The growth of plants containing pyrrolizidine alkaloids (PAs) in plots cultivated with medicinal aromatic plants (MAPs) and in their natural wild habitats in Kosovo

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Abstract: Thousands of plant species worldwide produce about 600 different pyrrolizidine alkaloids (PAs), which are known to cause disease in humans and animals. The plants known for their PAs content were investigated in 70 plots cultivated with 19 MAPs species and the natural habitats of 20 wild MAPs species. Most of the poisonous plants found in cultivated and the natural habitats of wild MAPs belong to the families of Asteraceae and Boraginaceae. In the cultivated MAPs plots, 22 plant species known for their PAs content were identified, including 7 from the Asteraceae, 13 from the Boraginaceae, and 1 species from the Convolvulaceae and Solanaceae families. Convolvulaceae and Solanaceae families were represented by only one species each. Most species from the Asteraceae family known for their PAs content identified in cultivated fields and natural habitats were from the genera Senecio and Jacobaea, while genera Myosotis, Pulmonaria and Symphytum from Boraginaceae family. In the plots cultivated with MAPs, Convolvulus arvensis L. known for its PAs content and tropane alkaloids (TAs) was the most prevalent.

Key words: plant species, pyrrolizidine alkaloids, medicinal plants, cultivated, wild

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Prisotnost rastlin, ki vsebujejo pirolizidinske alkaloide na rastiščih gojenih in samoniklih zdravilnih in aromatičnih rastlin na Kosovu

Izvleček: Več tisoč rastlin, razširjenih širom po svetu, tvori okrog 600 različnih pirolizidinskih alkaloidov (PAs), ki povzročajo bolezni pri ljudeh in živalih. V raziskavi so bile preučevane rastline, ki vsebujejo PAs na 70 ploskvah, kjer so se goji 19 zdravilnih in aromatičnih rastlin in na naravnih rastiščih 20 samoniklih vrst teh rastlin. Večina strupenih rastlin, najdenih na rastiščih gojenih in samoniklinih zdravilnih in aromatičnih rastlin pripada družinama Asteraceae in Boraginaceae. Na ploskvah gojenih zdravilnih in aromatičnih rastlin je bilo najdeno 22 vrst, ki vsebujejo pirolizidinske alkaloide, od tega 7 iz družine Asteraceae, 13 iz družine Boraginaceae in po 1 vrsta iz družin Convolvulaceae in Solanaceae. Na naravnih rastiščih samoniklih zdravilnih in aromatičnih rastlin je bilo najdjenih 34 vrst, ki vsebujejo pirolizidinske alkaloide, od katerih je 17 vrst v pripadku samoniklinih zdravilnih in aromatičnih rastlin, 15 vrst drža Asteraceae, 13 vrst Convolvulaceae in Solanaceae.

Ključne besede: rastlinske vrste, pirolizidinski alkaloidi, zdravilne rastline, gojene, samonikle
1 INTRODUCTION

