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MICROBIOLOGICAL AND CHEMICAL EVALUATION OF SEVERAL COMMERCIAL SAMPLES OF *TILIAE FLOS*

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Abstract: The aim of our study was to evaluate the chemical and microbiological composition of seven commercial samples of *Tiliae flos cum bracteis*. Sample S2 presents the maximum level of microbiological contamination with $1.2x10^4$ colony forming units (CFU) aerobic germs/g, $9.6x10^3$ CFU fungi/g, and 360 CFU *Salmonella sp.*/g. For all samples the number of colony forming units for each type of microorganisms was under the limits of European Pharmacopoeia, 8th edition. The amount of flavonoids ranges between 489±1.25 mg/100 g (S2) and 647±1.32 mg/100 g (S4). For polyphenols the quantities range between 663±2.12 mg/100 g (S2) and 1,169±2.76 mg/100 g (S3). The maximum levels of metals determined in mineralised extracts were: 10.35±0.22 mg Mn/100 g (S5), 22.66±0.23 mg Zn/100 g (S3), 1.36±0.12 mg Cu/100 g (S4), 9.92±0.12 mg Fe/100 g (S2), and 9.15±0.12 mg Pb/100 g (S3).

Keywords: flavonoids, metals, microorganisms, polyphenols, *Tiliae flos cum bracteis.*

Introduction

Tiliae flos cum bracteis, generally named lime flowers, represent the flowers and bracteis of different species of *Tilia*. Lime flowers are used in traditional medicine in feverish cold, respiratory diseases and migraine, but also for their sedative, antispasmodic, antioxidant and hepatoprotective effects [KARIOTI & al. 2014; YAYALACI & al. 2014].

The dichloromethane extract from *Tilia cordata* showed antiproliferative activity on lymphoma cell line, and the extract from *Tilia x viridis* proved antiproliferative effect on tumour lymphocytes and stimulated normal lymphocytes [MANUELE & al. 2008].

In cosmetics, extracts from lime flowers are used for hydrating and astringent effects. Aqueous extracts from *Tilia* are used in folk medicine to trait anxiety. Ethanol and dichloromethane extracts from *Tilia cordata* Mill. had antiproliferative activity on murine lymphoma cell line BW 5147 [BARREIRO ARCOS & al. 2006].

Tilia tomentosa Moench bud extracts (TTBEs) are used in folk medicine for their sedative effect. Compounds from these extracts have the capacity to fix on $GABA_A$ and benzodiazepine receptors from hippocampal neurons and to mimic agonist effects of GABA and benzodiazepines [ALLIO & al. 2015].

European Pharmacopoeia, 8th edition, sets rules for microbiological quality of medicinal plants that are used to prepare aqueous extracts, so the maximum limits are 10^7 colony forming units (CFU) aerobic germs/g vegetal product, and 10^5 CFU fungi/g vegetal product [EUROPEAN PHARMACOPOEIA, 2014].

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The aim of our study was to evaluate the chemical (polyphenols, flavonoids, heavy metals) and microbiological quality of lime flowers.

Material and method

In our study, we evaluated seven samples of commercial sorts of *Tiliae flos cum bracteis*.

Microbiological evaluation

For each sample, extracts have been prepared according to European Pharmacopoeia - 1 g sample was mixed with 10 mL peptone-phosphate buffer (pH 7). 1 mL of each extract and diluted extracts (1:10; 1:100) was inoculated on agar broth (aerobic germs), MacConkey broth (*Escherichia coli, Salmonella* sp.), and Sabouraud broth (fungi). The Petri dishes were incubated for 5 days at 37 °C (for bacteria) and 7 days at 25 °C (for fungi). The results were expressed in number of colony forming units/g sample (CFU/g) [EUROPEAN PHARMACOPOEIA, 2014].

Chemical evaluation

5 g of sample was extracted with 100 mL methanol at 80 °C and the extract was used for quantification of polyphenols and flavonoids.

Flavonoids were quantified by a spectrophotometric method based on the reaction of flavonoids with aluminium chloride and sodium nitrite resulting in a pink compound with a maximum absorbance at 510 nm. The results are expressed in mg rutoside/100 g sample [HATANO & al. 1988].

Polyphenols were determined by a spectrophotometric method based on the reaction with Folin-Ciocalteau reagent resulting in a blue compound with a maximum absorbance at 765 nm. The results are expressed in mg caffeic acid/100 g sample [SINGLETON & ROSSI, 1965].

Each determination was made in triplicate and the results represent the average ± standard deviation.

