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Pyrrolizidine Alkaloids in Some Algerian's Honeys

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ABSTRACT

In this research, some Algerian honey samples were collected from various geographical and botanical origins, and analyzed by LC/MS method for determination of pyrrolyzidine alkaloids. The study of 46 samples revealed the presence of PA in the honeys tested and that some contained high concentrations of this substance. 74% contained PAs with a total PAs' concentration ranging from 1 to 748 µg/kg of PA-positive samples. This study demonstrated that Algerian honeys may contain high amounts of PAs exceeding the current recommendations. Massive intoxication during honey consumption seemed unlikely. However, long-term consumption of large quantities of highly contaminated honey could lead to poisoning specially in possible additional exposure of consumer to PAs from other sources like medicinal plants, which is recognized as a potential threat to human health. For that reason, beekeepers have been advised to carry out more rigorous quality control tests to the evaluation of PAs in honeys.

Keywords: *Pyrrolizidine alkaloids, Melissopalynology, Algerian honey quality, alternative medical treatments, Food safety.*

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1. INTRODUCTION

Algeria comprises 2,381,741 square kilometers of land. The nature and the richness of the Algerian territory, unique by its location, its mountains, its forests and its Sahara desert, as well as the diversity of its climatic conditions, makes it possible to produce different kinds of honey. Honey is a food that humanity has known since prehistoric times. It is an extraordinary "food- medicine" that the ancients used in a variety of ways, and this has been often in connection with customs, legends and myths of humanity. It has been used in the diet, because of its very energetic character, its exceptional nutritional value and on the other hand, it has been widely used by the Algerian population in alternative to medical treatments. Indeed, virtues of honey in the traditional medicine have been widely attributed to its antioxidant and antimicrobial properties.

Beyond its aforementioned values, honey enjoys an important status in every monotheism, which symbolises prosperity, abundance, and blessing. As far as Islam is concerned, there is an entire sourate named *Sourat Al-Nahl* (Soute of the Bee) that is devoted to describe the virtues of bees and honey "And your Lord inspired the bees, saying: Take you habitations in the mountains and in the trees and in what they erect" (68) "Then, eat all fruits, and follow the ways of your Lord made easy (for you)." "There comes forth from their bellies, a drink of varying color wherein is for men. Verily, in this, there is indeed a sign for people who think" (69). Sourah of Bees 'AL Nahl 16', verses 68-69.

In spite of its numerous benefits and uses, honeys have been found to contain pyrrolizidine alkaloids (PAs) from bees foraging on PA-containing plants (Reinhard et al., 2009). The PAs are usually found in the form of naturally occurring plant toxins in an estimated 3 % of all flowering plants (more than 6,000 plants) which have been known to synthesize more than 660 different PAs (Wiedenfeld et al., 2008; Habs et al., 2018), in particular the families of Boraginaceae (all genera), most frequently inflowering plants of the members Asteraceae (tribes Senecioneae and Eupatorieae), Fabaceae (genus Crotalaria) families (Letsyo et al., 2017) and in some genera of the Orchidaceae family (Skoneczny et al. 2015 a). These plants consist of plant species of Echium, Senecio, Eupatorium, Heliotropium, Borago, Myosotis, Chromolaena, Petasites, Ageratum, Cynoglosum, Crotalaria, Tussilago and Symphytum (Edgar et al., 2011; Ozansoy and Küplülü, 2017).

Pyrrolizidine alkaloids (PAs) are one of the most common groups 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 (Moreira et al., 2018). They rarely occur in the free form as a pyrrolizidine base, being instead found as esters (mono-, di- or macrocyclic biesters) 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 (Moreira et al. 2018). The necine base can either be saturated or unsaturated (i.e. contain a double bond in the 1,2 position). The PAs may exist

as free bases or as N-oxides (PANOs), which act as distinct compounds, with contrasting physical properties (Molyneux et al., 2011; Lucatello et al., 2016).

The toxicity of PAs depends mainly on the nature of the bond in position 1,2 of the pyrrolizidine ring system. 1, 2-unsaturated PAs themselves are not toxic in their original forms but require metabolic activation to exert their toxicities (Fu et al., 2007). Numerous reports have indicated that the adverse effects of 1,2-unsaturated PAs - in experimental animals - include hepatotoxicity, developmental toxicity, genotoxicity, carcinogenicity (Dreger et al., 2009; EFSA, 2016), and sometimes pneumotoxic (Wiedenfeld, 2011a). PAs have also been associated with weak antileukemic and virustatic activity and embryotoxicity (Alali et al., 2008; Skoneczny et al., 2015 b). However, not all PAs lead to the synthesis of the toxic metabolite. Interestingly, some pyrrolizidine alkaloids with their glycosidase inhibitory activity make them an important compound for the treatment of diseases like cancer and diabetes and as a result, they have been of great interest in medical research fields (Kaur and Arora, 2015).

PAs exert hepatotoxicity through metabolic activation in liver which is the first target organ for PA poisoning. Only the esters of 1,2-unsaturated retronecine- and otonecine type PAs, which are bioactivated by hepatic cytochrome PF mixed function oxidase to toxic pyrrolic ester (dehydropyrrolizidine: DHP) (Prakash et al., 1999). Also, hepatic cytochromes P450s (CYPs) can bioactivate two types of toxic PAs to generate reactive intermediates which form pyrrole-protein adducts which provide a mechanism-based biomarker to assess PA toxicity (Ruan et al., 2014; Zhu et al., 2017). These bifunctional DHP molecules can react with a variety of nucleophilic intracellular macromolecules, resulting primarily in liver, lungs and blood vessels; associated with different types of PA poisonings and other tissue damage (Fu et al., 2002). Acute liver damage caused by Pas has been characterized by hepatic sinusoidal obstruction syndrome (HSOS) (Fu et al. 2002; Edgar et al. 2011), and HSOS has been considered pathognomonic for acute PA exposure (Roeder et al., 2015). Then the secondary damage can also be observed in kidney, gastrointestinal tract, pancreas and bone marrow (Ozansoy and Küplülü, 2017). Lungs in particular, have been known to develop chronic and progressive pulmonary arterial hypertension, leading to the right heart failure (Fu et al., 2002; Edgar et al., 2011; Roeder et al., 2015). However, not all PAs are toxic, and it is important to be able to distinguish between toxic and toxic PAs because those with saturated necine bases are considered to be non-toxic (Rosemann et al., 2014).

