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Polycyclic Aromatic Hydrocarbons in Traditionally Used Medicinal Plants from Varna Region, Bulgaria

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Abstract. Extracts of medicinal plants are often used for preparation of food supplements, pharmaceutical products or for direct preparation of teas, but they may contain toxic substances, such as polycyclic aromatic hydrocarbons (PAHs). The content of PAHs was determined in *Matricaria chamomilla* L., *Thymus serpyllum* L., *Tilia tomentosa* Moench, *Sambucus nigra* L. and *Achillea millefolium* L., collected from urban and rural region near Varna, Bulgaria. The aim of this study was to compare the levels of PAHs in traditionally used medicinal plants from different regions to assess environmental pollution. Benzo[a]anthracene, chrysene, benzo[b]fluoranthene and benzo[a]pyrene are among the 16 priority pollutants pointed out by The United States Environmental Protection Agency. The PAH levels were determined in extracts of medicinal plants by GC-MS after purification. Chrysene was registered as most abundant compound in all the plant species investigated. Benzo[a]pyrene, identified by the International Agency for Research on Cancer (IARC) as carcinogenic to humans, was not detected in the samples analyzed. The levels of investigated PAHs were significantly higher in samples from areas with intensive traffic compared to samples from suburban areas. The sum of the four PAHs in all plant species were found in the range of 0.68 (*Sambucus nigra* L.) to 6.82 μg/kg dw (*Tilia tomentosa* Moench) and was below the permissible limit of the European commission.

Key words: Polycyclic aromatic hydrocarbons, medicinal plants, GC-MS, Bulgaria.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a group of organic compounds that consist of two, three or more condensed aromatic rings. Some of them are classified as carcinogens to humans by the International Agency for Research on Cancer (IARC, 2010). The United States Environmental Protection Agency (US-EPA) determined 16 PAHs as priority pollutants based on their incidence and carcinogenicity (EPA, 1984). Vehicle traffic (Napier *et al.*, 2008), coal combustion (Yunker *et al.*, 2002) and other pyrogenic processes (Li *et al.*, 2006) were pointed out as significant sources of PAHs in the atmosphere.

People have benefited from the biologically active components of the herbs for many years. Dried herbs are often used for direct preparation of teas and tinctures

for oral administration or external treatment of skin disorders. Extracts of medicinal plants are included in food supplements, pharmaceutical products and cosmetics. Some of the herbs are used as spices in food processing (FSAI, 2001; WHO, 2018). Thus, the advantages of herbs are indisputable, but recent research showed that they may contain toxic substances, such as PAHs (Guillen *et al.,* 1994; EC, 2002; Šimko *et al.,* 2005; Jira *et al.,* 2008; Perelló *et al.,* 2008; Lawal *et al.,* 2017; Ilinkin *et al.,* 2020). The PAHs content in unprocessed plants depends mainly on the level of environmental pollution (Ciemniak *et al.,* 2019; EFSA, 2008). Therefore, in the EU, permissible limits for PAHs content in dry herbs were set, e.g. the levels of benzo[a]pyrene should not exceed 10 µg/kg, and the sum of the four PAHs: benzo[a]pyrene, benz[a]anthracene, benzo[b]fluoranthene and chrysene should not exceed 50 µg/kg (EC, 2015). An introduction of a new regulatory limit for sum of the four PAHs was necessary due to high levels of PAHs found in herbs and spices (EFSA, 2008). Recent studies reported high levels of PAHs in tea leaves (Ciemniak *et al.,* 2019) which were exceeding the regulatory limit (Zelinkova & Wenzl, 2015a; Londoño *et al.,* 2015).

In Bulgaria, there is a long-lasting tradition of harvesting, drying and use of herbs for medicinal purposes and as beverages.

Local studies showed that the most often used medicinal plant species in the area of the North Black Sea coast, Bulgaria are from the Lamiaceae, Asteraceae, Apiaceae and Rosaceae families (Cherneva *et al.,* 2017; Kozuharova *et al.,* 2013; Stoyanov *et al.,* 2018) and the most commonly used plant species were *Valeriana officinalis* L., *Matricaria chamomilla* L., *Tilia tomentosa* Moench, *Ginkgo biloba* L., *Sambucus nigra* L., *Mentha piperita* L. and *Thymus serpyllum* L. (Bachev & Yaneva, 2018).

