
# Quality control of honey: research and evaluation of pyrrolizidine alkaloids and phenolic compounds

Dissertação do 2º Ciclo de Estudos conducente ao grau de Mestre em Controlo de Qualidade

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# Abstract

Honey is a nutritious food consumed worldwide, which has been used by man since ancient times. Over the years, this foodstuff has been recognized for its obvious health-promoting properties and, consequently, its consumption is associated with a healthy diet. Besides the beneficial bioactive compounds present in honey, such as phenolics, some molecules with potentially harmful effects can be found, like pyrrolizidine alkaloids. Pyrrolizidine alkaloids have been extensively reported in literature, due to the toxicity caused in humans and livestock happened after the consumption of products containing them. The fact that these alkaloids are distributed in plants throughout the world, frequently in species relevant for human consumption made this a wider concern.

In order to evaluate Portuguese monofloral and multifloral honeys produced from *Echium plantagineum* L., this work is focused in the search of pyrrolizidine alkaloids and phenolic compounds in honey samples from *Serra da Estrela*, *Serra d'Aire* and *Parque Natural do Montesinho*, following by the assessment of their potential anti-inflammatory activity in macrophages (RAW 264.7), their potential cytotoxicity in gastric adenocarcinoma cells (AGS) and of their safety in fetal lung human fibroblasts (MRC-5). As a complement in the evaluation of the samples, pollen identification was also conducted, with the aim of identify the botanical origin of the pollen present in the samples.

None of the honey samples revealed the presence of pyrrolizidine alkaloids. However, concerning phenolic compounds, they were detected although in low concentrations.

In a general way, the samples tested were not cytotoxic for the cell lines used, neither demonstrated appreciable biological activity. Furthermore, the study of the potential anti-inflammatory of the alkaloid- and phenolic-targeted extracts in a cellular model of RAW 264.7, revealed decreases of the radical nitric oxide production of ca. 30 and 40%, respectively.


**Keywords:** Honey; *Echium plantagineum* L.; pyrrolizidine alkaloids; phenolic compounds.

# Resumo

O mel é um alimento nutritivo, usado desde sempre pelo Homem e consumido em todo o mundo. Ao longo dos anos, este alimento tem sido reconhecido pelas suas propriedades terapêuticas evidentes e é, por essa razão, associado a uma alimentação saudável. Apesar dos compostos bioativos com propriedades benéficas que o mel possui na sua composição, como é o caso dos compostos fenólicos, este alimento também contém outro tipo de compostos que poderão exercer efeitos prejudiciais, os alcaloides pirrolizidínicos são um exemplo. Estes alcaloides têm sido alvo de crescente atenção na literatura, devido a episódios de toxicidade reportados em humanos e animais, após o consumo de produtos contendo estes compostos. O facto de estarem presentes em plantas de todo o mundo, inclusive em espécies usadas na alimentação humana e animal, torna este assunto uma preocupação global.

Esta dissertação recai sobre a análise de méis portugueses, monoflorais e multiflorais, produzidos a partir da planta *Echium plantagineum* L., focando-se na pesquisa de alcaloides pirrolizidínicos e compostos fenólicos em amostras de mel da Serra da Estrela, da Serra d'Aire e do Parque Natural do Montesinho. Posto isto, centra-se na avaliação do seu potencial anti-inflamatório, com recurso a ensaios biológicos em linhas celulares de macrófagos, da sua citotoxicidade em células do adenocarcinoma gástrico e ainda da sua segurança em fibroblastos de pulmão. Como complemento do estudo destas amostras e com o intuito de identificar a origem botânica do pólen presente nas mesmas, foi também efetuada uma análise polínica.

Das três amostras estudadas, nenhuma revelou a presença de alcaloides pirrolizidínicos. Contudo, relativamente aos compostos fenólicos, ainda que em baixas concentrações, estes foram detetados nas amostras.

De uma forma geral, as amostras estudadas não foram tóxicas nem revelaram uma atividade biológica apreciável nas linhas celulares testadas. Além disso, o estudo do potencial anti-inflamatório dos extratos direcionados para alcaloides e compostos fenólicos em macrófagos demonstrou diminuições da produção do radical óxido nítrico de aproximadamente 30 e 40%, respetivamente.

**Palavras-chave:** Mel; *Echium plantagineum* L.; alcaloides pirrolizidínicos; compostos fenólicos.

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# Abbreviations

ACh – acetylcholine

AChE – acetylcholinesterase

CYP 450 – cytochrome P450

COX-2 – cyclooxygenase-2

DAD – diode array detector

DHPA – dehydropyrrolizidine alkaloid(s)

DMEM – Dulbecco's Modified Eagle Medium

DMSO – dimethyl sulfoxide

DNA – deoxyribonucleic acid

EFSA – European Food Safety Authority

GSH - glutathione

FBS – fetal bovine serum

HIV – human immunodeficiency virus

HMF – 5-hydroxymethylfurfural

HPLC – high performance liquid chromatography

HSOS - hepatic sinusoidal obstruction syndrome

iNOS – inducible nitric oxide synthase

LC-MS – liquid chromatography-mass spectrometry

5-LOX – 5-lypoxygenase

LOD – limit of detection

LPS – lipopolysaccharide

MEM – Minimum Essential Medium

MOE – margin of exposure

MTT – N-(1-Naphtyl) ethylenediamine, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide

NADH – nicotinamide adenine dinucleotide

NADPH – dihydronicotinamide-adenine dinucleotide phosphate

$^{\bullet}NO$ – nitric oxide

$O_2^{\bullet-}$ – radical superoxide anion

$ONOO^-$ – peroxynitrite

PA – pyrrolizidine alkaloid(s)

PANO – pyrrolizidine alkaloids N-oxide(s)

RNS – reactive nitrogen species

ROS – reactive oxygen species

SPE – solid phase extraction

UV – ultraviolet

UV-Vis – ultraviolet-visible

VOD – veno-occlusive disease

# *Chapter 1*

# 1. Introduction

## 1.1. Honey

Honey-bees (*Apis melifera*) collect nectar from flowers to produce a natural nutritious product: honey. Enzymes, such as invertase, glucose oxidase and diastase, present in the bees' stomach convert polysaccharides into monosaccharides to produce this foodstuff (1). Honey is constituted by water (15-20%) and sugars (80-85%), mainly fructose and glucose. Other disaccharides have been identified: maltose, sucrose, maltulose, turanose, isomaltose, laminaribiose, nigerose, kojibiose, gentiobiose and α,β-trehalose; trisaccharides include maltotriose, erlose, melezitose, centose, isomaltosylglucose, l-kestose, isomaltotriose, panose, isopanose, and theanderose (2). Also other types of compounds, such as proteins, aroma compounds, amino and organic acids, minerals, 5-hydroxymethylfurfural (HMF), phenolic compounds and other phytochemicals are part of honey's composition, which depends on its floral, geographical and entomological sources (3,4). However, it is influenced by several other factors, such as seasonal, environmental, processing and storage time, and conditions (3).

Physicochemical parameters of honey, such as water, sugar, HMF, acidity, ash (mineral content), density, electrical conductivity, invertase activity and diastase level are used to evaluate the quality of honey (5). The level of water indicates the density, the extraction method, and it is also related with the maturity of honey (6). If the water content increases, the density decreases. Sucrose is not completely converted to glucose and fructose by invertase, so the level of sucrose should not be more than 5% (7). Invertase and diastase activities and HMF are quality indicators, which show freshness and overheating of honey (5). A higher level of HMF can indicate that honey was overheated, stored under poor conditions or that is an aged honey, being the recommended limit by the European Union of 40 mg/kg, with the following exceptions: 80 mg/kg for honey from countries with tropical temperatures and 15 mg/kg for honey with low enzymatic levels (8). Honey should be consumed within one year of storage (8). The acidity of the honey is related with the presence of organic acids, esters, lactones and inorganic ions of chloride and phosphate, and the values should be between pH 3.4 – 6.1 (6). These values allows to maintain the honey flavour and avoids the spoilage by microorganisms (5). When these values exceed the limit, probably honey sugars are suffering fermentation into organic acids (6). Besides

that, the high concentration of sugars combined with a low moisture content is the main reason for avoiding the spoilage by microorganisms, because of the osmotic stress caused (9).

The electrical conductivity allows the distinction of the botanical origin of honey, since this criterion correlates significantly with the mineral content that is a known parameter for this determination (5). Nowadays, this measurement is generally replaced by the electrical conductivity, as it is considered the most useful parameter for the determination of the botanical origin of monofloral honeys, and for performing the distinction between blossom and honeydew honeys (5,10). Electrical conductivity can also be linked to the acid content, since the electrical conductivity increases as this parameter rises (6).

Over the years, honey has revealed to exhibit interesting and broad-spectrum pharmacological properties, such as anti-inflammatory, antimicrobial, anticancer, antidiabetic and antimutagenic activities (11–15). Despite all the benefits honey can exert, it can also bring harmful consequences, due to the presence of some constituents, as an example, HMF, heavy metals and alkaloids (16,17).

## 1.2. Pollen

Pollen grains are male microgametophytes, which produce male gametes. When pollen lands on a compatible female cone, it germinates, forming a pollen tube that allows the transference of the sperm to the ovule (18).

Pollen can appear in honey by two different events: when the honeybee interacts with the flower for collecting the nectar, or by the wind, being the pollen grains transported to the hive (19). The microscopical analysis of honey allows to obtain information about its geographical and floral origins (18). Consequently, it enables the distinction between monofloral and multifloral honeys, as well as it allows to control the verity of the botanical origin declared in the honey package.

In the pollen grain, there are two recognized plans, polar and equatorial (18). The pollen grain exhibits a hard coat, namely sporoderm (18). The generative cells are surrounded by a delicate wall of cellulose, known as endospore and intine (18). The external wall, exine or exospore, is composed largely of sporopollenin, and often has spines or warts, or is variously sculptured. The character of these marks is frequently of value for the identification of a plant family, a genus, or even a species (18).

Pollen apertures are regions of the pollen grain, which correspond to a thin or modified region of exine, through which the pollen tube will grow at germination (18). There are several sorts of apertures, such as slit-like apertures – colpi – that are usually three, crossing the equator of the grain perpendicularly; sulcus is an elongated polar aperture. When the aperture receives the name pore, it means that it is more circular (18).

## 1.3. *Echium plantagineum* L.

The *Echium* genus, which belongs to Boraginaceae family, presents two distinctive nodes: the woody species of the Macaronesian archipelago and the herbaceous species distributed in Europe and North Africa (20). Iberian Peninsula is considered the main centre of species diversity with about sixteen species, as well as North-West Africa (particularly Morocco) with about twenty species (20).

*E. plantagineum* L., or commonly called Salvation Jane or Paterson's Curse, is a plant native of Iberian Peninsula that can be found throughout Mediterranean region. In Portugal, it is recognized as *Chupa-mel*, *Língua-de-vaca* or *Soagem*. However, it has been introduced in other countries, such as Australia (21,22). The spread in this country is supposed to be a consequence of the similar type of climate, to the drought tolerance and the high concentration of defensive secondary metabolites that it supports, PA (21). This plant usually appears in degenerate, dry or wet lands, such as cultivation fields, pastures and margins of roads and sands (23). *E. plantagineum* grows preferentially in soils rich in nitrogen, which were left empty by other plants, and it blooms between the months of March and July (24).

*E. plantagineum* is an herb with commonly 20-60 cm high, but can grow until 1.5 m high; annual or occasionally biennial, with erect or ascending stems, simple or branched in the top (20,25). However, it can be sporadically single-stemmed. Young plants form rosettes with basal leaves that can be up to 25 cm long, broadly ovate, petiolate, with prominent lateral veins (20). The calyx is 8-10 mm at anthesis and up to 15 mm in the fruit (20). The corolla measures 20-30 mm, and it is trumpet shaped, curled and purple (20). The flowers consist in five petals with 2-3 cm long five stamens (20). This plant exhibits pollen of dark blue colour and a fruit composed by four nutlets, tuberculate, pale brownish-grey that is surrounded by persistent stiffly bristled calyx (20).

## 1.4. Alkaloids

The item 1.4 of the section “Introduction” will be presented as a scientific paper published in *International Journal of Molecular Sciences*

# *Pyrrolizidine Alkaloids: Chemistry, Pharmacology, Toxicology and Food Safety*

### 1.4.1. Introduction

Alkaloids are a diverse group of amino acid-derived and nitrogen-bearing molecules that display a wide range of roles in nature, where they occur in plants, microorganisms or animals [26]. In plants, alkaloids can be found in the form of salts of organic acids, mainly malate, acetate and citrate, or combined with other molecules, such as tannins [26]. Most alkaloids display basic properties and present a lipophilic character, being soluble in apolar organic solvents and alcohol [26].

Several alkaloids are known for their remarkable biological properties, which can be either marked toxicity or potent pharmacological capacity [27]. The class of alkaloids has a long history of use, both lawful and illicit, as pharmaceuticals, stimulants and narcotics [28].

Within the many known families of alkaloids, pyrrolizidine alkaloids (PA) have been receiving increasing attention due to their occurrence in several species relevant for human and animal nutrition, as well as for their toxicological and pharmacological properties. The increasing awareness of PA contamination in all sorts of foodstuff worldwide justifies the interest and concern around this topic. Although in most cases their levels are insufficient to cause acute poisoning, they are frequently consumed in quantities that exceed the maximum daily intake suggested by authorities, which can be a contributory factor to chronic diseases.

This work reviews the available information on PA, mainly regarding their chemistry, toxic and pharmacological properties, and food safety.

### 1.4.2. The Chemistry of PA

PA are a group of alkaloids derived from ornithine that are distributed in plants of certain taxa, being also found in insects that uptake them for defense against predators [26,29]. They rarely occur in the free form as a pyrrolizidine base, being instead found as esters (mono-, di- or macrocyclic diesters) formed by a necine base (amino alcohols) and one or more necic acids (mono- or dicarboxylic aliphatic acids), which are responsible for their structural diversity [26,30]. They are usually found in the form of tertiary bases or pyrrolizidine alkaloids *N*-oxides (PANO) (Figure 1) [26,30,31].



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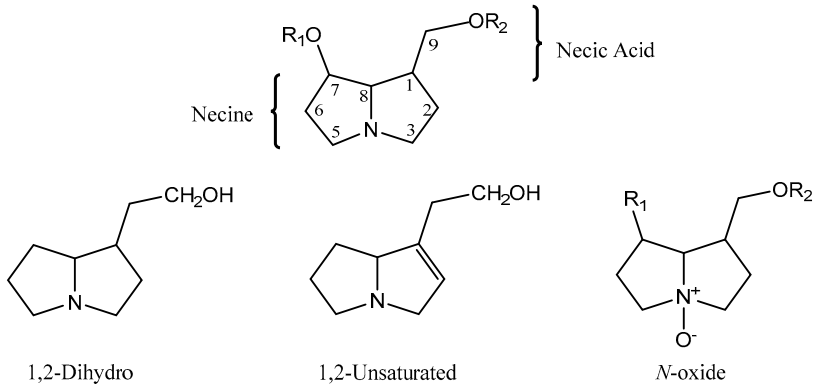


**Figure 1.** Structure of a PA and its different forms. $R_1$ and $R_2$ correspond to different necic acids.

Amino alcohols, or necines, are derived from pyrrolizidine. The pyrrolizidine core, comprising two saturated five-membered rings with a nitrogen atom between them, sometimes displays a double bond in the 1,2 position, which frequently results in enhanced toxicity [32]. They can also have a single alcohol at C-1, a second alcohol in position C-7 (di-hydroxylated) and less often a third in C-2 or C-6 (tri-hydroxylated) [33-35] . Esterification can take place at C-7 and/or C-9 positions [34].

According to the structure of the necine base, PA may be sorted into four groups: retronecine-, heliotridine-, otonecine- and platynecine-types (Figure 2) [36] . Retronecine-, otonecine- and heliotridine-types are unsaturated bases, while platynecine-type is saturated [37,38]. From a structural point of view, otonecine is the most distinct among all types, since it is oxidized at C-8 and displays a monocyclic ring, thus diverging from the other groups, which display a bicyclic ring [33,35,39] . Retronecine and heliotridine are diastereomers, with distinct orientation at position C-7 [40].

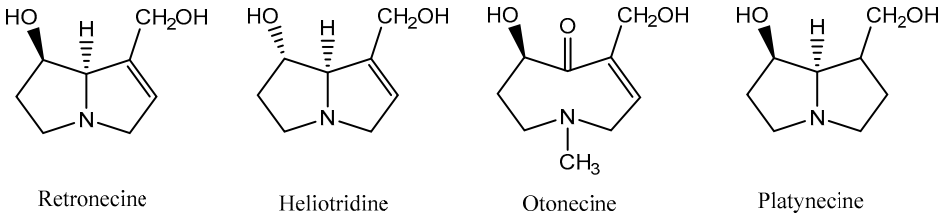


**Figure 2.** Groups of PA, according to the necine base.

Necic acids are aliphatic carboxylic acids that can be simple (angelic and tiglic acids), monocarboxylic acids with hydroxyisopropylbutanoic structures at C-7 (trachelantic and viridifloric acids) or dicarboxylic acids at C-8 or at C-10 (senecic and isatinecic acids) (Figure 3)[26].

The combination of the above-mentioned structures results in mono- or di-esters. Within the monocarboxylic acids, characteristic of the Boraginaceae family, some have a hydroxyl group at C-9 esterified by a hydroxyisopropylbutanoic acid, such as intermedine (Figure 4)[26]. In cases where there is a second necic acid, it usually occurs in the hydroxyl group of C-7, in the form of angelic acid or tiglic acid, as in echimidine (Figure 5)[26]. Macrocyclic diesters, characteristic from Asteraceae family, have also been described, which correspond to C-7 and C-9 esterified by a dicarboxylic acid (Figure 6)[26]. Unusually, necines may be esterified with aromatic or arylalkyl acids [26].

According to the most widely accepted pathway, the biosynthesis of the pyrrolizidine core begins with a $NAD^+$-dependent condensation of two molecules of putrescine. It should be highlighted that this initial step is disputed by some authors, which advocate the involvement of one molecule of putrescine and one molecule of spermidine, the latter providing the aminobutyl group [41-43] . Interestingly, it could be the case that both theories are correct, as suggested by the finding that bacterial homospermidine synthase is able to accept either putrescine and spermidine as a substrate [44] .

Regardless of the initial step, in both cases the reaction is catalyzed by homospermidine synthase and the result is the symmetrical intermediate homospermidine [26]. Subsequently, homospermidine is cyclized to the corresponding iminium ion, which is reduced and cyclized to trachelanthamidine and isoretronecanole (Figure 7A) [45] .

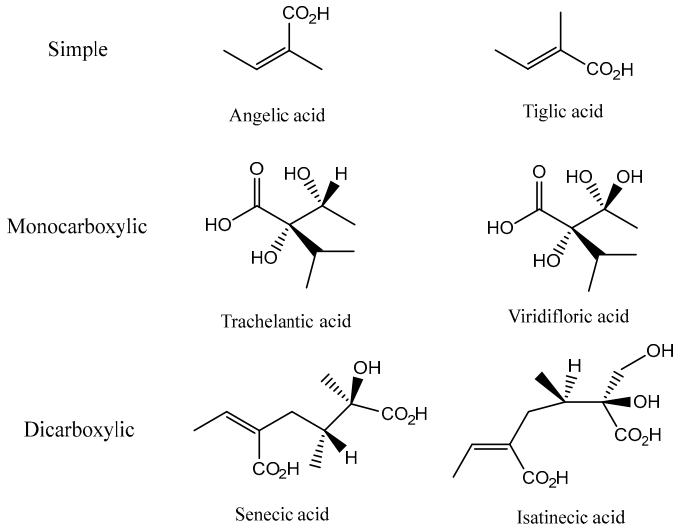


**Figure 3.** Chemical structures of necic acids.

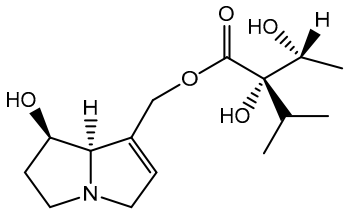

**Figure 4.** Chemical structure of intermedine, an ester of monocarboxylic acid.

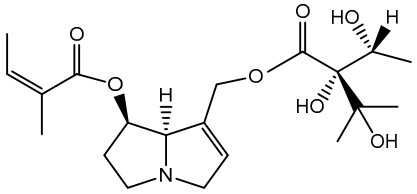

**Figure 5.** Chemical structure of echimidine, a diester of monocarboxylic acid.

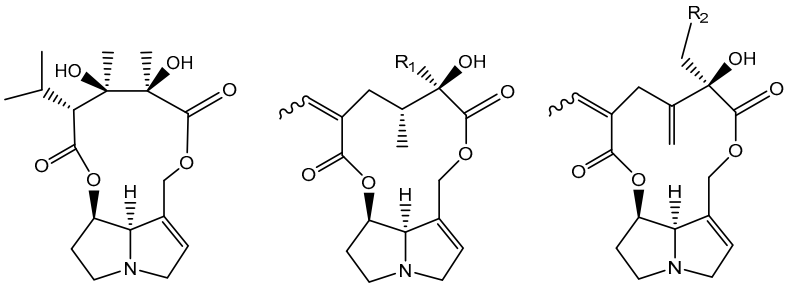

**Figure 6.** Chemical structure of tricodesmine, senecionine ($R_1$ = H)/retrorsine ($R_1$ = OH) and seneciphylline ($R_2$ = H)/riddeline ($R_2$ = OH), macrocylic diesters.