Pyrrolizidine alkaloids (PAs) are active substances of secondary metabolism in many plant species that belong to the families Asteraceae, Boraginaceae, and Fabaceae. Different authors confirm that more than 6000 vascular plant species in the world produce pyrrolizidine alkaloids (Macel, 2010; Schrenk, 2020). Based on EFSA's opinion on pyrrolizidine alkaloids in food and feed, there are approximately 600 different PAs (EFSA 2011). The potentially toxic PAs, with their N-oxides, are identified in 3-5 % of flowering plants (Smith & Culvenor, 1981). The plants known for their PAs content can contaminate soil and water as well as non-PAs-producing plants (Günthardt et al. 2020). PAs-producing plants are considered to be among the most frequent natural toxins that can endanger the health of people, animals, and wildlife, especially people who use herbal medicines (Longhurst et al. 2019). There is evidence that PAs caused many outbreaks of poisoning cases, which resulted in thousands of deaths, documented in many countries, (Chauvin et al., 1994). PAs contamination has been reported also in honey in various countries (Kempf et al., 2009, Griffin et al., 2014, He et al., 2017). Many authors found the presence of pyrrolizidine alkaloids (PAs) in the species belonging to the family Asteraceae, especially in the genera: Senecio, Jacobaea, Tanacetum, Adenostyles, Eupatorium, and Tussilago (Pestchanker & Giordano, 1986; Witte et al., 1992; Suaa et al., 2002, Christov and Evstatieva, 2003; Hol et al., 2003; Macel et al., 2004; Pelser et al., 2005; Kostova et al., 2006; Kirk et al., 2010; Cheng et al., 2017; Smyrskas-Wieleba et al., 2017; Lebada et al., 2000; Adamczak et al., 2013; Nedelcheva et al., 2015; Klevenhusen et al., 2022). In addition to the species of the Asteraceae family, PAs were also found in the genera and species belonging to the Boraginaceae Family; in particular, the species of the genera Anchusa, Anchusella, Cynoglossum, Echiun, Heliotropium, Lithospermum, Myosotis, Pulmonaria and Symphytum (Hendriks et al., 1988 Pfister et al., 1992; Van Dam et al., 1994; El-Shazly et al., 1996; Oberlies et al., 2004; O’Dowd and Edgar, 2006). Different researches show that the concentration of PAs in plant organs differs depending on their age; the young shoots of Heliotropium europacum L. had more PAs than older ones, while the youngest leaves of Cynoglossum officinale L. contained up to 190 times higher levels of PAs than older leaves (Van Dam et al., 1994; O’Dowd & Edgar, 2006). Since both, wild and cultivated MAPs may grow in association with PAs containing plants, the risk of their mixing during the harvesting time is high. The risk of occurrence of PAs in herbal products used in medicine or as medicinal tea may be a consequence of contamination caused by weeds (German Federal Institute for Risk Assessment, 2013).

For medicinal plants that naturally contain PAs, there are regulations and recommendations on contamination, including the EMA’s (EMEA, 2006) guidance document „Good Agricultural and Collection Practice”.

After the Kosovo war, the MAPs sector became a source of income for the rural population and has developed into a sector that exports semi-finished products to international markets. About 3000 taxa of vascular plants grow in the Republic of Kosovo, (Millaku et al., 2013), of which about 300 (10 %) are wild MAPs. Currently, about 70 species of wild plants are collected in Kosovo, 20 of which have high economic potential. According to a report from 2022 (Anonymous, 2022), in addition to the collection of wild plants, there are also 928 hectares in Kosovo where MAPs are cultivated. About 90 % of these wild and cultivated products are exported mainly as semi-processed products to the EU and the USA, where they are used as raw materials in the pharmaceutical, food, and cosmetic industries.

Recently, some exporters on MAPs from Kosovo have faced export difficulties, because in some cases the analyzed MAPs samples exceeded the standards or the maximum permitted levels of EU Regulation 2020/2040 (Commission Regulation (EU) 2020/2040), which regulates the maximum content of pyrrolizidine alkaloids in certain food products. The detection of PAs levels above the maximum permitted levels mobilized the Association „Organika” (Kosovar Association of Processors and Exporters of NWFP) and the exporters of these products, who called for a survey of toxic plants containing PAs in the plots cultivated with MAPs, as well as in the natural habitats of wild medicinal and aromatic plants (MAPs). The objective of this study was the inventory of PAs plants known for their PAs content in cultivated as well as in natural MAPs growing habitats.