Since the first documented outbreak of 80 PA-induced liver injury (PA-ILI) cases in South Africa (Willmot and Robertson, 1920) , numerous PA- poisoning cases have been reported worldwide (Lin et al., 2011; Ruan et al., 2015; Zhuge et al., 2018).

Although over 6,000 plant species have been reported to contain PAs, both the composition and concentration of PAs may fluctuate according to climatic and environmental conditions and the age and the variety of the plants (Hoogenboom et al., 2011).

Due to the presence of PAs 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 grains, honey and pollens (Kempf et al., 2011; Moreira et al., 2018). Cases of intoxication and direct poisonings in man and animals seemed to be associated with only a few species containing PAs contaminated cereals, teas, and salads which have been extensively reported (Kakar et al., 2010; Wiedenfeld and Edgar, 2011).

Thus, several studies on food chemistry and food safety have shown that many of the PAs can contaminate many biological products, such as foodstuff, beverages (milk, teas, and herbal infusions), and herbal remedies (Mulder et al., 2015; Habs et al., 2017). Even though, the toxicity of PAs has been well documented, and the high concentrations of PAs have been detected in various products, there has been no official limit for the maximum allowable level of PAs in food and feed (Kowalczyk and Kwiatek, 2018).

Honey can contain PAs, because when bees visit areas, in which PA plants are abundant, PAs can be transferred into honey because nectar and pollen of PA plants contain alkaloids (Detzel et al., 1993; El-Shazly and Wink, 2014). For this reason, honey is one of the best studied food products with respect to PA contamination (Lucchetti et al., 2016). Still, PAs have been found in honey from various botanical and geographical origins (Kempf et al., 2008). However, PAs contamination in honey has been widely studied in many countries (Boppre et al., 2005; Kempf et al., 2010; Dübecke et al., 2011; EFSA, 2011; Cramer et al., 2012; Bodi et al. 2014; Griffin et al., 2015; Lucatello et al., 2016, Martinello et al., 2014; Kowalczyk and Kwiatek, 2018) and it has been demonstrated that honey for human consumption was contaminated with natural occurring, plant derived pyrrolizidine alkaloids. However, there have been no data available on PA levels in Algerian honeys. The present study focused on PAs contamination in honey sold in several regions of Algeria (Souk Ahras, Annaba, Guelma, Taref, Skikda, Media, Blida, Chlef, Djelfa and Algiers) in order to obtain some information on PA levels in Algerian honeys in order to have a potential health threat in the light of increasingly retail honey consumption in the country.

2. MATERIAL AND METHODS

2.1. Chemical and solvents

All solvents used had HPLC grade purity. Sulphuric acide was purchased from Carlo Erba (Milan, Italy). Acetonitrile was obtained from VWR (Leicesters Hire, UK) for HPLC. Acetonitrile LC-MS gradient, Formic acid eluent additive for LC-MS and Zink dust (purity ≥ 98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). QuEChERS salt EN method, Dispersive SPE, High Pigment, EN and ceramic homogenizers were acquired from Agilent Technologies (Santa Clara, CA, USA). Pure water was prepared from Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Sample preparation

2.2.1. Pyrrolizidine alkaloid (PA) standards

Pyrrolizidine alkaloid references were purchased from a range of distributors. The following seven (7) PAs standards were obtained from phytolab GmbH & Co. KG (Vestenbergsgreuth, Germany): ECHI, HELIO, SENEPHY, SENECIO, LYCO, LASIOC and SENKIR (purity > 88%). The internal standard senecionine-D_3 (SEND3) was purchased from Toronto

Research Chemicals (Toronto, ON, Canada) (purity 95%) and RETRO (purity ≥ 94%) from Sigma-Aldrich (St. Louis, MO, USA). Individual primary stock solutions and working standard solution of PAs were prepared and were stored at -20°C.

2.2.2. Honey

Forty-six (46) honey samples produced in various regions of Algeria (Fig. 1) were obtained from the Beekeeping industry (apiculture), small vendors, local open air markets, and enterprises.

All honey samples were labeled either according to their geographical and botanical origin given by the beekeepers. The samples were stored in airtight plastic containers, and kept in dry conditions at room temperature until the analysis.

