated molecular ion at m/z 741. Its MS^2 spectrum exhibited an ion at m/z 609, produced by the loss of a pentosyl residue ([(M-H)-132]$^-$) (**Figure 27**). Thus, it was considered as a pentosyl derivative of quercetin-3-*O*-rutinoside (**9**). The presence of the ion at m/z 300 (corresponding to the deprotonated aglycone [quercetin-2H]$^-$) as base peak indicated that only one phenolic hydroxyl was glycosylated (272). An ion at m/z 591 ([(M-H)-150]$^-$) was also observed.

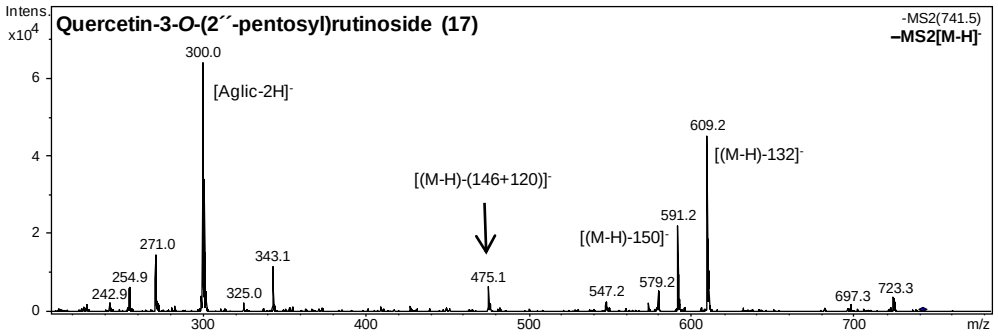
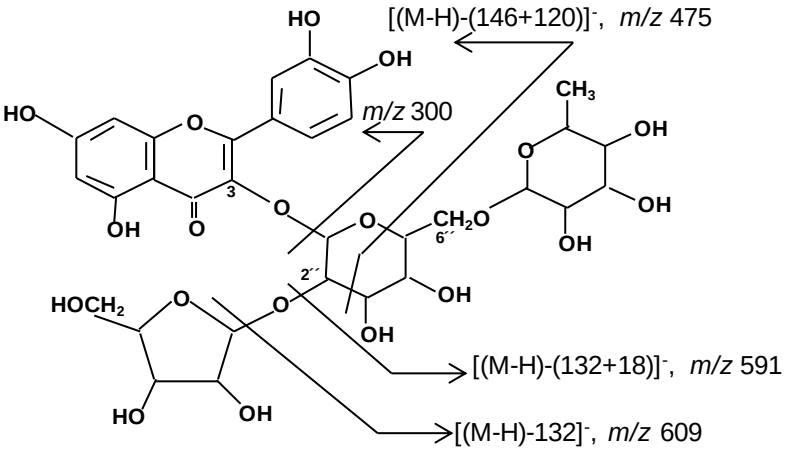


Figure 27. MS^2 [M-H]$^-$ of quercetin-3-*O*-(2´´-pentosyl)rutinoside (**17**) (negative ionisation mode).

The loss of sugar plus water (132+18=150), characteristic of a linkage between a sugar and a non-phenolic hydroxyl, probably at the 2 position (307), indicated that the pentose was linked to the rutinosyl residue. Furthermore, the loss of the fragment of 266 u (146+120), resulting from the internal cleavage of glucose and containing the rhamnosyl residue (**Figure 28**), to originate the ion at m/z 475 ([(M-H)-(146+120)]$^-$) confirmed that the pentose was not linked to the hydroxyl groups at positions 3 or 4 of glucose, nor on rhamnose.

According to the above mentioned this compound was tentatively characterized as quercetin-3-*O*-(2´´-pentosyl)rutinoside (**17**) and may correspond to quercetin-3-*O*-(2´´-*O*-*β*-apiofuranosyl-6´´-*O*-α-rhamnopyranosyl)-*β*-glucopyranoside, reported before in *L. esculentum* fruits (148).



[M-H]$^-$, *m/z* 741 (**17**)

Figure 28. MS fragmentation pathway of quercetin-3-*O*-(2´´-pentosyl)rutinoside (**17**).

On the other hand, caffeoyl-hexoside acid (**1**), kaempferol-3-*O*-rutinoside (**10**) and the possible quinic acid derivative eluting at 25.2 min (**11**) were not found in “bull’s heart” variety. In addition, *p*-coumaroyl-hexoside acid (**2**), sinapoyl-hexoside acid (**4**), the hydroxycinnamoylquinic acids, namely *p*-coumaroylquinic acid isomers (**3** and **6**), 5-*O*-feruloylquinic acid (**5**) and the tentatively assigned feruloylquinic acid isomer (**8**), as well as quercetin-3-*O*-rutinoside (**9**) and compounds **7** and **12**, were common to both varieties.

None of compounds **2-6**, **8**, **15**, **17** and **18** were described before in *L. esculentum* leaves. In addition, none of the free phenolic acids previously described in this matrix, namely *p*-hydroxybenzoic, protocatechuic, vanillic, *cis*-*p*-coumaric, *trans*-*p*-coumaric, caffeic, sinapic and *trans*-ferulic acids (172), was found.

Concerning the materials corresponding to the several stages of *S. littoralis* life cycle, they were analysed under the conditions used for *L. esculentum* host plant. The results revealed that phenolic compounds were absent in those samples. In addition, MS was also used to check for the presence of glucuronate and/or sulphate derivatives, by the extraction of the ions exhibiting losses of 176 or 80 u, respectively ("constant neutral loss chromatogram"). These compounds often occur as consequence of metabolic processes,

as already reported for other Lepidoptera (273, 276), leading to more polar compounds that are excreted. However, no such derivative was observed.

The absence of phenolic compounds in *S. littoralis* materials may be explained by the presence of polyphenol oxidases (PPO) in *L. esculentum* leaves. In most plant species PPO and phenolic compounds are stored in different compartments (PPO are found in plastids and phenolics in the vacuole). They come into contact only after cellular rupture caused by wounding, senescence, or attack by insects or pathogens (308, 309). After decompartmentalization, a large amount of quinones is readily generated. Quinones are highly reactive intermediates that rapidly polymerize. At high pH, nucleophilic Michael addition reactions of quinones and the covalent addition of quinines to cellular nucleophiles may occur (295), turning the detection of phenolic compounds impossible.

For instance, 5-*O*-caffeoylquinic acid is a substrate for these enzymes, being rapidly converted to chlorogenoquinone, a highly reactive molecule that is known to covalently bind to nucleophilic -NH_2 and -SH groups of molecules like amino acids and proteins, present in the gut of several larvae, such as *Helicoverpa zea* (Boddie) and *Spodoptera exigua* (Hubner). Additionally, quinones can also lead to the production of superoxide radical, which is toxic to the larvae of several species of Lepidoptera (43).

Thus, the alkaline pH conditions of the midguts of many Lepidoptera, like *S. littoralis* (297), may favour the establishment of covalent bonds and cross-linking between quinones and amino acids/proteins, rendering them unusable by the digestive and absorption systems. This is considered to be a mechanism of defence of the insect against phenolics toxicity (278), precluding the detection of these compounds.

2.2. HPLC-DAD-ESI-MS^n steroidal alkaloids analysis

For the HPLC-DAD-ESI-MS^n screening of steroidal alkaloids in *L. esculentum* leaves the same hydromethanolic extracts were used. Detection was performed at 202 and 205 nm and MS positive ionisation mode was applied (**Figure 29**).

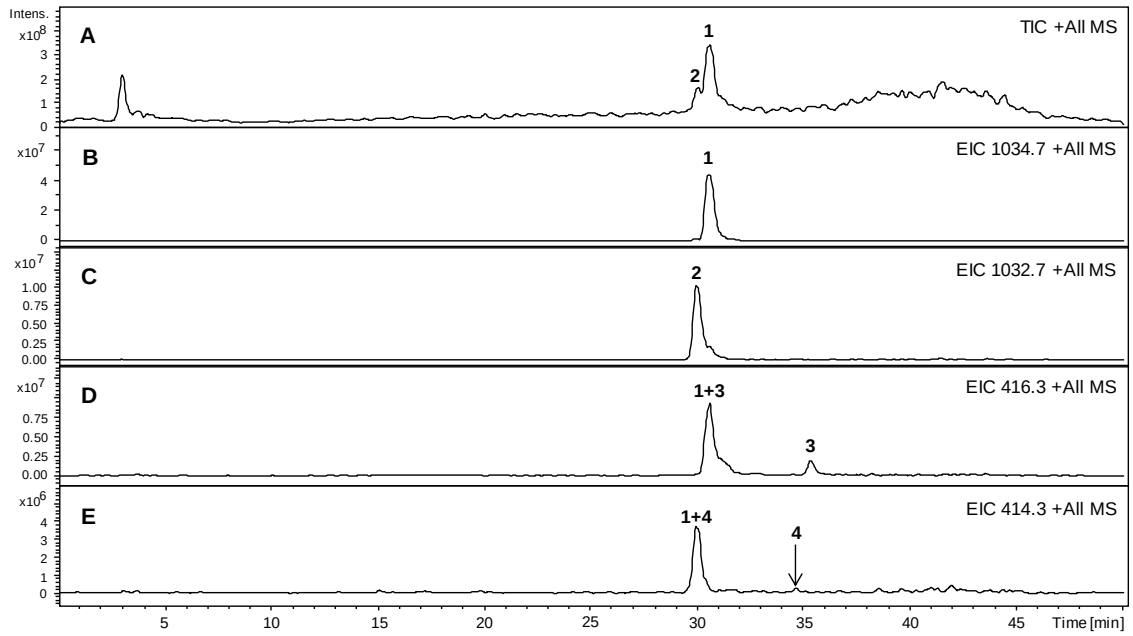


Figure 29. HPLC-MS chromatogram (positive ionisation mode) of the hydromethanolic extract of *L. esculentum* leaves from "cherry" variety. (**A**) Total Ion Chromatogram; (**B**) Extraction Ion Chromatogram (EIC) m/z 1034.7; (**C**) EIC m/z 1032.7; (**D**) EIC m/z 416.3; (**E**) EIC m/z 414.3. In **D** and **E** compounds **3** and **4** were noticed at *R*t 30.5 min and 29.9 min, close to **1** and **2,** respectively, produced as artefacts from **1** and **2**, respectively, in the process of transfer of ions. Peaks: (**1**) α-tomatine; (**2**) dehydrotomatine; (**3**) tomatidine; (**4**) tomatidenol.

The UV chromatograms were not well resolved and the UV spectra could not be properly observed. As so, compounds' characterization was based on their chromatographic behaviour, MS fragmentation in ESI conditions and on the comparison with authentic standards of α-tomatine and tomatidine (as well as with dehydrotomatine and tomatidenol, which, as referred before, were also present in the commercial α-tomatine and tomatidine standards, respectively), using the Extraction Ion Chromatogram (EIC). The Total Ion Chromatogram (TIC) of the hydromethanolic extract of *L. esculentum* leaves from "cherry" variety showed a large peak in the area in which α-tomatine and dehydrotomatine eluted (30.5 and 29.9 min) (**Figure 29A**). The EIC of the ions at m/z 1034.7 and 1032.7 (α-tomatine (**1**) and dehydrotomatine (**2**), respectively) (**Figures 29**), as well as that of their aglycones, tomatidine (**3**) (m/z 416.3, *R*t 34.5 min) (**Figures 29**) and tomatidenol (**4**) (m/z 414.3, *R*t 33.7 min) (**Figures 29**), indicated that these compounds eluted at the same retention time as the reference standards.