2 MATERIALS AND METHODS

2.1 METHODOLOGY FOR THE INVENTORY OF PLANTS CONTAINING PAS IN PLOTS CULTIVATED WITH MAPS

During the vegetation period 2021-2022, the plants known for their PAs content were investigated in 7 regions of the Republic of Kosovo, in plots cultivated with 19 species of MAPs. Those were: Allium ampeloprasum L., Alcea rosea L., Althaeae officinalis L., Calendula officinalis L., Centaurea cyanus L., Lavandula angustifolia Mill., Levisticum officinale W.D.J. Koch, Malva sylvestris L., Matricaria chamomilla L., Melissa officinalis L., Mentha x piperita L., Mentha spicata L., Ocimum basilicum L., Origanum onites L., Origanum vulgare L., Rosmarinus
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officinalis L., Salvia officinalis L., Thymus vulgaris L. and Urtica dioica L.). A total of 70 cultivated plots with MAPs in 7 regions (10 plots for each region) were studied. Of 70 researched plots, 50 were organically certified, while the other 20 plots were undergoing the certification. The identification of PAs-containing plants in plots with an area of less than 1 ha was done on the whole plot, while in plots with an area of more than 1 ha, 10 survey points with an area of 10 x 10 m² were randomly selected. The inventory of PAs-containing plants in plots with annual MAPs started after germination and lasted until the time of harvest to eliminate them after identification. On the other hand, in plots with perennial herbaceous MAPs, the inventory of plants known for their PAs content was made before the harvest to prevent their mixing with MAPs.

2.2 METHODOLOGY FOR THE INVENTORY OF PLANTS CONTAINING PAS IN NATURAL HABITATS

The inventory of the plants known for their PAs content was also conducted in naturally growing habitats of 20 MAPs with high economic potential (Achillea millefolium L., Alchemilla vulgaris L., Althaea officinalis L., Allium ursinum L., Artemisia absinthium L., Bellis perennis L., Capsella bursa-pastoris (L.) Medik., Centaurium erythraea Rafn., Epilobium angustifolium L., Hypericum perforatum L., Origanum vulgare L., Plantago lanceolata L., Primula veris L., Rubus plicatus Weihe & Nees, Rubus idaeus L., Satureja montana L., Thymus pulegioides L., Urtica dioica L., Verbascum thapsus L., and Viola tricolor L.). The inventory included 5 areas in the Republic of Kosovo certified as areas of organic MAPs cultivation. The plants known for their PAs content in zone I include Istog mountains (Alps 1) (34,850 ha), zone II includes Shala e Bajgorës mountains (55,000 ha), zone III Lipé-Gjakovë/Alps 2 (62,488 ha), zone IV - Sharri mountains (110,000 ha) and zone V - Gollaku 3 (Novobërde, Gjilan, Viti, Kamenica). Maps for certified organic areas of wild MAPs and data for cultivated and certified organic plots were obtained from the Association „Organika”. Figure 1 shows an example of a part of a zone map (zone IV /5 - Sharri Mountains).

In the areas surrounded by a red line, harvesting of wild MAPs is allowed, while in the areas surrounded by a yellow line, harvesting is prohibited (zonal map IV/5). Plant taxa identification and nomenclature were based mostly on Flora Europaea (Tutin & al., 1968-1980, 1993), Flora of Serbia (Josifović, 1970-1977; Sarić & Diklić, 1986; Stevanović, 2012), Flora of Macedonia (Micevski, 1985-2005; Matevski, 2010) and Flora of Albania (Barina et al., 2018; Paparisto et al., 1988; Qosja et al., 1992, 1996; Vangjeli et al., 2000), while finally their nomenclature was updated according to Euro+Med Plantbase (Euro+Med 2006+). Jaccard similarity index (Jaccard, 1912) was used to compare the similarities and diversity of plants containing PAs in cultivated plots of 7 regions of Kosovo.

3 RESULTS AND DISCUSSION

3.1 PLANT SPECIES CONTAINING PYRROLIZIDINE ALKALOIDS (PAS) FOUND IN CULTIVATED PLOTS

The inventory of the plants known for their PAs content in 70 plots cultivated with 19 species of MAPs plants resulted in the identification of 22 plant species known to contain PAs.