Metals content

1 g of sample was mineralized with nitric acid and the metals were determined by atomic absorption spectrophotometry. For each sample, an aqueous extract was prepared according to the rules for water extracts used as pharmaceutical solutions (6 g vegetal sample extracted with 100 mL of boiling water). The results are expressed in mg metal/100 g sample for each extract

Results and discussion

Firstly, we analysed the macroscopic quality of the samples. Because in our samples the vegetal product is minced, it is difficult to identify the species of linden that have been harvested. According to the European Pharmacopoeia, linden flowers are represented by inflorescence of *Tilia cordata* Miller, *Tilia platyphyllos* Scop., *Tilia* × *vulgaris* Heyne or a mixture of these [EUROPEAN PHARMACOPOEIA, 2014.]. Romanian producers indicate on the label of the box of medicinal product the presence of flowers from *Tilia cordata* and *Tilia platyphyllos* but it is very difficult to establish the species when samples are minced.

Microbiological quality

The results of the microbiological evaluation are presented in Tab. 1.

For all samples, the level of microbiological contamination is under the limits of European Pharmacopoeia, 8th edition [EUROPEAN PHARMACOPOEIA, 2014.]. Sample 2, having the highest level of microbiological contamination, also presented a lot of brown fragments indicating sample processing deficiencies during drying of vegetable product or during storage.

Tab. 1. Microbiological evaluation of *Tiliae flos cum bracteis* samples

Sample	Microorganisms (CFU/g)		
	Aerobic germs	*Salmonella* sp.	Fungi
S 1	$2.4x10^2$	0	$1.8x10^2$
S 2	$1.2x10^4$	$3.6x10^2$	$9.6x10^3$
S 3	4.510^3	0	$3.6x10^3$
S 4	$3.6x10^3$	20	$3x10^3$
S 5	$4.2x10^3$	10	$4x10^3$
S 6	$5.4x10^3$	10	$4.8x10^3$
S 7	$3.8x10^3$	16	$3.2x10^3$

Microbiological contamination is an important parameter for the quality of medicinal herbs because microorganisms, by their enzymes, could modify bioactive phytochemicals. On the other hand, the microorganisms could contaminate aqueous extracts prepared from medicinal herbs, modify their composition and influence the patient's health.

Fungal strains identified in tested samples are different but *Rhizopus nigricans* was found in all samples. Other fungal strains were identified: *Mucor sp.* in S 1 and S 3, *Aspergillus niger* in S 3 and S 5, *Penicillium* sp. and *Aspergillus niger* in S 2.

Chemical composition

The quantities of flavonoids and polyphenols determined in lime flower samples are presented in Fig. 1 and 2.

Flavonoids and polyphenols are compounds with antioxidant properties and could pass in water extracts. By HPLC-DAD and HPLC-ESI-MS analysis, Karioti and Ieri identified in water extracts from *Tilia platyphylos* different quantities of epicatechin, tiliroside, quercetin and kaempferol derivatives and ferulic acid [IERI & al. 2015; KARIOTI & al. 2014].

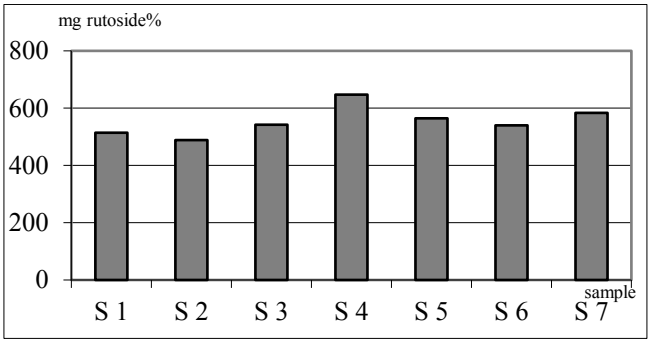

Fig. 1. Flavonoid content in *Tiliae flos cum bracteis* samples (mg rutoside/100 g sample)

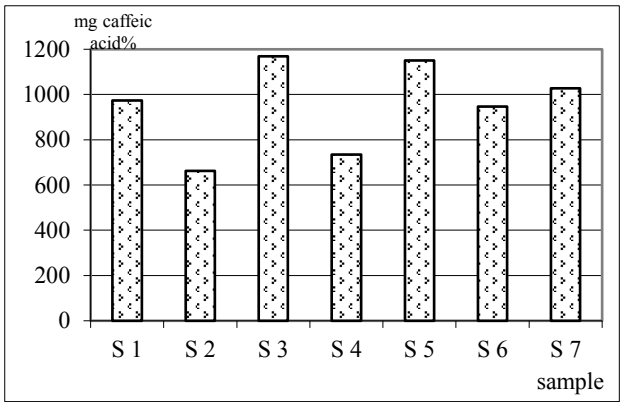

Fig. 2. Polyphenol content in *Tiliae flos cum bracteis* samples (mg caffeic acid/100 g sample)

Flavonoids and polyphenols present antioxidant properties. These compounds have hydroxyl groups that neutralize reactive species of oxygen or different radicals involved in oxidative processes.