Regarding necic acids, they are mostly derived from L-valine, L-leucine, L-isoleucine and L-threonine [45].

The formation of monocarboxylic acids with five carbon atoms, such as angelic, tiglic and sarracinic acid, takes place through the metabolization of threonine, which in turn proceeds from via α-ketobutyric acid, also called 2-oxobutanoic acid. The interaction between this compound and pyruvate yields isoleucine [45].

With respect to senecioic, viridifloric and trachelanthic acids, the precursor involved is valine, which suffers a conversion into these necic acids, via an acyloin reaction with activated acetaldehyde [45].

In the case of dicarboxylic acids such as senecic acid, with ten carbon atoms (Figure 7B), cyclization of the open-chain monocarboxylic acid diesters takes place [45]. The biosynthesis occurs in the roots, where they are formed as PANO [46]. Afterwards, due to their high solubility in water, they are easily transported to the aerial parts so they can be stored in cell vacuoles [46].

Concerning the chemical synthesis, there has been an immense amount of research conducted on the partial and total synthesis of numerous naturally-occurring PA and related non-natural analogues [47,48].

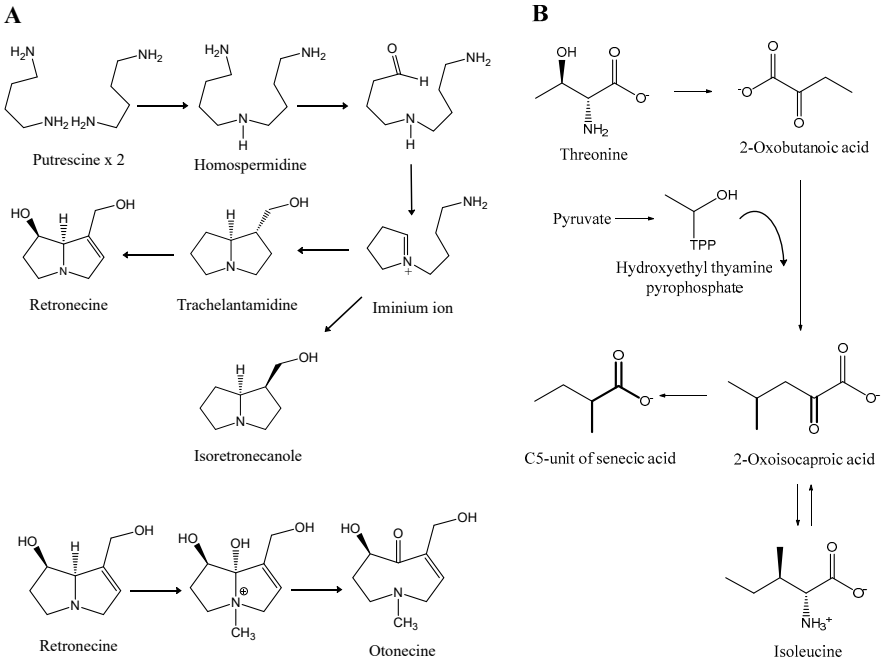


**Figure 7.** Biosynthesis of necines (**A**) and of senecic acid (**B**). Adapted from [26,41,45,49].

### 1.4.3. **Biological Activity of PA**

#### *1.4.3.1. Pharmacokinetics*

Concerning PA pharmacokinetics, after oral ingestion these compounds are absorbed from the gastrointestinal tract [50]. Most of them, around 80%, are excreted in urine, feces and milk, a few being able to pass the placenta due to their high lipophilicity [50-52]. Bioactivation occurs mostly in the liver and, for this reason, this organ is the most affected by toxicity [53]. Other organs have been identified as targets, namely the lungs and kidneys [54]. The lung is the second most affected organ by the pyrroles formed after metabolic activation in the liver, since they can travel to the lungs through blood [54]. For PA to be excreted or exert toxicity, as with many xenobiotics, biotransformation must occur.

There are three principal pathways for the metabolic activation of PA, namely hydrolysis to produce necines and necic acids, *N*-oxidation to form PANO, and oxidation that leads to the formation

of pyrrolic esters or dehydropyrrolizidine alkaloids (DHPA). Hydrolysis is an important detoxification route, promoting the clearance of these compounds [39], as well as *N*-oxidation, which allows the formation of PANO that can be conjugated for excretion. However, PANO can reverse back into PA and suffer oxidation into DHPA [54]. This route is carried out mostly by cytochrome P-450 (CYP450) monooxygenases. In fact, the activity of these enzymes can partly explain the distinct susceptibility of different species to PA [54]. The isoforms of CYP450 involved in the metabolism leading to DHPA are generally CYP3A and CYP2B [55]. In the case of hydrolysis, liver microsomal carboxylesterases are involved [55]. However, only retronecine-type and heliotridine-type PA are capable of suffering *N*-oxidation, otonecine-type PA being unable to generate PANO owing to their methylation in the nitrogen [39].

The balance between the formation of DHPA and the formation of detoxification compounds, such as necines, necic acids and PANO, is also important in explaining the distinct susceptibility of different species to these compounds [56].

The formation of DHPA happens through hydroxylation of the necine base at C-3 and C-8 positions, in the specific case of retronecine- and heliotridine-types [39]. In otonecine-type, an oxidative *N*-demethylation is necessary [39]. After these highly reactive metabolites are formed, they can bind to glutathione (GSH) to form GSH conjugates and in doing so, they can be eliminated [57], which is the reason that conjugation to GSH is considered a detoxification route [57]. In the same way, pyrrolic esters can bind to proteins and deoxyribonucleic acid (DNA) and, consequently, they can form adducts. These metabolites can also suffer hydrolysis and be transformed in dehydronecines, which are also toxic metabolites, but are less reactive than the previously mentioned form [57]. Figure 8 illustrates the metabolism of PA.

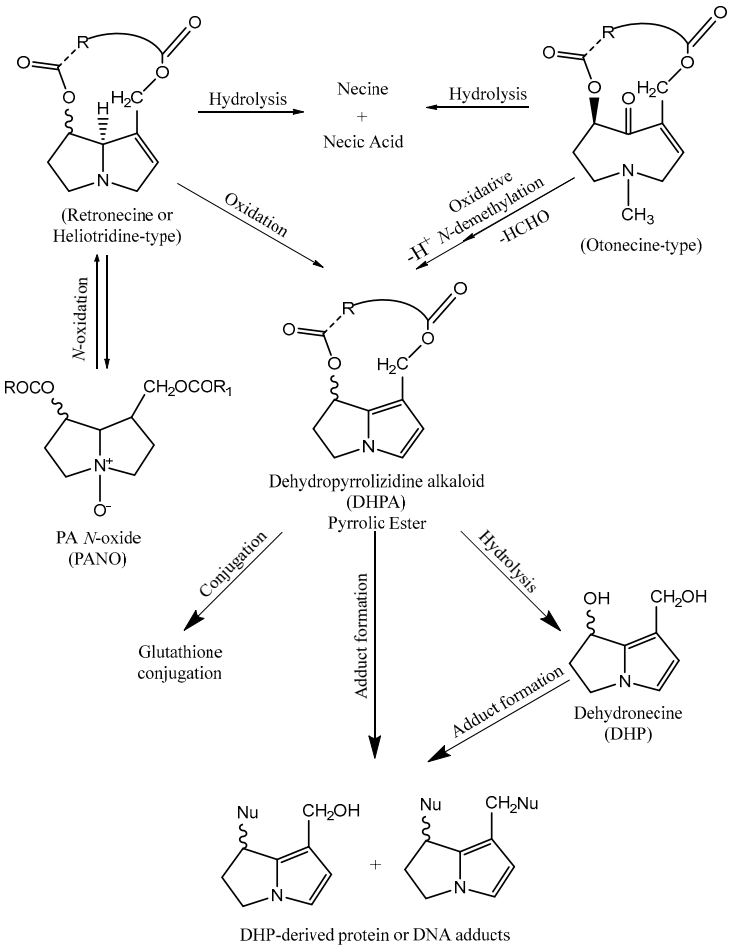


**Figure 8.** Metabolism of PA.

*1.4.3.2. Pharmacological Properties*

Despite the toxicity described in some experimental models, which will be discussed later, PA exhibit an interesting spectrum of biological properties, which can be exploited in drug discovery programs.

1.4.3.2.1. Anti-Microbial Activity

Many alkaloids have been described as effective anti-microbials, which is in line with the defensive role of this class of secondary metabolites in plants [58].

In the specific case of PA, the anti-microbial activity of usaramine, monocrotaline and azido-retronecine against some bacteria has been demonstrated [59]. Usaramine was analyzed concerning its ability to inhibit biofilm formation in *Staphylococcus epidermidis* and *Pseudomonas aeruginosa*. Although the mechanism of action of usaramine remained unclear, it was possible to observe that it prevented the formation of biofilm by *S. epidermis* by about 50% at 1 mg/mL. However, no effect was detected in the formation of biofilm by *P. aeruginosa*. Furthermore, monocrotaline and azido-retronecine demonstrated anti-*Trichomonas vaginalis* activity (concentrations up to 1 mg/mL), being lethal to 70% and 85% of bacterial cells, respectively, while was devoid of toxicity towards *T. vaginalis*. Interestingly, no detectable damage in vaginal epithelial cells was found, a selectivity trait that may be relevant for the development of new drugs, such as topic anti-microbial agents.

In another study, the effects of PA from *Senecio jacobaea* L. were investigated for their effect on the growth of nine plant-associated fungi (five strains of *Fusarium oxysporum*, two of *Fusarium sambucinum* and two of *Trichoderma* sp.) [60]. A PA mixture consisting of senecionine (12%), seneciphylline (22%), jacobine (24%) and jaconine (24%) was highly effective, however high concentrations were required, the effective range of each individual PA varying from 0.33 mM to 3.33 mM, the most sensitive fungus belonging to the *Trichoderma* genus.

1.4.3.2.2. Anti-Inflammatory Activity

The inflammatory process is a physiological response of the body in order to eliminate, neutralize and/or destroy *stimuli* resulting from infection or tissue damage [61].

In inflammatory processes, the upregulation of inducible nitric oxide synthase as a consequence of pro-inflammatory mediators, such as cytokines, results in increased levels of nitric oxide (·NO), which plays an important role as a mediator in the inflammatory response [62]. Therefore, the regulation of its production in tissues may be important for the treatment of inflammation.

In a study by Huang et al., six new PA and two that were already known were isolated from *Liparis nervosa* (Thunb.) Lindl. and evaluated for their inhibitory capacity towards ·NO production by lipopolysaccharide (LPS)-challenged RAW 264.7 macrophages. The new molecules tested were nervosine I, nervosine II, nervosine III, nervosine IV, nervosine V, nervosine VI, and the previously-described PA were lindelofidine and labumine. Overall, all molecules were effective in this model, with $IC_{50}$ values ranging from 2.16 to 38.25 μM [63].

Another study with the same cell line led to the conclusion that PA present in an ethanol extract of the plant *Heliotropium digynum* (Forssk.) C. Chr inhibited the production of ·NO by 78% at 25 μg/mL [64]. In this work, the $IC_{50}$ values found for heliotrine, heliotrine *N*-oxide, 7-angelyolsincamidine *N*-oxide and europine were 52.4, 85.1, 105.1 and 7.9 μM, respectively.

Crotalaburnine was evaluated for its activity against increased vascular permeability and oedema induced by formaline, carrageenin, 5-hydroxytryptamine, dextran, bradykinin and prostaglandin [65]. This alkaloid was also tested against the formation of granulation tissues by cotton-pellet in rats. Its effects were compared with the activity of different compounds known for their anti-inflammatory properties, such as hydrocortisone [65]. Results showed that this PA was only efficient against acute

edema induced by carrageenin and hyaluronidase, with a dose of 10 mg/kg [65] . In the cotton-pellet granuloma test it was shown that crotalaburnine was two times more potent than hydrocortisone [65] .

#### 1.4.3.2.3. Anti-Cancer Activity

In 1992, researchers in the area of pediatric cancer treated 31 children with acute lymphoblastic leukemia with indicine-*N*-oxide at two dose levels (2000 mg/m$^2$/day and 2500 mg/m$^2$/day) for 5 consecutive days [66] . Among the 12 patients treated with 2000 mg/m$^2$/day, 1 achieved a complete response after 6 months. On the other hand, of the 16 patients treated with 2500 mg/m$^2$/day, 1 reached a similar response after 1 month. The patient with chronic myelogenous leukemia displayed a partial response in 4 months. These results suggested that indicine-*N*-oxide is active in the treatment of acute lymphoblastic leukemia of children. However, it has a narrow therapeutic index and a very steep dose response curve. At the doses tested, mild acute hepatotoxicity was registered. However, the administration of doses $\geq$3000 mg/m$^2$/day for 5 days caused severe hepatotoxicity. Another study involving patients with ages between 4 and 67 years confirmed that indicine-*N*-oxide can induce remissions in cases of acute and chronic leukaemia at the concentration of 3000 mg/m$^2$ administered daily for 5 days. In this study, only 1 out of 22 cycles of treatment resulted in liver failure [67] .

In a study using different human cancer cell lines (cervical, breast, prostate and cervical squamous) indicine *N*-oxide from *Heliotropium indicum* L. inhibited the proliferation of the previous referred cancer cell lines, with $IC_{50}$ values ranging from 46 to 100 μM [68] . At these concentrations, cell cycle arrest at mitosis was detected, without noticeable changes in the organization of the spindle or interphase microtubules.

#### 1.4.3.2.4. Anti-HIV Activity

Polyhydroxylated PA have been described as capable of interacting with human immunodeficiency virus (HIV) activity [69] . Australine and alexine, isolated from *Castanospermum australe* A. Cunn. & C. Fraser ex Hook and *Alexa Leiopetela* Sandwith, are examples of these polyhydroxylated PA that in concentrations between 0.1 and 10 mM inhibited, in distinct degrees, the activity of glycosidases, particularly the nitrogen-linked glycosylation process of HIV [69] . This event ultimately results in reduced cell fusion with the virions and, consequently, restricted syncytium formation [70] .

A study from Taylor et al. with alexine and other four PA isolated from *A. leiopetala* and *C. australe*, respectively, also showed inhibitory activity against HIV-1 [71] . The positive results were obtained with 7,7a-diepialexine and an $IC_{50}$ of 0.38 mM was found. This anti-HIV activity was correlated with the inhibition of pig kidney α-glucosidase 1 and the diminished cleavage of the precursor HIV-1 glycoprotein gp160.

#### 1.4.3.2.5. Acetylcholinesterase Inhibitors

Acetylcholinesterase (AchE) is an enzyme that catalyzes the hydrolysis of acetylcholine (ACh) and other esters that act as neurotransmitters [72] . It plays an important role in neural function and it is mainly present in the synaptic gaps of central and peripheral nervous system, being responsible for terminate nerve impulses [72] . Overstimulation of ACh receptors can lead to disorders like depression. However, when present in low amounts, other diseases can manifest, namely Alzheimer and *Myasthenia gravis* [72,73] . For this reason, inhibitors of this enzyme are exploited as therapeutic targets [72] .

Benamar et al. isolated four PA from *Solenanthus lanatus* DC., including a new one named 7-*O*-angeloylechinatine-*N*-oxide, together with 3′-*O*-acetylheliosupine-*N*-oxide, heliosupine-*N*-oxide, and heliosupine [74] . All of these compounds inhibited AChE, with $IC_{50}$ values between 0.53 and 0.60 mM. A more recent study, from the same author, with 7-*O*-angeloyllycopsamine-*N*-oxide, echimidine-*N*-oxide, echimidine, and 7-*O*-angeloylretronecine isolated from *Echium confusum* Coincy showed the inhibition of AChE, with $IC_{50}$ values ranging from 0.275 to 0.769 mM [75] .

#### 1.4.3.2.6. Miscellaneous

A work with the leaves and inflorescences from *Senecio brasiliensis* (Spreng.) Less., performed by Toma et al. on mice and rats, shed a light on the possible use of PA in the treatment of ulcerogenic disease and stomach pain [76]. The therapeutic doses of PA were assessed by the administration of hydrochloric acid/ethanol to induce gastric ulcer. It was possible to perceive that the extent of the lesion induced was significantly reduced by 32.9%, 42.5% and 66.8% with concentrations of 12.5, 25 and 50 mg/kg of PA extract (containing senecionine, integerrimine, retrorsine, usaramine and seneciphylline), respectively. In the same work, a dose of 12.5 mg/kg of the same PA extract was shown to ameliorate nonsteroidal anti-inflammatory drugs-induced gastric ulcer [76].

### 1.4.4. **Toxicity**

The toxicity of PA is largely documented [77,78], being almost exclusively associated to their metabolites.

In 1968, Mattocks introduced what is now considered the main mechanism responsible for the toxicity of PA, namely the binding of DHPA with groups containing sulphur, nitrogen and oxygen present in proteins, to form adducts, such as 2,3-dihydro-1H-pyrrolizineprotein [78], mainly in the site of formation [54]. Pyrroles can also penetrate the nucleus and react with DNA, ultimately causing DNA cross-links and DNA-protein cross-links with abnormal functions, which will be the cause of damage, mainly in the hepatocyte. They can pass to the adjacent Dissé space and into the sinusoidal lumen, where they attack sinusoidal cells [54]. The injury caused by the toxic metabolites in hepatocytes and in the walls of hepatic veins, for example, is what leads to veno-occlusive disease (VOD), called nowadays hepatic sinusoidal obstruction syndrome (HSOS) [54].

After that, several studies have been conducted to add to the knowledge of this toxicity mechanism. In a study by Lin et al., serum protein adducts were detected in a PA-induced HSOS patient for the first time [79]. The authors developed an analytical approach based on liquid chromatography-mass spectrometry (LC-MS) to study these adducts and have concluded that pyrrole-protein adducts could be potential biomarkers of PA-induced HSOS. In this specific study, the observed HSOS were confirmed to arise from the consumption of a PA-containing plant, *Gynura segetum* (L.) DC. Another study with PA-induced liver injury led to the conclusion that pyrrole-protein adducts were present in the blood of all the patients, further strengthening the case for their use as biomarkers for this kind of liver injury [80].

A study by Zhu et al. showed that these adducts can also be used as a biomarker of liver tumor formation [81]. As a result, they decided to carry a study to clarify the basic kinetics of PA-derived DNA adducts, namely their persistence in vivo. The conclusion was that they can be used to monitoring or predicting chronic liver diseases, since DHPA-derived DNA adducts have sufficient stability and persistence. In the single-dose exposure, the PA-derived DNA adducts exhibited dose-dependent linearity and persisted for up to 4 weeks. Following multiple dose treatment, they persisted more than 8 weeks. In addition, they exhibit correlation with the progression of liver damage caused. Another group achieved the same conclusion, with five hepatocarcinogenic PA (lasiocarpine, retrorsine, riddelliine, monocrotaline and heliotrine) and their corresponding PANO [82]. All of them being able to produce DNA adducts, through rat liver microsomal metabolism.

#### *1.4.4.1. Acute and Chronic Intoxications*

As previously mentioned, the liver is the main target of toxicity caused by PA, mainly because bioactivation occurs mostly in this organ. VOD is the clinical manifestation most frequently found, being considered a marker for PA intoxication [39]. The symptoms include vomiting, enlargement of the liver and bleeding diarrhea [39].

PA intoxication can be acute, sub-acute and chronic, each of them presenting different symptoms. Acute intoxication is characterized by hemorrhagic necrosis, hepatomegaly and ascites; in sub-acute

there is a blockage of hepatic veins, which leads to HSOS (primary sinusoidal damage and parenchymal cell dysfunction [83]) [84]. Chronic PA exposure is characterized by necrosis, fibrosis, cirrhosis and proliferation of the bile duct epithelium [85,86]; liver failure and death is the highest level of this toxicity [84].

*1.4.4.2. Genotoxicity and Tumourigenicity*

In 1954, Schoental et al. discovered that retrorsine was capable of inducing tumors in experimental studies in animals [55,87]. Tumors developed in liver, lung, bladder, skin, brain, spinal cord, pancreas and gastrointestinal tract were found [50]. All PA known to have this effect belong to heliotridine-, retronecine- and otonecine-types.