Their MS^2 fragmentations also agreed with them (**Figure 30** and **Table 15**) and with data from other authors (89, 310), evidencing their presence in *L. esculentum* leaves. Besides these, other ions at m/z 416.3 and 414.3 were also observed in the zone in which steroidal alkaloids **1** and **2** eluted (**Figures 29**). Their MS fragmentations were consistent with those of the aglycones tomatidine and tomatidenol. Nevertheless, they corresponded to artefacts produced during the ion transfer process from α-tomatine and dehydrotomatine, respectively (ions formed by spontaneous fragmentation at the ESI source set at 300 ºC).

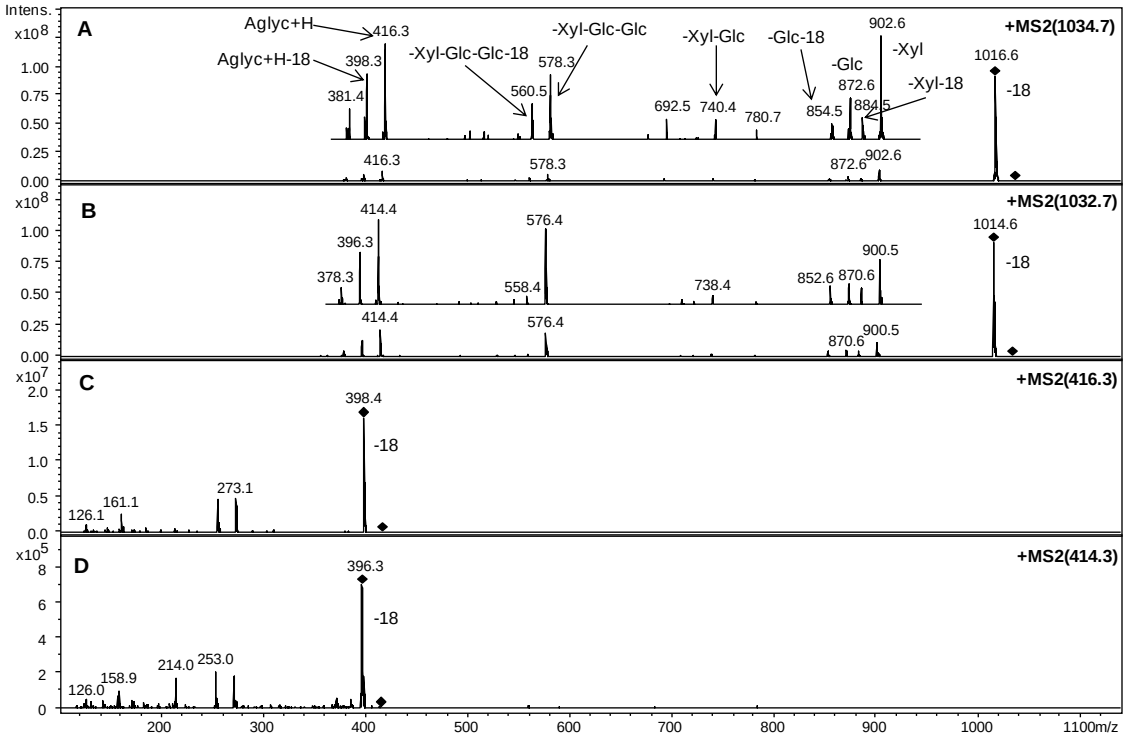


Figure 30. The produced ion spectra (positive ionisation mode) of (**A**) α-tomatine, (**B**) dehydrotomatine, (**C**) tomatidine and (**D**) tomatidenol.

The MS^2 fragmentation of α-tomatine and dehydrotomatine (**Figures 30** and **31**) was similar (they differed only in 2 u) and, besides the base peak resulting from the loss of water from the protonated molecular ion ($[(M + H) -18]^+$), there could be observed several ions arising from the loss of sugars (-Xyl: -132; -Glc: -162; -Xyl-Glc: -294; -Xyl-Glc-Glc: -456 u), as well as others resulting from the loss of sugar plus water (-150, -180, -474) and the protonated ion of the aglycone (**Table 15**).

Table 15. *R*t and MS data ([M+H]$^+$, MS^2 [M+H]$^+$) of alkaloids from hydromethanolic extract of *L. esculentum* leaves.

Compound	*R*t	[M+H]$^+$	MS^2[M+H]$^+$, m/z (%)									
			-18	-132	-150	-162	-180	-294	-456	-474	Agl+H	Agl+H-18
α-Tomatine	27.9	1034.7	1016.6(100)	902.6(10)	884.6(2)	872.6(4)	854.5(1)	740.4(2)	578.3(6)	560.5(3)	416.3(9)	398.3(6)
Dehydrotomatine	26.8	1032.7		900.5(12)	882.6(4)	870.6(5)	852.6(5)	738.4(2)	576.4(20)	558.4(2)	414.4(23)	396.3(14)
			-18	-143	-143-18							
Tomatidine	34.5	416.3	398.4(100)	273.1(29)	255(29)							
Tomatidenol	33.7	414.3	396.3(100)	271.0(25)	253.0(30)							

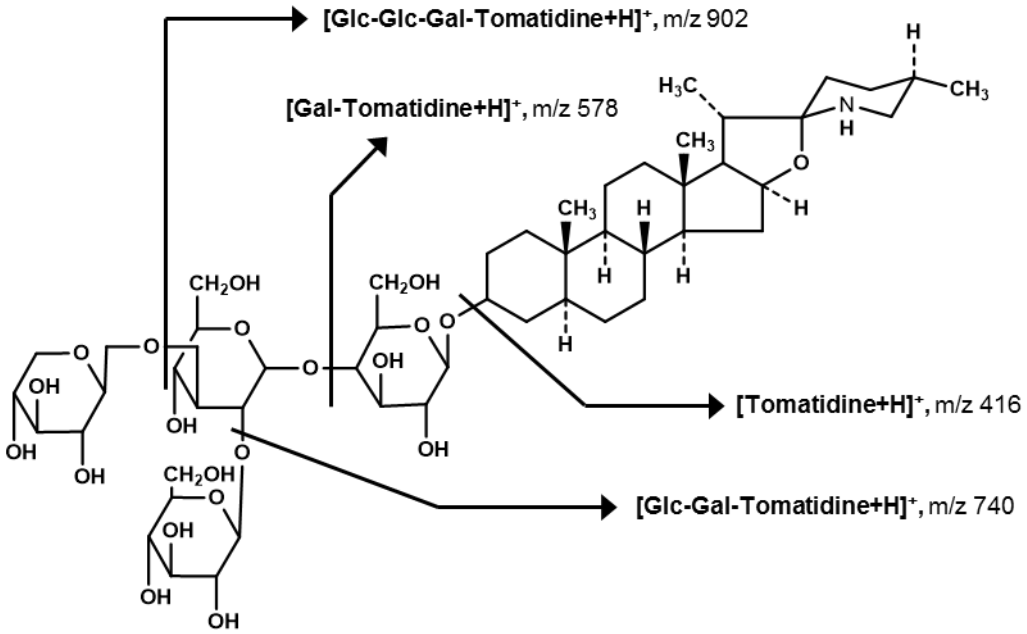


Figure 31. Fragmentation profile of α-tomatine.

α-Tomatine was predominant in *L. esculentum* leaves from "cherry" variety, followed by dehydrotomatine and tomatidine, tomatidenol being found in trace amounts (**Figure 31**). In "bull's heart" variety α-tomatine was also more abundant than dehydrotomatine, while tomatidine was found only in trace amounts and tomatidenol was not detected.

The screening of these alkaloids in *S. littoralis* materials reared on *L. esculentum* leaves from "cherry" variety indicated that α-tomatine, tomatidine and dehydrotomatine were present in small or trace amounts in larvae, excrements and exuviae, while tomatidenol was detected only in excrements (**Table 16**). In *S. littoralis* adult no alkaloid was identified. In the materials of *S. littoralis* reared in "bull's heart" variety only glycoalkaloids were found and just in larvae and excrements.

As observed for host plant leaves, α-tomatine also dominated over dehydrotomatine in both insect materials. In addition, alkaloids content was higher in excrements than in larvae reared in both varieties.

As for phenolic compounds, the presence of sulphate and/or glucuronate derivatives was also investigated in *L. esculentum* leaves and in *S. littoralis* materials by EIC, but no such compound was noticed. The same was done regarding glycoalkaloids with a lower glycosylation degree, but they were not detected in these samples.

Table 16. Alkaloids in *S. litoralis*/*L. esculentum* system[a].

Compound	"Cherry"					"Bull's heart"				
	Leaves	**Larvae**	**Excrements**	**Exuviae**	**Adults**	**Leaves**	**Larvae**	**Excrements**	**Exuviae**	**Adults**
α-Tomatine	+	+	+	+	-	+	+	+	-	-
Dehydrotomatine	+	+	+	+	-	+	+	+	-	-
Tomatidine	+	+	+	t	-	t	-	-	-	-
Tomatidenol	t	-	t	-	-	-	-	-	-	-

[a] (+) present; (-) not detected; t: traces.

These results indicate that *S. littoralis* is able to detoxicate α-tomatine, which, as referred above, constitutes a defence of *L. esculentum*. Glucosidases have been suggested to be involved in fungal detoxification of α-tomatine, by cleavage of sugar residues attached to the basic alkaloid skeleton. The removal of one or more sugars from the tetrasaccharide moiety of α-tomatine destroys its ability to bind 3β-hydroxy sterols, namely cholesterol (311).

As previously mentioned, steroidal alkaloids form a 1:1 complex with sterols, causing cell membrane disruption. Furthermore, the presence of glycolipids in cholesterol containing membranes is known to increase membrane destruction: lipid sugar groups establish hydrogen bonds with sugar moieties of membrane-associated steroidal alkaloids, thereby prolonging the presence of these compounds in the membrane and enhancing the opportunity to destroy it (311, 312). Thus, the removal of sugar residues reduces the toxicity of the compound.

May be *S. littoralis* possesses enzymes that promote deglycosylation of these alkaloids. In fact, the presence of *β*-glucosidases in another *Spodoptera* species (*Spodoptera frugiperda*) was already reported (313), which seems to corroborate this detoxification hypothesis. The expression profile of each of these genes and the information about their putative functions is important to evaluate the detoxification capacity of *S. littoralis*. Moreover, as the detoxification potential of insects is strongly enhanced by their commensal gut bacteria or by endosymbionts, also known to have detoxifying enzymes, symbiotic relationships with such microorganisms cannot be excluded (314).

3. *L. esculentum* leaves as source of bioactive compounds

We intended to develop rapid and sensitive HPLC-DAD methods for the determination of phenolics and steroidal alkaloids in *L. esculentum* leaves, regarding the possible industrial use of this by-product. The chromatographic conditions were optimized and validated for both types of compounds.

3.1. Phenolic compounds

3.1.1. HPLC-DAD analysis optimization

For this work an elution system composed by methanol and acetic acid 1% was chosen. In order to optimize the chromatographic separation of the hydroalcoholic extracts, two gradients were tested (**Table 5**). Both gradients were able to separate very efficiently the target compounds. Nevertheless, as gradient A was rather long (45 min), adjustments were performed in order to shorten the run duration without sacrificing the chromatographic goals. Gradient B fulfilled these criteria, being 10 min shorter (**Table 5**).

The choice of the optimal flow rate was based on a compromise between compounds' separation, peak width and column backpressure, which was achieved when elution was performed at 0.8 ml/min.