The aim of this study was to determine and compare the levels of PAHs in traditionally used medicinal plants - *Matricaria chamomilla* L. (chamomile), *Achillea millefolium* L. (white yarrow), *Tilia tomentosa* Moench (linden), *Sambucus nigra* L. (elder) and *Thymus serpyllum* L. (thyme) collected from a rural region and a suburban area (town of Varna).

Materials and Methods

Collection of the plant material

Two regions were selected to assess the impact of intensive traffic on PAH levels in medicinal plants. The urban area was defined in a main city road in Varna, Bulgaria. It is characterized by a heavy traffic – 2124 vehicles/h (MC - Annex A, 2017). The rural region - Fichoza is located about 15 km southeast of Varna. It is characterized by very light traffic – the closest vehicle counting point has registered 120 vehicles/h (MC - Annex B, 2017). The second station was selected in order to compare the levels of PAHs in plant species from both regions.

Plant species *Matricaria chamomilla* L., *Achillea millefolium* L., *Tilia tomentosa* Moench, *Sambucus nigra* L. and *Thymus serpyllum* L. were hand-picked from their natural habitats in the months of May - July 2018 following the guidelines of the Bulgarian Ministry of Environment and Water (MOEW, 2004). The average height of harvested plants was about 25-40 cm (including leaves, stems and flowers). The plant species were determined by "Field guide to the vascular plants in Bulgaria " (Kozhuharov, 1992).

From every plant species was prepared an average sample from at least 5–7 randomly selected individual plants. Collected herbs were dried in a ventilated and dry place at room temperature. The samples were prepared by grinding the leaves, stems and flowers of the dried plants in a mortar. A representative portion of the ground sample (1.5 g) was transferred in a glass centrifuge tube (50 mL).

Extraction, cleanup and chemical analysis

Samples were prepared according to a previously described procedure (Kowalski *et*

al., 2015) with some modifications. The dried samples (1.5 g) of each plant species was homogenized with 15 mL distilled water. Next, a mixture of hexane: acetone (8:2, v/v) (10 mL) was added. Samples were then vortexed for 30 minutes. Thereafter 4 g of anhydrous $MgSO_4$ and 1 g of NaCl were slowly added. The resulting mixture was shaken again and centrifuged for 20 minutes at 3000 rpm. The supernatant was subjected to a cleanup by column chromatography.

A chromatographic glass column (10x250 mm) was loaded in the following sequence - 1 g of Na_2SO_4, a pre-prepared mixture of 5 g of silica gel (G60) and 0.4 g of primary-secondary amine (PSA) sorbent (silica gel with polymer-bonded ethylenediamine-N-propyl phase) and 1 g of anhydrous Na_2SO_4. The loaded column was conditioned with hexane. The supernatant was transferred into the chromatographic column and eluted with 20 mL hexane/dichloromethane (7: 3, v/v).

The eluate was concentrated at 40 °C on a rotary evaporator (Hei-Vap Precision Heidolph, Heidolph Instruments GmbH & CO. KG, Germany) and was transferred to a gas chromatography vial. The eluate was evaporated under nitrogen to near dryness and reconstituted in 0.5 mL hexane.

The analytical determination of PAHs was performed by gas chromatograph (GC FOCUS, Thermo Electron Corporation, USA), with a POLARIS Q Ion Trap mass spectrometer and AI 3000 autosampler. Experimental mass spectrometry parameters: transfer line and ion source temperatures were 250 °C and 220 °C, respectively. The injector was in splitless mode, with a temperature of 250 °C. The temperature program of the oven was as follows: 40 °C (1 min), 40 °C/min to 130 °C (3 min), 12 °C/min to 180 °C, 7 °C/min to 280 °C, 10 °C/min to 310 °C with a final hold for 5.0 min. The volume of sample injected was 1 µL. A TG-5ms capillary column with a length of 30 m, 0.25 mm ID and a film thickness of 0.25 µm was used. Helium was used as carrier gas at a flow rate of 1 mL/min.

All measurements were performed in triplicate in order to ensure accuracy of the analytical procedures. The measured compounds were: acenaphthylene (ACL), anthracene (AN), benz[a]anthracene (BaA), benzo[b]fluoranthene (BbFA), benzo[k]fluoranthene (BkFA), benzo[ghi]perylene (BghiP), benzo[a]pyrene (BaP), chrysene (CHR), dibenzo[a,h]anthracene (DbahA), fluorene (FL), indeno[1,2,3-cd]pyrene (IP), phenanthrene (PHE) and pyrene (PY).