The mechanism responsible for the formation of tumors was clarified by Yang et al., which established that riddelline (retronecine-type) form DNA adducts, in the form of DHPA [88]. In addition, it was also demonstrated by other authors that the levels of DNA adducts induced by DHPA were associated with the appearance of tumors, so they can be used as biomarkers of the tumourigenicity caused by PA [89]. Besides the formation of DNA adducts, these compounds can also react with proteins and trigger DNA cross-linking, sister chromatid exchange and chromosomal aberrations [34,39,90].

Furthermore, PA were associated to skin cancer, since they can lead to photosensitization in animals upon their consumption and metabolism [91]. It is thought that phylloerythrin, a porphyrin derived from the damage of chlorophyll by microorganisms present in gastrointestinal tract, passes to the circulation and is excreted by the liver into the bile. However, a PA-damaged liver is unable to eliminate phylloerythrin, resulting in its accumulation in the blood and skin. In this case, when phylloerythrin is exposed to sunlight, the resulting metabolites can cause oxidative stress and lipid peroxidation in skin tissues and ultimately trigger the formation of tumors [91].

We were unable to find any reports of cancer cases in humans as a direct consequence of PA consumption. However, it has been shown before that the metabolism of riddelline in human liver microsomes is similar to that of rodents, including the formation of DNA adducts [92]. Since this PA induces liver tumors in rodents via formation of DNA adducts, it is plausible to conclude that this PA may also be genotoxic and tumorigenic to humans [92]. In fact, the National Toxicology Program in the United States has declared that riddelliine is "*reasonably anticipated to be a human carcinogen*" [93].

The potential role of PA in diseases such as cancer, pulmonary hypertension, congenital anomalies and liver diseases has been reviewed before [94]. These alkaloids are genotoxic and can slowly initiate diseases of this sort, which is problematic because clinicians are unware of PA dietary exposure. The authors defined six indicators that can suggest a dietary dehydroPA etiology, appointing, for example: "cirrhosis, especially if associated with HSOS and/or accumulation of copper in the liver" and "cancers and/or congenital anomalies where there is evidence of overt or asymptomatic HSOS, pulmonary arterial hypertension (PAH), bone deformities, or immunological deficiencies". If several of these indicators are present, the authors affirm that it is possible that a dietary exposure to PA is involved in the disease etiology.

*1.4.4.3. Other Types of Toxicity*

Lungs can also be a target of injury, since DHPA can travel from the liver into pulmonary arterioles, producing damage similar to the VOD [95]. After reaching this organ, thrombi in vessels and thickening in their walls leads to occlusion and inflammation [54]. Overall, the combination of these phenomena ultimately trigger pulmonary hypertension and subsequent congestive heart failure [54]. In a study by Culvenor et al. carried out on hooded Wistar rats, it was demonstrated that PA can elicit lung lesions, as result from low-level (0.025 mmoles/kg body weight) and long-term exposure to PA [96]. Two types of lung lesions were observed: intravascular accumulation of mononuclear cells ultimately resulting in venous occlusion, and extravascular alteration, in which the alveolar septa were thickened,

and the number of cells increased. The authors also concluded that rats developing lung lesions always presented chronic liver lesions.

Neurotoxicity was also reported as a part of the poisoning by these substances, particularly by tricodesmine, including symptoms like encephalitis, characterized by vertigo and headaches, which could progress to delirium and loss of consciousness [95] . At the central nervous system, necrotic lesions have been described [97] .

There are also reports of teratogenicity in the literature, justified by the fact that some PA can pass the placenta, as referred above. For example, a case of hepatic VOD in a newborn of a woman who consumed herbal tea prepared from *Tussilago farfara* L. was described [98] . Also, in Australia, the consumption of *Senecio madagascariensis* Poir. by a mare was reported to lead to hepatic failure in a foal of two months [99] .

A study with clivorine isolated from *Ligularia hodgsonii* Hook, in concentrations between 10 and 100 μM, showed that this PA can induce DNA fragmentation, compatible with apoptosis, in human foetal hepatocyte line and mouse hepatocytes, with $IC_{50}$ of 40.8 μM[100,101].

#### *1.4.4.4. Chemical and Biological Aspects That Influence the Toxicological Profile*

The structural basis for the toxicological effects of PA have been described in some works. The presence of the 1,2 double bond, as found in retronecine-, heliotridine- and otonecine-types, has been associated with the toxic effects of PA [77], as well as the presence of one or two hydroxyl groups attached to the pyrrole ring [54] . Several studies also suggest that the presence of a methyl group at C-1 is relevant, as is the presence of two esterified groups and branching in at least one of the carboxylic acids [54] . For this reason, PA that exert the highest toxicity are cyclic diesters, monoesters being the ones that cause the lowest level of injuriousness; between them are the open-chain diesters, which cause an intermediary toxicity [50] . The existence of relationship between the esterification level and the toxicity has been suggested, as, for example, macrocyclic DHPA were revealed to be more toxic than open chain diesters [53] .

Toxicity of PA can be influenced by age and gender, since members of masculine sex are a group of risk, as well as children and fetuses, which are the most vulnerable group . There are also toxicological differences between distinct PA within a species and of the same PA in different species [102].

PA poisoning is exacerbated with bacteria and metals. A study from Yee et al. showed that the simultaneous exposure to low doses of monocrotaline, which would not normally cause damage, and LPS elicited hepatotoxicity [103]. In this case, centrilobular and midzonal liver lesions were registered. Aston et al. studied the impact of a copper-rich diet in PA toxicity [104]. The results showed that retrorsine and copper together led to a more serious liver damage than retrorsine alone, a result that was confirmed in another work [105].

### 1.4.5. **Human and Animal Consumption of PA**

#### *1.4.5.1. Legal Framework*

With the increasing consumption of herbal medicines, PA poisoning has begun to be regarded as a public health problem. Consequently, some countries established regulations about PA in foodstuff. In the United States of America, the Food and Drug Administration ordered the ban of all PA-containing comfrey preparations from the market [106]. The German Federal Department of Health restricted the use of these preparations to 6 weeks and in a level of less than 1 μg/day; if the use was prolonged in time, the daily limit should be reduced to 0.1 μg [107]. Another regulation implemented was the labeling of these products with the following statement: "*Not to be used in pregnancy and during the lactation period*.", due to the susceptibility of fetuses and children to diseases instigated by PA [107,108]. In the European Union, the European Food Safety Authority (EFSA) determined that the ingestion of toxic PA induces VOD and that they have carcinogenic effects in rodents [109]. In 2011, EFSA concluded

that no tolerable daily intake could be established. They followed the margin of exposure (MOE) approach, a "ratio of two factors, which assesses for a given population the dose at which a small but measurable adverse effect is first observed and the level of exposure to the substance considered". The MOE defined was of 1:10,000 for an exposure of 7 ng/kg of body weight per day. As an example, for a 70 kg individual, this corresponds to a daily exposure of approximately 500 ng of PA [110]. The European Medicines Agency, based on toxicological considerations and the available guidelines for assessment/management of genotoxic carcinogens, also showed concern about the hazards of PA, recommending a maximum daily intake of 0.35 μg PA/day for a person with a body weight of 50 kg and life-long exposure [111]. Austria excluded all products with PA from the market, and in the Netherlands, all foodstuff, herbal preparations, and extracts of plants known to have PA were limited to 1 μg/kg or 1 μg/L in the ending product [112].

Risk assessments of PA are based on animal studies and, for this reason, different approaches were suggested to translate animal doses to human exposure risks. Guidance documents have been developed taking into account differences between species that can influence the toxicity, namely the metabolic pathways [113,114]. Some groups reviewed the relevance of animal models to predict the effects of PA in humans [115]. The findings highlight that direct comparison between animal and human results is not always possible. For example, the PA-induced tumourigenicity previously reported for animals has not, to this day, been demonstrated in humans. Anyway, it is still an open question whether the differences between species should exclude the results in animals for quantitative risk assessment in humans [116].

As extracted from the conclusions drawn by the several risk assessment authorities, there is no consensus in the PA daily intake limit, although they all concur that PA are a class of undesirable compounds in food. For this reason, quality control of foodstuffs is pivotal and can be important for establishing legally binding limits. The first step should be the choice of an appropriate and universal analytical method for PA, as it was requested by EMA to the European Pharmacopeia [116]. As far as we could determine, this is being undertaken at the moment [117].

#### *1.4.5.2. Data from Literature*

Due to the presence of PA in several species relevant for human and animal nutrition, they may pose a threat to human health through their presence in herbal teas, herbal medicines, dietary supplements, vegetables, cereals, wheat grain, honey and pollen [118-123]. Cases of intoxication by contaminated cereals, teas, and salads have been extensively reported [122,124,125].

In 1903, it was recognized by Gilruth that tansy ragwort (*S. jacobaea*) produced chronic liver disease in cattle [126-127]. Afterwards, in 1956, a study by Bull and Dick showed that species from *Crotalaria* spp. led to comparable diseases [128]. A serious outbreak with the consumption of bread made from wheat contaminated with seeds of *Heliotropium* sp. plants, which contain PA, happened in Afghanistan, in 1974–1975 [129]. The patients exhibited ascites and emaciation, typical of hepatic VOD. Equally, in Tajikistan (1993), an epidemic was observed involving wheat contaminated by *Heliotropium lasiocarpum* Fisch. & C.A.Mey. As consequence, 3906 cases of liver diseases were registered, leading to over 60 deaths [130].

In 1989, the International Program on Chemical Safety, an agency of the World Health Organization and Food and Agriculture Organization, published the "Pyrrolizidine Alkaloids Health and Safety Guide" [108]. This guide contained statements about the hazards for humans and animals and the confirmation that contaminated grain, herbal medicines, beverages, foodstuff or grazing with PA could cause acute or chronic illness [108].

PA poisoning was initially a problem, mainly in developing countries, as result of the use of traditional medicines containing PA (Table 1) [125]. However, in the last years, there has been a growing focus on this type of medicine in industrialized countries, thus making this problem a wider concern [125]. In Europe, chronic toxicity due to long-term consumption of food or herbal medicines containing these alkaloids is now a reality [131].

**Table 1.** Medicinal species containing PA [42,45] .

| Family | Plant | Reference |
|---|---|---|
| Apiaceae | *Foeniculum vulgare* Mill.; *Pimpinella anisum* L.; *Carum carvi* L. | [132] |
| Apocynaceae | *Amphineurion marginatum* (Roxb.) D. J. Middleton; *Alafia* cf. *caudata* Stapf | [133] |
| Asteraceae | *Eupatorium cannabinum* L.; *Adenostyles alliariae* (Gouan) Kern; *Emilia sonchifolia* (L.) DC.; *Petasites hybridus* (L.) PH Gaertn., B. Mey & Scherb.; *Petasites spurius* (Retz) RCHB; *S. jacobaea*; *Senecio vulgaris* L.; *T. farfara; Senecio nemorensis* L.; *Ageratum conyzoides* L.; *Chromolaena odorata* (L.) R. M. King & H. Rob.; *Eupatorium chinense* L.; *Eupatorium fortunei* Turcz.; *Eupatorium japonicum* Thunberg ex Murray.; *Cacalia hastata* L.; *Cacalia hupehensis* Hand.-Mazz.; *Crassocephalum crepidioides* (Benth.) S. Moore; *Farfugium japonicum* (L.) Kitam.; *Gynura bicolor* (Roxb. ex Willd.) DC.; *Gynura divaricata* (L.) DC.; *G. segetum; Ligularia dentata* (A.Gray) Hara; *Petasites japonicus* (Siebold & Zucc.) Maxim.; *Senecio argunensis* Turcz.; *Senecio integrifolius* (L.) Clairv.; *Senecio scandens* Buch.-Ham. Ex D. Don; *Syneilesis aconitifolia* (Bunge) Maxim.; *Matricaria chamomilla* L.; *Gynura pseudochina* (L.) DC.; *Gynura japonica* (Thunb.) Juel; *Packera candidissima* (Greene) W. A. Weber & Á. Löve; *Solanecio mannii* (Hook.f.) C. Jeffrey; *Solanecio tuberosus* (Sch. Bip. ex A. Rich.) C. Jeffrey var. tuberosus; *Bidens pilosa* L.; *Senecio longilobus* Benth. | [85,132,134-143] |
| Boraginaceae | *Alkanna tinctoria* (L.) Tausch; *Anchusa officinalis* L.; *Borago officinalis* L.; *Cynoglossum officinale* L.; *Heliotropium arborescens* L.; *Lithospermum officinale* L.; *Myosotis scorpioides* L.; *Symphytum asperum* Lepech; *Symphytum caucasicum* Bieb.; *Symphytum officinale* L.; *Symphytum tuberosum* L.; *Symphytum* × *uplandicum* Nyman; *Arnebia euchroma* (Royle) I. M. Johnst.; *Cordia myxa* L.; *Cynoglossum amabile* Stapf & J. R. Drumm; *Cynoglossum lanceolatum* Forssk.; *Cynoglossum zeylanicum* (Vahl) Brand; *Cynoglossum grande* Dougl. ex Lehm.; *Cynoglossum virginianum* L.; *Arnebia benthamii* (Wall. ex G.Don.) Johnst.; *H. indicum*; *Lappula intermedia* (Ledeb.) Popov; *Lithospermum erythrorhizon* Siebold & Zucc. | [144-148] |
| Fabaceae | *Crotalaria albida* Roth; *Crotalaria assamica* Benth.; *Crotalaria pallida* Aiton; *Crotalaria sessiliflora* L.; *Crotalaria tetragona* Andrews | / |
| Lamiaceae | *Melissa officinalis* L. | [132] |
| Orchidaceae | *L. nervosa* | / |
| Urticaceae | *Urtica dioica* L. | [132] |

Several studies on food chemistry and food safety have shown that many of the foodstuff currently consumed are sources of this type of alkaloids. A recent study from Mulder et al. showed that the contamination of eggs and meat products with PA seems to be rare in the European Union [149] . Nevertheless, PA are sometimes found in milk, albeit in very low concentrations, since milk suffers from extensive processing, during which these compounds are diluted [149] . The class of PA found in milk revealed that *Senecio* spp. and species from the Boraginaceae family could be the origin of their occurrence [149] .

In the last few years it has been reported that even herbal teas and teas not prepared from plants known to have PA in their composition, such as *M. chamomilla* and *Mentha* × *piperita* L., can have high amounts of these compounds, as consequence of cross-contamination [132,150] . When studying the distribution of PA in herbal teas, namely green, black, peppermint, rooibos, chamomile and one mix of herbs, it was observed that the most frequent was the senecionine-type (senecionine-, retrorsine-, seneciphylline-, senecivernine-*N*-oxides and their respective free bases) [149] . Lycopsamine- and heliotrine-types were less frequently found, intermedine being the most common, followed by lycopsamine-*N*-oxide and heliotrine-*N*-oxide [149] . The highest average concentration was

from senecionine-*N*-oxide (1.73 μg/L and 64% frequency), followed by retrorsine-, seneciphylline-, senecivernine-*N*-oxides and the corresponding free bases [149]. Together, these PA accounted for 76% of total PA content found in herbal teas; lycopsamine- and heliotrine-types accounted for 24%, while monocrotaline-type was not present [149]. Moreover, PANO were found in higher concentrations than the corresponding free bases. High amounts of PA were also revealed in tea, namely in black and green tea from retail market, an unexpected finding [149]. As described before, different chemical types were identified, open-chain diesters being mainly perceived in fennel (*F. vulgare*) infusion and cyclic PA in black tea [132].

Concerning food supplements, the samples analyzed were often contaminated with PA, being the amounts highly variable [149]. The analysis was made considering three types of food supplements: supplements based on plants not known to produce PA (*Valeriana officinalis* L., *Hypericum perforatum* L.), supplements based on plants known to produce PA (*B. officinalis*, *Eupatorium perfoliatum* L., *Eupatorium odoratum* L., *L. officinale*, *Pulmonaria officinalis* L., *S. officinale*, *Petasitis* sp., *P. hybridus*, *T. farfara*), supplements containing bee products (pollen, propolis and royal jelly). Food supplements made from plant material known for their content in PA revealed the highest PA levels, those from lycopsamine-type (lycopsamine, intermedine, echimidine) being the more common [149]. However, the supplements made from plants not known to produce PA similarly demonstrated to have these compounds, probably due to cross-contamination. Supplements made of oil-based extracts of PA-producing plants were devoid of PA, whereas the presence of PA in supplements containing bee products was also confirmed [149].

Several studies have shown that the distribution of PA subclasses varies with the vegetal material [151]. For example, while pollen is richer in seneciphylline-type, in flower heads retrorsine- and usaramine-types are more common [151].

Among the several food products that can harbor these toxins, honey is one of the most studied and important [151-153]. PA have been found in honey from various botanical and geographical origins [154]. Senecionine, echimidine and lycopsamine, in particular, were present in *Echium* spp. honey samples coming from Spain [155]. Considering the concentrations found by Kempf et al. in honey samples (0.019–0.120 μg/g) and that a common dose is 1 or 2 table spoons per day (10–20 g), it is possible to conclude that a honey consumer can easily exceed the recommended limit: maximum of 1.0 μg of PA per day [154,156-160]. A study from Lucchetti et al. [152] revealed the presence of PA in nectar from *Echium vulgare* L. Echimidine corresponded to half of the PA content found and acetylechimidine, vulgarine, echiuvulgarine and acetylvulgarine were the other half. They also concluded that pollen frequently exhibited higher levels of PA than nectar, but the proportion of the diverse types of these compounds found in honey was more closely related to that found in nectar compared to that present in pollen. For this reason, there are some doubts about the origin of these toxins in honey, since it is composed by nectar, but also contains traces of pollen.

PA-containing plants known to be used in the production of honey can be found in Table 2.

Studies with pollen from *S. vernalis* revealed that PA-free honey can be contaminated when this pollen is added to it, probably by diffusion from pollen to honey [118]. For some authors, it was also clear that pollen contained much higher levels of PA than honey and that pollen appeared in it at low doses [151]. However, a study showed the opposite, specifically that a relationship between the concentration of pollen in the honey and its PA levels is not always found, since honeys with considerable amounts of PA on their composition are revealed to have low levels of pollen [154].

**Table 2.** Plants containing PA used in the production of honey in several countries.

| Country | Plant | Reference |
|---|---|---|
| Argentine | *Senecio grisebachii* Baker | [161] |
| Australia | *Echium plantagineum* L.; *E. vulgare*; *Eucryphia lucida* (Labill) Baill; *Heliotropium amplexicaule* Vahl; *Heliotropium europaeum* L. | [156] [166] |
| Brazil | *C. pallida; Eupatorium* sp. | [162-163] |
| Bulgaria | *T. farfara* | [164] |
| China | *E. plantagineum*; *E. vulgare*; *Senecio* spp.; *C. officinale; Tussilago* spp. | [165] |
| Ethiopia | *Solanecio angulatus* (Vahl) C. Jeffrey | [134] |
| Germany | *E. vulgare*; *Phalaenopsis* sp.; *S. jacobaea*; *Senecio vernalis* Waldst. & Kit. | [151] |
| Ghana | *C. odorata*; *Eupatorium* spp.; *Ageratum* spp. | [166] |
| India | *Crotalaria juncea* L. | [101,167] |
| Italy | *Echium* sp.; *Senecio erucifolius* L.; *Senecio inaequidens* DC; *S. jacobaea*; *S. vulgaris*; *Robinia pseudoacacia* L. | [168-170] |
| New Zealand | *E. vulgare*; *B. officinalis*; *Echium* spp. | [159,165] |
| Portugal | *Echium* sp. | [168] |
| South Africa | *S. inaequidens*; *Senecio pterophorus* DC | [168,170] |
| Spain | *E. plantagineum*; *E. vulgare* | [160] |
| Switzerland | *E.vulgare*; *Eupatorium* sp.; *Senecio* sp. | [165,171] |
| Thailand | *E. odoratum* | [172] |
| Turkey | *Myosotis* sp. | [173] |
| United Kingdom | *Borago* sp.; *S. jacobaea* | [165,174] |
| United States | *C. officinale*; *E. vulgare*; *S. jacobaea*; *S. vulgaris*; *S. officinale* | [175,176] |
| Uruguay | *E. plantagineum* | [177] |

### 1.4.6. **Conclusions**

PA are a widespread group of secondary metabolites that can, in certain situations, pose a life threat to humans and animals, once they are present in a variety of foodstuff. These compounds have became known for their toxicity, as per several outbreaks that were registered, mainly in developing countries. However, in the last years, industrialized countries began to face this reality, when the use of traditional medicines increased. Despite this, some PA can also be useful, since they demonstrate pharmacological properties which can be further exploited by relying in medicinal chemistry strategies that can maintain bioactivity while reducing toxicity.

Thereby, for the sake of human and animal health protection, it is of great importance to further develop the information regarding the chemistry, pharmacology and toxicology of PA.