3.1.2. Method validation

For the validation of the proposed analytical method, linear regression analysis was performed by using external calibration curves. The parameters for the calibration curves are shown in **Table 17.**

An excellent linearity was found for all the analytes peak areas at the tested concentrations ($r^2 \geq 0.998$). LOD and LOQ values were experimentally determined with standard solutions of quercetin-3-*O*-rutinoside (LOD = 0.22 µg/ml; LOQ = 0.72 µg/ml) and 5-*O*-caffeoylquinic acid (LOD = 1.95 µg/ml; LOQ = 6.50 µg/ml).

The accuracy of the analytical procedure was evaluated by recovery tests. Since the recovery rates obtained were higher than 95% this method can be considered to be accurate (**Table 17**). The low intra- and inter-day variations of peak areas (RSD ≤ 5.03%) indicate a high precision of the chromatographic system (**Table 17**).

Thus, the proposed methodology was sufficiently sensitive and had a good linearity, accuracy and precision for quantification of phenolic compounds present at low concentrations in extracts of *L. esculentum* leaves, being suitable for this purpose.

Table 17. Validation results for HPLC-DAD analysis of phenolic compounds in *L. esculentum* leaves.

Phenolics	Regression equation	r^2	Linearity range (mg/ml)	LOD[a] (µg/ml)	LOQ[b] (µg/ml)	Intra-day precision (RSD)[c]	Inter-day precision (RSD)[c]	Recovery (%)
5-*O*-Caffeoylquinic acid	y = 1459226110.382 x - 842337.106	0.998	0.200 – 0.003	1.95	6.50	3.32	5.03	98.33 – 99.35
Quercetin-3-*O*-rutinoside	y = 1077062938.646 x - 274869.635	0.999	0.160 – 0.003	0.22	0.72	2.05	3.07	95.24 – 98.52

[a] LOD: limit of detection.

[b] LOQ: limit of quantification.

[c] RSD: relative standard deviation.

3.1.3. Quantification

The method described above was applied to the quantification of phenolics in *L. esculentum* leaves hydromethanolic extract from the two varieties (**Figure 32**).

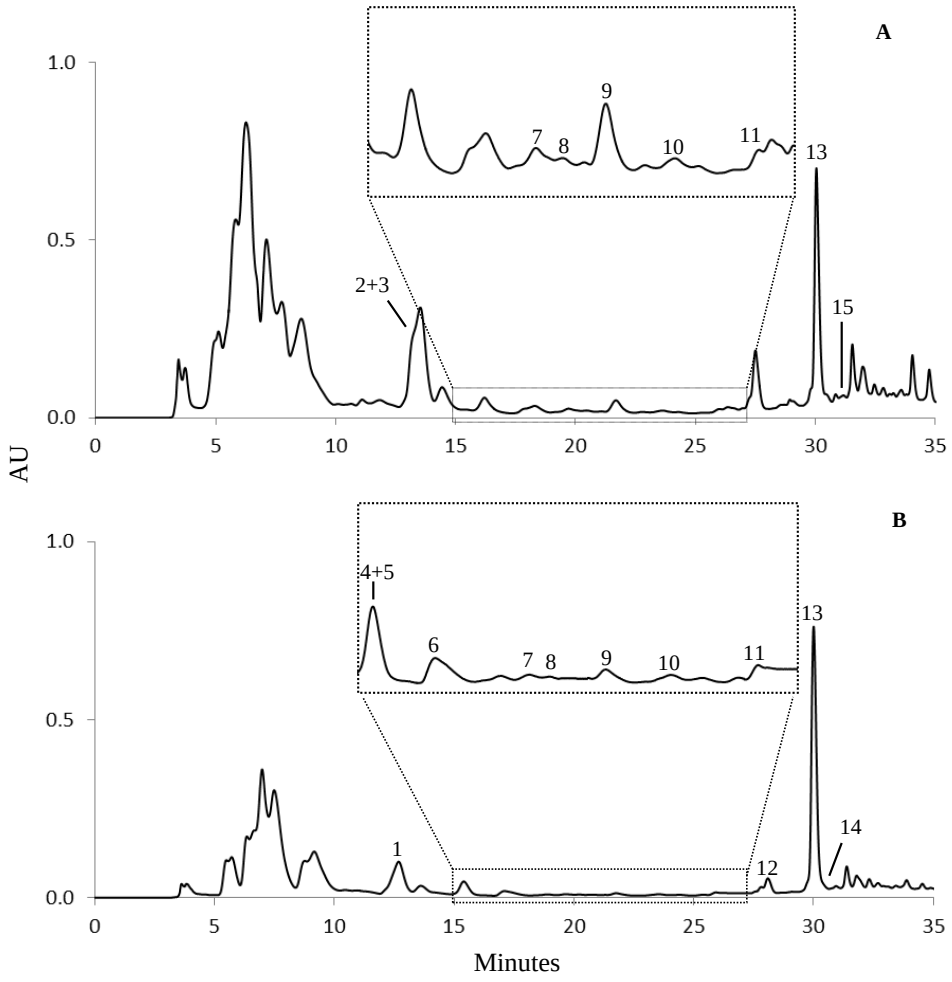


Figure 32. HPLC-DAD chromatogram of phenolic compounds of *L. esculentum* leaves obtained with the optimized method (gradient B, 0.8 ml/min). Detection at 330 nm. (**A**) "cherry" and (**B**) "bull's heart" varieties. Peaks: (**1**) 3-*O*-caffeoylquinic acid; (**2**) caffeoyl-hexoside acid; (**3**) *p*-coumaroyl-hexoside acid; (**4**) feruloyl-hexoside acid; (**5**) sinapoyl-hexoside acid isomer; (**6**) 5-*O*-caffeoylquinic acid; (**7**) *p*-coumaroylquinic acid; (**8**) sinapoyl-hexoside acid; (**9**) 5-*O*-feruloylquinic acid; (**10**) *p*-coumaroylquinic acid isomer; (**11**) feruloylquinic acid isomer; (**12**) quercetin-3-*O*-pentosyl-rutinoside; (**13**) quercetin-3-*O*-rutinoside; (**14**) sinapoyl derivate; (**15**) kaempferol-3-*O*-rutinoside.

Some differences were found between the leaves of “cherry” and “bull’s heart” varieties (**Figure 32**). *p*-Coumaroylquinic acid (**7**), sinapoyl-hexoside acid (**8**), 5-*O*-feruloylquinic acid (**9**), *p*-coumaroylquinic acid isomer (**10**), feruloylquinic acid isomer (**11**) and quercetin-3-*O*-rutinoside (**13**) were common to both varieties. 3-*O*-Caffeoylquinic acid (**1**), feruloyl-hexoside acid (**4**), sinapoyl-hexoside acid isomer (**5**), 5-*O*-caffeoylquinic acid (**6**), quercetin-3-*O*-pentosyl-rutinoside (**12**) and a sinapoyl derivative (**14**) were found only in “bull’s heart” variety. On the other hand, caffeoyl-hexoside acid (**2**), *p*-coumaroyl-hexoside acid (**3**) and kaempferol-3-*O*-rutinoside (**15**) were present just in “cherry” variety (**Table 18**).

Table 18. Phenolic composition of *L. esculentum* leaves (mg/kg of dried leaves)[a].

	Compounds	**“Cherry”**	**“Bull’s heart”**
1	3-*O*-Caffeoylquinic acid	-	512.2 (25.2)
2+3	Caffeoyl-hexoside acid + *p*-Coumaroyl-hexoside acid	790.7 (10.7)	-
4+5	Feruloyl-hexoside acid + Sinapoyl-hexoside acid isomer	-	84.0 (2.0)
6	5-*O*-Caffeoylquinic acid	-	92.1 (1.9)
7	*p*-Coumaroylquinic acid	28.0 (2.3)	nq
8	Sinapoyl-hexoside acid	7.6 (1.6)	nq
9	5-*O*-Feruloylquinic acid	42.9 (2.4)	9.7 (0.5)
10	*p*-Coumaroylquinic acid isomer	3.7 (0.0)	24.4 (2.3)
11	Feruloylquinic acid isomer	5.8 (0.6)	5.8 (3.3)
12	Quercetin-3-*O*-pentosyl-rutinoside	-	60.6 (7.2)
13	Quercetin-3-*O*-rutinoside	1155.3 (3.2)	1913.9 (4.3)
14	Sinapoyl derivative	-	nq
15	Kaempferol-3-*O*-rutinoside	36.0 (0.1)	-
	∑	2070.1	2702.7

[a] Results are expressed as mean (standard deviation) of three determinations. nq: not quantified. ∑: sum of the determined phenolic compounds.

As observed in the seeds (**Tables 7** and **8**), “bull’s heart” variety presented a higher content of phenolic compounds (about 23.4% more than “cherry” variety). Quercetin-3-*O*-rutinoside (**13**) was the major compound in both varieties (**Table 18**).

Some studies using HPLC-MS were already performed for the characterization of phenolics in *L. esculentum* leaves (38, 193, 315). Nevertheless, as this technique is not available in all laboratories and as the quantitative analysis of complex matrices can be affected by ionisation suppression problems (316), the developed method, based on

HPLC-DAD (a more common methodology), constitutes an advantage, since it can be more easily implemented, allowing an easy quantification of these compounds.

Baker and co-workers (317) have published a method for phenolics determination in *L. esculentum* leaves using ultra-performance liquid chromatography. However, no compound identification was provided: the authors just pointed to their chemical class based on the analysis of their UV spectra. Panina and collaborators (318) reported an HPLC-DAD method that allowed the identification of only three compounds in *L. esculentum* leaves, in a 40 min elution. Van der Rest and co-workers (38) have identified ten compounds in a 60 min HPLC-DAD analysis. So, comparing with these last two, the developed method provides the identification of more compounds (fifteen), in a shorter analysis time (35 min) (**Figure 32**). In addition, none of the works above mentioned (38, 317, 318) presents any validation data. Thus, to the best of our knowledge, the proposed method constitutes the first fully validated one for phenolics determination in *L. esculentum* leaves.

Comparing both *L. esculentum* materials studied, leaves present approximately ten times more phenolic compounds than seeds (**Tables 7, 8** and **18**). This higher content can be due to the UV protective role of phenolics in plant (319).

3.1.4. Antioxidant potential

The *L. esculentum* leaves hydromethanolic extracts were evaluated as radical scavengers against DPPH$^{\bullet}$, $^{\bullet}$NO, and $O_2^{\bullet-}$. A dose-dependent response was observed in all assays (**Figure 33**) and IC_{50} values are presented in **Table 19.**

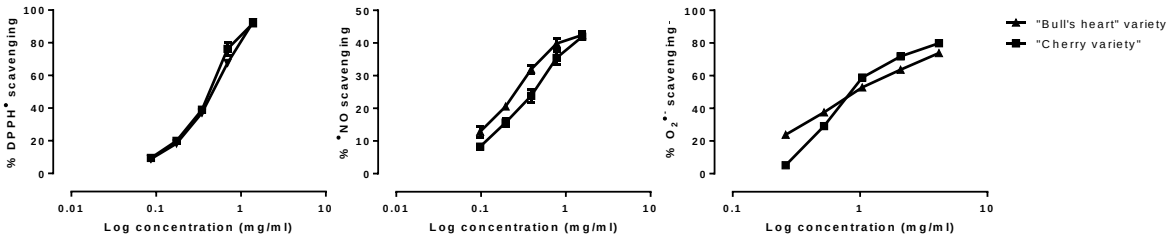


Figure 33. Effect of *L. esculentum* leaves hydromethanolic extracts against DPPH$^{\bullet}$, $^{\bullet}$NO and $O_2^{\bullet-}$. Values show mean ± SE from three experiments performed in triplicate.