Quality control

Pure reference standard solution (EPA 525 PAH Mix B, 500 µg/mL of each component in acetone, Supelco) was used for instrument calibration, quantification of compounds and recovery determination. Procedural blanks were analyzed between each 5 samples to monitor possible laboratory contamination. The limit of detection (LOD) of the method was calculated from 0.15 to 0.36 µg/kg and limit of quantification (LOQ) from 0.46 to 1.09 µg/kg. Based on the low concentrations of the analytes in plant species the method limits of detection (LOD) were estimated as 3 times the standard deviation and LOQ is the analyte concentration corresponding to ten times standard deviation. LOD for individual PAHs: 0.23 (ACL), 0.16 (FL), 0.36 (PHE), 0.34 (AN), 0.27 (PY), 0.17 (BaA), 0.20 (CHR), 0.31 (BbFA), 0.31 (BkFA), 0.15 (BaP), 0.27 (IP), 0.30 (DbahA) and 0.19 (BghiP) µg/kg.

Data analysis

Concentrations of the 13 investigated PAHs were calculated on a dry weight basis (µg/kg dw). Statistical significant differences in the mean levels of detected PAHs in samples from the two regions were tested at $\alpha = 0.05$ with Student's t-Test (Excel for Microsoft Office Professional Plus 2013).

Results and Discussion

In the present work, the levels of 13 PAHs were determined in five wild herbs: *Matricaria chamomilla* L., *Achillea millefolium* L., *Tilia tomentosa* Moench, *Sambucus nigra* L. and *Thymus serpyllum* L. collected from urban (Varna) and rural (Fichoza) regions in Bulgaria. The concentrations (µg/kg dw) of 13 PAHs in medicinal plants in two different locations are presented in Table 1.

Table 1. PAH levels (µg/kg dw) in the studied medicinal plants. *Legend:* LOD - Limit of detection, PAHs: polycyclic aromatic carbons; ACL: acenaphthylene, AN: anthracene, BaA: benz[a]anthracene, BbFA: benzo[b]fluoranthene, BkFA: benzo[k]fluoranthene, BghiP: benzo[ghi]perylene, BaP: benzo[a]pyrene, CHR: chrysene, DbahA: dibenzo[a,h]anthracene, FL: fluorene, IP: indeno [1,2,3-cd]pyrene, PHE: phenanthrene and PY: pyrene.

	ACL	FL	PHE	AN	PY	BaA	CHR	BbFA	BkFA	BaP	IP	DBahA	BghiP	Sum of 13 PAHs
Tilia tomentosa Moench **(**n=6)														
rural region	< LOD	88.26 ± 0.14	< LOD	< LOD	< LOD	< LOD	1.43 ± 0.07	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	89.69
urban area	< LOD	379.42 ± 0.72	1239.61 ± 1.00	64.76 ± 0.20	13.95 ± 0.20	0.52 ± 0.10	6.30 ± 0.30	< LOD	< LOD	< LOD	< LOD	< LOD	1.29 ± 0.11	1705.9
Achillea millefolium **L.** (n=5)														
rural region	< LOD	30.94 ± 0.31	< LOD	< LOD	< LOD	< LOD	2.37 ± 0.12	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	33.30
urban area	< LOD	251.48 ± 0.52	945.10 ± 1.01	84.29 ± 0.21	37.66 ± 0.17	< LOD	5.43 ± 0.10	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	1324.0
Sambucus nigra **L.** (n=6)														
rural region	< LOD	97.53 ± 0.21	180.43 ± 0.22	< LOD	< LOD	< LOD	0.68 ± 0.11	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	278.64
urban area	< LOD	239.50 ± 0.71	779.89 ± 0.24	85.99 ± 0.22	19.81 ± 0.25	< LOD	5.04 ± 0.09	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	1130.2
Thymus serpyllum **L.** (n=4)														
rural region	< LOD	105.53 ± 0.10	225.69 ± 0.24	10.66 ± 0.06	< LOD	< LOD	0.82 ± 0.08	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	342.70
urban area	< LOD	292.45 ± 0.23	977.84 ± 0.51	63.37 ± 0.20	20.59 ± 0.17	< LOD	3.96 ± 0.11	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	1358.2
Matricaria chamomilla **L.** (n=5)														
rural region	< LOD	3.23 ± 0.10	< LOD	< LOD	< LOD	< LOD	0.86 ± 0.08	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	4.10
urban area	< LOD	163.12 ± 0.34	419.33 ± 0.29	4.20 ± 0.09	7.20 ± 0.10	< LOD	1.80 ± 0.07	< LOD	< LOD	< LOD	< LOD	< LOD	0.60 ± 0.08	596.25