## 1.5. Pyrrolizidine alkaloids in *E. plantagineum*

The concern with the hazards of *E. plantagineum* increased with the emergence of intoxications after the consumption of this plant. An example of an unusually proliferation of *E. plantagineum,* after a fire, occurred in Australia, in December 2003, resulting in several horse deaths with liver disease (178). As a result of this and the honeys that arise thru the spread of the plant, the Food Standards Australia New Zealand has been worried about that and issued food advisories, since 2004 (178,179). They recommended to the consumers, do not eat exclusively Paterson's Curse honey, if they eat more than two tablespoons of honey *per* day (179). The perception of the presence of considerable levels of PA, compared with the recommended doses for human consumption, has been increasing as studies have emerged in this area and thus it led to the mixing of honey from *E. plantagineum* with honey from other species to reduce the risks (180). The seeds of this plant are also a source of oils used to produce supplements with healthiness benefits, because they are rich in alpha- and gamma-linolenic acids (180).

Based on studies carried out in products obtained from the plant *E. plantagineum*, the following table was constructed to provide information about the identity of PA or PANO found in this plant (Table 3).

**Table 3.** PA present in *E. plantagineum*.

| ***E. plantagineum*** | **PA** | **Reference** |
|---|---|---|
| **Honey** | Acetylechimidine<br>Echimidine<br>Echiumine<br>7-*O*-acetyl lycopsamine<br>7-*O*-acetyl intermedine | (156) |
| **Pollen** | Echimidine-*N*-oxide<br>Echiumine-*N*-oxide<br>Echiuplatine-*N*-oxide | (181) |
| **Flower/Leaf** | Echimidine-*N*-oxide<br>Echimiplatine-*N*-oxide<br>Echiumine-*N*-oxide<br>Echiuplatine-*N*-oxide<br>Intermedine-*N*-oxide<br>Leptanthine-*N*-oxide<br>Lycopsamine-*N*-oxide<br>Uplandicine-*N*-oxide<br>3′-*O*-Acetylechimidine-*N*-oxide<br>3′-*O*-Acetylechiumine-*N*-oxide<br>7-*O*- Acetyllycopsamine/intermedine<br>7-*O*-Acetyllycopsamine/intermedine-*N*-oxide<br>7-Angeloylretronecine-*N*-oxide<br>9-O-Angelylretronecine-*N*-oxide | (156) |

## 1.6. Phenolic compounds

### 1.6.1. Chemical structure

Phenolic compounds are secondary metabolites of plants and they are the main class of antioxidants in human diet (182). They are classified in two main groups: phenolic acids and flavonoids (figure 9), being subsequently sorted in different subclasses (26).

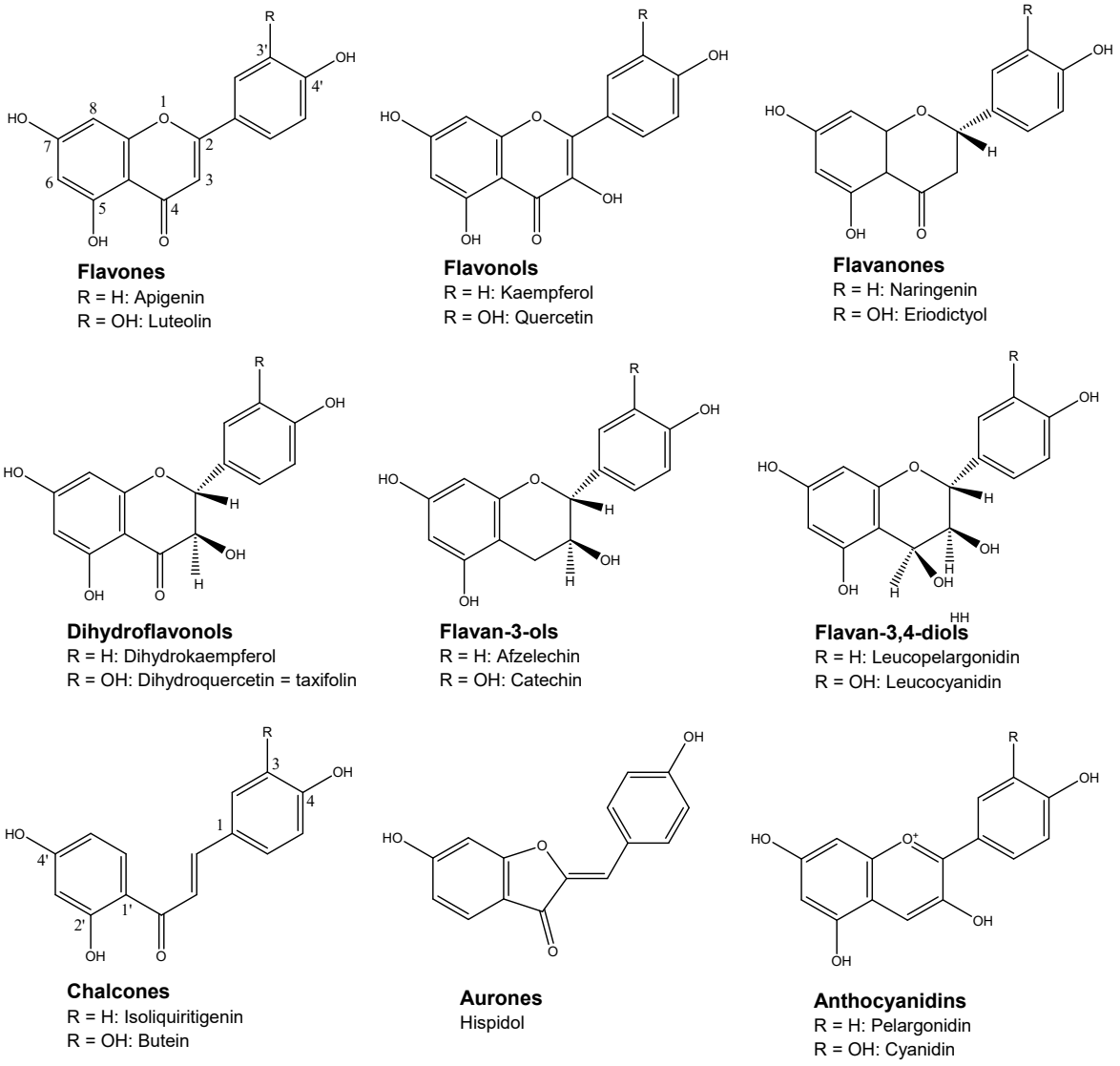


**Figure 9.** Chemical structures of the different subclasses of flavonoids (26).

Flavonoids are compounds responsible for certain colours of flowers, fruits and sometimes leaves (26). Yellow flavonoids (e.g., chalcones, aurones, yellow flavonols) and red, blue, or purple anthocyanins are examples of these compounds (26). They are plant secondary metabolites displaying a C6-C3-C6 skeletal system, including a chromane ring

(ring C) and a second aromatic ring B in position 2,3 (isoflavones) or 4 (neoflavonoids) (183). Flavonoids with ring B attached to position 2 can be sorted in different groups according to structural features of the ring C: flavones, flavonols, flavanones, flavanonols, flavanols, and anthocyanidins (183). In all sorts of flavonoids, there are frequently three phenolic hydroxyl groups in positions 5-, 7- and 4'- of the aglycone (26). However, one of these may also not be present (26). In plants, flavonoids usually occur glycosylated, mostly with glucose or rhamnose, but they can appear also with other sugars; usually from one to three glycosyl moieties (183).

Phenolic acids are organic compounds with at least one carboxyl group and one phenolic hydroxyl group (26). However, nowadays, it is frequent to call the term phenolic acid to benzoic and cinnamic acid derivatives (26).

Phenolic acids derived from benzoic acid (C6-C1) are constituted by a benzene ring and a carboxyl group linked to it, such as *p*-hydroxybenzoic acid (figure 10) (26,183). They usually occur in their free state, however they may also occur in the form of esters or glycosides (26).

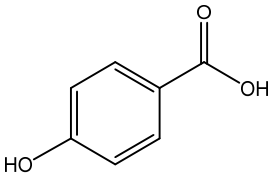


**Figure 10.** Chemical structure of *p*-hydroxybenzoic acid.

The phenolic acids which derive from cinnamic acid (C6-C3) are formed by an aromatic ring and a three-carbon chain, as an example: *p*-coumaric acid (figure 11) (26,183). These compounds are very often found as esters of aliphatic alcohols and of quinic acids, however rarely appear in the free form (26). They can also be amides or they can occur combined with sugars (26).

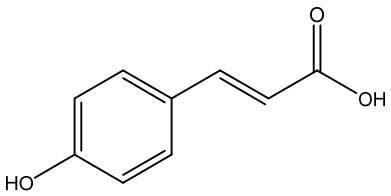


**Figure 11.** Chemical structure of *p*-coumaric acid.

### 1.6.2. Biosynthesis

The synthesis of the benzene ring is made only by plants and microorganisms. These compounds can come from two principal pathways, named shikimate and acetate, as can be seen in figure 12 (182). In the shikimate pathway, there is formation of aromatic amino acids, like phenylalanine and tyrosine (182). The deamination of the last ones leads to the formation of cinnamic acids and to compounds that derive from them (26). In the other pathway, the formation of β-keto-esters leads to the appearance of polycyclic compounds (isocoumarins, xanthones, quinones), by the Claisen condensation (26). The mevalonate pathway, despite less frequent, can also participate in their biosynthesis, forming, for example, quinones, which derive from shikimate and mevalonate (26). The structural diversity of these molecules results from the possibility of coming from two pathways simultaneously: shikimate and acetate (26).

The first reaction in shikimate pathway is the condensation of phosphoenolpyruvate with erythrose-7-phosphate, which yields 3-deoxy-*D*-arabinoheptulo-sonate-7-phosphate (26). After this, cyclization of the last compound to 3-dehydroquinate involves the elimination of the phosphate group and an intramolecular aldol condensation (26). The conversion of 3-dehydroquinate to 3-dehydroshikimate starts with the dehydration of 3-dehydroquinate, forming a Schiff base between a lysine residue and the carbonyl group and the elimination of a water molecule (26). Subsequently to the reduction of 3-dehydroshikimate and to shikimate phosphorylation, the condensation with another molecule of phosphoenolpyruvate takes place, yielding 5-enolpyruvylshikimate 3-phosphate, which leads to chorismate (26). Via phenylpyruvate, chorismate will allow the formation of the aromatic amino acids referred above, which gives rise to cinnamic acid and their derivatives (26). Benzoic acids result in general from side chain degradation of the cinnamic acids, but they can also arise from 3-dehydroshikimic acid or from chorismate (26). The majority of benzophenones come from acetate and malonate metabolisms (26).

The biosynthesis of flavonoids is characterized by the condensation of three molecules of malonyl-CoA with an ester of coenzyme A and a hydroxycinnamic acid, by chalcone synthase (26). This step allows the formation of a chalcone, a 4,2',4',6'-tetrahydroxychalcone, or a 6'-deoxychalcone, 4,2',4'-trihydroxychalcone, when in the presence of an dihydronicotinamide-adenine dinucleotide phosphate (NADPH)-dependent polyacetate reductase (26). Chalcone isomerizes to racemic flavanone, a precursor of flavonoids, which is catalysed by chalcone isomerase (26).

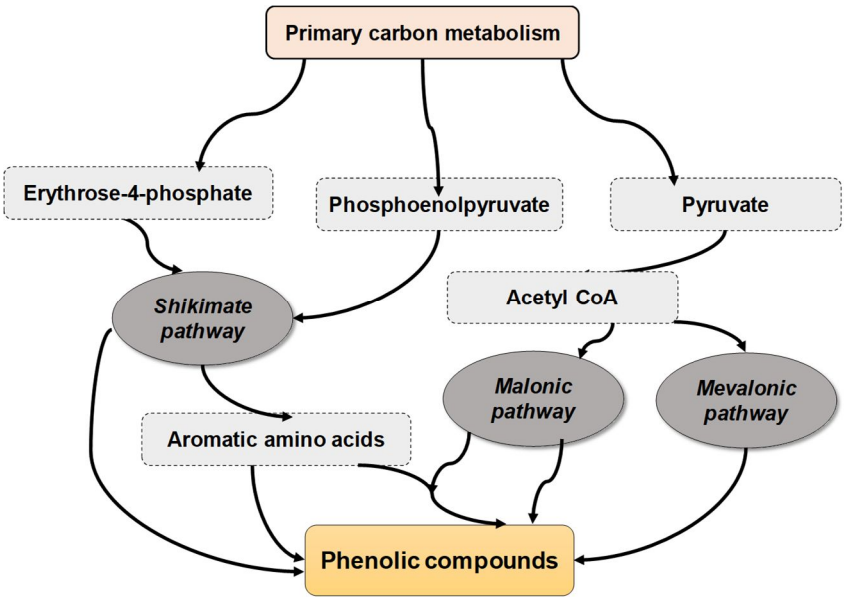


**Figure 12.** Biosynthetic pathways of phenolic compounds (182).

### 1.6.3. Biological activity

As referred above, phenolic compounds are among the most relevant antioxidants in human diet (182). They have been suggested to play a preventive role in several diseases caused by oxidative stress, such as cancer, Alzheimer, Parkinson and multiple sclerosis (183–186). In the human body, the balance between reactive species and antioxidants is required, and when this equilibrium is disturbed, vital components can be affected. Thereby, the regular consumption of food rich in phenolic compounds can ameliorate the protection of vital components and, consequently, can bring health benefits (187). This fact is linked to the capacity of avoiding the oxidative damage through scavenging of free radicals from cell metabolism, and the ability to protect against the harmful effects of reactive oxygen species (ROS) (188-191). The antioxidant properties of these compounds are dependent on the rearrangement and number of functional groups, since they are potent hydrogen donators (192).

Apart from the antioxidant activity recognized, other mechanisms of action can explain the biological effects of phenolics, such as the ones based on the specific structural and conformational features of some polyphenols (193). These compounds have the ability to inhibit the activity of a broad number of enzymes, namely enzymes with purines as substrates and enzymes with NADPH as cofactor (193). An example is the interaction between the *O*-methylated metabolite of flavan-3-ol(-)-epicatechin with the enzyme NADPH-oxidase, which leads to a decrease in the production of the radical superoxide

anion ($O_2^{\bullet-}$) (193). Likewise the interaction of phenolics with receptors can explain some biological activities that they reveal (193). For example, the similarity of isoflavones structures with animal estrogens offers the possibility of interacting with its receptors, acting as estrogen agonists or antagonists (194).

Other pharmacological properties have been reported for these compounds, namely the anti-inflammatory, antiallergic, anti-hypertensive and antimicrobial activities, among others (195-198).

## 1.7. Inflammation

The inflammatory process is a physiological response of the body in order to eliminate, neutralize and/or destroy *stimuli* resulting from infection or tissue damage (198). The main goal of inflammation is to resolve infection/repair damage in order to achieve homeostasis equilibrium (200,201).

An inflammatory process can be acute or chronic. When the response is rapid and can lead to the destruction of the foreign agent and sometimes of the host tissue, lasting only a few days, it is called acute inflammation (200). However, when this response does not resolve and becomes disordered, occurs chronic inflammation (202). This inflammatory process can be localized, but sometimes progresses to disabling diseases. This happens in consequence of the persistence of inflammatory cells, hyperplastic stroma and tissue destruction, which leads to organ dysfunction (202). This type of inflammation is characterized by causing more damage to the host than to the foreign agent, and for the presence of lymphocytes, plasma cells and macrophages (200).

Inflammation is characterized by four classical signs: redness, heat, pain and swelling, as a consequence of events such as vasodilation, increased permeability of blood vessels, leukocyte infiltration, extravasation of blood plasma, proteolytic activity, formation of ROS and reactive nitrogen species (RNS), necrosis, apoptosis and phagocytosis (199,203). The inflammatory process starts with the increased movement of defence cells from the bloodstream into affected tissues, and the development of these events is regulated by a cascade of molecular interactions and reactions (61). Inflammation is driven by the vascular system in tandem with the immune system and different cell types. The inflammatory response is initiated by inducers (exogenous or endogenous) responsible to trigger the production of inflammatory mediators, by activating specialized sensors (201). Inducers can be, for example, fungi, viruses, LPS and even cells of the body upon damage and death (203). As an example of these specialized sensors, there are the macrophages and mast

cells, which prove to be crucial in the entire process. They are accountable to produce inflammatory mediators, such as chemokines, cytokines, vasoactive amines, eicosanoids and products of proteolytic cascades (201). In turn, the production of these mediators elicits the movement of plasma proteins and leukocytes from the blood vessels to the extravascular tissues at the site of infection/injury (201). When they reach the affected tissue, in an attempt to eliminate the originator agent, neutrophils release ROS, RNS, proteinase 3, cathepsin G and elastase (204). However, it is important to state that the overproduction of mediators is involved in various diseases, namely rheumatoid arthritis and pulmonary fibrosis (199).

$^{\bullet}NO$ is involved in the regulation of diverse physiological and pathophysiological mechanisms in the cardiovascular, nervous and immunological systems (199). However, in pathological conditions, the upregulation of inducible nitric oxide synthase (iNOS) as a consequence of pro-inflammatory mediators, such as cytokines, results in increased levels of $^{\bullet}NO$, which plays an important role as mediator of the inflammatory response (62). The levels of $O_2^{\bullet-}$ also increase, resulting in the formation of peroxynitrite ($ONOO^-$), which is a powerful cellular oxidant.

Mediators derive from distinct sources, such as the metabolism of phospholipids and arachidonic acid, or they can be preformed and stored in cytoplasmic granules (202). Arachidonic acid is generated by one of two pathways: hydrolysis of cell membrane phospholipids *via* phospholipase $A_2$, or the metabolism of phosphatidylinositol phosphates to diacylglycerol and inositol phosphates by phospholipase C (202). After the formation of arachidonic acid, it is metabolized through cyclooxygenation by cyclooxygenase-2 (COX-2), forming prostaglandins and thromboxanes, or through lipoxygenation by 5-lipoxygenase (5-LOX) to produce leukotrienes and lipoxins (202).

# *Chapter 2*

## 2. Objectives

The aim of this work was to analyse honeys of national origin, evaluating the presence of PA and phenolic compounds, as well as to assess their toxicity and possible therapeutic properties in macrophages (RAW 264.7), gastric adenocarcinoma (AGS), and human fetal lung fibroblasts (MRC-5) cell lines.

The specific objectives were:

- Identify the botanical origin of the pollen present in the samples;
- Conduct the chemical characterization of the alkaloid-targeted and phenolic-targeted extracts of the honey samples;
- Assess the cytotoxicity of the extracts in AGS cells;
- Assess the potential anti-inflammatory activity of the extracts in RAW 264.7;
- Assess the safety of the extracts in MRC-5 cell lines;
- Contribute to quality control profile of Portuguese honeys.

# *Chapter 3*

# 3. Materials and Methods

## 3.1. Standards and reagents

Methanol, acetonitrile, 2-propanol were obtained from Merck (Darmstadt, Germany). Ammonia, hydrochloric acid and dimethyl sulfoxide (DMSO) were obtained from Fischer Scientific (Loughborough, UK). Sulphuric acid, sulphanilamide, N-(1-Naphtyl) ethylenediamine, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT), LPS of *Salmonella enterica* and trypan blue, chrysin, pinocembrin, hyperoside, quercetin-3-*O*-rutinoside, quercetin-3-*O*-glucoside, galangin, chlorogenic, ellagic, caffeic, gallic, *p*-coumaric, *p*-hydroxybenzoic and ferulic acids, were obtained from Sigma-Aldrich (St. Louis, MO, USA); orto-phosphoric acid from Scharlab S.L. (Barcelona, Spain). Dulbecco's Modified Eagle Medium (DMEM), Minimum Essential medium (MEM), heat inactivated fetal bovine serum (FBS), Pen Strep solution (Penicillin 5000 units $mL^{-1}$ and Streptomycin 5000 mg $mL^{-1}$) were obtained in Gibco Invitrogen™ (Grand Island, NY, USA). Echimidine *N*-oxide was obtained from Carl Roth (Germany) and echimidine from PhytoLab (Vestenbergsgreuth, Germany).

## 3.2. Samples

Honey samples (table 4) were acquired online. Two of them were sold as multifloral honey, produced from various plants, including *E. plantagineum*, and they were from *Serra d'Aire* and *Parque Natural do Montesinho* in Portugal. The other honey sample was classified as monofloral honey of *E. plantagineum*, produced in *Serra da Estrela*. Samples were stored in a cool, dry place and protected from the light.

**Table 4.** Description of the samples analysed.

| Samples | Acquisition year | Origin | Type of honey | Plant species |
|---|---|---|---|---|
| *Domimel* | 2017 | *Serra da Estrela* | Monofloral | *E. plantagineum* |
| *Mel de Montanha* | 2017 | *Serra d'Aire* | Multifloral | *Rosmarinus officinalis* L.<br>*E. plantagineum* |
| *Bagas Bravas* | 2017 | *Parque Natural de Montesinho* | Multifloral | Ericaceae<br>*Thymus vulgaris* L.<br>*Castanea sativa* Mill.<br>*E. plantagineum* |

## 3.3. Pollen identification of the samples

For the analysis of the pollen present in honey samples, one drop of each honey was directly placed in a microscope slide, and they were observed and photographed in a microscope. The pictures were obtained with a total magnification of 400 x, and they were compared with an Atlas (18). An expert opinion was also taken (Prof. Paula B. Andrade).