Concerning the potential of *L. esculentum* hydromethanolic leaves extracts as $O_2^{\bullet-}$ scavengers, better results were obtained for "bull's heart" variety. A lower scavenging activity was observed for $^{\bullet}NO$, not reaching 50 % radical interception.

Table 19. IC_{50} (μg/ml) of *L. esculentum* leaves hydromethanolic extracts against $DPPH^{\bullet}$, $^{\bullet}NO$ and $O_2^{\bullet-}$.

Variety	$DPPH^{\bullet}$	$^{\bullet}NO^{a}$	$O_2^{\bullet-}$
"Bull's heart"	494	369	887
"Cherry"	450	430	944

[a] IC_{25}

Overall, "bull's heart" variety seems to be more active against the tested reactive species. This higher activity can be associated to its higher content of phenolic compounds, mainly quercetin-3-*O*-rutinoside (**Table 18**).

In addition, comparing both *L. esculentum* materials studied, leaves have a higher antioxidant potential than seeds, which can also be related to their higher content of phenolics (**Figure 20**; **Tables 7, 18** and **19**).

3.2. Steroidal alkaloids

3.2.1. Optimization of SPE purification

Several difficulties have been reported concerning the chromatographic separation of steroidal alkaloids, such as complex interactions between solute, mobile and stationary phases (320). In addition, due to the low specificity of their UV absorption, a previous purification method is commonly applied (87), as above mentioned.

Therefore, the methodology for steroidal alkaloids analysis includes extraction and enrichment steps, followed by chromatographic determination of the target analytes (35). Concerning this, the use of acetic acid for steroidal alkaloids extraction is an advantage, since it can be applied into most of the SPE columns. In acetic solution steroidal alkaloids are found in their ionised form, which is a prerequisite for their retention in ion-exchange sorbents (87).

In our study, SPE was employed to eliminate possible matrix interferences: target compounds interact more with the SPE sorbent and the interferences are washed away. However, good recoveries of the steroidal alkaloids are required. We cannot theoretically predict which sorbent is better because several interactions between the alkaloids and the stationary phase are established: separation efficiency cannot be explained only by ionic interactions in SCX columns, by Van der Waals forces between the steroidic and C_{18} groups in reversed-phase packings, or by possible hydrogen bonding between hydroxyl groups and CN and NH_2 in normal phase and combined ones (87). Furthermore, interactions between free silanol groups in silica-based sorbents and basic steroidal alkaloids may render low recoveries. In addition, the amounts of residual surface silanols are dependent on the sorbent manufacturer, which can also influence SPE performance (87). Therefore, in this work several sorbents were compared (**Figure 34** and **Table 4**).

The elution conditions were adjusted for the different sorbents and the elution volume was adapted taking into account the sorbent quantity. It has been reported that steroidal alkaloids should be eluted with 100% methanol or ammonia-methanol to avoid acidic environment, which can lead to compounds degradation (88). Nevertheless, they do not elute from CN and NH_2 SPE columns when methanol is used as elution solvent, so 25% acetic acid in methanol has to be applied (88).

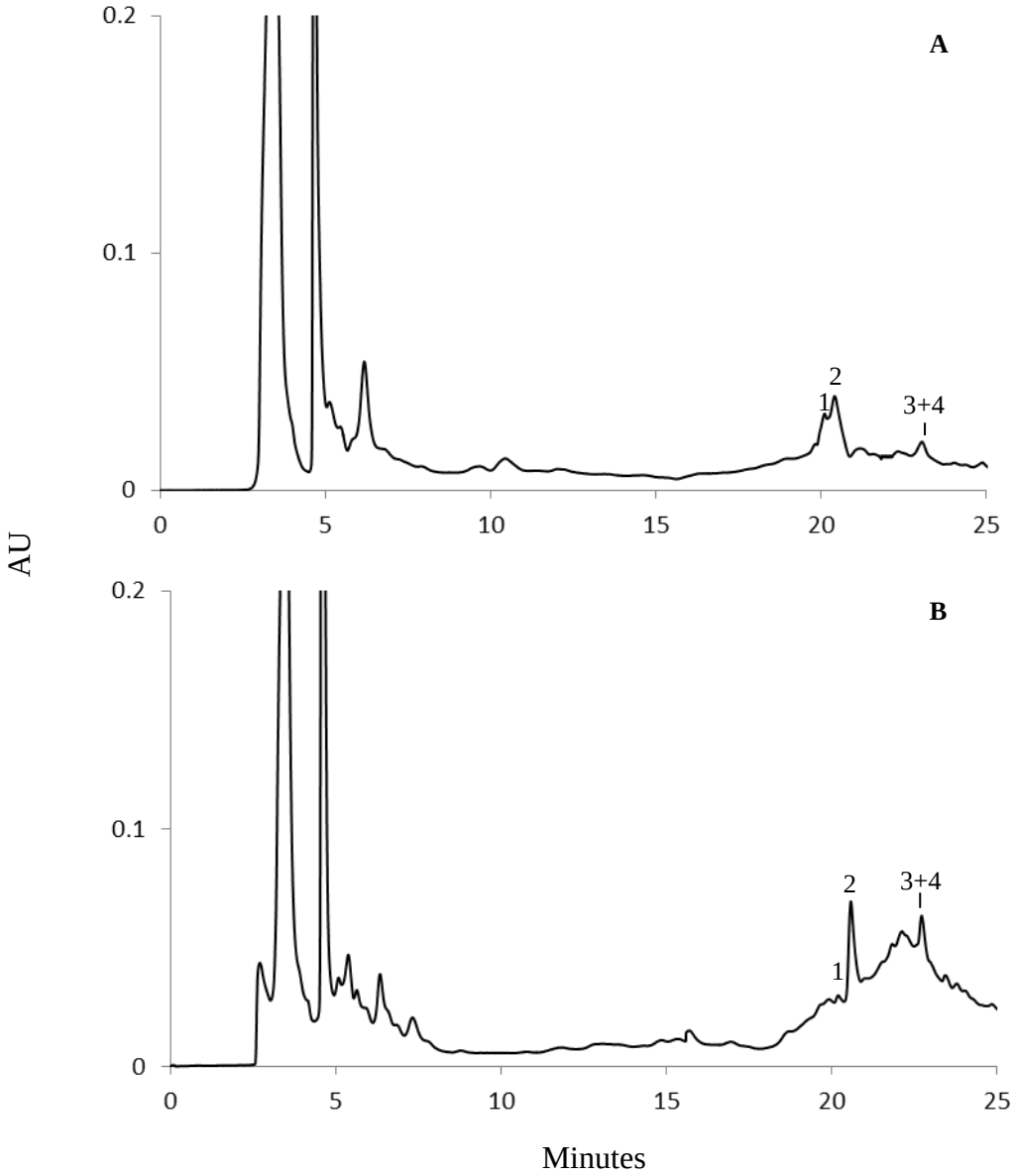


Figure 34. HPLC-DAD steroidal alkaloids profile of *L. esculentum* leaves of "cherry" variety, obtained with (**A**) SCX and (**B**) CN SPE columns, using gradient A. Detection at 205 nm. Peaks: (**1**) dehydrotomatine; (**2**) α-tomatine; (**3**) tomatidenol; (**4**) tomatidine.

As referred above, SPE columns are used to remove undesired compounds (**Figure 34**). Comparing all obtained chromatograms, the most efficient was SCX column (**Figure 34A**), which is in accordance with results published before (87) for extraction of potato glycoalkaloids. SCX column contains silica with aliphatic sulfonic acid groups that are bonded to the surface. The sulfonic acid group is strongly acidic and attracts or exchanges cationic species (basic), such as alkaloids, which could explain its higher efficiency (87). Thus, this SPE column was used for further optimization of chromatographic conditions.

3.2.2. HPLC-DAD analysis optimization

The gradient conditions initially tested (Gradient A, **Table 5**) were similar to those reported by Kuronen et al. (320). Acetonitrile was employed as the basic organic modifier of the mobile phase, because of its low UV cut-off, which allows the use of 205 nm as detection wavelength for alkaloids. The use of TEAP buffer (pH 3.0) provides that both basic functional group of steroidal alkaloids and acidic silanol groups on the silica support are fully protonated, minimizing ionic interactions between them and leading to more reproducible separations (321). Additionally, the use of triethylammonium ions contributes to a better chromatographic separation (320).

With gradient A steroidal alkaloids eluted only after 20 min run (**Figure 34**). Thus, we tried to reduce the retention time of the target compounds and improve the chromatographic separation. On analysing **Figure 34**, we can see that some steps of gradient A (**Table 4**) could be eliminated in order to reach earlier a higher percentage of acetonitrile. Using gradient B a reduction of approximately 10 min of the compounds' retention time was observed (**Table 5**), as well as an improvement of the chromatographic separation.

Concerning the flow rate, the choice of the best performance was based on a compromise between compounds' separation, peak width and column backpressure, which was achieved using 0.8 ml/min.

The optimized chromatographic conditions were used for the steroidal alkaloids quantification (**Figure 35**).

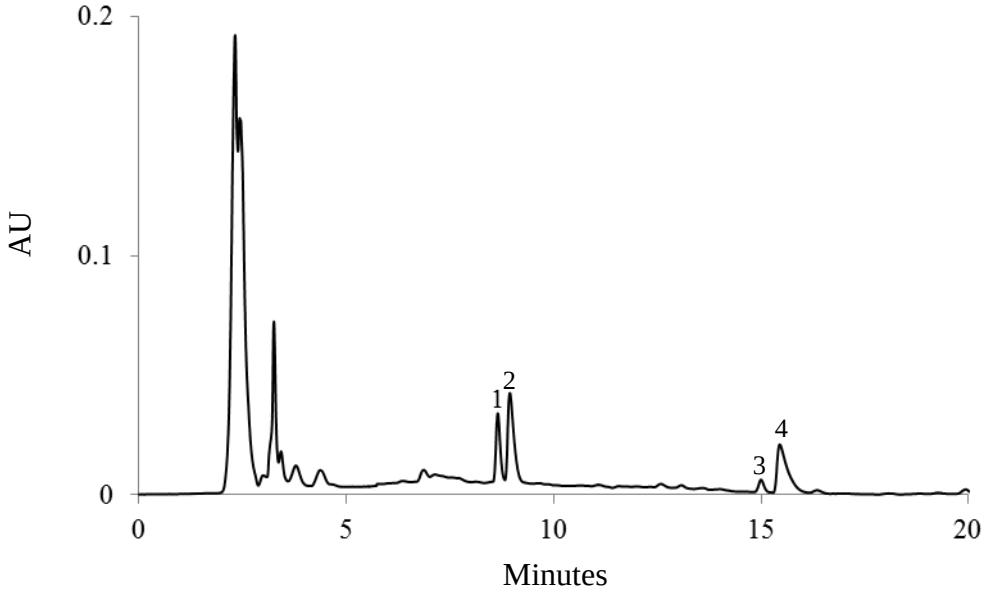


Figure 35. HPLC-DAD chromatogram of steroidal alkaloids in *L. esculentum* leaves of "cherry" variety obtained with the optimized method (SCX SPE column; gradient B, 0.8 ml/min). Detection at 205 nm. Peaks: (**1**) dehydrotomatine; (**2**) α-tomatine; (**3**) tomatidenol; (**4**) tomatidine.

