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RESEARCH ARTICLE

Galantamine content in Leucojum aestivum populations grown in northwest Albania

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Abstract. Although chemical synthesis of galantamine is feasible, plants are still the main sources of production for this natural product. Galantamine, a reversible acethylcholinesterase inhibitor, has been used for decades for various medicinal purposes. More recently, it has been successfully applied to the treatment of symptoms in Alzheimer's disease. Generally, sources for galantamine isolation are the plants of Amaryllidaceae family, usually from the Leucojum and Narcissus genus. The bulbs of Leucojum aestivum L., known in Albania as "zambaku i kënetës" or "bilbilbardha", are used for galantamine isolation. Leucojum is included in the IUCN Red List of endangered species. It is known for the diversity of alkaloid fraction, dependent on genotype and geographical position. Our study aims to investigate alkaloid fraction, with a focus on the galantamine content in the bulbs of Leucojum aestivum L. populations, grown near the Buna river banks. The samples were collected during two different periods of vegetation: bloom and fructification. Thin layer chromatography and high performance liquid chromatography were the analytical techniques used for the qualitative and quantitative determination. The number of alkaloids detected in different bulb extracts was eight, with galantamine as the dominant compound. The linearity of HPLC method was checked by injecting solutions containing 20 up to 320 µg/ml of galantamine. The average galantamine content was found to be respectively 0.13% and 0.14% for samples M_1 and M_2, with reference to the dried material.

Key words: *Galantamine, Leucojum aestivum L., TLC, HPLC.*

Introduction. Leucojum aestivum L. (summer snowflake), a plant species of Amaryllidaceae family, known as "zambaku i kënetës" or "bilbilbardha", grows in wet environment [1]. In Albania the natural habitats are: the Buna river delta, the Derven-Krujë zone and the outskirts of the village Gjergjevicë, near the mountain Ostrovicë. The plant is considered as an endangered plant species [2]. For decades the bulbs of L. aestivum have been used as a valuable source for the industrial isolation of the alkaloid. Galantamin was first isolated in 1952 from Galantus woronowii. The pharmacological properties soon attracted the attention of the pharmaceutical industry. Due to their small size and the variability of galantamine content, Galantus species were soon replaced by L. aestivum [3]. Although the chemical synthesis of galantamine has been achieved, plant material remains the main source for galantamin isolation. In Europe nowadays it is being extracted from Leucojum aestivum and from Narcissus cultivars [4]. Depending on the geographical position and the plant genotype, the alkaloid pattern as well as the galantamine content and its percentage in the alkaloid mixture, varies widely [5, 6]. In relation to the geographical location of the populations distributed along Balkan peninsula, Leucojum aestivum alkaloid profile is dominated by galantamin, lycorine or hemantamin. The production cost of galantamine is determined by the quality of plant raw material. One of the first products of the pharmaceutical industry was traded under the name of "Nivalin" (Sopharma).

Galantamine has been used for decades for various indications such as the treatment of poliomyelitis, myasthenia gravis and other neuromuscular disorders. [7]. Galantamin is a long acting, selective, reversible and competitive acetylcholinesterase inhibitor, marketed as a hydrobromide salt [8, 9]. Currently galantamine is used for the treatment of mild to moderate Alzheimer's disease. It allows levels of acetylcholine to be increased in the parts of the brain associated with intellectual function. Galantamin hydrobromide is one of the three acethylcholinesterase inhibitors approved by regulatory agencies EMA and FDA for the symptomatic treatment of Alzheimer's disease, together with donepezil and rivastigmine. [10, 11]. Recently the monographs of galantamine hydrobromide have been included in the new editions of official pharmacopoeias (USP 2011, Ph. Eur 7). There are numerous studies relating to the evaluation of L. aestivum alkaloid pattern, especially to the galantamine assessment. Hyphened instrumental techniques have been applied for the compounds characterization [12, 13, 14, 15].