Regarding the regulated four PAHs (benzo[a]pyrene, benz[a]anthracene, benzo[b]fluoranthene and chrysene), chrysene was determined as most abundant compound in all the plant species investigated. The measured concentrations of chrysene varied from 0.68 µg/kg (*Sambucus nigra* L.) to 6.30 µg/kg (*Tilia tomentosa* Moench) and were found lower compared to other studies. Drabova (2012) reported the highest level of chrysene in tea samples - 41.9 µg/kg (black tea) and 21.2 µg/kg (green tea) (Drabova *et al.,* 2012). Zelinkova & Wenzl (2015) determined chrysene in medicinal plants and food supplements in the range <0.25 to 280.1 µg/kg (mean 13.6 µg/kg) (Zelinkova & Wenzl, 2015b). Among the regulated four PAHs chrysene was determined as prevalent compound in a study in Croatia. The mean value of chrysene in dry herbs analyzed was 10.15 µg/kg (Bogdanovic *et al.,* 2019).

Benzo[a]pyrene (BaP) was classified by the International Agency for Research on Cancer as a human carcinogen (IARC, 2010). Therefore, the EU legislation set a maximum limit for BaP of 10 µg/kg in dry herbs. In our research BaP and BbFA were found below limit of detection (<LOD) for both herbs sampled from urban and rural region. Benzo[a]anthracene was found only in *Tilia tomentosa* Moench from urban area.

Among the unregulated PAHs, phenanthrene (PHE) was found as predominant compound in all samples. The maximum concentration of PHE was determined in *Tilia tomentosa* Moench (1239.6 µg/kg in urban area) and the lowest in *Matricaria chamomilla* L. (419.33 µg/kg in urban area) (Table 1). These findings were in accordance with research by Yu et al. (2012) who reported phenanthrene as prevalent contaminant of 16 PAHs investigated in nine Chinese medicinal herbs.

Phenanthrene, fluorene, anthracene and pyrene were added to the list of priority PAHs, due to their high frequency of occurrence and potential for human exposure (US EPA, 1984; Zelinkova & Wenzl, 2015b). The presented PAHs profile with dominant less-toxic PAHs (PHE, FL, AN and PY) was in accordance to results from other studies (Ciemniak *et al.,* 2019; Bogdanovic *et al.,* 2019)

The sum of 4 PAHs in all plant species from rural region was in the range of 0.68 (*Sambucus nigra* L.) to 2.37 μg/kg dw (*Tilia tomentosa* Moench) and were below the permissible limit - 50 μg/kg of the European commission (EC, 2015). The results for herbs from the urban area showed maximum level in *Tilia tomentosa* Moench – 6.82 μg/kg dw and minimal values in *Chamomilla* L. (1.8 μg/kg) – Fig. 1. In recent study by Ciemniak (2019) was shown the highest values of the sum 4 PAHs in linden – mean 213.5 μg/kg, compared to other herbs investigated (Ciemniak *et al.,* 2019).

The sum of the 4 PAHs in medicinal plants was found to be significantly higher in samples from areas with intensive traffic (urban area) compared to samples from rural region (Fig. 1). Potential sources of toxic chemicals and contaminations in herbs and herbal products can be environmental growing conditions, method of drying (FAO, 2009), storage conditions and manufacturing processes (Chan *et al.,* 2003; Kosalec *et al.,* 2009). The higher content of pollutants in herbs from urban areas (Fig. 1) is probably due to heavy car traffic as the main source of PAHs.

The total levels of PAHs (as sum of 13 PAHs) in medicinal plant species from rural areas varied from 4.10 μg/kg (*Matricaria chamomilla* L.) to 342.70 μg/kg (*Tilia tomentosa* Moench). Our results were lower compared to data from recent studies (Ishizaki *et al.,* 2011; Yu *et al.,* 2012). Ishizaki (2011) reported the total content of 15 PAHs (in five teas and 29 medicinal herbs) in the range from 6.5 to 1112.1 μg/kg, and Yu (2012) have investigated 16 PAHs in nine Chinese medicinal herbs and the sum of 16 PAHs varied from 98.2 μg/kg (cassia seed) to 2245 μg/kg (eucommia bark) (Yu *et al.,* 2012).