The following 7 species were identified from the Asteraceae (Compositae) Family: Eupatorium cannabinum L., Jacobaea erratica (Bertol.) Fourr., Jacobaea vulgaris Gaertn., Senecio leucanthemifolius subs. vernalis (Waldst. & Kit.) Greuter, Senecio vulgaris L, Tanacetum vulgare L. and Tussilago farfara L. The plants known for their PAs content from the Asteraceae family were present in certain periods within the year. The species Senecio vulgaris was present in the cultivated areas from late February to December. Tussilago farfara was present from early March to November. The species Jacobaea erratica and Eupatorium cannabinum occurred from June to September, especially in the plots planted with mint.
(Mentha x piperita L. and Mentha spicata L.), which were located near rivers and had a lot of moisture.


The most frequent species were: Buglossoides arvensis (L.) I. M. Johnst. recorded in 19 plots, Anchusa officinalis L. in 18 plots, Cynoglossum officinale L. in 17 plots, Echium vulgare L. in 15 plots, while the species Heliotropium europaeum L. and Myosotis arvensis (L.) Hill, were present in 10 plots. The species Symphytum officinale L. was present in 2 plots cultivated with Mentha x piperita L. in the Kamenica region and 2 plots cultivated with Mentha spicata L. in the Dukagjini region (Istog locality). The species Echium rubrum Forssk. was found only in one locality (Kishnica) on soils and rocks of volcanic origin (serpentine) planted with Thymus vulgaris L. and Melissa officinalis L.

In addition to the identified species from the fami-
The growth of plants containing pyrrolizidine alkaloids (PAs) in plots cultivated with medicinal aromatic plants (MAPs) ... lies Asteraceae and Boraginaceae, one species from the family Convolvulaceae and the family Solanaceae was also identified.

*Convolvulus arvensis* L., which is known for its production of pyrrolidine alkaloids (Todd et al. 1995) was the most frequent in plots cultivated with MAPs and was found from June to November in 48 cultivated plots (Figure 2). Mechanical removal was most difficult in the plots cultivated with MAPs because the underground part (rhizome) was deeply rooted in the soil, while the above-ground part enveloped the body of the medicinal plants. Over the harvest period of MAPs (*Mentha x piperita*, *M. spicata*, *Melisa officinalis*, *Urtica dioica*, *Levisticum officinale*, *Origanum onites*, *Origanum vulgare*, *Thymus vulgaris* and *Salvia officinalis*), this species was recorded during the harvesting and drying process.

The summary results of identified plants known for their PAs content in the MAPs-cultivated plots are shown in Figure 3.

The similarities and diversity of PAs-containing plants in plots cultivated with MAPs in the 7 regions of Kosovo were analyzed using the Jaccard similarity index (Table 1).

The greatest similarities in the composition of plant species appeared between regions II and III with an index value of 0.66 (66 %); the biggest differences appeared between regions III and VI where the Jaccard index is 0.31 or 31 %.

### 3.2 PLANT SPECIES CONTAINING PYRROLIZIDINE ALKALOIDS (PAS) FOUND IN NATURAL HABITATS RICH IN MAPS

During the research of plant species known for their PAs content in natural habitats rich in MAPs in the 5 surveyed areas, 34 such species were identified. The following 17 plant species known for their PAs

| Table 1: Jaccard's similarity index for plants containing PAs in 7 regions of Kosovo |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | II   | III  | IV   | V    | VI   | VII  |
| I                | 0.611111 | 0.555556 | 0.555556 | 0.450000 | 0.526316 | 0.526316 |
| II               | 0.666667 | 0.388889 | 0.333333 | 0.388889 | 0.388889 | 0.444444 |
| III              | 0.333333 | 0.562500 | 0.562500 | 0.562500 | 0.562500 | 0.562500 |
| IV               | 0.388889 | 0.562500 | 0.562500 | 0.562500 | 0.562500 | 0.562500 |
| V                | 0.444444 | 0.529412 | 0.529412 | 0.529412 | 0.529412 | 0.529412 |
| VI               | 0.529412 | 0.470588 | 0.470588 | 0.470588 | 0.470588 | 0.470588 |