Although L. aestivum represents a medicinal plant of special importance, for the populations grown in Albania only few studies have been carried out, dated years ago. The species was briefly described in the book "Përcaktues Bimësh", published in 1979 [2]. Regarding the chemistry of the plant, the only study which chiefly deals with galantamine isolation was published in the periodical press of the year 1967 [16].

Herein we report the results of our study on the identification and quantitative assessment of galantamine in the bulbs of Leucojum aestivum L. populations grown near the Buna river banks.

Matherial and Methods. ***Plant material*** Samples were collected in the first week of April (at the flowering stage: M_2) and by the end of June (during the fructification: M_1), in the years 2013, 2014 and 2015. A voucher specimen of the species was deposited in the National Herbarium of Tirana University. After washing with running water and removal of foreign parts, the separated bulbs were cut in thin slices (Fig. 1 A). The plant material was dried at room temperature, protected from sun. The leaves were treated in the same manner. ***Alkaloid extraction*** 1 g powdered dry plant material (sieve 500) was mixed in a covered glass dish with 25 ml methanol and left for maceration for 24 hours. Extraction was carried out in an ultrasonic bath, for 15 minutes. The sonication procedure was repeated for another 15 minutes. After centrifugation, the plant residues were rinsed with 5 ml methanol and the combined extracts were evaporated under vacuum. The dry extract was dissolved in 4 ml of 3% sulfuric acid and defatted with diethyl ether (3 x 5 ml) in a separating funnel. After basification to pH 9-10 with 25% ammonia, the alkaloids were extracted with chloroform (3 x 5 ml). The combined chloroform layers were dried over a small quantity of anhydrous sodium sulphate and evaporated to dryness. The obtained dried alkaloid fraction was dissolved in 1 ml methanol for further TLC and HPLC analysis (a). ***Thin layer chromatography (TLC).*** An aliquot of 10 µl of sample solution (a) and 10 µl of galantamine hydrobromide standard solution (2.5 mg/ml) were spotted on a silica gel 60 F_{254} pre-coated plate (10 x 20 cm, 0.2 mm layer) and were developed with the mixture chloroform : methanol (8:2) - migration distance 10 cm. After the solvents evaporation at room temperature, the plate was examined under the UV light, at 254 nm. Alkaloid compounds were visualized after spraying with Dragendorff's reagent [17]. Pre-coted plates and the reagents were Merck products. The reference substance used was galantamine hydrobromide (Martin Bauer, PhytoLab – Germany). ***High performance liquid chromatography (HPLC).*** *Equipment*: HPLC analysis system Shimadzu. Software: LabSolution, detector DAD, auto sampler: Sil-20A.

The chromatographic assay was performed on a Lichrosorb C_{18}

column (250 x 4.6 mm) reversed phase matrix (5 µm), column temperature: 30^{0}C. Isocratic elution was carried out with the mobile phase acetonitrile: methanol: buffer pH 4.5 (10: 10: 80), flow rate: 1 ml/min. Detector was set at 230 nm. [18]. Volume of injection was 20 µl. ***Buffer solution*****:** in a volumetric dish of 1L, 1 ml triethylamine was mixed with the solution of 3.45 g/L monobasic sodium phosphate in water and filled with the same solution up to the mark. pH was adjusted with phosphoric acid to pH 4.5. Standard stock solution with the concentration of 16 mg/ml galantamine in methanol (10.2 mg galantamine hydrobromide in 50 ml methanol), was diluted several times to obtain the concentrations of 20, 40, 80, 160 and 320 µg/ml. The number of replications at each calibration level was four.
Sample solutions (a) from M_1 and M_2 were diluted four times before being injected. Galantamin identification in sample solution was carried out by comparison of retention time, while the quantitative evaluation of it by measuring the surface under the corresponding peak.