The distribution pattern of PAHs with 3-6 rings is shown in Figure 2. PAHs with 3 rings (PHE, FL, AN) were predominant in all plant species and account for 96.8-98.7% of the total PAHs content, while PAHs with 4 rings (BaA, CHR, PY) and 6 rings (BghiP) were found below 2% (Fig. 2). Yu (2012), Ciemniak (2019) and Lin (2005) reported similar mass distribution. In all of these studies, low molecular weight PAHs (PHE, FL, AN and PY) were found as the dominant pollutants (Yu *et al.,* 2012, Ciemniak *et al.,* 2019, Lin *et al.,* 2005).

In samples from rural region were found only PAHs with 3 rings (PHE, FL, AN) in levels lower than concentrations in plants species from urban area (Table 1).

The molecular ratio of specific hydrocarbons is often used to investigate the anthropogenic sources of PAHs (Lin *et al.,* 2005). Main emissions of PAHs in atmosphere originated primarily from combustion processes and release of petroleum products (Kucuksezgin *et al.,* 2020).

The molecular distribution of PAHs and specific ratios in environmental and food samples are used for determination of possible sources and processes that generate these pollutants (Table 2) (Lin et al., 2005; Kucuksezgin et al., 2020). The ratios commonly used in the literature are FLUR/PY, IcdP/BPe, PHE/AN, FLU/FLU+PY (Baumard *et al.,* 1998; Yunker *et al.,* 1996; Benlahcen *et al.,* 1997; Kucuksezgin *et al.,* 2020).

The present study evaluated the PHE/AN ratio in medicinal plants from urban areas whereas the results from rural region for AN were found below LOD and no PHE/AN ratio might be calculated. The PHE/AN values of urban samples were higher than 10 (ranged from 9.07 to 19.14) showing prevalence of petrogenic source.

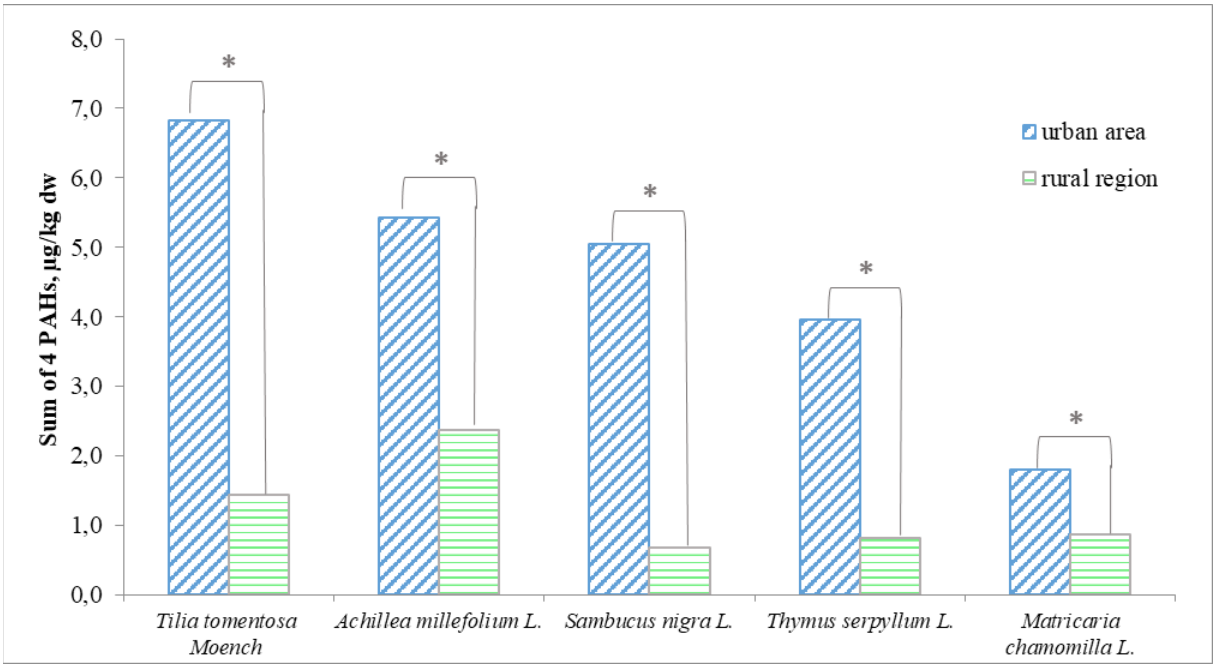

Fig. 1 The sum of 4 PAHs in samples from areas with intensive traffic and from suburban areas (* indicates statistical significant differences of $p < 0.05$).