3.2.3. Method validation

The method proposed for the determination of steroidal alkaloids in *L. esculentum* leaves was further validated. An excellent linearity ($r^2 \geq 0.997$) was observed for the peak areas at the tested concentrations. This method also presented low values of LOD and LOQ, pointing to its utility for the quantification of steroidal alkaloids in this matrix. Besides the good capacity for removing interfering compounds, SCX column led to a good recovery of the alkaloids (**Table 20**).

The chromatographic system was precise, with low values of intra- and inter-day variations. Additionally, considering the calibration slopes and the good recovery values it can be inferred that this method is reproducible and accurate for the determination of steroidal alkaloids in *L. esculentum* leaves extracts (**Table 20**).

Table 20. Validation results for HPLC-DAD analysis of steroidal alkaloids in *L. esculentum* leaves.

Alkaloids[a]	Regression equation	r^2	Linearity range (mg/ml)	LOD[b] (µg/ml)	LOQ[c] (µg/ml)	Intra-day precision (RSD)[d]	Inter-day precision (RSD)[d]	Recovery (%)
Tomatine	y = 92234591.959 x + 177356.472	0.997	3.100-0.097	17	58	2.03	5.87	92.34-93.75
Tomatidine	y = 74720096.655 x – 418816.229	0.999	3.500-0.109	29	97	3.96	6.14	95.47-98.14

[a] α-Tomatine and dehydrotomatine were determined together as tomatine and tomatidine plus tomatidenol were determined together as tomatidine.
[b] LOD: limit of detection.
[c] LOQ: limit of quantification.
[d] RSD: relative standard deviation.

3.2.4. Quantification

The validated method was then applied to *L. esculentum* leaves. α-Tomatine, dehydrotomatine, tomatidine and tomatidenol were identified in “cherry” and “bull’s heart” varieties (**Table 21**). The first presented higher levels of steroidal alkaloids. In addition, it was observed that the quantity of glycosylated steroidal alkaloids in both varieties was higher than that of their aglycones. These compounds were quantified for the first time in *L. esculentum* leaves. However, it was already reported that *L. esculentum* fruits from “cherry” variety present higher amounts of steroidal alkaloids (35) and, according to the results obtained, it seems that the same happens with the leaves.

Table 21. Steroidal alkaloids composition of *L. esculentum* leaves (mg/kg of dried leaves)[a].

Compounds[b]	**“Cherry"**	**“Bull’s heart”**
Tomatine	9620.3 (201.5)	6030.4 (303.1)
Tomatidine	140.1 (10.8)	80.8 (10.3)
∑	9760.4	6111.2

[a] Results are expressed as mean (standard deviation) of three determinations. ∑: Sum of the determined steroidal alkaloids.
[b] α-Tomatine and dehydrotomatine were quantified together as tomatine and tomatidine plus tomatidenol were quantified together as tomatidine.

A previous study with different tomato materials (fruits, stems, flowers, calyxes and leaves) (98) reported the use of a reversed-phase HPLC column to separate the glycoalkaloids and of a normal phase one for their aglycones. Similar LOD and LOQ were obtained (98). However, with our methodology using only one reversed-phase HPLC column we could separate in a single run both steroidal glycoalkaloids and their aglycones. Besides, the authors used two methods with 40 min each, while we can separate both analytes in only 20 min. Thus, we avoid the need of two different columns and perform the analysis in 1/4 of the time used by Kozukue and colleagues (98), which turns our analytical methodology cheaper.

On the other hand, when comparing our results with those obtained by Kuronen and colleagues (320), they were also able to simultaneously separate steroidal glycoalkaloids and their aglycones. However, our analysis time is 5 min shorter and run conditions lead to a better chromatographic separation. Additionally, that study (320) was performed only with standard compounds, so no matrix interference could be

evaluated and no analytical validation was provided by the authors. Thus, the proposed method shows a clear advance for the qualitative and quantitative analyses of this class of compounds in *L. esculentum* leaves.

3.2.5. Non-human cholinesterases' inhibition

Under the assay conditions, the purified extract of *L. esculentum* leaves rich in steroidal alkaloids exhibited a dose-dependent response against non-human AChE and BChE (**Figure 36**).

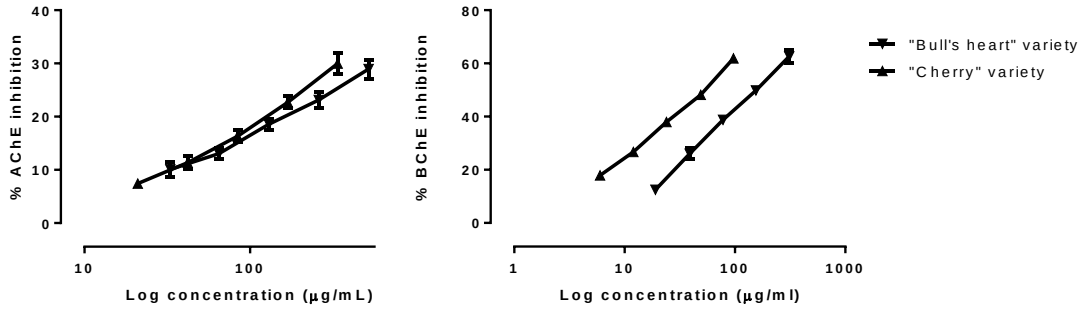


Figure 36. Effect of *L. esculentum* leaves extracts against non-human AChE and BChE. Values show mean ± SE from three experiments performed in triplicate.

Overall, "cherry" variety exhibited a higher activity against both enzymes than "bull's heart" one (**Table 22**).

Table 22. IC_{25} values (µg/ml) of *L. esculentum* extracts against non-human AChE and BChE.

Variety	AChE	BChE
"Cherry"	133.0	10.8
"Bull's heart"	170.1	41.5

Both extracts revealed to be more effective against BChE, which can be a promising result regarding their application in AD. Nevertheless, these assays were performed with non-human enzymes, which can lead to misinterpretations. As so, further studies with human enzymes were performed.

4. Steroidal alkaloids and Alzheimer's disease

ChE inhibition capacity of steroidal alkaloids (tomatine and tomatidine) and tomato leaves purified extracts, as well as their neuroprotective potential against glutamate-induced toxicity, were also evaluated in human derived SH-SY5Y cells. *L. esculentum* leaves were chosen once they are the vegetable material with higher levels of glycoalkaloids (35). Taking into account the gastrointestinal toxicity commonly ascribed to steroidal glycoalkaloids (67), the effect of compounds and extracts on the viability of neuronal, gastric and intestinal cells lines was assessed.

4.1. Human cholinesterases' inhibition

Human neuroblastoma SH-SY5Y cells were used as source of AChE and BChE. Tomatine and *L. esculentum* leaves purified extracts revealed a promising potential as ChE inhibitors, especially against BChE (**Table 23**).

Table 23. IC_{25} values of steroidal alkaloids and *L. esculentum* leaves extracts against human ChE.

Compound/Extract	AChE	BChE
Tomatine[a]	49.7	10.9
Tomatidine	-	-
"Cherry"[b]	104.9	73.1
"Bull's Heart"[b]	139.5	71.5

[a] Values expressed in µM.
[b] Values expressed in µg/ml.

The extracts of tomato leaves, rich in these steroidal alkaloids, were also tested, in order to verify if they retained the same biological potential of the isolated compounds. The extracts were active against the two enzymes, that from "cherry" variety being more active against AChE than the "bull's heart" one (**Table 23**). In addition, a stronger effect against BChE was noticed, as observed with the non-human enzymes (**Table 22**).

The inhibitory results can be attributed to the steroidal alkaloids, which are present in higher amounts in the extract of "cherry" variety leaves (**Table 21**). Although the anti-cholinesterase activity of these compounds is associated with the steroid nucleus and

its amine group, which act as a binding site for the enzyme, it has been previously demonstrated *in vitro* that glycosylated compounds (like tomatine) present higher activity against AChE than the aglycones (322).

As referred above, both enzymes seem to be legitimate therapeutic targets to ameliorate the cholinergic deficit and increase the levels of acetylcholine in the brain.

4.2. Effect on cell viability

As previously mentioned, in spite of the steroidal alkaloids ability to inhibit ChE, they are also known by their toxicity (67). Actually, one of the main drawbacks for their possible therapeutic utilization is their claimed toxicity on gastric and intestinal cells (35). Nevertheless, toxicity is dependent on the chemical structure of the compound (aglycone, type and number of sugars), dosage, time of exposure, co-exposition with other compounds, among other aspects (67).

The toxicity of steroidal alkaloids is not yet fully understood; however, it was suggested that it may be the result of multiple molecular events, including the formation of complexes of steroidal alkaloids with cholesterol located within cell membranes, leading to membrane disruption (67).

As above referred, tomatine is described to strongly bind to cholesterol, but its ability to disrupt cellular membranes is not as high as expected, considering other glycoalkaloids with similar cholesterol binding potential. Among steroidal alkaloids, the ones found in tomato are the less prone to exert cytotoxicity by membrane disruption (67).

In fact, there are high quantities of tomatine in green tomato fruits (up to 500 mg/kg), which have long been eaten fried and pickled and their alkaloids seem to be relatively harmless to people. The limited toxicity of tomatine is also reinforced by the fact that Peruvians consume a high tomatine variant of *L. esculentum* (var. *cerasiforme*, in which it can reach 5000 mg/kg) without deleterious effects (35). So, the alkaloids present in tomato (spirosolane skeleton and lycotetraose glycoside chain) appear to be safer for humans than the potato alkaloids (solanidane skeleton and chacotriose or solatriose glycoside chains).

To evaluate the possible use of these compounds/extracts in therapeutics, their cytotoxicity was screened in SH-SY5Y, AGS and Caco-2 cell lines. Cell viability was evaluated by the MTT reduction and LDH release assays. Each compound/extract revealed quite similar toxic profiles for each cell line, SH-SY5Y generally being the most sensitive (**Figures 37** and **38**).

Nevertheless, under the assay conditions, tomatine and tomatidine up to 1.59 and 126.25 µM, respectively, were not cytotoxic to any of the cell lines used (**Figure 37**). A range of non-cytotoxic concentrations was also found for the tested leaves' extracts: only concentrations higher than 197.5 µg/ml of both "cherry" and "bull's heart" varieties significantly reduced cells viability (**Figure 38**).

Tomatine revealed to be the most toxic compound for all cell lines, presenting an LC_{50} of 4.64 µM for SH-SY5Y cells (**Figure 37**). On the other hand, tomatidine was only toxic for SH-SY5Y cells (LC_{50} = 457.12 µM; **Figure 37**).

Regarding both extracts, the cytotoxic profile observed for SH-SY5Y cells was similar to that displayed for AGS and Caco-2 cells (**Figure 38**), the extract obtained from "cherry" variety (LC_{50} = 422.57 µg/ml) being more toxic than "bull's heart" one (LC_{50} = 517.07 µg/ml).

As expected, tomatine was more toxic than its aglycone for all cell lines (**Figure 37**). In fact, it was already described (120) that tomatidine has little effect on AGS cells, while tomatine is more toxic. Regarding the extracts, the one from "cherry" variety was more toxic (**Figure 38**). This higher toxicity can be related to its higher tomatine content (**Table 21**). Studies comparing tomatine with tomatidine showed that the glycosidic residue was required for membrane-disrupting activity (67). These results reinforce the importance of the carbohydrate side chain to the biological effect.