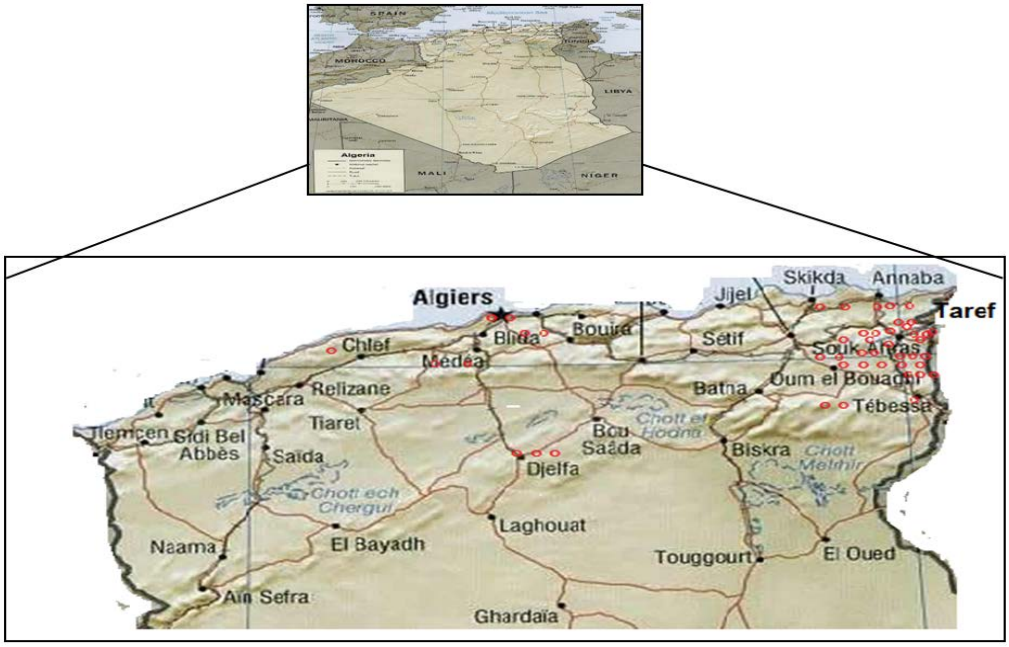

Figure 1. Map of Algeria showing locations of honey collection

2.2.3. Sample preparation for LC-MS analysis

Honey samples were prepared as described by Martinello et al. (2014). 2.5 g of well homogenized honey was weighed in a 50ml polypropylene tube and was extracted with 10ml of sulphuric acid (0.05M). After that, about 1g of zinc dust was added to the sample solution, and weakly agitated for 90 min. Then after the agitation, the mixture was centrifuged (5,000 rpm, 10min) in order to remove insoluble particulates. Supernatant was poured into a clean 50mL QuEChERS extract tube. A ceramic homogenizer, acetonitrile (10mL) and QuEChERS salt EN method (sodium citrate 1g, sodium hydrogen citrate sesquihydrate 0.5g, magnesium sulphate 4g and sodium chloride 1g) were added and vigorously shaken for 5min. The mixture was centrifuged (5,000 rpm, 10 min), and 8mL of supernatant was transferred into a 15 mL tube containing purification dispersive SPE High Pigment EN salts (PSA 0.15 g, magnesium sulphate 0.9 g and carbon 0.045 g). This solution was vortexed (5min) and centrifuged, and 6 mL of purified supernatant were transferred into a clean tube and evaporated to dryness under a stream of nitrogen at 45°C. The residue was dissolved in 1mL of reconstitution solution composed of acetonitrile (13%), formic acid 0.5% in water (87%) and the internal standard senecionine-D_3 10 ng/g (Martinello et al., 2014).

2.2.4. Quantification of PAs

The samples were analysed using a Shimadzu ultra fast LC system (UFLC-XR) (Kyoto, Japan), equipped with binary solvent delivery system, continuous vacuum degasser, autosampler and thermo-stated column compartment. The chromatography was performed on a Supelco Analytical (Bellafonte, PA, US) and Ascentis Express C18 column installed (10 cm x 2.1 mm, 2.7 µm- particles). The mobile phase was composed of (A) formic acid 0.5% in water and (B) acetonitrile. The UFLC eluting conditions were optimized as follows: linear gradient from 5.5% to 90% B (0-10min), and then re-equilibration to 5.5% B for a further 7.1 min. The flow rate was 0.8mL min^{-1} , and the injection volume was 10 µL. The column was thermostated at 34°C (Martinello et al., 2014).

The PAs detection was performed by target analysis using an UFLC system, allowing the detection of nine different PAs at the same time. However, LC-MS analysis was performed as described by Martinello et al. (2014), using a Shimadzu LCMS-2010 EV, with a single quadruple analyzer, using an ESI source in a positive ion mode. Both the temperatures of the heated capillary (CDL) and of the heat block were set at 250°C. The nebulising gas flow was set to 1.5 L min^{-1} , and the detector voltage was set to 1.5 kVm/z. PAs and senecionine-D_3 (IS) were analysed in a single UPLC run, in single ion monitoring (SIM) mode, and the selected m/z which have been shown in Figure 2.