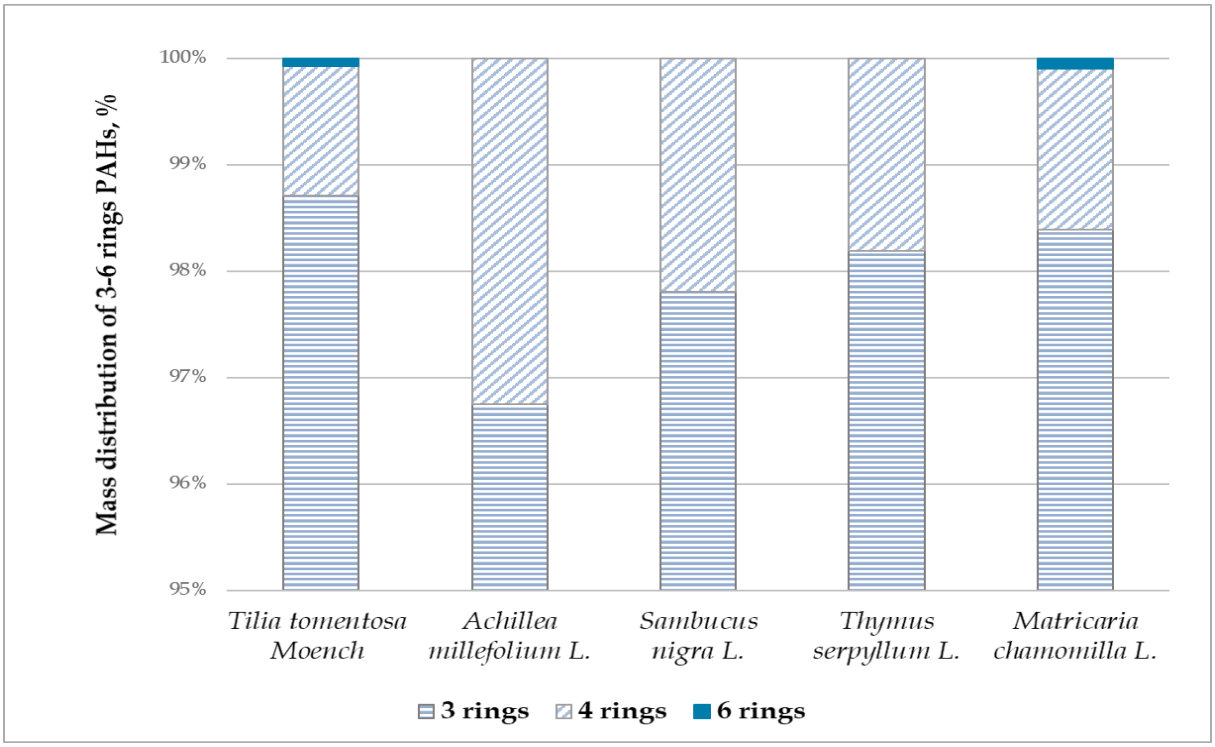

Fig. 2 Distribution pattern of PAHs with 3-6 rings in plant species from urban area.

Table 2 Potential source of pollution depending on values of molecular ratio of specific PAHs.

Ratio	Value	Potential source	Reference
FLUR/PY	> 1.0	pyrolytic origin	Baumard *et al.*, 1998
FLUR/PY	< 1.0	petroleum hydrocarbons	Baumard *et al.*, 1998
IcdP/BPe	> 1.0	combustion sources	Yunker *et al.*, 1996
IcdP/BPe	< 1.0	petrogenic sources	Yunker *et al.*, 1996
PHE/AN	< 10.0	combustion sources	Benlahcen *et al.*, 1997
PHE/AN	> 10.0	petrogenic sources	Benlahcen *et al.*, 1997
FLU/FLU+PY	< 0.4	petrogenic origin	Kucuksezgin *et al.*, 2020
FLU/FLU+PY	> 0.5	wood and coal combustion	Kucuksezgin *et al.*, 2020

Conclusion

Thirteen PAHs were determined in five medicinal plants collected from different areas near Varna. The maximum overall PAH's contamination was found for *Tilia tomentosa* Moench, while *Matricaria chamomilla* L. proved to have the minimum contamination. The levels of PAHs were found to be significantly higher in samples from urban area compared to samples from rural region. The sum of four PAHs in all traditionally used medicinal plant did not exceed the permissible limit of the European commission. The results of the present study showed that the cultivation and collection of medicinal plants from areas close to the roads with heavy traffic leads to a possible risk of accumulation of high concentrations of PAHs. The data from present research can be useful in further studies for assessing the human exposure to PAHs.

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