Comparing the two methods for viability assessment used in this work (**Figures 37** and **38**) the MTT assay appeared to be more sensitive in detecting early toxicity for these compounds and extracts when compared to LDH leakage assay. In fact, cell viability evaluated by the two assays was different for some tested concentrations (**Figures 37** and **38**). This can be explained by the nature of each assay. The MTT assay is based on the conversion of this compound to formazan, mainly by mitochondrial enzymes (323), whereas the LDH leakage assay is based on the release of this cytoplasmic enzyme into the culture medium after cell membrane damage (324).

So, it can be speculated that the tested compounds/extracts exert their toxicity at the mitochondrial level first, followed by cell membrane disruption. As so, the MTT assay was chosen to measure cell viability in the experiments designed to evaluate the neuroprotective effect in glutamate-insulted SH-SY5Ycells.

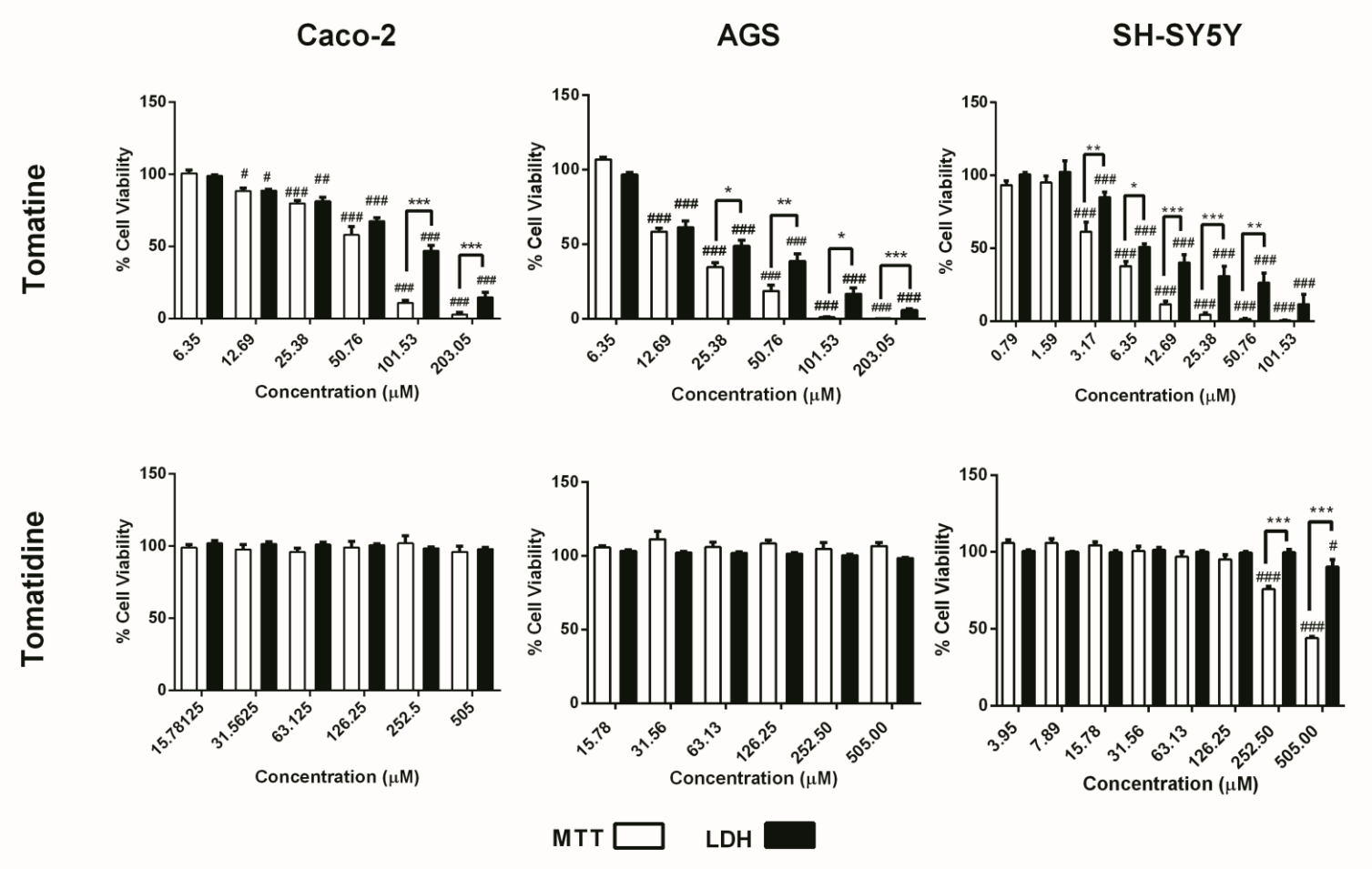


Figure 37. Cytotoxic effect on SH-SY5Y, AGS and Caco-2 cells of tomatine and tomatidine. Results are presented as mean ± SEM of four independent experiments (triplicates were performed in each experiment). # Compared to control (#$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$). *Comparison between MTT and LDH assays for the same compound concentration (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

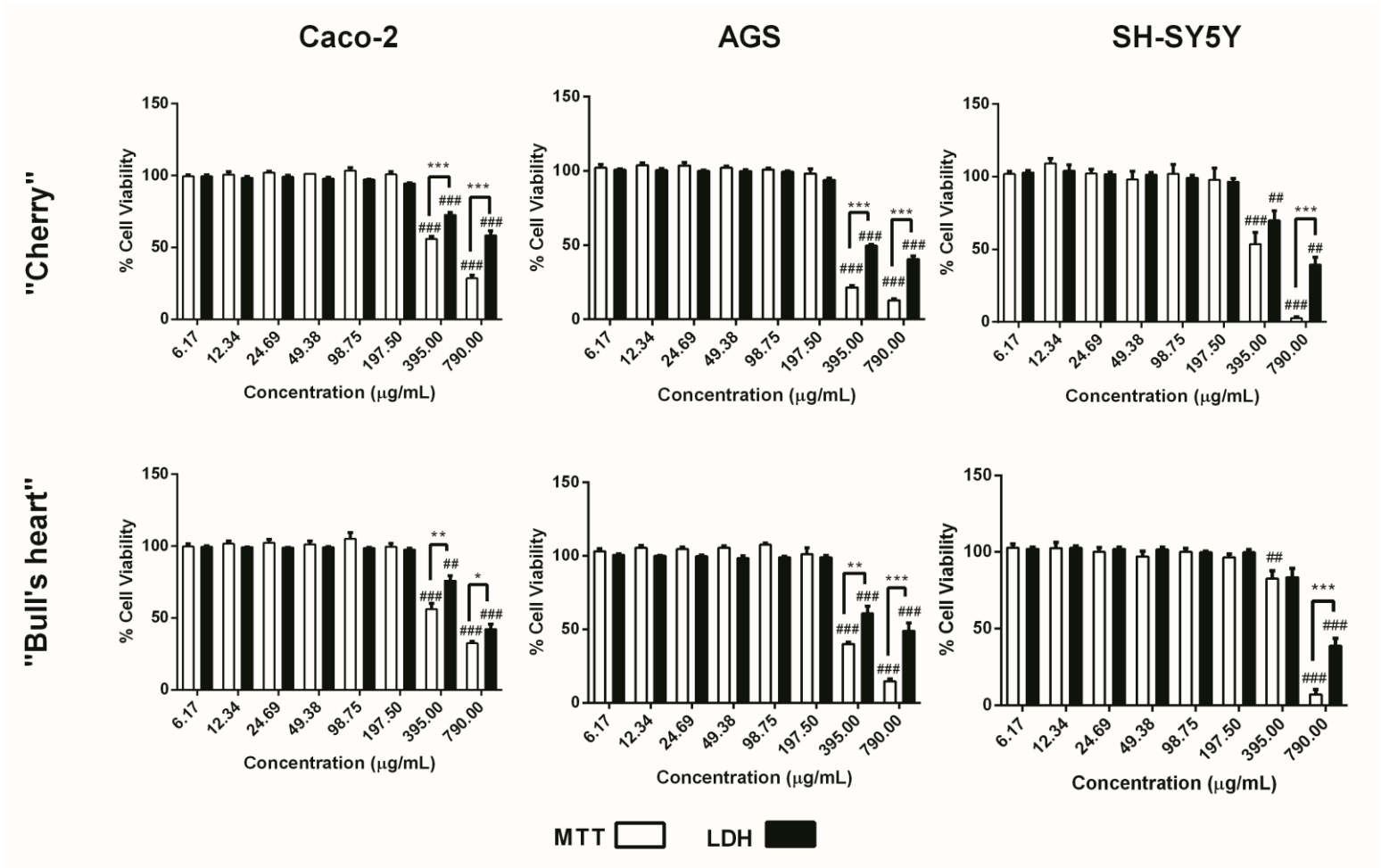


Figure 38. Cytotoxic effect on SH-SY5Y, AGS and Caco-2 cells of purified extracts from *L. esculentum* leaves of “cherry” and “bull’s heart” varieties. Results are presented as mean ± SEM of four independent experiments (triplicates were performed in each experiment). # Compared to control (#$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$). *Comparison between MTT and LDH assays for the same extract concentration (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

4.3. Neuroprotective effect against glutamate-induced toxicity

AD is a prevalent neurodegenerative disorder with multifactorial causes that requires multitargeted treatment. As referred above, AChE inhibitors are well established in the therapy of AD, but patients also benefit from the reduction of pathologic glutamate-induced Ca^{2+}-mediated excitotoxicity by NMDA antagonists. New drugs that simultaneously affect both cholinergic transmission and glutamate-induced excitotoxicity may further improve AD treatment. Thus, the neuroprotective effect of the steroidal alkaloids and *L. esculentum* leaves purified extracts against glutamate-induced toxicity was evaluated in SH-SY5Y neuroblastoma cells (**Figure 39**). The concentrations of 1.59 µM of tomatine, 126.25 µM of tomatidine and 197.50 µg/ml of both extracts were used in the assays, since they revealed to be not cytotoxic for AGS, Caco-2 and SH-SY5Y cells (**Figures 37** and **38**).

Maximal cell death occurred at 100 mM glutamate concentration, both with and without compounds/extracts co-incubation. Below this concentration, significant rightward shifts of the glutamate concentration-cell death response curves, accompanied by significant increases of the LC_{50} values (representing the half-maximum lethal effect concentrations from the fitted curves) were observed ($p < 0.05$) for all the tested compounds/extracts (**Figure 39**).

The protective effect was more evident for glutamate concentrations between 25-75 mM. Bellow these concentrations no significant cell death was observed both with and without compounds/extracts co-incubation. Tomatidine was the less active compound, a LC_{50} increase of 4.14 mM being observed when this compound was co-incubated with glutamate; all the others increased the LC_{50} value of glutamate to around 13.5% (8.22 mM) (**Figure 39**).

As the observed protection could be exerted through different mechanisms, other experiments were performed to evaluate if mitochondrial protection, antioxidant capacity or ability to interact with AChR were involved.