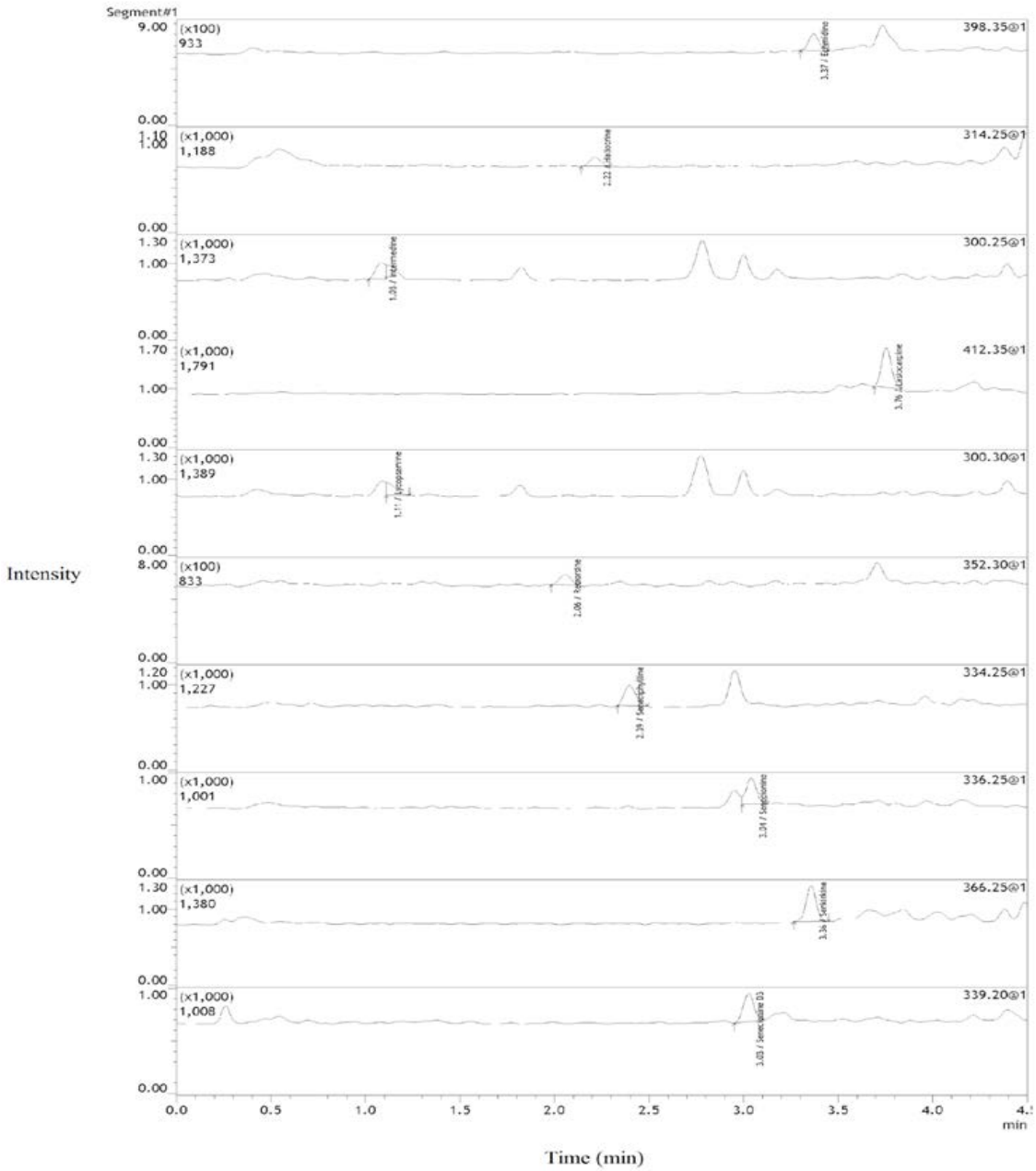

Figure 2. HPLC-MS ion profile of spiked samples (5 µg/kg)

2.2.5. Pollen analysis

The quantification of pollen in honey was performed according to Maurizio's method cited by Louveaux et al. (1978). The examination under the microscope was carried out in order to microscopically count the number of pollens present in the honey sediment after centrifuging a honey solution (400 to 1000 rpm, 15min). The number of pollen counted was greater than 500 grains of pollen per sample analyzed. The results were classified according to the categories proposed by Louveaux et al. (1978): dominant or predominant pollen (> 45%), accompanying pollen (16 - 45%), important isolated pollen (3 - 15%), and rare isolated pollen (1 - 3%).

3. RESULTS AND DISCUSSION

3.1. Pollen analysis

According to Louveaux (1968), five classes were considered: class I (honeys low in < 20,000/10 g), class II (normal honeys 20,000-100,000/10 g), class III (honeys rich in pollen 100,000-500,000/10 g), Group IV (honeys extremely rich in pollen 500,000-1,000,000/10 g), Group V (pressed honeys >1,000,000/10 g). According to the total number of plant elements, the honey samples were distributed into four classes (Fig. 3). The honeys analyzed were rich in pollen, and the dominant class was class III.

This variation can be the consequence of various factors such as the diversity and difference in vegetation cover, and the variation of the climate between the Algerian zones.

In the current study, it has been tried to specify and give the systematic level of determination (species, genus, and family) for each of the taxa and for the indeterminate forms, their percentage in each spectrum. Consequently, the difficulty of identifying the indeterminate forms was due to the bursting of the pollen grains, and the alteration and abortion of these grains before they reach full maturity because of exogenous (climatic) or endogenous conditions. The variability of the topology influenced the composition of the floristic procession (Chefrour, 2007; Draiaia, 2016).

It was observed that 59 botanical families were visited by bees in the different study areas (Draiaia, 2016). Indeed, it was the result of the grouping of taxa in their families. Therefore, the families most represented compared to the totality were: the *Fabaceae* family with a frequency of 79.10% (53 taxa) followed by *Asteraceae* with a number of 52 taxa (77.61%) represented the two families that were ubiquitous in most of the honeys studied. The third position was occupied by *Rosaceae* with a frequency of 52.24% (35 taxa).