## 3.4. Alkaloid-targeted extracts

### 3.4.1. Preparation

For the extraction, it was used a method adapted from the described by Griffin *et al.* (205). Honey was firstly homogenised in a water bath at 40ºC and then it was weighed 20 g into centrifuge tubes. After this step, it was had 20 mL of 0.05 M sulphuric acid solution. Samples were vortexed until homogenised, and after that they were centrifuged at 6000 rpm for 10 min, and the supernatant recovered. Solid phase extraction (SPE) benzenesulfonic SCX (200 mg/3 mL) cartridges were preconditioned with 2 mL of methanol and 2 mL of 0.1% formic acid. Subsequently, the supernatant recovered (2.5 mL) was loaded onto the cartridge. The cartridge was washed with 2 mL of 0.05% formic acid and 2 mL of methanol. For last, the elution was made with 9 mL of 0.1% ammoniated methanol. Then, the elute was evaporated under reduced pressure in a water bath at 30ºC. After that, the extract was dissolved in methanol and filtered with a PTFE filter of 0.45 µm.

### 3.4.2. Alkaloid precipitation tests for alkaloid detection

The presence of alkaloids in the extracts was evaluated by the means of the general alkaloid precipitation tests, by the addition of three drops of Dragendorff's (solution of potassium bismuth iodide), Mayer's (potassium mercuric iodide solution) and Bertrand's (silicotungstic acid solution) reagents to the extract, having one tube as control (206).

### 3.4.3. HPLC-DAD analysis

Different high performance liquid chromatography-diode array detector (HPLC-DAD) methods were tested. The first one consisted in a chromatographic analysis of the alkaloid-targeted extracts of honey with a *Hypersil* ODS column (20 × 0.4 mm; particle size 5 µm), with a flow of 0.2, 0.4 and 0.6 mL/min (different attempts) and a gradient elution described in table 5 (205).

**Table 5.** Gradient elution used in the first method applied to the analysis of alkaloid-targeted extracts by HPLC-DAD.

| Time (min) | 0.05% $HCO_2H$ (%) | 100 % $CH_3CN$ (%) |
|---|---|---|
| 0 – 2 | 80 | 20 |
| 2 – 15 | 80-50 | 20-50 |
| 15 – 17 | 50 | 50 |
| 18 – 30 | 80 | 20 |

After this, another method was tested using a *Waters Spherisorb* ODS 2 column (4.6 × 250 mm; particle size 5 µm) with a flow of 0.9 mL/min and the gradient elution represented on table 6 (207).

**Table 6.** Gradient elution used in the second method applied to the analysis of alkaloid-targeted extracts by HPLC-DAD.

| Time (min) | 0.05% $HCO_2H$ (%) | 100 % $CH_3CN$ (%) |
|---|---|---|
| 0-5 | 0 | 100 |
| 5-15 | 0-15 | 100-85 |
| 15-25 | 15-30 | 85-75 |
| 25-35 | 30-70 | 70-30 |
| 35-40 | 70-50 | 30-50 |
| 40-50 | 0 | 100 |

The last method employed in this analysis was performed with a reverse phase column *Luna* C18 (2) (250 × 4.6 mm; particle size: 5 µm), and it was used the gradient elution represented in table 7 (208).

**Table 7.** Gradient elution performed in the third method applied to the analysis of the alkaloid-targeted extracts by HPLC-DAD.

| Time (min) | $H_2O$ pH 2.0 ($H_3PO_4$) (%) | 90% $CH_3CN$ (%) |
|---|---|---|
| 0 | 90 | 10 |
| 15 | 50 | 50 |
| 17 | 90 | 10 |
| 35 | 90 | 10 |

The column was kept at 23 ºC, using a flow rate of 0.8 mL/min. The extracts of monofloral honey of *E. plantagineum*, multifloral honey from *Serra d'Aire* and multifloral honey from *Parque Natural do Montesinho* were injected in various concentrations. Sample injections consisted in 20 µL and the needle was washed with 100% methanol between injections to eliminate carryover. The detection was done at 280, 320, 350 and 223 nm and the software used was *Clarity* (Europa Science Ltd, Cambridge, Reino Unido).

## 3.5. Phenolic-targeted extracts

### 3.5.1. Preparation

Honey was weighed into a flask (50 g) and then 50 mL of $H_2O$ acidified with HCl (pH = 2) were added, until completely dissolved. Samples were sonicated for approximately 5 min. After that, Chromabond C18 octadecyl-modified silica column (70 mL/10 000 mg) was preconditioned with 30 mL of methanol and 70 mL of acid water. The samples were applied to the column, and washed with 25 mL of acid water, so that sugars and polar compounds can be eluted with the aqueous solvent. The phenolic compounds were eluted with 60 mL of methanol. Eluted samples were evaporated under reduced pressure, in a water bath at 40ºC. Then, the extracts were dissolved in methanol, filtered with a 0.45 µm PTFE filter, and stored at -20 ºC.

### 3.5.2. HPLC-DAD analysis

For the analysis of phenolic compounds, a gradient elution in a *Spherisorb* ODS 2 column (25.0 cm x 0.46 cm, 5 µm particle size; Waters) was conducted, using a flow rate of 0.9 mL/min (table 8) (209).

**Table 8.** Gradient elution performed in the analysis of the phenolic-targeted extracts by HPLC-DAD.

| Time (min) | 5% $HCO_2H$ (%) | 100 % $CH_3OH$ (%) |
|---|---|---|
| 0-3 | 95 | 5 |
| 3-13 | 85-25 | 15-25 |
| 13-25 | 75-70 | 25-30 |
| 25-35 | 70-65 | 30-35 |
| 35-42 | 65-55 | 35-45 |
| 42-47 | 55-45 | 45-55 |
| 47-56 | 45-25 | 55-75 |
| 56-65 | 25-0 | 75-100 |
| 65-66 | 0-95 | 100-5 |
| 66-80 | 95 | 5 |

The extracts of monofloral honey of *E. plantagineum*, multifloral honey from *Serra d'Aire* and multifloral honey from *Parque Natural do Montesinho* were injected in a volume of 20 µL with concentrations of 85.5, 296.8 and 300.4 mg/mL, respectively. Between injections, the needle was washed with 100% methanol to eliminate carryover.

The detection was done at 280, 320, 350 and 500 nm and the software used was *Clarity* (Europa Science Ltd, Cambridge, Reino Unido).

## 3.6. Cell culture

RAW 264.7 and AGS were cultured in GIBCO® DMEM + GlutaMAX™. MRC-5 was cultured in GIBCO® MEM + GlutaMAX™ also supplemented with 1% penicillin/streptomycin and 10% FBS. All cells were cultivated in a humidified atmosphere under 37ºC and 5% of $CO_2$.

### 3.6.1. Cell viability assay

The impact of alkaloid- and phenolic-targeted extracts upon cell viability was evaluated with the MTT assay. Cells were seeded in a 96-well plate and incubated for 24h, varying the cell density according to the cell line used: 25 000 for RAW 264.7, 15 000 for AGS and 20 000 for MRC-5. Different concentrations of each extract were added, and cells incubated for 24h. At the end of the incubation period, MTT was added to each well (final concentration = 0.5 mg $mL^{-1}$). After 1h15 of incubation, the media was removed and the formazan was dissolved in 200 μL of DMSO:isopropanol (3:1). The plate was read at 570 nm.

### 3.6.2. •NO assay

RAW 264.7 cells were seeded in 96-well plates at 35 000 cells for well and allowed to attach for 24h. Then, the extracts were added, and 2h later, 2 μL of LPS (1 μg/mL) were used to stimulate the cells, being added to each well. After 22h, 75 μL of cell supernatant was transferred to another 96-well plate and mixed with 75 μL of Griess reagent. The plate was incubated for 10 min in the dark, and the absorbance was measured at 540 nm.

### 3.6.3. Statistical analysis

All the results were analysed with *GraphPad Prism 6* software (San Diego, California, USA). To evaluate the distribution of the data, the Shapiro-Wilk normality test was conducted, and to determine the presence of outliers, Grubb's test was used. After this analysis, the determination of the statistical significance of each concentration in comparison to control was performed using the parametric method of one-way ANOVA and

Bonferroni's multiple comparisons test. In all cases, *p* values lower than 0.05 were considered statistically significant.

# *Chapter 4*

# 4. Results and discussion

## 4.1. Pollen identification of the samples

With the aim to improve the quality control made in the honey samples, it was done a qualitative analysis of the pollen present in them. This way, it was possible to understand the truth of the affirmations present in the label of these products.

### 4.1.1. Monofloral honey of *E. plantagineum*

All the pollen grains in the monofloral honey analysed revealed the presence of one major type of pollen: *Echium* sp. (figure 13).

The results obtained are in accordance with the expected, since the sample is sold as a monofloral honey, in other words, a honey that comes predominantly from the nectar of one plant species. However, it does not imply that pollen grains coming from other species are not present. Honey is classified as monofloral when is produced mainly from one specie, and if their predominant pollen is present in a proportion equal or greater than 45%. In this sample, it was also found pollen from Oleaceae family (figure 14).

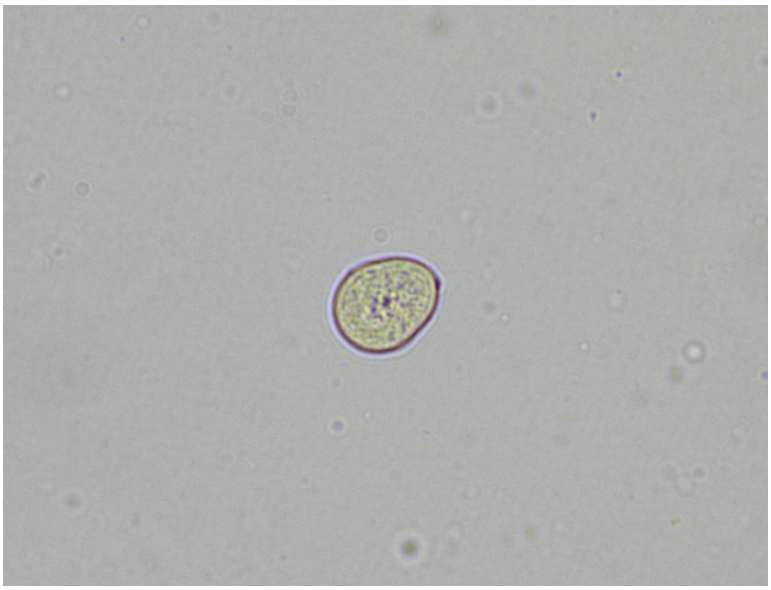

**Figure 13.** Pollen grain of *Echium* sp. (total magnification: 400 x).

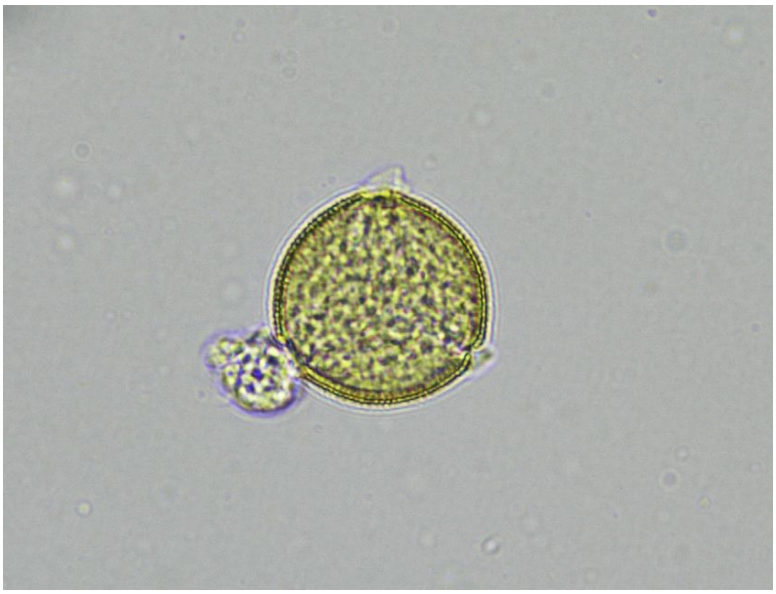

**Figure 14.** Pollen grain of Oleaceae family (total magnification: 400 x).

### 4.1.2. Multifloral honey from *Serra d'Aire*

The analysis of the pollen grains of multifloral honey from *Serra d'Aire* revealed pollen from different plant species, as it was expected, due to the type of honey. Pollen grains of *Echium* sp. were found as well, probably of the specie *E. plantagineum*, the one referred by the producer. Pollen grains of other genera and families were also part of this honey, namely: *Bidens* sp., *Eucalyptus* sp. and Lamiaceae and Oleaceae families (figures 15-19).

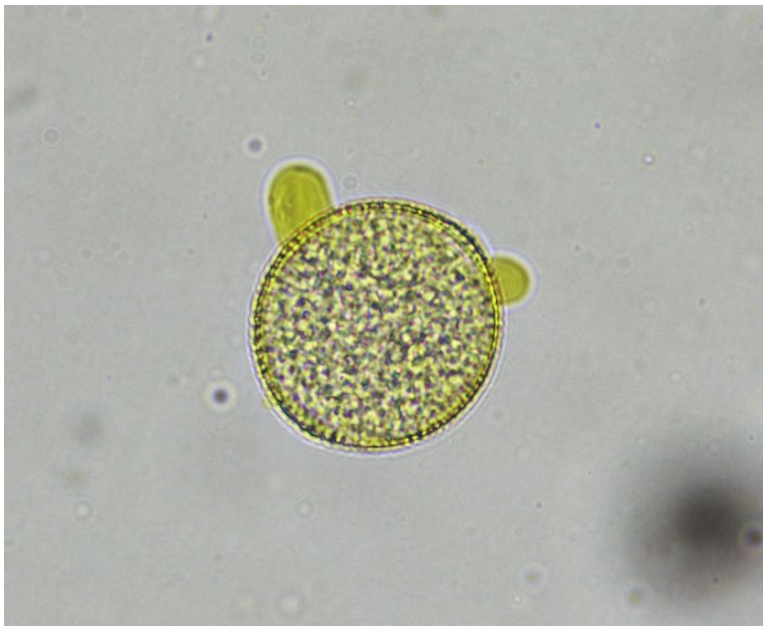

**Figure 15.** Pollen grain of Oleaceae family (total magnification: 400 x).

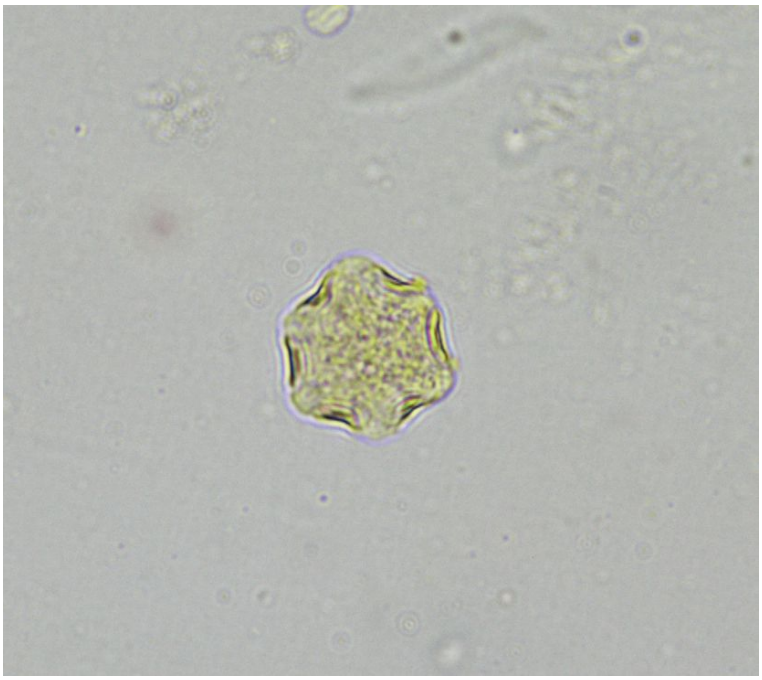

**Figure 16.** Pollen grain of Lamiaceae family (total magnification: 400 x).

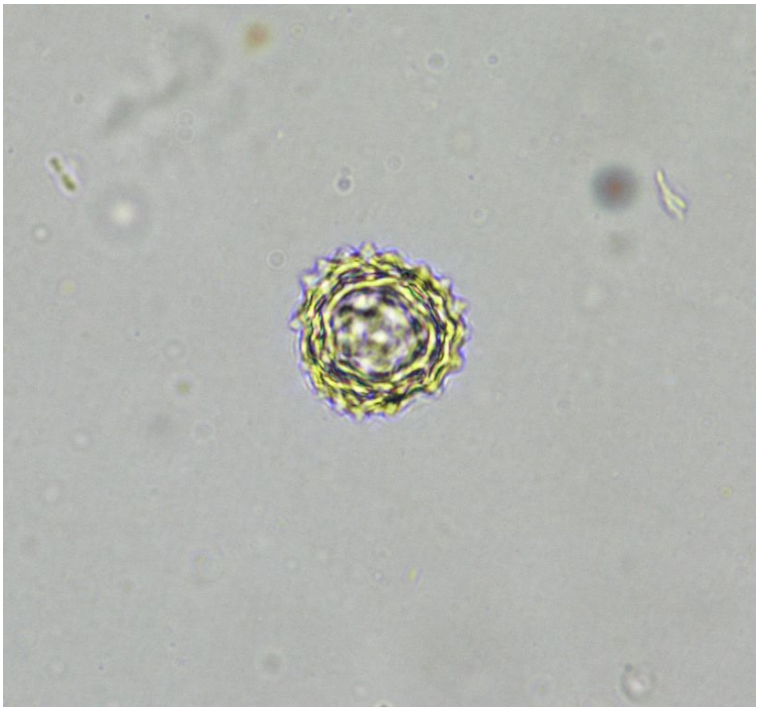

**Figure 17.** Pollen grain of *Bidens* sp. (total magnification: 400 x).

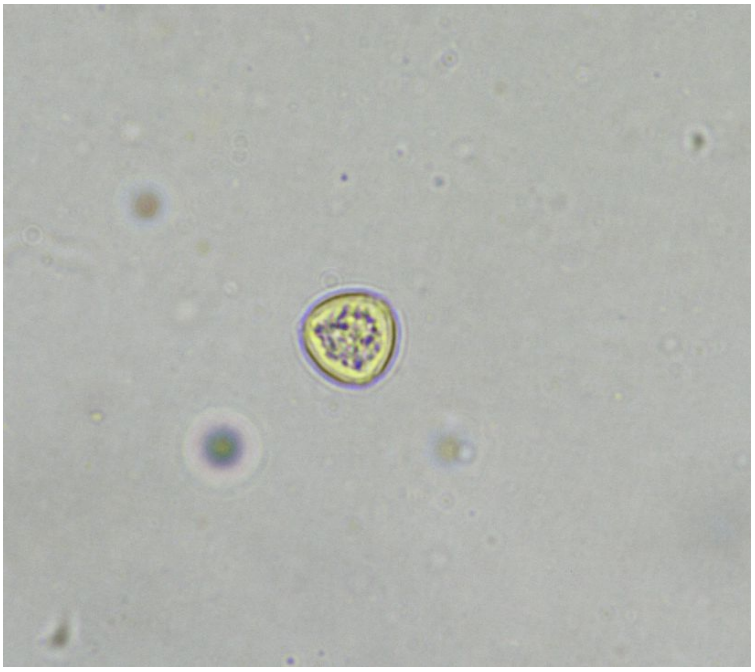

**Figure 18.** Pollen grain of *Eucalyptus* sp. (total magnification: 400 x).

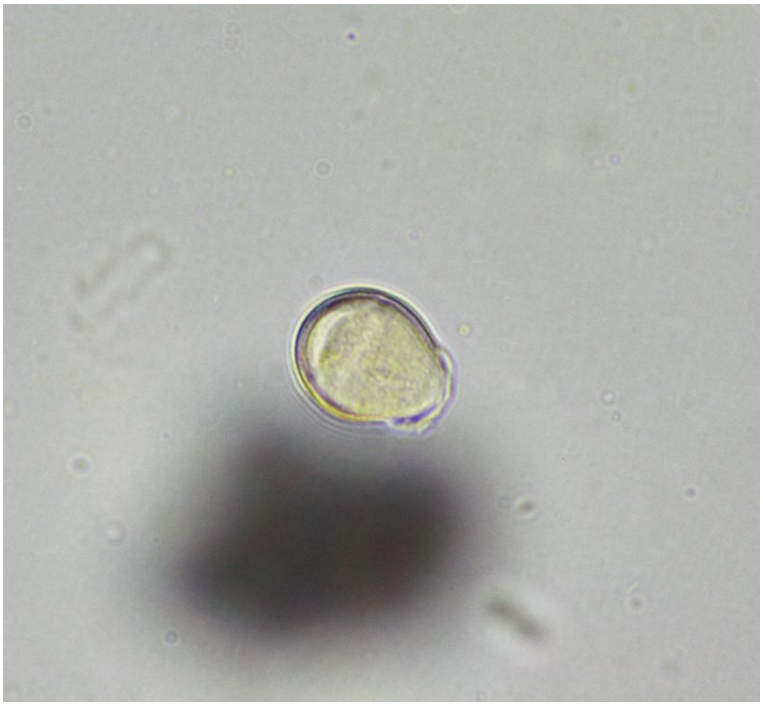

**Figure 19.** Pollen grain of *Echium* sp. (total magnification: 400 x).

As described by the producer in the label of this honey, there are two species particularly responsible to produce it: *E. plantagineum* and *R. officinalis* (Lamiaceae family). By the analysis of the pollen grains present in the sample, it is possible to conclude that pollen from the genera *Echium* sp. (figure 19) and from the family Lamiaceae (figure 16) appear in the honey, which means that the information contained in the label can be considered truth.