Metals content

The metals content in samples depends on the type of metal and its properties. The results are presented in Tab. 2.

Tab. 2. The metals content of *Tiliae flos cum bracteis* samples

Sample	mg metal/100 g sample				
	Mn	Zn	Cu	Fe	Pb
S 1	3.17±0.15	4.56±0.08	0.93±0.03	3.76±0.09	0.93±0.02
S 2	6.52±0.18	4,41±0.10	0.99±0.07	9.92±0.12	0.19±0.02
S 3	6.10±0.19	22.66±0.23	0.79±0.08	5.99±0.10	9.15±0.12
S 4	9.83±0.24	5.61±0.12	1.36±0.12	4.81±0.19	4.52±0.11
S 5	10.35±0.22	8.59±0.15	0.90±0.06	7.86±0.18	0
S 6	7.45±0.18	6.24±0.09	0.82±0.05	6.42±0.12	0.35±0.04
S 7	7.01±0.15	7.93±0.22	0.91±0.04	5.91±0.13	0

European Pharmacopoeia indicates the necessity to evaluate the metals content of vegetal samples used as medicinal herbs but it does not specify limits for metals content.

In mineralised extracts, there have been identified some metals that could induce toxic effects on humans such as: manganese, copper, iron, zinc, and lead.

In water extracts, a small quantity of metals passes because these metals have low water solubility and the method used for extraction does not destroy the complexes between metals and other cell constituents.

The ratio of water extraction depends on metals: manganese - between 7.35% (S 2) and 25.92% (S 3); zinc between 4.53% (S 2) and 20.42% (S 5); copper between 9.33% (S 1) and 14.11% (S 5); iron between 0% (S 1, S 2) and 4.95% (S 4). Water extracts didn't contain lead.

In small quantities, these metals, excepting lead, are necessary for the body because they are involved in many biochemical processes. Romanian laws do not mention limits for the content of metals in medicinal plants, but limit the metals content in foods. Thus, the

limits are 0.3 mg% for lead, 5 mg% for zinc and 5 mg% for copper [REGULATION OF HEALTH MINISTRY, 1998].

Cells of the body could use metals present in aqueous extract in different biochemical processes. So, *zinc* is used for immunity, enzymes structure, hormones synthesis and insulin structure. Normal daily intake of zinc for adults is 7-10 mg. The excess of zinc (over 15 mg/day) might affect the nervous system and heart [DART, 2004].

CELECHOVSKA analysed samples of *Tiliae flos cum bracteis* and found zinc contents between 13.8 and 32.5 μg/g. Similar to our evaluation, the yield of water extraction for metals from vegetal samples was under 25% [CELECHOVSKA & al. 2004].

Copper is present in the structure of enzymes that catalyze dopamine and epinephrine synthesis, melanin synthesis and in enzymes of respiratory chain. Normal daily intake for adults is 1-1.5 mg and an intake over 4 mg/day for a long time could induce Wilson's disease.

Manganese is a metal used for enzymes structure, collagen synthesis and glucose synthesis. In excess, this metal will affect neurons that produce dopamine and, by this mechanism, could induce symptoms similar to Parkinson's disease [DART, 2004]. Most of countries have different limits for manganese in medicinal herbs with maximum value of 1 mg%.

Iron is used for haemoglobin synthesis and also for ATP synthesis. The normal daily intake is 10 mg for men and 15 mg for women. Iron excess induces oxidative processes and affects the structure of DNA and lipids. When iron is present in water extracts, it could modify the colour of these extracts or could precipitate active compounds in extracts. The yield of water extraction for iron is low because it reacts with polyphenols from vegetal samples or is present on vegetal samples as powder with low water solubility.

Lead is a toxic metal that blocks haemoglobin synthesis and affects neurons [DART, 2004]. Lead is present in samples but boiling water extraction is not enough to separate it. The WHO standard is 1 mg% for lead in raw materials for herbal medicines [WHO, 1998]. Samples S 3 and S 4 contain lead over this limit.

Heavy metals are present in plants that have been harvested from polluted area and sometimes are used like biomonitors to assess the level of atmospheric pollution [ANNAN & al. 2013; HÜLSMANN & al. 2005]. Tomašević and Marković analysed linden leaves and bark from different areas and they found a direct correlation between the level of heavy metals and air, respectively soil pollution of the area where these trees are growing [MARKOVIĆ & al. 2013; TOMAŠEVIĆ & al. 2004].

Comparing the results for metals, we observe differences between values depending on metal, so this could indicate the type of pollution of area from where flowers have been harvested.

Conclusions

The chemical composition will influence the quality of plant extracts obtained from them and ultimately, the therapeutic effects.

Microorganisms and metals present in plant samples can alter the biological properties of plant extracts or can cause toxicities in some patients.

By increasing the time and temperature for water extraction, more important quantities of metals could pass in herbal extracts.

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