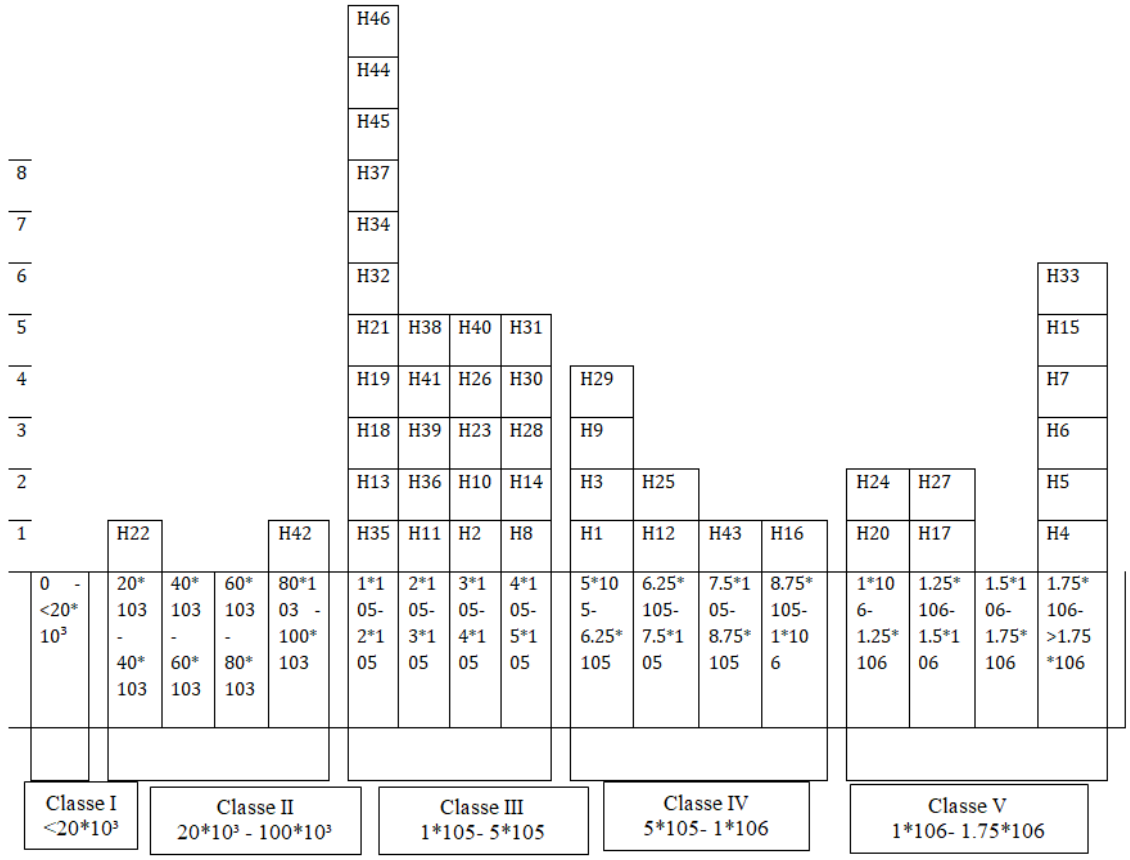

Figure 3. Presentation of pollen quantitative analysis according to the classification of Louveaux (1968).

Brassicaceae (31taxons) and *Apiaceae* (represented by 27 honey taxa) were present with relative frequencies of 46.27% and 40.30% of the honeys analyzed. Regarding the *Lamiaceae* family, it represented a relative frequency of 31.34% (21 botanical taxa) followed by *Boraginaceae* with 19 taxa (28.36%), *Scrophulariaceae* (16.42%) with a number of 11taxons, *Myrtaceae* (10.45%). The rest of the other families were present but to a small degree.

The results from the pollen analysis (pollen spectrum), showed that the majority of honeys were polyfloral, only one was monofloral because pollen type was 45 percent of the total pollen grain number found in honey samples.

The list of melliferous species drawn up for the study areas revealed that the honey flora was mainly spontaneous, since the majority of the apiaries have been installed in natural pastures thus favoring its wealth and diversification.

The plants reported to be associated with poisonings in humans were *Crotalaria, Gynura, Heliotropium, Senecio, Symphytum and Trichodesma* species (Committee on Herbal Medicinal Products, European Medicines Agency, 2016; FEHD, 2017).

Algeria adopted an ambitious strategy of honey exportation, that targeted stable markets, such as the European one; a market that has represented 20% of the world's honey consumption (Kowalczyk et al., 2018). However, as determined by many authors, the European ambition to import honey in important quantities, has been highly challenged by the contamination of the world's honey with considerable PAs

concentration (Dübecke et al., 2011; Martinello et al., 2014; Griffin et al., 2015; Kowalczyk et al., 2018). Knowing that the EU authorities have been responsible to provide an appropriate level of consumer protection, it has been hard to imagine an alternative other than drastically checking the PAs presence in the honey introduced on its territories (Kempf et al., 2008). In these circumstances, a non PAs contaminated Algerian honey could represent a win-win solution for both parties.

3.2. PAs analysis

In this study, 46 samples of the Algerian honeys, collected from various geographical and botanical origins, used in alternative medical treatments and considered as medicinal products or destined to food consumption because of its good quality, were analyzed for the detection of 9 available PAs.

Indeed, the results of the honey sampling (Table 1) revealed the presence of PAs in the majority types of honey and 46 Algerian honey samples tested, that 74% contained one or more PAs, indicating the contamination of honey with PA that was commonly produced by plants. Although, Algerian honeys did not generally contain high levels of PAs except the samples of H32, H10, H13 and H29. The ΣPAs' concentration detected ranged from 1 to 742 µg PA/ kg of PA-positive samples.

In fact, the frequency of PA contamination of the honeys analyzed was high especially for Echimidine and Intermedine (Fig. 4). These two latter were identified as the most predominant PAs in positive honey samples with 20 positive samples for echimidine, and 15 positive samples for intermedine. In terms of concentration, echimidine was the most important PA detected with a maximum of 232 µg PA/kg for the sample (H32). The latter results were in accordance with the previous reports (Kempf et al., 2011; Dübecke et al., 2011; Kast et al., 2014; Martinello et al., 2014; Kowalczyk et al., 2018).