### 4.1.3. Multifloral honey from *Parque Natural do Montesinho*

The analysis of multifloral honey from *Parque Natural do Montesinho* revealed pollen grains from the genus *Echium* sp., and pollen belonging to Ericaceae, Oleaceae, Carophyllaceae, Lamiaceae and Fagaceae families (figures 20-25). When the analysis was made, fungal spores were also found (figure 26), which is a normal finding. They are honeydew elements commonly used to help in the identification of the geographical and botanical origin of honey (210). The results obtained are in accordance with the information reported about the plants responsible for the production of this honey, because as described in table 4, the family and species referred match with some pollen grains identified, namely the ones represented in figures 20, 21, 22 and 23.

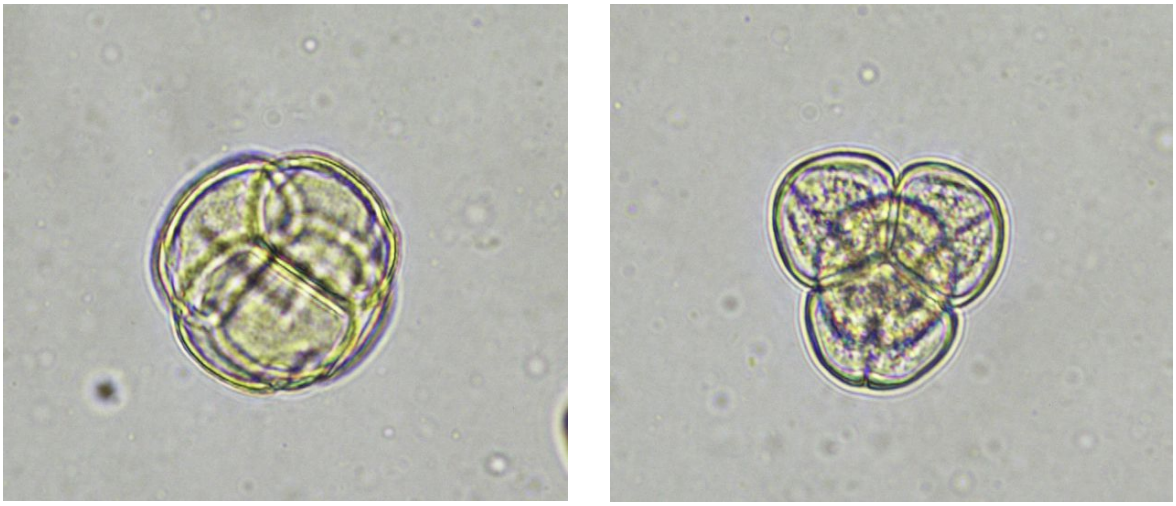

**Figure 20.** Pollen grains of Ericaceae family (total magnification: 400 x).

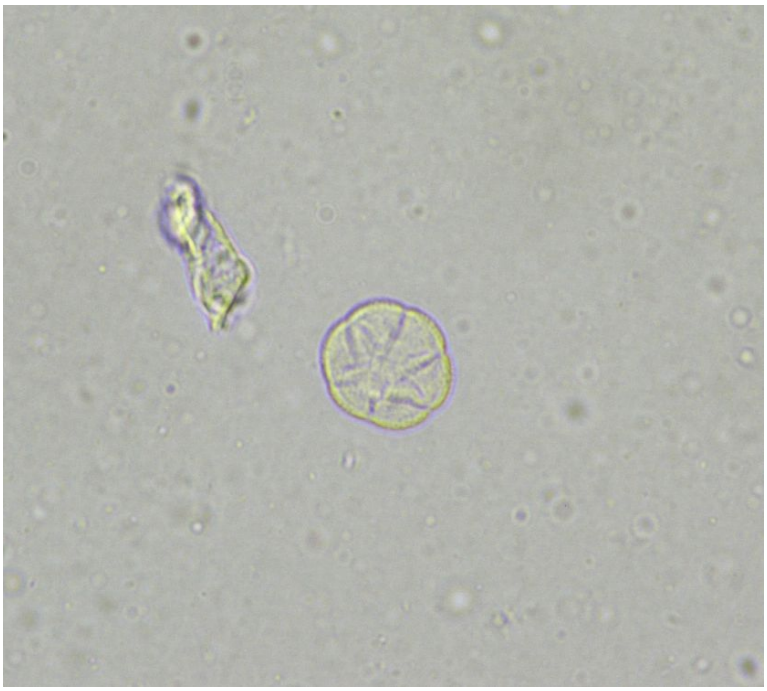

**Figure 21.** Pollen grain of Lamiaceae family (total magnification: 400 x).

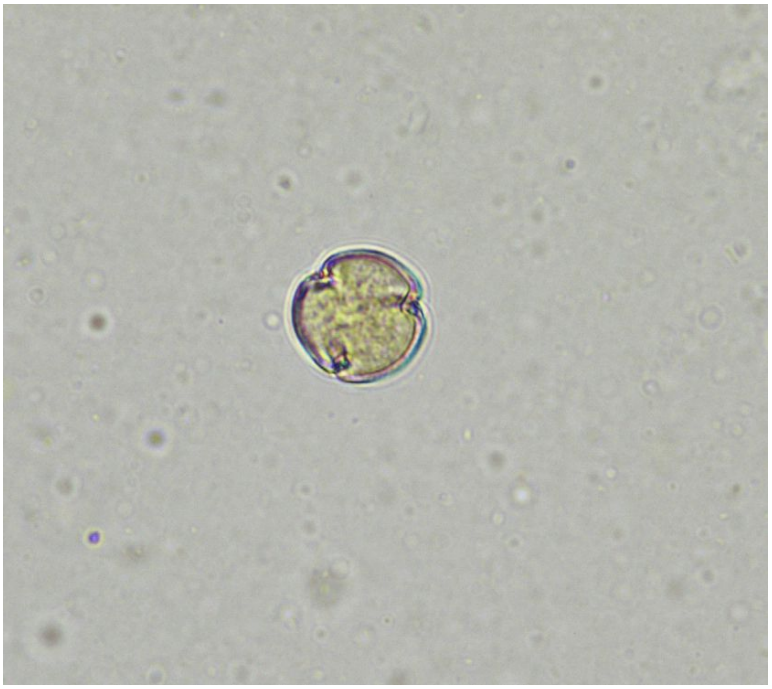

**Figure 22.** Pollen grain of Fagaceae family (total magnification: 400 x).

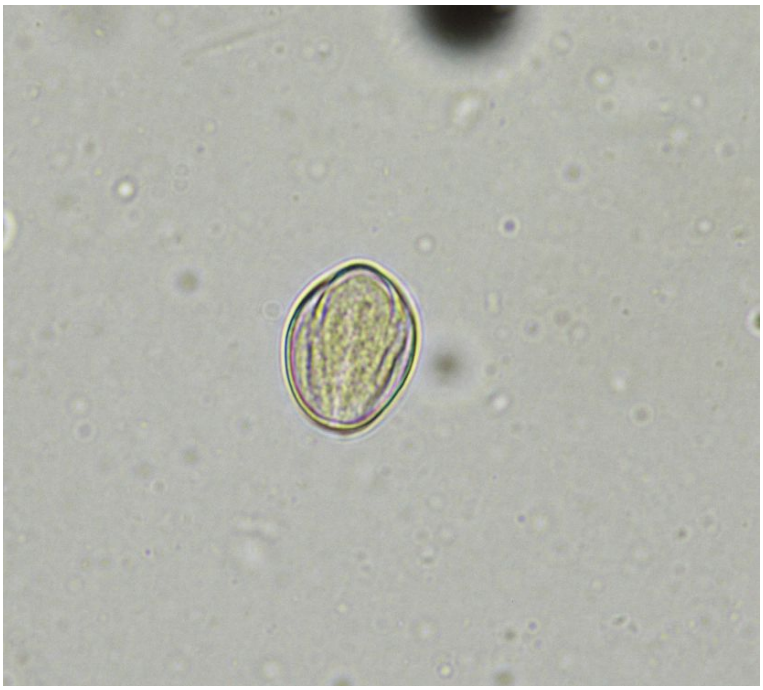

**Figure 23.** Pollen grain of *Echium* sp. (total magnification: 400 x).

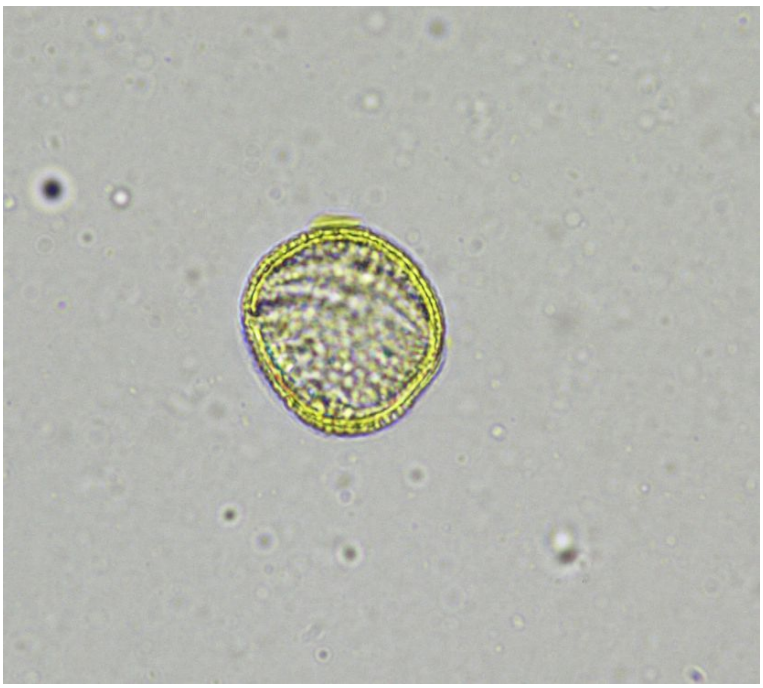

**Figure 24.** Pollen grain of Oleaceae family (total magnification: 400 x).

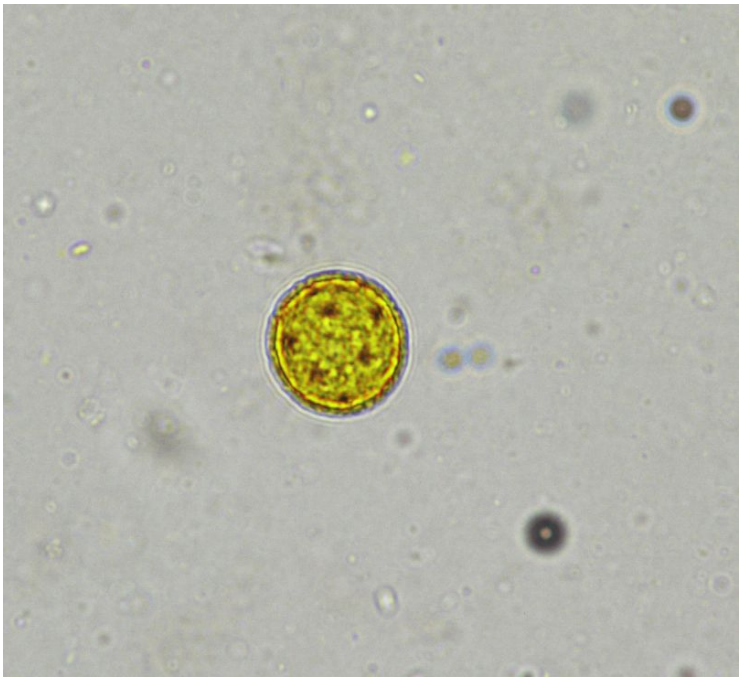

**Figure 25.** Pollen grain of Caryophyllaceae family (total magnification: 400 x).

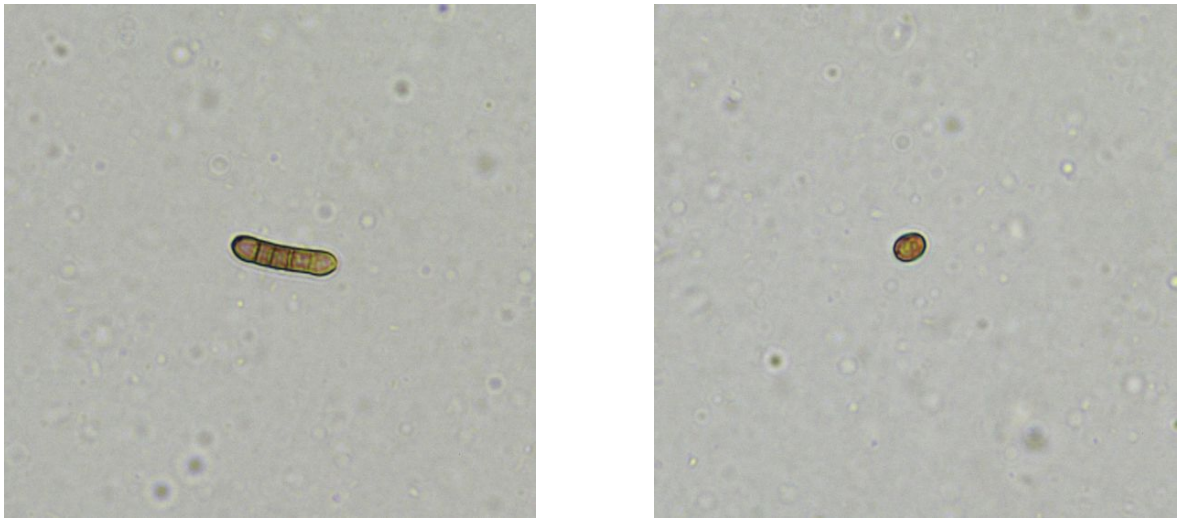

**Figure 26.** Fungal spores found in multifloral honey from *Parque Natural do Montesinho* (total magnification: 400 x).

## 4.2. Alkaloids precipitation tests

After extraction and purification of the alkaloid-targeted extracts, a screening test was conducted to assess the presence of these molecules. For such purpose, chemical reagents based on the solubility of alkaloids salts in acid solutions were used: Dragendorff's (solution of potassium bismuth iodide), Mayer's (potassium mercuric iodide solution) and Bertrand's (silicotungstic acid solution) reagents.

In light of the absence of precipitates (figure 27), it was possible to conclude that either the molecules were not present in the extracts or their concentration was below the limit of detection (LOD) of the technique.

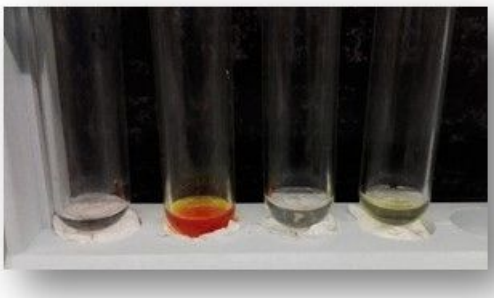

**Figure 27.** Result for alkaloids precipitation tests: control, Dragendorff, Mayer and Bertrand, respectively.

## 4.3. Chemical characterization of the alkaloid-targeted extracts by HPLC-DAD

The chromatographic profile of the alkaloid-targeted extracts was analysed by HPLC-DAD. The method used that revealed better results was performed with the reverse phase *Luna* C18 and the gradient elution described in table 5, being the subsequently concentrations those that allowed improved results: 25.6 mg/mL (monofloral honey), 65.4 mg/mL (multifloral honey from *Serra d'Aire*), 83.4 mg/mL (multifloral honey from *Parque Natural do Montesinho*).

By analysing the ultraviolet-visible (UV-Vis) spectra of the compounds present in the chromatograms of the extracts analysed, and their retention time in the gradient used, it was not possible to identify any alkaloid (figures 29-31).

Pyrrolizidine alkaloids usually present a maximum absorption at a nonspecific wavelength, 220 nm (211). Therefore, for confirmation, two alkaloids that are usually present in honey produced from *E. plantagineum*: echimidine and echimidine *N*-oxide, which exhibit a maximum absorption at 223 nm, were injected and searched (figure 28).

The conclusion withdrawn with such results is that the samples analysed by HPLC-DAD do not have alkaloids or they have these compounds in concentrations behind the LOD of this method, which is important for their safety profile. The results obtained in the HPLC-DAD method are in accordance with the previous referred screening done with the three precipitation reagents of alkaloids: Dragendorff, Mayer and Bertrand.

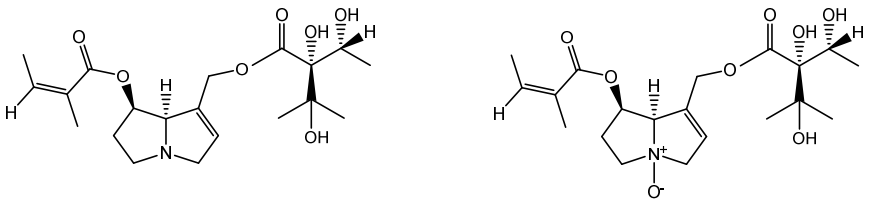

**Figure 28.** Chemical structures of echimidine (left) and echimidine *N*-oxide (right).

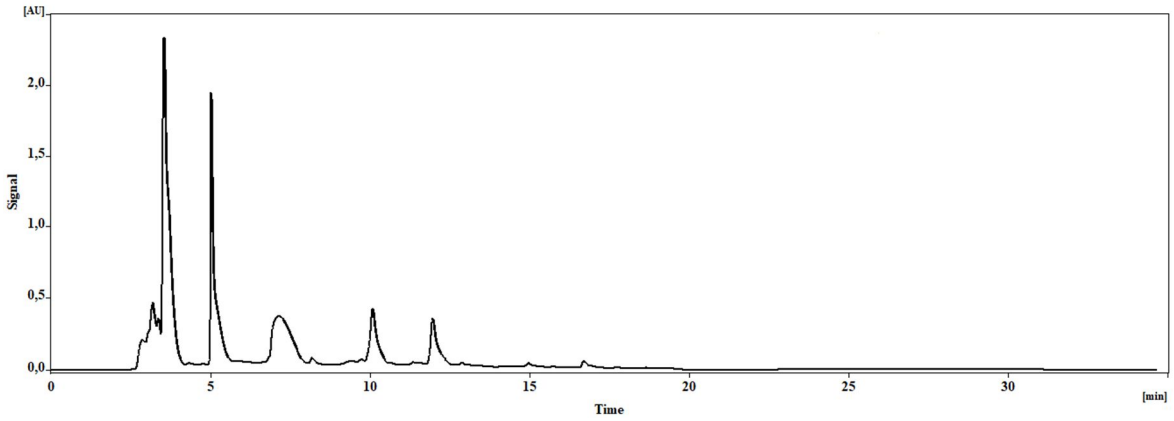


**Figure 29.** Chromatographic profile of the alkaloid-targeted extract of monofloral honey of *E. plantagineum* by HPLC-DAD at 223 nm.

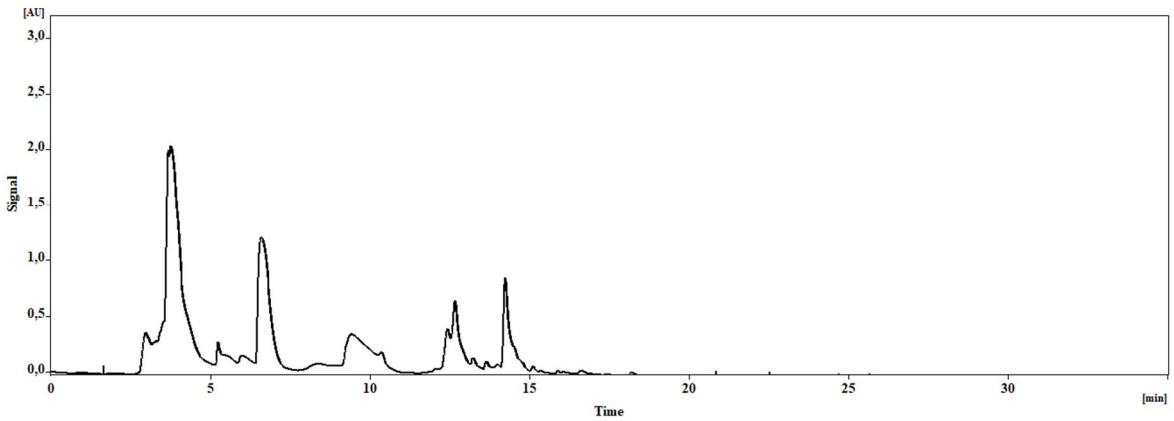


**Figure 30.** Chromatographic profile of the alkaloid-rich extract of multifloral honey from *Serra d'Aire* by HPLC-DAD at 223 nm.