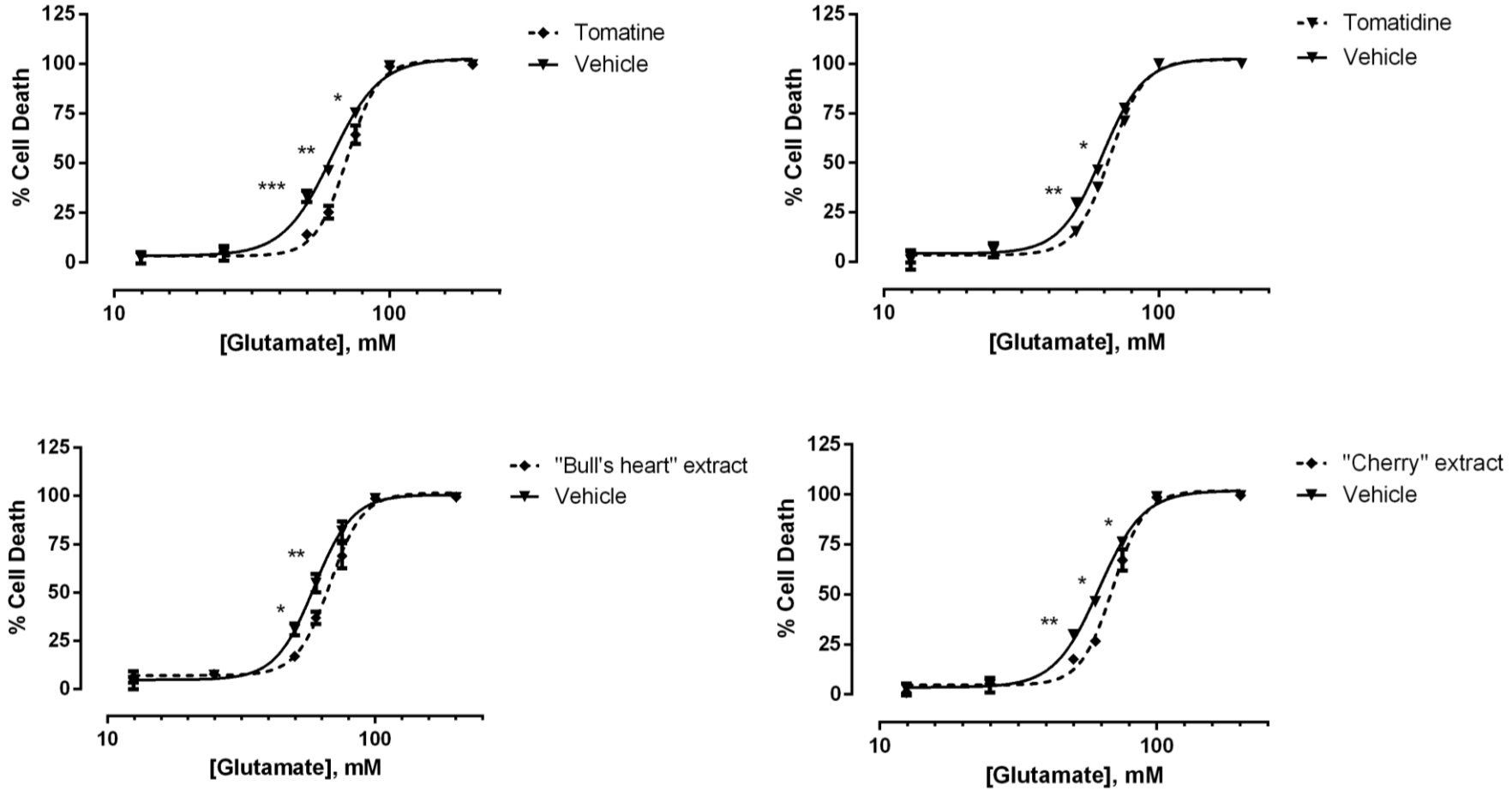


Figure 39. Glutamate concentration-cell death response curves (by MTT assay) with or without compounds/extracts co-incubation (1.59 µM of tomatine; 126.25 µM of tomatidine; 197.50 µg/ml of each *L. esculentum* leaves purified extract). Results are presented as mean ± SEM from four independent experiments (triplicates were performed in each experiment). Concentration-response curves were fitted using least squares as the fitting method and the comparisons between curves (bottom, top and log LC_{50}) were made using the extra sum-of-squares F test. *Compared to the same concentration of glutamate with or without compound/extract (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

4.4. Effect on mitochondrial membrane potential

Cells were exposed to 25 mM of glutamate and the mitochondrial membrane potential was evaluated over time (data not shown). After 4 h exposition the cells revealed a significant decrease of rhodamine 123 fluorescence to 78% of the control level ($p < 0.001$). As so, the protection of the compounds/extracts was assessed under these conditions. The co-incubation of glutamate-insulted cells with compounds/extracts resulted in the preservation of the mitochondrial membrane potential, with significant differences ($p < 0.001$) relatively to glutamate-only exposed cells (**Figure 40**).

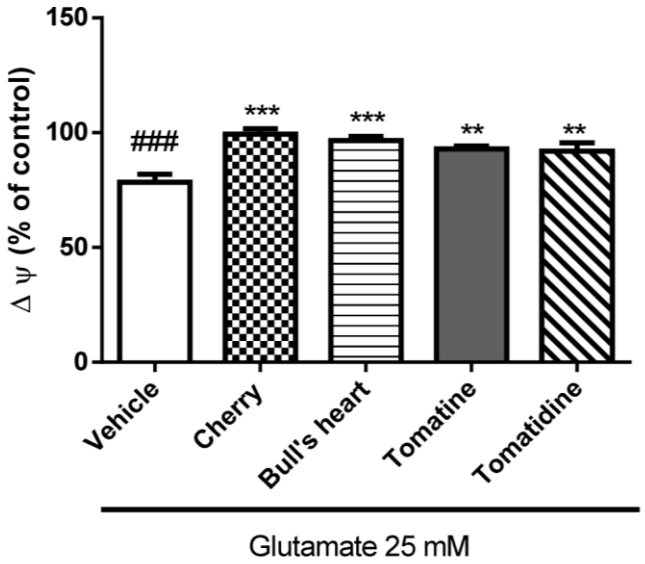


Figure 40. Evaluation of mitochondrial membrane potential variation of SH-SY5Y cells by rhodamine 123 assay. Cells were exposed to glutamate with or without compounds/extracts co-incubation (1.59 µM of tomatine; 126.25 µM of tomatidine; 197.50 µg/ml of each *L. esculentum* leaves purified extract). Results are presented as mean ± SEM from four independent experiments (triplicates were performed in each experiment). # Compared to control (###$p < 0.001$). *Compared to glutamate-only exposed cells (**$p < 0.01$, ***$p < 0.001$).

All the compounds/extracts revealed to have mitochondrial protective capacity, preserving the membrane potential (**Figure 40**). Taking into account that apoptosis signalling cascades can be initiated by mitochondria, the maintenance of the membrane potential and mitochondrial functionality in glutamate exposed cells by the tested extracts and compounds can explain the increased cells' survival. Some authors suggest that the mitochondrial dysfunction provoked by glutamate plays an important role in the pathological changes associated to AD (245). The results obtained point to the possible utility of these compounds/extracts in this disorder.

4.5. Scavenging of reactive species

In this work the reactive species formed by cells exposure to 25 mM of glutamate were measured by the DCFH-DA assay during time (data not shown). It was observed that 20 min of glutamate exposure lead to a significant increase ($p < 0.001$) of reactive species production. As so, these conditions were used to evaluate the compounds/extracts protection. The co-incubation of glutamate insulted cells with compounds/extracts resulted in a significant decrease of reactive species ($p < 0.001$) when compared to the glutamate-only exposed cells (**Figure 41**). In fact, the levels of reactive species of cells co-exposed to glutamate and to the compounds/extracts were similar to that of control cells (**Figure 41**).

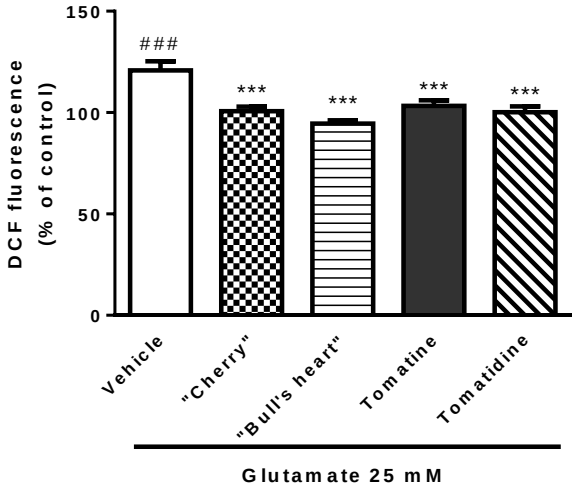


Figure 41. Evaluation of reactive species levels in SH-SY5Y cells by DCFH-DA. Cells were exposed to glutamate with or without compounds/extracts co-incubation (1.59 µM of tomatine; 126.25 µM of tomatidine; 197.50 µg/ml of each *L. esculentum* leaves purified extract). Results are presented as mean ± SEM from four independent experiments (triplicates were performed in each experiment). # Compared to control (###$p < 0.001$). *Compared to glutamate exposed cells (***$p < 0.001$).

As referred above, glutamate also leads to an increase of oxidative stress, which seems to be involved in the aetiology of AD (225). Concerning the antioxidant capacity, all the compounds/extracts were able to reduce the oxidative species (**Figure 41**), which can also involve those produced by mitochondria, thus contributing to the preservation of the mitochondrial membrane potential of cells 4 h after glutamate exposition, as seen in **Figure 41**. This result is particularly significant since pre-exposition to the extracts/compounds was not necessary for protection against glutamate-induced oxidative stress.

4.6. Implication of acetylcholine receptors in the neuroprotective effects

Glutamate-induced toxicity can also be prevented by modulation of nAChRs. This modulation can have therapeutic applications once most drugs currently employed in AD act through ChE inhibition and also as allosteric modulators of nAChRs. In fact, this dual mechanism is observed with galantamine, donepezil and tacrine (248). Under the experimental conditions, tomatine and tomatidine revealed capacity to interact with α7-nAChRs, which seems to be responsible for their neuroprotection capacity (**Figure 42**).

SH-SY5Y cells express functional AChR receptors (233) and their involvement in the protective effects of the compounds and extracts was assessed using mecamylamine, a nAChR antagonist. When the cells were co-exposed to 1 μM mecamylamine the neuroprotective effect of tomatine and tomatidine previously observed (**Figure 39**) was significantly antagonized (**Figure 42**). However, the protection afforded by leaves extracts rich in steroidal alkaloids was not affected by mecamylamine (data not shown).

The effect of the α7-nAChR antagonist MLA on the neuroprotective effect of the compounds was further evaluated, being observed that the protection afforded by tomatine and tomatidine was reverted (**Figure 42**).

α7-nAChR is known to display memory-enhancing activity and is abundantly expressed in the neocortex, hippocampus and basal ganglia (325). Previous studies have reported that neuroprotection mediated through α7-nAChR (326) is correlated with the activation of PI3K and Erk 1/2 pathways. PI3K in turn, phosphorylates Akt (327) and up-regulates the expression of Bcl-2, an anti-apoptotic protein that protects cells from a variety of toxic insults, including glutamate (328). The cytoprotective effect of Bcl-2 may be due to is ability to modify cell's Ca^{2+} homeostasis, particularly by reducing the depletion of Ca^{2+} from its endoplasmic reticulum store (329). However, no effect on these receptors was observed with both extracts. This lack of activity on nAChRs when cells were treated with the extracts could be explained by the fact that the concentrations of tomatine and tomatidine in "cherry" (304.1 nM and 4.4 nM, respectively) and "bull's heart" (176.8 and 2.6 nM, respectively) extracts were lower than the ones used in the assays with the compounds.