Figure 4: The presence of plants known for their PAs content in the natural habitats of wild MAPs in the researched regions of Kosovo
content were identified from the Boraginaceae family: *Anchusa azurea*, *Anchusa officinalis*, *Anchusella cretica*, *Cynoglossum officinale*, *Echium italicum*, *Echium vulgare*, *Lycopsis arvensis*, *Myosotis arvensis*, *Myosotis scorpioides*, *Myosotis suaveolens*, *Myosotis sylvatica*, *Pulmonaria angustifolia*, *Pulmonaria obscura*, *Pulmonaria officinalis*, *Symphytum bulbosum*, *Symphytum ottomanum* and *Symphytum tuberosum*. From the Asteraceae family, 15 plants known for their PAs content were identified, as following: *Adenostyles alliariae*, *Eupatorium cannabinum*, *Jacobaea erucifolia*, *Jacobaea erratica*, *Jacobaea othonnae*, *Jacobaea paludosa*, *Jacobaea subalpina*, *Jacobaea vulgaris*, *Senecio leucanthemifolius var. vernalis*, *Senecio nemorensis*, *Senecio ovatus*, *Senecio squalidus var. rupestris*, *Senecio vulgaris*, *Tanacetum vulgare* and *Tussilago farfara*.

Figure 4 shows the occurrence of species known for their PAs content in the natural habitats where certain MAPs grow.

Most plants known for their PAs content (14 species) occurred in natural habitats where *Achillea millefolium* and *Artemisia absinthium* species grow. High numbers of the plants known for their PAs content were also found in the natural habitats where the following species grow: *Urtica dioica* (12 species-Fig.5A), *Verbascum thapsus* (11 species), *Hypericum perforatum* (10 species), *Rubus idaeus* (9 species-Fig.B), *Epilobium angustifolium* (8 species).

This study showed that 18 plants known for their PAs content (*Eupatorium cannabinum*, *Jacobaea erratica*, *Jacobaea vulgaris*, *Senecio leucanthemifolius* var. *vernalis*, *Senecio vulgaris*, *Tanacetum vulgare*, *Tussilago farfara*, *Anchusa azurea*, *Anchusa officinalis*, *Anchusella cretica*, *Cynoglossum officinale*, *Echium italicum*, *Echium vulgare*, *Myosotis arvensis*, *Symphytum officinale*, *Convolvulus arvensis*, *Solanum nigrum*) were found in both, plots cultivated with MAPs and in natural habitats where MAPs grow.

4 DISCUSSION

Analysis of data from 70 plots cultivated with MAPs showed that there were 7 species known for their PAs content from the Asteraceae family, of which the most common was the species *Senecio vulgaris*, present in 46 plots, followed by the species: *Tanacetum vulgare*, *Tussilago farfara* and *Jacobaea vulgaris*.

Guidelines for dietary supplements in Europe state that plant species from the genus *Senecio* may have significant concentrations of PAs. In an area of one hectare with 60,000 plants, only 6 plants with a PAs load of 1,310 mg kg$^{-1}$ yield 0.1310 mg kg$^{-1}$ of the dried harvested crop.

The presence of the species *Senecio vulgaris* in 66% of the plots cultivated with MAPs, as well as other PAs-known for their PAs content from this family, indicates the risk of their co-harvesting with MAPs if not removed before harvesting. Of the 13 plants known for their PAs content from the Boraginaceae family, *Lithospermum arvense*, *Anchusa officinalis*, *Cynoglossum officinale*, *Echium vulgare* and *Heliotropium europaeum* were the most common species.

The results of a study (Abd El-Razik et al., 2019) of toxic plants growing in association with medicinal plant fields in Egypt are similar to our findings where the *Senecio* was most problematic precisely because of its content of PAs. Their findings are similar to the results of this study regarding the presence of the species *Convolvulus arvensis*. Another study (Ljevnaić-Mašić et al., 2022)
of weed occurrence in conventionally and organically grown MAPs shows that *Senecio vulgaris* was among the most common weed species in both MAPs cropping systems.