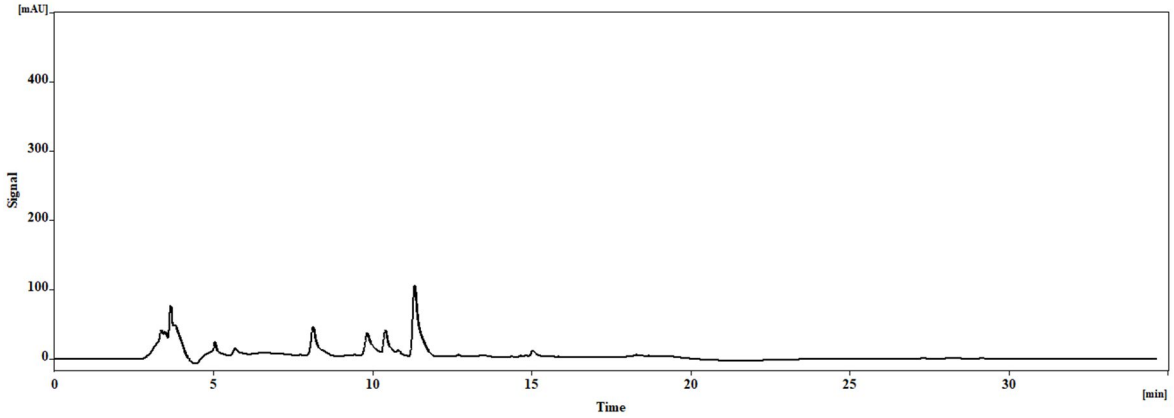


**Figure 31.** Chromatographic profile of the alkaloid-targeted extract of multifloral honey from *Parque Natural do Montesinho* by HPLC-DAD at 223 nm.

## 4.4. Chemical characterization of phenolic-targeted extracts by HPLC-DAD

After extraction and purification of the phenolic-targeted extracts of each sample, the chromatographic profile of these extracts was evaluated by HPLC-DAD. By the observation of the UV-Vis spectra of these compounds and chromatographic profile, it was possible to conclude that they could be phenolic acids and flavonoids. In other words, the retention time presented by the compounds and the maximum absorptions exhibited in the UV-Vis spectra were coincident with phenolic compounds. Some peaks showed an absorption maximum at 320 nm, which allowed to suspect of the presence of phenolic acids, while others showed two major absorption peaks characteristic from bands I (300-380 nm) and II (240-280 nm) from flavonoids.

The analysis of the UV-vis spectra was crucial to do a correct identification of these compounds. Phenolic compounds contain aromatic conjugated systems and multiple double bonds, which make polyphenols strong chromophores and explains the absorption of UV radiation (212). Consequently, they are frequently detected using UV-Vis and DAD detectors at wavelengths ranging from 190 to 600 nm (25). As each class of phenolic compounds has a specific UV spectrum, the advantage of using DAD is the fact that a single run will provide a broad range of information (212). Hydroxybenzoic acids present a maximum absorbance between 235 and 325 nm; hydroxycinnamic acids absorb in two zones of the UV spectrum, exhibiting a first maximum absorbance between 225 and 235 nm, and two others between 290 and 330 nm, while flavonoids show two major absorption peaks at 300-380 nm (band I) and 240-280 nm (band II) (212-214). Band I represents the absorption due to ring B and C and band II to ring A (212). This knowledge was helpful in

the choice of the standards injected to proceed with the identification. Therefore, for the confirmation, the standards were injected, and the subsequent compounds were identified.

### 4.4.1. Phenolic compounds in monofloral honey of *E. plantagineum*

The analysis of the chromatographic profile obtained by HPLC-DAD allowed the identification of 6 compounds in the extract of monofloral honey of *E. plantagineum*. The chromatogram showed *p*-hydroxybenzoic acid, caffeic acid, *p*-coumaric acid, ferulic acid, chrysin and hyperoside (figure 32).

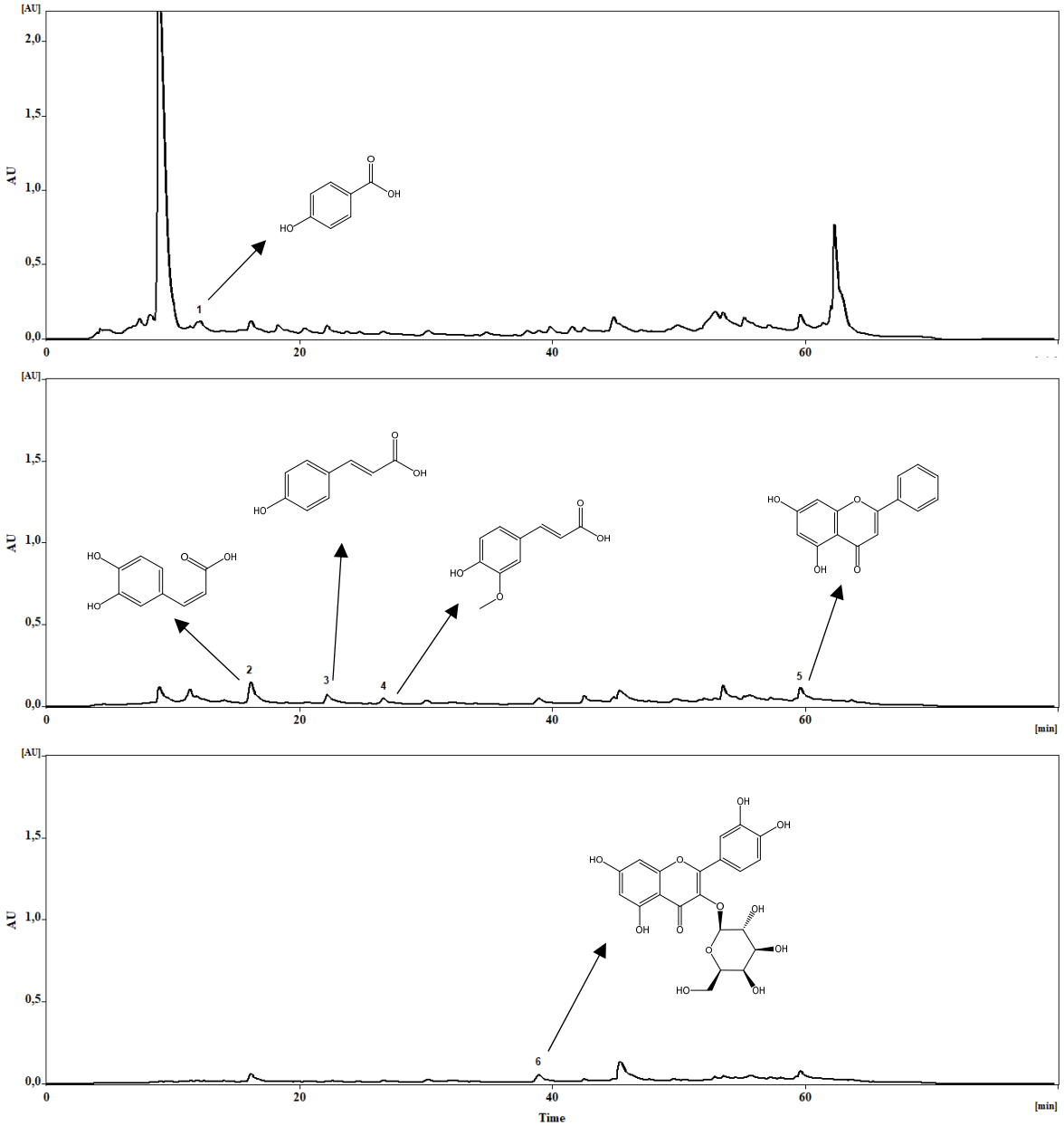


**Figure 32.** Chromatographic profile of the extract of monofloral honey of *E. plantagineum* by HPLC-DAD at 280, 320 and 350 nm, respectively. Peaks: 1. *p*-hydroxybenzoic acid; 2. caffeic acid; 3. *p*-coumaric acid; 4. ferulic acid; 5. chrysin; 6. hyperoside.

After the identification of 6 compounds present in the extract of monofloral honey, the quantification was done, using an external standard of known concentration to quantify each one of the compounds identified. The standards were injected three times, as well as the sample.

The extract of the sample was injected in a concentration of 85,5 mg/mL, and the results obtained for each one of the compounds quantified are shown in table 9.

**Table 9.** Phenolic compounds quantified in the methanolic extract of monofloral honey of *E. plantagineum*. The results obtained are represented as value ± standard deviation of the mean.

| | **Molecule** | **Concentration (mg/mL)** | **Concentration in the sample (mg/g)** |
|---|---|---|---|
| 1 | *p*-Hydroxybenzoic acid | 0.230 ± 0.004 | 0.0046 ± 0.00009 |
| 2 | Caffeic acid | 0.096 ± 0.001 | 0.0019 ± 0.00003 |
| 3 | *p*-Coumaric acid | 0.028 ± 0.001 | 0.0006 ± 0.00002 |
| 4 | Ferulic acid | 0.009 ± 0.0001 | 0.0002 ± 0.000002 |
| 5 | Chrysin | 0.010 ± 0.001 | 0.0002 ± 0.00003 |
| 6 | Hyperoside | 0.115 ± 0.007 | 0.0023 ± 0.0001 |

### 4.4.2. Phenolic compounds in multifloral honey from *Serra d'Aire*

The analysis of the chromatographic profile (figure 33) obtained by HPLC-DAD allowed the identification of 4 compounds of the extract of multifloral honey coming from *Serra d'Aire*.

After identification of 4 compounds present in the extract of multifloral honey from *Serra d'Aire*, the quantification was done, using an external standard of known concentration to quantify each one of the compounds identified. The standards were injected three times, as well as the sample.

The extract of the sample was injected in a concentration of 296.8 mg/mL, and the results obtained for each one of the compounds quantified are shown in table 10.

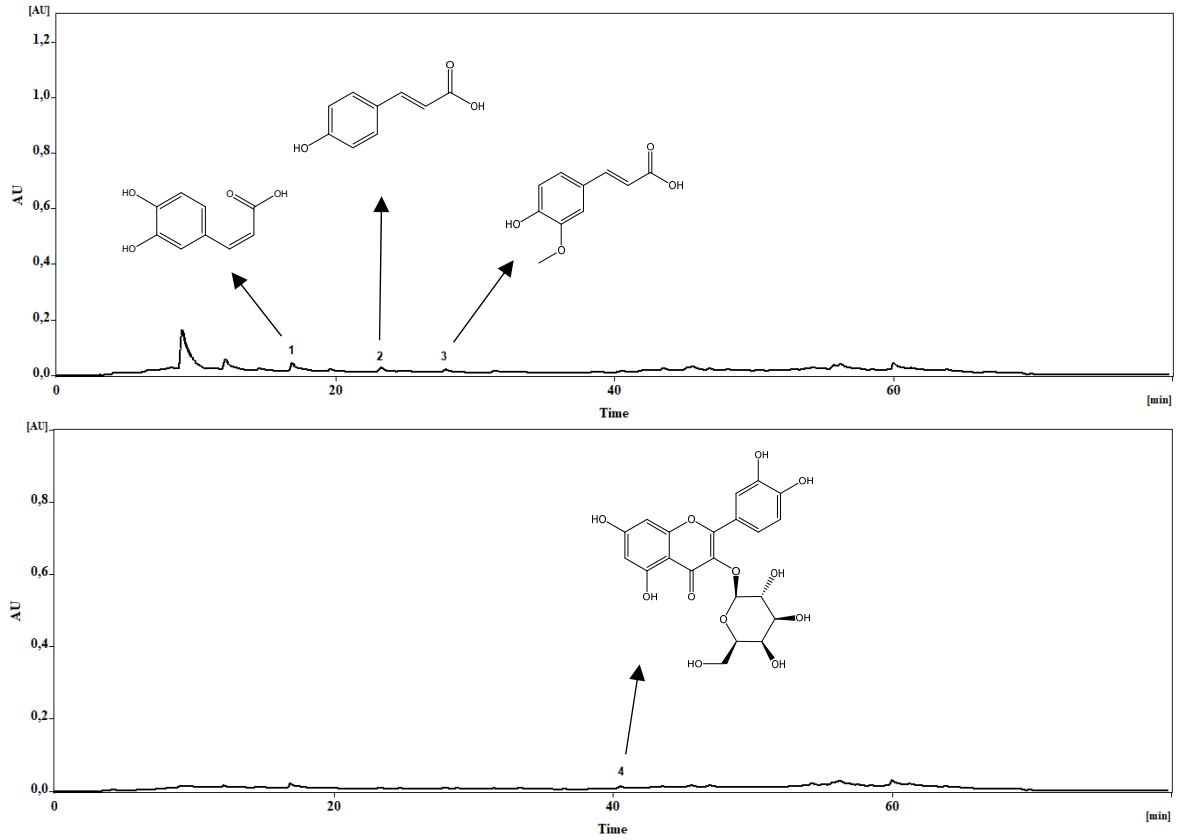


**Figure 33.** Chromatographic profile of the extract of multifloral honey from *Serra d'Aire* by HPLC-DAD at 320 and 350 nm, respectively. Peaks: 1. caffeic acid; 2. *p*-coumaric acid; 3. ferulic acid; 4. hyperoside.

**Table 10.** Phenolic compounds quantified in the methanolic extract of multifloral honey from *Serra d'Aire.* Values are expressed as concentration ± standard deviation of the mean.

| | **Molecule** | **Concentration (mg/mL)** | **Concentration in the sample (mg/g)** |
|---|---|---|---|
| 1 | Caffeic acid | 0.011 ± 0.0001 | 0.00021 ± 0.000003 |
| 2 | *p*-Coumaric acid | 0.008 ± 0.0004 | 0.00016 ± 0.00001 |
| 3 | Ferulic acid | 0.003 ± 0.00005 | 0.00005 ± 0.000001 |
| 4 | Hyperoside | 0.013 ± 0.0005 | 0.00025 ± 0.00001 |

### 4.4.3. Phenolic compounds in multifloral honey from *Parque Natural do Montesinho*

The analysis of the chromatographic profile obtained by HPLC-DAD allowed the identification of 3 peaks of the extract of multifloral honey coming from *Parque Natural do Montesinho*. The chromatogram showed caffeic acid, *p*-coumaric acid and chrysin (figure 34).

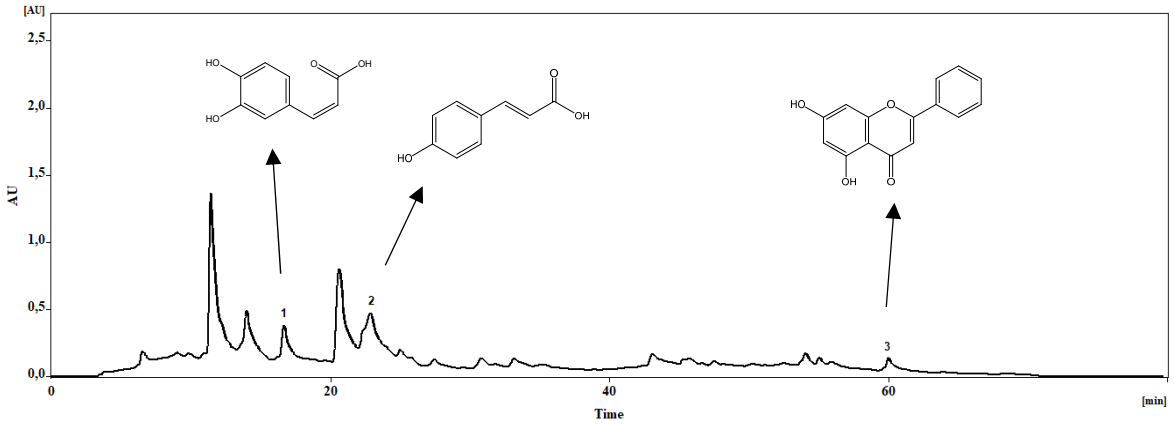


**Figure 34.** Chromatographic profile of the extract of multifloral honey from *P. N. Montesinho* by HPLC-DAD at 320 nm. Peaks: 1. caffeic acid; 2. *p*-coumaric acid; 3. chrysin.

After identification of 3 compounds present in the extract of monofloral honey, the quantification was carried out in the same way as it was performed for the other extracts. The extract of the sample was injected in a concentration of 300.39 mg/mL, and the results obtained for each one of the compounds quantified are shown in table 11.

**Table 11.** Phenolic compounds quantified in multifloral honey from *Parque Natural do Montesinho*. Values are shown as concentration ± standard deviation of the mean.

| Molecule | | Concentration (mg/mL) | Concentration in the sample (mg/g) |
|---|---|---|---|
| 1 | Caffeic acid | 0.237 ± 0.001 | 0.00471 ± 0.00002 |
| 2 | *p*-Coumaric acid | 0.354 ± 0.009 | 0.00705 ± 0.0002 |
| 3 | Chrysin | 0.021 ± 0.0004 | 0.00042 ± 0.00001 |

Comparing the chromatographic profile of the three extracts, it was possible to conclude that there are two compounds common to all of them: caffeic and *p*-coumaric acids. In addition, the extracts of monofloral honey and honey from *Serra d'Aire*, when compared, present four compounds in common: caffeic, *p*-coumaric acid, ferulic acid, and hyperoside. Besides, the three chromatographic profiles, when compared to each other, are different.

The extract where a bigger number of compounds was identified was the phenolic-targeted extract of honey from *E. plantagineum*. There is one compound that only appear in monofloral honey: *p*-hydroxybenzoic acid.

Comparing the results obtained, it is also possible to conclude by the analysis of the concentration present in the samples that the honey with the highest concentrations of phenolic compounds is the multifloral from *Parque Natural do Montesinho*. Otherwise, multifloral honey from *Serra d'Aire* revealed to be the honey with the lowest levels of phenolics.

## 4.5. Biological activity of the alkaloid-targeted extracts

### 4.5.1. Effect on macrophages

#### 4.5.1.1. Cell viability

For the analysis of the cytotoxic effect of the alkaloid-targeted extracts on RAW 264.7, the MTT assay was used. MTT assay is a colorimetric test based on the metabolic reduction of MTT, since only metabolic active cells are capable of this (215). After entering in the cells, MTT is reduced to formazan by nicotinamide adenine dinucleotide (NADH) reductase and other enzymes (215). The violet crystals of formazan can then be extracted with organic solvents and their absorbance is read at 570 nm. This method is one of the most frequently employed for the evaluation of cell viability, owing to its reliability, low cost and fastness (216). However, a study with the purpose of comparing different viability assays questioned the reliability of this test, declaring that honey sugars can reduce the MTT reagent (217). For this reason, it was decided to test the influence of the honey sugars of the samples analysed in the reduction of the MTT, and the conclusion was that no significant differences were found. A possible explanation for this is the fact that, as referred above, a SPE procedure was employed to prepare the samples for the analysis. Therefore, the majority of the sugars present in the samples was removed.

The cell line was exposed to the extract in the range 0.0625-1 mg/mL, in order to define the concentrations that did not elicit toxicity. As can be seen in figure 35, the extracts under study did negatively impact cell viability.

From the three extracts, it is possible to conclude that the one that induces a minor increase of cell viability is the extract of multifloral honey from *Parque Natural do Montesinho* and the extract that causes the highest increase is the extract of monofloral honey of *E. plantagineum*.

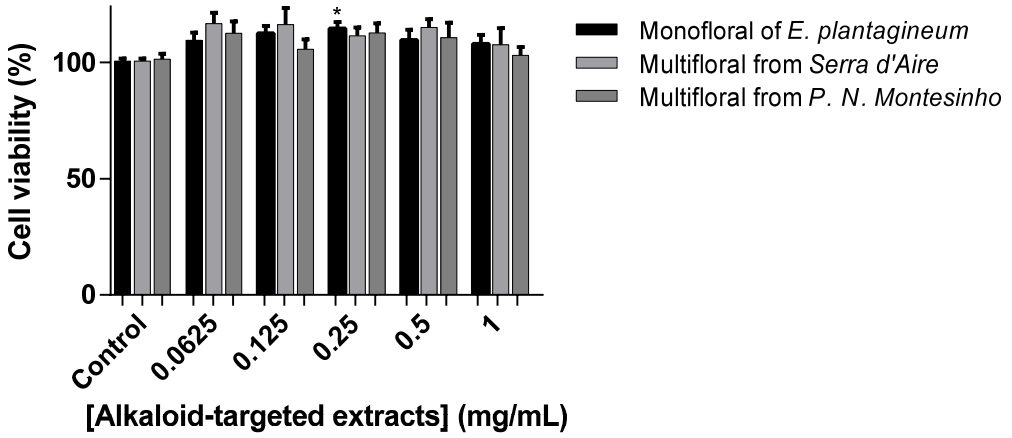


**Figure 35.** Viability of RAW 264.7 after 24h of incubation with the alkaloid-targeted extracts from honey. The results are expressed as mean ± standard error of the mean of three independent experiments made in triplicate. *p<0.05.