In addition to the chemical PA assays, and after analyzing the samples with quantitative and qualitative pollen analysis as described above, the potential botanical sources, including the botanical families *Asteraceae, Boraginaceae and Fabaceae,*were identified by pollen analysis, and confirmed that *Echium* pollen was already identified in honey H32 that was harvested from a region where many common vipers flourished. Moreover, the presence of *Echium* pollen in the majority of positive honeys was confirmed, which further supported the concept of *Echium* as the source of contamination of these honeys. *Echium* has been a very attractive nectar and pollen source for honey bees. The results of this study were similar to some works indicating that pollen analysis suggested Echium-occurrence in major honey exporting from regions like Australia / New Zealand, South America and southern Europe (Kempf et al., 2008; Kempf et al., 2010).

In this study, it was found that sample H32 in Ain Assal (Taref) and H10 in Guelma contained the highest amount of *Echium* pollen, 95 pollen grains/g and 99 pollen grains/g ; respectively. The findings also demonstrated, in general, that honey samples with higher PA pollen count also showed the high total PA amounts.

However, the amount of *Echium* pollen grains did not always quantitatively correlate with the concentration of PAs in the honeys. For instance, honey sample H13 from Chlef with a high PA content of 30 µg/kg showed only 14 *Echium* pollen grains/g, whereas honey sample H3 Hammammet Tebessa contained 6 *Echium* pollen grains/g , and PAs was at 6 µg/kg. In addition, only 8 *Echium* pollen grains/g was found in honey H4 from Ain Ouessara with a PA concentration of 2 µg/kg PAs.

These apparent discrepancies between total PA content and PA pollen count possibly showed that the presence of PA pollen in a sample was obligatory, but not the single sufficient parameter to explain the higher PA content of a given honey sample (Letsyo et al., 2017). The results suggested that pollen might play a small role in the PA contamination of honey, and that a small proportion of PAs might be released from pollen into honey, as previously suggested by (Lucatello et al., 2016).

The results of this study were in accordance with some European honeys because PAs from *E. vulgare* have been frequently detected in European honeys (Dübecke al., 2011; Martinello et al., 2014), which pointed to a comparable botanical setting in terms of PAs plants in these countries. In fact, this plant has been known to produce copious amounts of nectar and pollen that have been harvested by bees between May and September when the plant is flowering (Lucatello et al., 2016). A multitude of *Echium* species produced toxic PAs especially *E. vulgare* and *E. plantagineum* (the main *Echium* species found in Algeria and Mediterranean ecosystems).

It has to be kept in mind that marker PAs cannot be attributed to a single family of plants, and a study showed that the relationship between the concentration of pollen in the honey and its PA levels was not always found, since honeys with considerable amounts of PA on their composition were revealed to have low levels of pollen (Kempf et al., 2008). The honey sample H32 from Ain Assal (Taref) with the highest concentration of PA (ΣPA 258 µg PA/ kg) and the lowest concentration of PA (ΣPA 1 µg PA/ kg) was observed in two samples of H34 and H44. Comparing with other countries, much higher PA concentrations were detected in the honeys of African and Asian origin. However, the results obtained for Algerian honeys were relatively low compared to Ghanaian honeys (85%) which were PA positive, with one-third of these had PA concentrations above 200 µg/kg. The average concentration was 283 µg/kg, while the highest level of 2639 µg/kg was found in a light-amber honey from Hoezo, a town in the Deciduous rain forest zone of the Volta region, and another high PA concentration (1,741 µg/kg) was also found in extra-light amber honey from the Deciduous rain forest zone of Adansi (Ashanti region), whereas 1,508 µg/kg was measured in the honey purchased from a supermarket (Letsyo et al., 2017) and honeys originating from some tropical countries of Central and South America (average 67 µg/kg; highest value 1,087 µg/kg (Dübecke et al., 2011).

From the results of this study and the few studies that were done before, it could be declared that there were indications that the PA concentrations and composition in plant species were genotype dependent but also affected by the environment (Joosten et al.; 2009; Macel and Klinkhamer, 2010).

Table 1. Content of individual PAs and calculated total PA-content for all Algerian samples

Sample	ECHI (µg/Kg)	HELIO (µg/Kg)	INTERM (µg/Kg)	LASIOC (µg/Kg)	LYCOP (µg/Kg)	RETROR (µg/Kg)	SENECIPH (µg/Kg)	SENECIO (µg/Kg)	SENKIRKIN (µg/Kg)	Total PA (µg/Kg)
H1	0	0	0	0	0	2	0	0	0	2
H2	2	0	1	0	1	1	0	1	0	6
H3	0	0	1	0	0	1	0	1	0	3
H4	0	0	0	0	0	2	0	0	0	2
H 5	0	0	0	0	0	21	0	0	0	21
H6	0	0	2	0	4	1	0	0	0	7
H7	2	0	0	0	0	1	0	0	0	4
H8	6	0	3	0	5	1	0	0	0	15
H9	0	0	0	0	0	15	0	0	0	15
H10	92	0	15	0	21	6	0	3	0	137
H11	0	0	0	0	0	0	0	0	0	0
H12	22	0	1	0	0	3	0	0	0	26
H13	30	0	3	6	0	0	0	0	1	40
H14	0	0	0	0	0	0	0	0	0	0
H15	7	0	4	0	4	0	11	1	0	27
H16	0	0	0	0	0	0	0	0	0	0
H17	0	0	0	0	0	0	0	0	0	0
H18	0	0	2	1	0	0	0	1	0	4
H19	0	3	1	0	0	0	0	1	0	5
H20	0	0	0	0	0	0	0	0	0	0
H21	6	0	4	0	0	0	0	0	1	11
H22	1	0	1	1	0	0	0	0	2	5
H23	0	0	0	0	0	0	0	0	0	0
H24	0	0	0	0	0	0	0	0	0	0
H25	0	0	0	0	0	0	0	0	0	0
H26	4	1	0	1	0	0	0	0	0	6
H27	0	0	0	0	0	0	2	3	0	5
H28	3	2	0	0	0	0	0	0	0	5
H29	28	0	7	0	0	0	0	0	0	35
H30	18	1	0	0	0	0	0	0	0	19
H31	0	8	0	0	0	0	0	0	0	8
H32	232	0	26	0	0	0	0	0	0	258
H33	4	1	0	0	0	0	0	0	0	5
H34	0	1	0	0	0	0	0	0	0	1
H35	4	0	4	0	0	0	0	0	0	8
H36	6	0	0	0	0	0	0	0	0	6
H37	0	0	0	0	0	0	0	0	0	0
H38	6	0	0	0	0	0	0	0	0	6
H39	0	0	0	0	0	0	0	0	0	0
H40	0	0	0	0	0	0	0	0	0	0
H41	9	0	0	0	0	0	0	0	0	9
H42	0	0	0	0	0	0	0	0	0	0
H43	0	0	0	3	0	0	0	0	0	3
H44	12	0	0	0	0	0	0	0	9	21
H45	0	0	0	0	0	16	0	0	0	16
H46	0	0	0	0	1	0	0	0	0	1