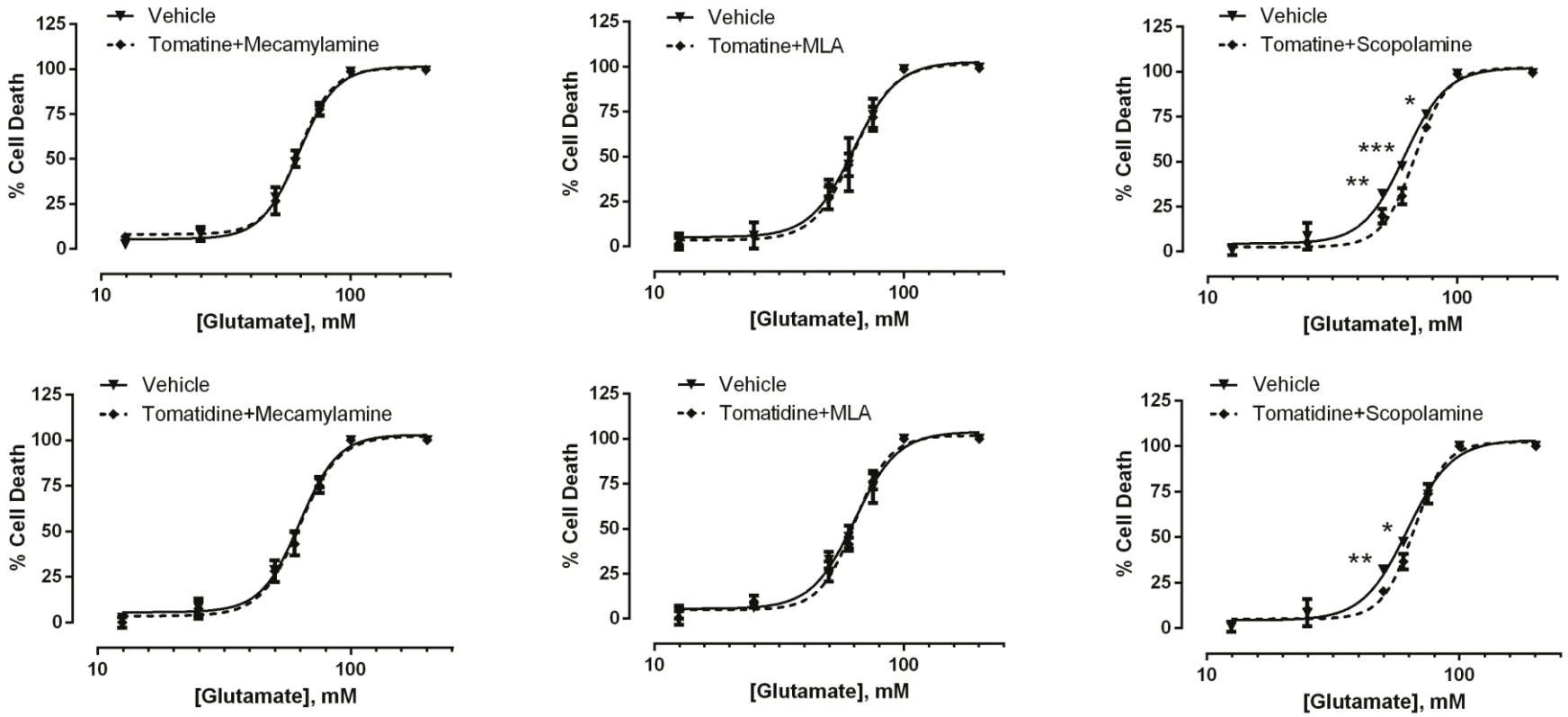


Figure 42. Glutamate concentration-cell death response curves (by MTT assay) with or without co-incubation with tomatine (1.59 µM) or tomatidine (126.25 µM) and receptors inhibitors (1 µM) mecamylamine, methyllycaconitine (MLA) or scopolamine. Results are presented as mean ± SEM from four independent experiments (triplicates were performed in each experiment). Concentration-response curves were fitted using least squares as the fitting method and the comparisons between curves (bottom, top and log LC_{50}) were made using the extra sum-of-squares F test. In all cases, p values <0.05 were considered statistically significant. *Compared to the same concentration of glutamate with or without tomatine plus receptor inhibitor (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Finally, when scopolamine (a muscarinic receptor antagonist) was used, the neuroprotective effect of the compounds (**Figures 39**) was maintained (**Figure 42**). The same result was observed for the leaves extracts (data not shown). Although the modulation of the M1 subtype of muscarinic receptors is important in reducing cognitive impairment in individuals suffering from AD, adverse side effects are observed when non-specific activation of M2 and M3 mAChRs occurs (330). The results obtained with SH-SY5Y cells, which mainly express the M3 muscarinic receptor (331), may indicate a lack of protection *via* muscarinic receptors.

An overview of the possible neuroprotective effect of steroidal alkaloids/extracts is presented in **Figure 43**.

In addition to these results, and since it is reported an association between AD and high LDL-cholesterol levels and inflammatory conditions, other preventive effects can be expected due to the cholesterol-lowering and anti-inflammatory ability of steroidal alkaloids (67). A decrease of the activity of cyclooxygenases and inhibition of prostaglandin synthesis can decrease reactive species generation and diminish NMDA-glutamate excitotoxicity (332).

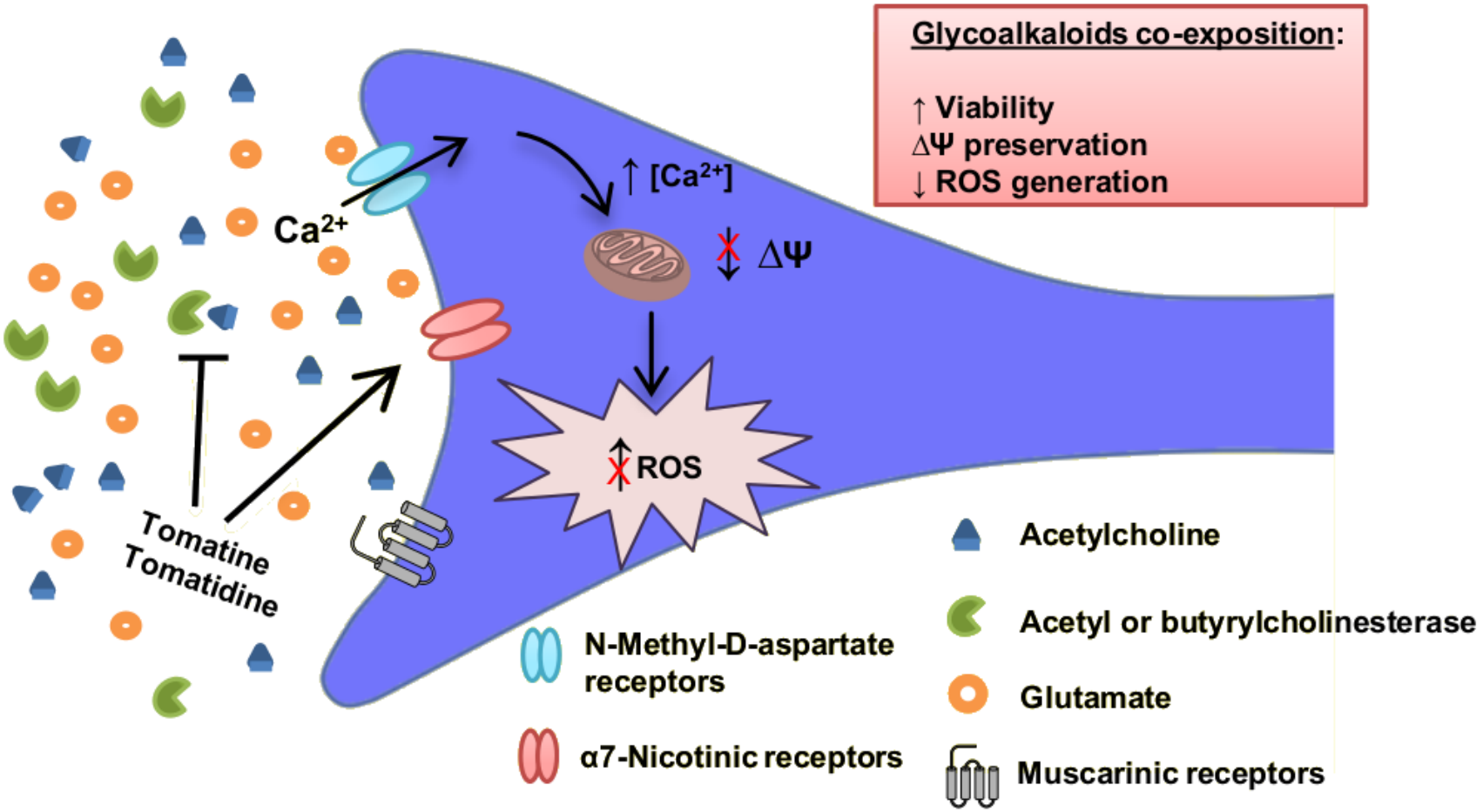


Figure 43. Neuroprotective effect of steroidal alkaloids: overview.

Chapter IV
Conclusions

Conclusions

With the work performed in this dissertation, the knowledge about the chemical composition and the biological potential of *L. esculentum* seeds and leaves, two common industrial by-products, was increased and new perspectives for their utilization were open. New insights into *S. littorlis*/*L. esculentum* ecological duo were provided. The main conclusions are indicated below.

***S. littoralis*/*L. esculentum* system:**

- The study, for the first time, of phenolic compounds and steroidal alkaloids fate revealed no phenolic compound in the *S. littoralis* materials, indicating that covalent bonds and cross-linking between them and amino acids/proteins, render them unusable by the digestive and absorption systems.

- *S. littoralis* showed the presence of steroidal alkaloids with a lower degree of glycosylation, which can constitute a possible mechanism of detoxification.

***L. esculentum* seeds:**

- Fourteen flavonoids were identified in the aqueous extract, including quercetin, kaempferol, and isorhamnetin derivatives, 13 of them being reported for the first time.

- The aqueous extract revealed to have antioxidant capacity, especially against $O_2^{\bullet -}$, ChE inhibition proprieties and cytotoxic activity against RBL-2H3 cell line.

- Chloroform, methanol, ethyl acetate, hexane and sulphuric acid extracts are active against Gram-positive bacteria and fungi, no significant differences being observed between them.

***L. esculentum* leaves:**

- Two simple and fast reversed-phase HPLC-DAD methods for qualitative and quantitative analysis of phenolics and steroidal alkaloids in *L. esculentum* leaves were successfully developed.

- Fifteen phenolic compounds were determined in *L. esculentum* leaves hydromethanolic extract, nine of them described for the first time.

- The hydromethanolic extract showed promising antioxidant properties against $DPPH^{\bullet}$, $^{\bullet}NO$ and $O_2^{\bullet-}$.

- Tomatine and the purified extracts rich in steroidal alkaloids showed AChE and BChE inhibition capacity, with higher selectivity for the latter. Additionally, both compounds and extracts revealed neuroprotective effects on glutamate-induced toxicity in SH-SY5Y neuroblastoma cells, without gastrointestinal toxicity, by preserving the mitochondrial membrane potential and reducing oxidative species.

- Tomatine and tomatidine showed capacity to interact with nicotinic receptors, namely those of α7 type. No effect on muscarinic receptors was observed.

- This dissertation demystifies the applicability of these steroidal alkaloids in therapeutics, by demonstrating that their toxicity was overestimated for long time. In addition, the neuroprotective effects observed are worthwhile exploring further and they may open new perspectives to the development of new AD-modifying agents.

Chapter V

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