The presence of 22 plant species known for their PAs content in plots managed with MAPs is a strong indication that there is a risk of inadvertent co-harvesting of PAs-producing weeds if the PAs-containing species are not removed prior to harvest or during MAPs harvest. Species of the genera *Senecio, Jacobaea, Tussilago, Tanacetum, Anchusa, Echium, Heliotropium,* *Myosotis* and *Symphytum* grew not only in MAPs-managed areas but also in large fields and in uncultivated areas adjacent to cultivated areas. Although these species were not found in some croplands, there is a possibility that PAs-containing plant seeds are already present in the soil or are introduced from adjacent fields or roadsides. (Anonymous, 2020). Therefore, the selection of land for the cultivation of MAPs is of particular importance. In the natural habitats of 20 wild-harvested herbaceous MAPs from the family Asteraceae, most species known for their PAs content were from the genera *Senecio* (5 species) and *Jacobaea* (4 species). The most common species were *Senecio vulgaris,* *Senecio squalidus* subsp. *rupestris,* *Jacobaea vulgaris,* and *Tanacetum vulgare.* The species *Eupatorium cannabinum* occurred in the habitats of the medicinal plant species *Althaeae officinalis,* *Rubus plicatus,* *Rubus idaeus* and *Urtica dioica,* while the species *Adenostyles alliariae* was found only in the habitat of the species *Alchemilla vulgaris,* which grows in Kosovo mainly in the subalpine and alpine areas.

Species known for their PAs content [*Jacobaea pancicii* (Degen) Vladimir & Raab-Straube (*Jacobaea abrotanifolia* subsp. *carpathica* (Herbich) B. Nord. & Greuter, *Jacobaea subalpina* (W. D. J. Koch) Pelser & *Jacobaea othonnae* (M. Bieb.) C. A. Mey., *Tephrosferis papposa* subsp. *wagnerii* (Degen) B. Nord. and *Senecio rupestris* Waldst. & Kit.], which are rare species for southeastern Europe (Christov & Evstatieva, 2003; Mandic et al., 2009) were not found in the natural habitats of the researched wild MAPs.

In the natural habitats of wild MAPs from the Boraginaceae family, *Myosotis arvensis* was the most common found species in 15 wild MAPs habitats whereas the species *Symphytum ottomanum* was found in 6 and *Pulmonaria officinalis* in 5 habitats of wild MAPs.

Most plant species (14 species) known for their PAs content were found in *Achillea millefolium* and *Artemisia absinthium* habitats, 12 in *Urtica dioica,* 11 in *Verbasum thapsus,* and 10 in *Hypericum perforatum* habitat. Only 3 species were found in the habitats of *Althaeae officinalis* and *Satureja montana.*

The fact that 34 plants known for their PAs content were present in the natural habitats of the 20 wild-harvested MAPs indicates the risk and possibility of the inadvertent mixing of collected MAPs material. Several publications (Letsoy et al., 2017; Chmit et al., 2019; Steinhoff, 2019; Suparni et al., 2020) show that PAs-containing plants contaminate raw materials used in the production of food and herbal medicines.

## 5 CONCLUSIONS

As a conclusion from this study, we can say that a considerable number of plants known for their PAs content were found in the plots cultivated with MAPs and in the natural habitats of wild MAPs. The identification of 22 plant species known to contain PAs in the cultivated plots of MAPs and 34 species of such plants identified in their natural habitats indicates that there is a possibility that these plant species were not handled with due care during harvest. One way to avoid co-harvesting of plants known for their PAs content is to train collectors to visually identify plants that produce PAs and to provide catalogs or atlases of such plants. The identification of numerous plants species known for their PAs content, including the most common species *Convolvulus arvensis,* shows that the application of good agricultural practices on cultivated land is insufficient and that strict application of these rules is necessary.

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### 5.2 AUTHOR CONTRIBUTIONS

Conceptualization and research designing, F.M. (Fadil Millaku) and A.R.; methodology, F.M. and N.B.; writing—original draft F.M. (Fadil Millaku); A.R and N.B were involved in the revision of this paper. All three authors approved the present manuscript.
5.3 CONFLICTS OF INTEREST

No potential conflict of interest was reported by the authors.

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