Moreover, it can also be said , PA can vary due to the environmental factors such as soil conditions or flowering stage of the plant grains, and the climatic conditions might affect the amount of PA produced by the plants, and thus, the PA content in pollen (Boppre et al., 2005).

Concerning the intermedine, it has been a typical representative for species belonging to the *Boraginaceae* family including the genera of *Anchusa, Borago, Symphytum,* and *Echium (*identified by pollen analysis presented above), and also *Eupatorium* species (family of Asteraceae) (Hartmann and Witte, 1995; El-Shazly and Wink, 2014). The concentration was detected with a maximum of 26 µg PA/ kg for the sample (H32) with ΣPA, and it almost ranged from 1 to 74 µg PA/ kg of PA-positive samples. Also, for lycopsamine, the frequency of PA contamination in honeys was 8% with a maximum of 21 µg PA/ kg for the sample (H10). Both species of *E. plantagineum* and *E.vulgare* produced lycopsamine type PAs, mainly echimidine and echimidine-derived isomers. However, intermedine and lycopsamine were among the least toxic PAs (Huybrechts and Callebaut, 2015).

It is worth of note that in this research, it was also found that the frequency retrorsine contamination in honeys was 15 %; senecionine was 9% and for seneciphylline PAs had slightly lower incidence rate of 3% (Fig.4), which was consistent with the results reported for EU honey by Huybrechts and Callebaut (2015) and Kowalczyk and Kwiatek (2018).

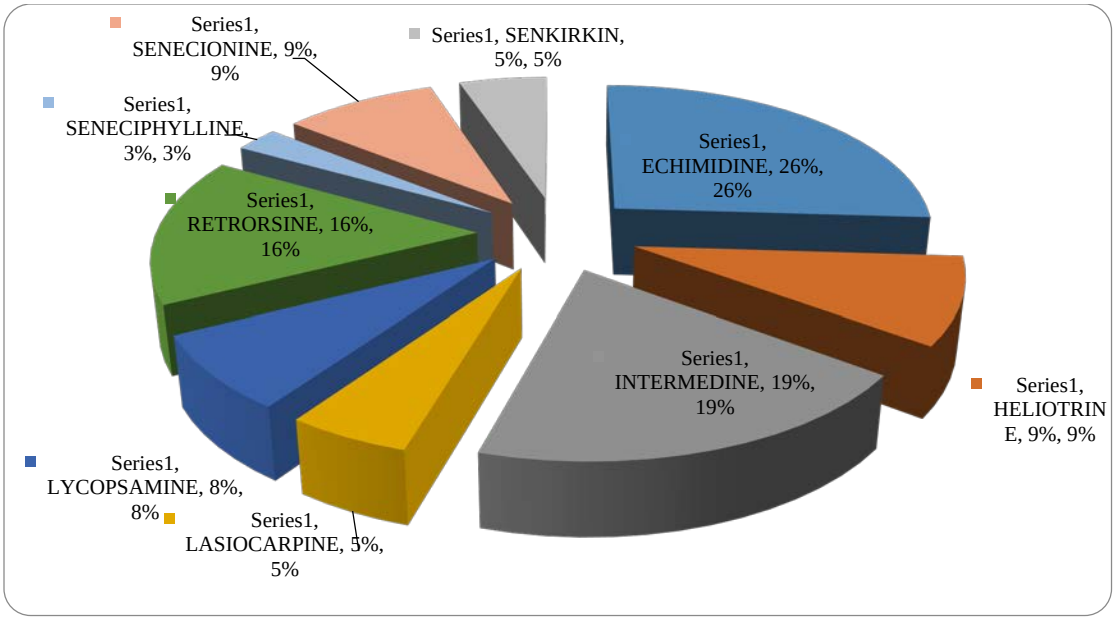

Figure 4. Total amount of PAs in positive Algerian honey samples

Regarding senkirkine, its contribution in the honey samples was with a maximum of 9 µg PA / kg for the sample H44, and lasiocarpine with a maximum of 6 µg PA / kg. The latter has been among the most toxic of the PAs that have been tested, and the BMDL10 for the induction of liver haemangiosarcomas in male rats was used as RP in the previous EFSA opinion (EFSA, 2011), the frequency contamination in our honeys was about 5% with 4 positive samples. Therefore, retrorsine was detected in 12 positive samples, and in three samples, there were relatively high levels with a maximum of 21 µg PA / kg for the sample H5. The results detected were in accordance with other findings (Dübecke et al., 2011; Griffin et al., 2015; Huybrechts and Callebaut, 2015). In 1954, retrorsine was found to induce liver tumors in rats (Zhao et al., 2011). Although most PAs have not been extensively tested in experimental animals or *in vitro* systems, information on the tested PAs included hepatotoxicity, developmental toxicity, genotoxicity and carcinogenicity (EFSA, 2017). The CONTAM Panel assessed both chronic and acute risks related to the human dietary exposure to PAs. For the chronic effects, the Panel concluded that all 1,2-unsaturated PAs shared a common metabolic pathway leading to the formation of genotoxic and carcinogenic reactive pyrroles.