### 4.5.1.2. ·NO inhibition

Since the extracts were shown to be devoid of toxicity towards the cells used, the potential anti-inflammatory of the extracts was evaluated. LPS was used in order to trigger a pro-inflammatory status in macrophage cells (61).

In inflammatory processes, the upregulation of iNOS as a consequence of pro-inflammatory mediators, such as cytokines, results in increased levels of ·NO, which plays an important role as a mediator in the inflammatory response (62). This free radical is involved in several physiological and pathophysiological mechanisms, in systems, as the cardiovascular and nervous systems (199). Therefore, the regulation of its production in tissues may be important for the treatment of inflammation. For this assessment, non-toxic concentrations of the extracts were tested in a cellular model of LPS-induced macrophages. The cells were incubated for 24h with the extracts, of which 22h they were stimulated with

LPS. After this time, •NO levels were determined by spectrophotometric methods, with support of Griess reaction. The results are shown in figure 36.

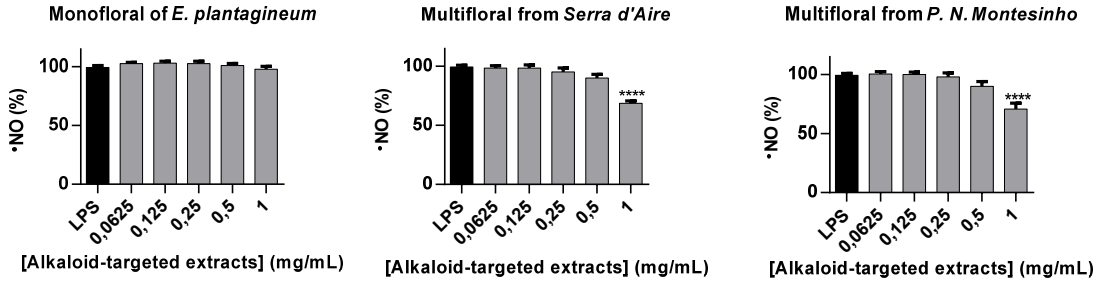


**Figure 36.** Effect of the three extracts (monofloral honey of *E. plantagineum*; multifloral honey from *Serra d'Aire*; multifloral honey from *P. N. Montesinho*) on •NO production LPS-stimulated macrophages for 22h. Results are expressed as mean ± standard error of the mean of four independent experiments performed in triplicate. ****p<0.0001.

As can be seen, the extract that revealed more activity in the decrease of •NO production was the extract of multifloral honey from *Parque Natural do Montesinho*, with the highest concentration (1 mg/mL) inhibiting approximately 30% of the free radical production. The less active extract was the extract of monofloral honey of *E. plantagineum* with a decrease of the •NO production not statistically significant.

Taking into account that no molecules could be identified in the extract by HPLC-DAD, the discussion of results is limited. Notwithstanding, if PA were present the results could be more relevant, demonstrating significant inhibitory activities against •NO production, as have been show by some studies (63).

### 4.5.2. Effect on gastric adenocarcinoma cells

#### 4.5.2.1. Cell viability

The cytotoxicity of the alkaloid-targeted extracts was also evaluated in AGS cells using the MTT assay. The choice of this cell line lies on the fact that honey is a foodstuff, so the use of gastric cells allows to mimic its route.

As can be seen in figure 37, none of the extracts demonstrated a high level of loss of viability against AGS. The extract that caused the statistically more significant decrease of viability was the extract from monofloral honey of *E. plantagineum,* with approximately 24% of loss of viability in a concentration of 1 mg/mL. Otherwise, the extract that caused lesser loss of viability was the one of multifloral honey from *Serra d'Aire*.

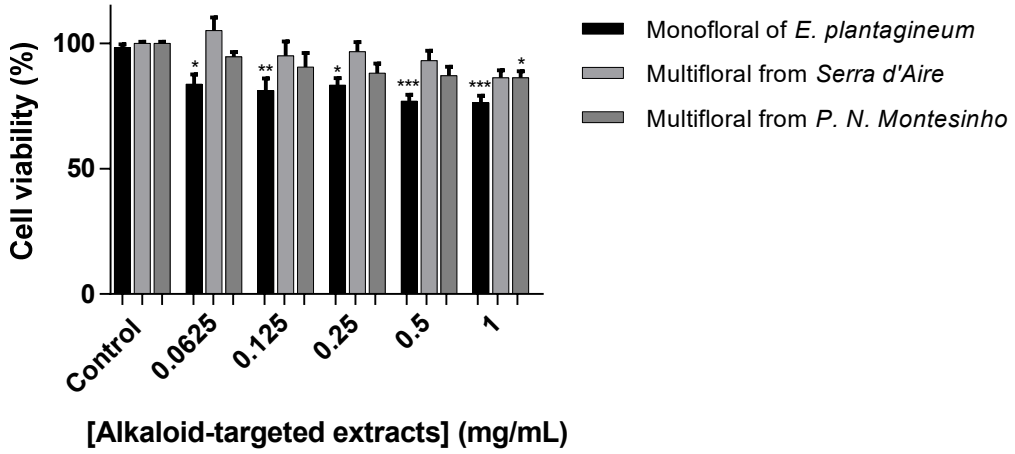


**Figure 37.** Viability of AGS in the presence of alkaloid-targeted extracts (monofloral honey of *E. plantagineum*; multifloral honey from *Serra d'Aire*; multifloral honey from *P. N. Montesinho*) for 24h. Values are shown with mean ± standard error mean of three independent experiments performed in triplicates. *p≤0.05; **p≤0.01; ***p≤0.001.

### 4.5.3. Effect on human fetal lung fibroblasts

#### 4.5.3.1. Cell viability

In an attempt to complete the analysis of the biological activity done, evaluation of the toxicity of the extracts in the non-cancer cell line MRC-5 was conducted. The conditions were similar to the other cell lines, using a range of concentrations between 0.0625 and 1 mg/mL, and an incubation time of 24h.

As expected by the analysis of the previous results in the other cell lines and by the HPLC-DAD analysis, no toxicity was registered, as represented in figure 38.

In the extracts studied, it was not possible to identify any type of PA, however it is possible to affirm that if they were present in considerable amounts it would be predictable to obtain a similar result. As referred above, the lungs are the second most affected organ by the metabolites produced, but only after metabolic activation of PA in the liver (54). The lungs are not able to metabolize PA to DHPA, their toxic metabolites, in consequence, cytotoxicity is not expected in a cell culture of fibroblasts.

The only extract that revealed a statistically significant difference was the extract from monofloral honey of *E. plantagineum* after 24 hours, in a concentration of 1 mg/mL.

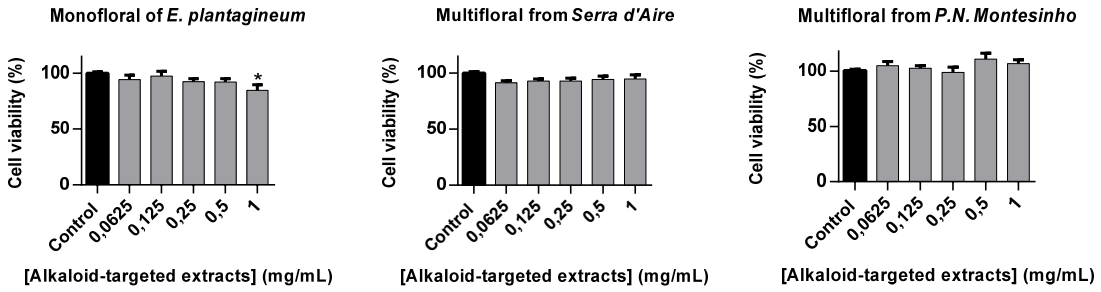


**Figure 38.** Viability of the three honey extracts of monofloral honey of *E. plantagineum*, multifloral honey from *Serra d'Aire* and multifloral honey from *P. N. Montesinho*. The results are shown as mean ± standard error of the mean of three independent experiments performed in triplicate. *p<0.05.

## 4.6. Biological activity of the phenolic-targeted extracts

### 4.6.1. Effect on macrophages

#### 4.6.1.1. Cell viability

The cytotoxicity of the phenolic-targeted extracts was also evaluated in macrophages by the MTT reduction assay.

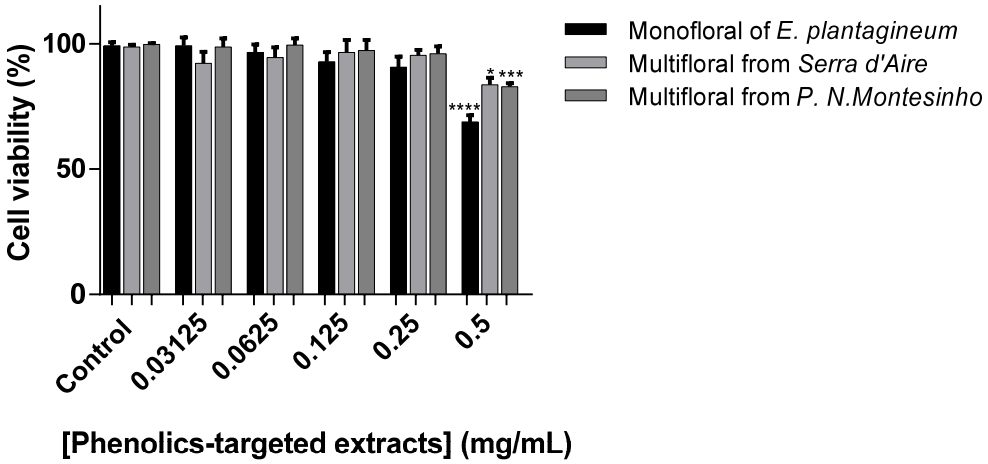


**Figure 39.** Viability of macrophages (RAW 264.7) with three phenolic-rich methanolic extracts of honey. Results are shown as mean ± standard error mean of three independent experiments performed in triplicate. *p ≤ 0.05.

The extract that caused a statistically significant decrease of cell viability was from monofloral honey produced from *E. plantagineum*, which resulted in a loss of viability ca.

30% at 0.5 mg/mL. The other two extracts instigated a maximum of 20% in loss of viability, as can be seen in figure 39.

A possible explanation for these results can be the fact that flavonoids in their oxidized form can act as pro-oxidant compounds, despite their known antioxidant activity (218). This class of compounds have been shown to act as pro-oxidants when in the presence of redox-active metals, which catalyse the redox cycling of phenolics ending in the formation of ROS and phenoxyl radicals that can damage DNA, lipids and other molecules (219). Flavonoids with a phenolic B ring, such as chrysin and hyperoside found in the extracts, may suffer oxidation by peroxidases/$H_2O_2$ to phenoxyl radicals, accompanied by GSH co-oxidation and oxygen activation, which results in $O_2^{\bullet-}$ and $H_2O_2$ formation (218). The formation of ROS may lead to lipid peroxidation, and consequently affect cell integrity, leading to cell death. Another hypothesis to explain the results obtained is the presence of other molecules in the extracts capable of triggering this loss of viability.

#### 4.6.1.2. $^{\bullet}$NO inhibition

After the results obtained in the cell viability assay, which revealed no loss of viability statistically significant to RAW 264.7 in the range 0.03125 - 0.25 mg/mL, it was decided to carry an assay to test the inhibition of $^{\bullet}$NO production. The results obtained are represented in the graphic of figure 40.

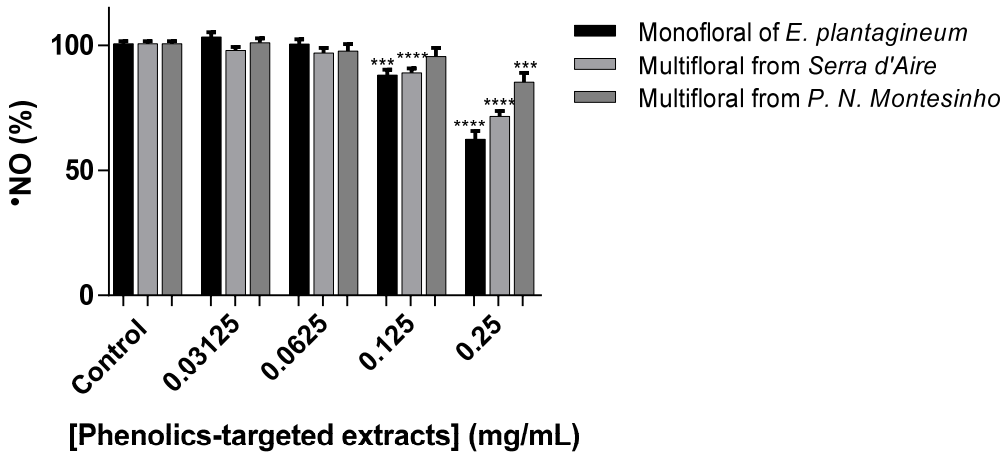


**Figure 40.** Effect of the three extracts of phenolic compounds (monofloral honey of *E. plantagineum*; multifloral honey from *Serra d'Aire*; multifloral honey from *P. N. Montesinho*) on $^{\bullet}$NO production LPS-stimulated macrophages for 22h. Results are expressed as mean ± standard error mean of three independent experiments realized in triplicate. ***p ≤ 0.001; ****p ≤ 0.0001.

The conclusion achieved was that the most active extract was the honey extract of *E. plantagineum*, which revealed a $^{\bullet}$NO decrease near to 40%. Regarding the alkaloid-targeted

extract of the same honey sample, it is possible to confirm that it was the least active in the inhibition of •NO production. On the other hand, the alkaloid-targeted extract from *Parque Natural do Montesinho* was the most active of this type of extract. Regarding the phenolic-targeted extract of this honey, it was the least active.

### 4.6.2. Effect on gastric adenocarcinoma cells

#### 4.6.2.1. Cell viability

The phenolic-targeted extracts were also tested against AGS. As can be seen in the graphic represented in figure 41, none of the extracts displayed relevant toxicity against this cancer cell line, exception being the extract from multifloral honey from *Serra d'Aire* in a concentration of 0.5 mg/mL, albeit decreasing cell viability solely by 12%.

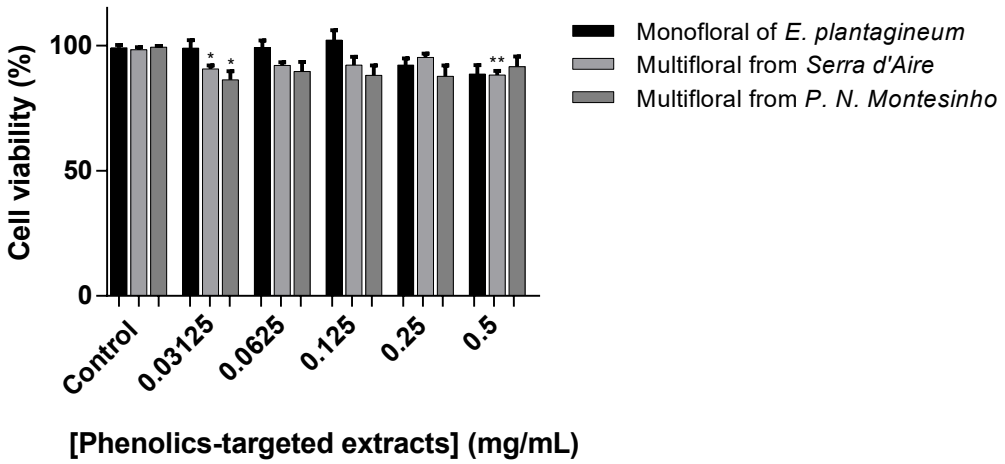


**Figure 41.** Viability of AGS in the presence of phenolic-targeted extracts (monofloral honey of *E. plantagineum*; multifloral honey from *Serra d'Aire*; multifloral honey from *P. N. Montesinho*) for 24h. Values are shown with mean ± standard error mean of three independent experiments performed in triplicate. *p ≤ 0.05; **p ≤ 0.01.

In the literature, there are reports about phenolics that induce growth inhibition and cell death in this cell line. For example, the results obtained in a study with malvidin, allowed to achieve the conclusion that it has a potent anti-proliferation effect on AGS cells in a time- and dose-dependent manner, which results from apoptosis (220). In other study, also polyphenolic-targeted extracts from *Hibiscus sabdariffa* L. in a concentration of 0.95 mg/mL inhibited the growth by 50% (221).Therefore, the results obtained with the phenolic-targeted extracts from honey can be explained by the fact that the three extracts analysed are very

diluted, having very low levels of bioactive compounds that could trigger an important activity against this type of cells.

### 4.6.3. Effect on human fetal lung fibroblasts

#### 4.6.3.1. Cell viability

Similar to the alkaloid-targeted extracts, phenolic-targeted extracts were tested in the non-cancer cell line MRC-5, in the concentration range of 0.03125 - 0.5 mg/mL.

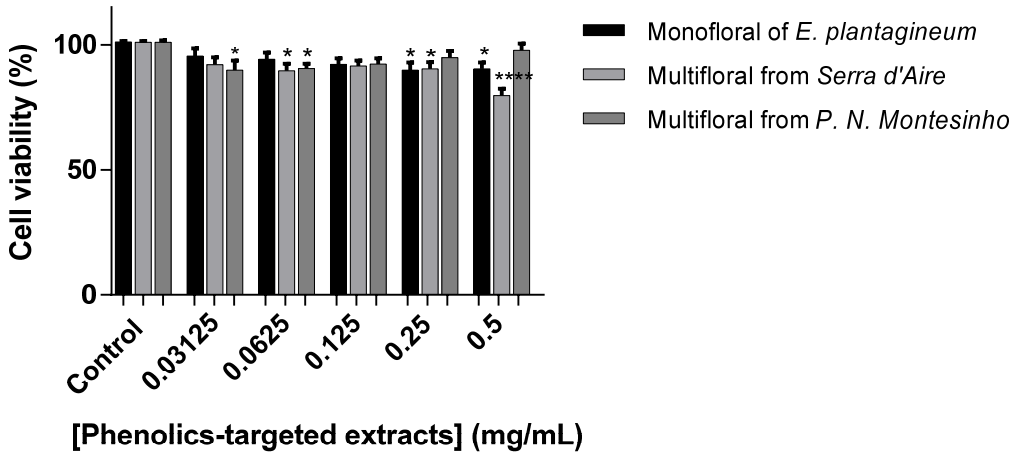


**Figure 42.** Viability of MRC-5 in the presence of phenolic-targeted extracts (monofloral honey of *E. plantagineum*; multifloral honey from *Serra d'Aire*; multifloral honey from *P. N. Montesinho*) for 24h. Values are shown with mean ± standard error mean of three independent experiments performed in triplicadte. *p<0.05; ****p<0.0001.

Once again, these extracts did not reveal a relevant loss of viability, a maximum of ca. 20% was obtained with the phenolic-targeted extract of multifloral honey from *Serra d'Aire*, which revealed the most statistically significant difference, as described in figure 42.

The results obtained are in accordance with previously described studies, which revealed that besides the cytotoxic effects against cancer cells, phenolic-targeted extracts do not statistically affect MRC-5 cells (222,223).

# *Chapter 5*

## 5. Conclusions and future perspectives

The analysis of the three Portuguese honey samples allowed to achieve important conclusions regarding the safety of these products. The search of PA, which are plenty reported in literature for being present in honey produced from the plant *E. plantagineum*, revealed that Portuguese honeys analysed do not possess these harmful compounds, at least in concentrations detectable by the method employed. On the other hand, they have compounds recognized for their beneficial properties, even if they occur in low concentrations: phenolics.

The biological studies performed with the two sorts of extract demonstrated no cytotoxicity for the non-cancer cell line (MRC-5) and no appreciable biological activity in AGS. In macrophages, the extracts presented ability to inhibit the $^{\bullet}$NO production although in a maximum decrease of 40%. An explanation for these results can be the low content of bioactive compounds, as a result of processing, during which these compounds are diluted.

Although pollen identification of honey was not one of the purposes of this work, it revealed to be of great importance in the quality control, since it allowed the authentication of the samples, providing information about its botanical origin.

In conclusion, this work represents an important contribution to the quality control of Portuguese honeys, since foodstuff with these secondary metabolites can pose a life threat to humans and animals when chronically consumed. Thereby, it is of great importance to further develop the information of the consumer in this area, providing analysis of products known for having harmful compounds, such as the ones referred above.

# *Chapter 6*

## 6. References

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