This study revealed that many of the tested honey samples e.g. H29 and H32 would exceed the suggested tolerable daily intake for these substances (see below) and could cause a risk to human health. Furthermore, it has been preferable to know the type of PA present in honey for toxicological consideration, since toxicity of the different PAs varied largely, e.g. lycopsamine was about 10 times less toxic than echimidine (Kast et al., 2014).

Unlike medicinal plants, no maximum limit has been set so far for PAs in foodstuffs, either in Algeria or in the EU. However, several international committees have developed recommendations (COT, 2008; BfR, 2011; 2016, EFSA; 2011; 2017; JECFA, 2015).

The maximum intake of PAs has been proposed by the Committee on Toxicity and the Federal Institute of Risk Assessment (EFSA,2011), and also the limit of exposure of 0.007 µg/kg/day recommended by BfR was derived from the same point of departure applying a margin of exposure (MoE) of 10,000 as recommended by EFSA for the safety assessment

of substances which have been both genotoxic and carcinogenic (COT, 2008; EFSA 2011).

However, the level was calculated according to a BMDL10 (the lower confidence limit on the benchmark dose associated with 10% response) of 73 µg/ kg b.w. per day that was the result of a carcinogenicity study of lasiocarpine in rats, and with the MOE (margin of exposure) of 10 000 (EFSA, 2017).

The CONTAM Panel established a new reference point of 0.237 mg/kg bw per day to assess the carcinogenic risks of PAs, derived from a more recent carcinogenicity study on riddelliine (Gottschalk et al., 2018).

Based on this new BMDL10, the most recent recommendation has been not to consume more than 0.0237 µg of 1, 2-unsaturated PA per day per kg of b.w. This revised value has quite recently been adopted by the BfR (Gottschalk et al., 2018).

In other words, considering a person of 60 kg b.w. consuming 20 g of honey per day and that, with this portion, he/she uptakes at most half of the recommended PA limit, for that, the maximum PA concentration of that honey should not exceed 71.1µg/kg and 24.2 µg/kg for adults and children; respectively (Kowalczyk and Kwiatek, 2018). In this respect, two of the tested honeys exceeded the concentration of 71.1 µg/kg, and would exceed the recommended limit of daily intake by adults in their 20 g per day consumption, while the PA concentrations in the other 44 honeys were below this concentration (Tab.1 and 2). Nevertheless, it should be pointed out that in this context, in case of children, the average consumption of 20 g might be somewhat overestimated.

It is important however to notice that the PA content of food has been probably undervalued because it has not been currently possible to accurately determine all types of PA. Nevertheless, it should be pointed out in this context that the toxicity of PAs has been mainly demonstrated for purified PAs and their metabolites. The herbal complexity also explained the highest sensitivity to PA poisoning. Acute poisoning with pyrrolizidine alkaloids in human cases and experimental animals has been characterised by HSOS, and could lead to liver cirrhosis and liver failure (Edgar et al., 2015). For instance, PA intoxication in humans has not been only related to the amount and the duration of the exposure but also to age and gender (males react more sensitively than females and foetuses and children, especially neonates or infants) (Wiedenfeld, 2011b).

4. CONCLUSION

To the authors' knowledge, this has been the first study on PA occurrence in Algerian honey. The aim of this study was to obtain a clearer view on PA levels in some Algerian honey. 74% (34/46) of the investigated honeys contained PAs, but did not generally contain the high levels of PAs except the samples of H32, H10 and H 29.

This study revealed that many of the tested samples (in particular H10 and H32) would exceed the suggested tolerable daily intake for these substances, and could lead to undesirable and negative health effects when the honeys are consumed, especially if they are associated with medicinal plants or used as herbal remedies and/or food supplements that contained PA-plants. Concerning phytopharmaceuticals, several European countries have regulated the use of these preparations (Kempf et al., 2010). However, in Algeria, until now, through self-medication and folk- and ethnomedicine, some of these plants have still been in use, and there has not been a limit set yet (Li & Wang, 2005). Indeed, the popular misconception that all 'natural' products are safe has tended to discourage the investigation into their potential toxicity (Margină et al., 2015; Seremet et al., 2018).

Furthermore, it is necessary to know that this study focused on a few number of honey samples, and only a limited number of PAs have been analyzed, possibly other PAs can be present in Algerian honeys. Thus, the results can be considered as preliminary values, which are likely to increase with the number of PAs and with the number of samples in order to identify the potential source plants of PAs in honeys. For that, it is important to continue this work, and to widen it in the maximum of Algerian wilayas (departments). It is also important to establish a cartography of the big and important melliferous areas in order to determine a biogeographical reference frame that would facilitate the choice of hives' installations

However, during production, it is difficult to control the actual foraging of these plants and consequently the concentration of alkaloids in honey because it is a product closely related to the natural environment of the hive. But at least, a PA monitoring and control plan can be put in place prior to the sale of honey to identify those that contain too much of these products.

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