Results and Disscussions. In the above TLC experimental conditions, after twice repeated migration, a good separation of galantamine from the other alkaloid compounds was achieved. Observed under UV light, as well as after visualization with Dragendorff's reagent, the spot of galantamine (Rf 0.58) in both chromatograms M_1 and M_2 was the main component (Fig.1B). Alkaloid patterns were similar in both analyzed samples, showing eight separated alkaloids. Considering the color intensity and the spot size, another alkaloid (Rf 0.32) seemed to be in relatively large quantities, especially in the chromatogram M_2. On a second plate were spotted aliquots of 20 µl, as well as the methanol extract of leaves prepared in the same manner (G). When TLC chromatograms were examined under UV light, galantamine was visible on the chromatogram G, but in smaller quantities compared with M_1 and M_2. There were also few of the other alkaloid constituents.
Among the HPLC chromatographic systems proven on the galantamine separation from the other constituents present in the herbal extracts, good results were achieved applying the system described by the USP 2012 for the dissolution test of galantamine tablets. The HPLC chromatogram of the standard solution is given in Fig 2A, while in Fig 2B the chromatogram of plant extract, where a well separated galantamine could be seen (retention time of 6.5 min.). The relative standard deviation for the standard solution injected several times was about 0.2%. The calibration curve (Fig. 3) showed linearity in concentrations between 20 – 320 µg/ml galantamine, with a correlation factor 0.9998.

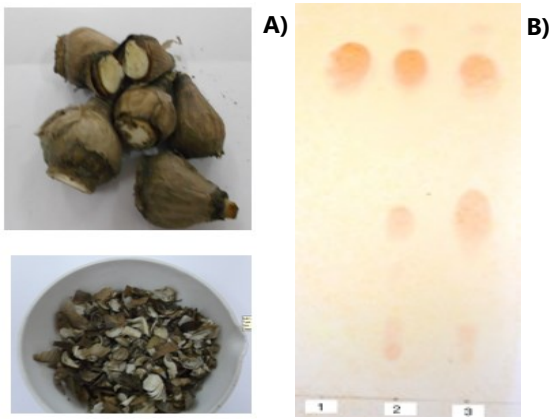

Figure 1 A) *L. aestivum* bulbs**. B)** TLC chromatograms. Galantamine, lane 1 L. aestivum extracts M_1 in lane 2 and M_2 in lane 3)

It remains to be verified, however considering the relative retention time of galantamine versus the peak with the retention time 3.7 min. and comparing it with values given by other authors (in similar HPLC systems) [19, 20], it is probable that the peak belongs to lycorine.
Sample M_1 and M_2 general parameters resulted as follows. The average loss on drying was 9.5% and 10.8% respectively, these values were taken into consideration in calculations. The mean values for the total ash were also close: 6.2% and 6.5%.
The average galantamine content in bulbs from plants collected in April (M_1) and those collected in June (M_2) does not vary widely, it was found to be 0.13% and 0.14%, with reference to the dried substance.
Generally, the average galantamine content in Leucojum aestivum populations gathered from natural habitats and used for industrial production was found to vary from 0.1 - 0.2% (dry substance) [5]. Investigations on L. aestivum plants grown in the Balkan peninsula have revealed chemodiversity in both alkaloid patterns and galantamine content. In relation to their origin, the alkaloid pattern can be dominated by galantamine, lycorine or haemanthamine. The average galantamine content in summer snowflake bulbs varied from traces to 0.3%, also the galantamine percentage in the total alkaloid mixture ranged from 4 to 99% [5, 6]. The relatively heigh content of galantamine in Leucojum aestivum bulbs in the study, as well as the domination of galantamine in the alkaloid mixture, makes the plant material suitable for the industrial production of galantamine. The results suggest the need for further agro-biological and chemical studies on L. aestivum populations grown in Albania.
Due to the diversity of alkaloid content in Amaryllidaceae species [21, 22] and a remarkable increased demand for galantamine in the last few years [23], the European Council approved the project "SUPROGAL" with international participation, to support the research on high - galantamine producing plants. The plants Leucojum aestivum and Narcissus confusus have been previously selected [24].

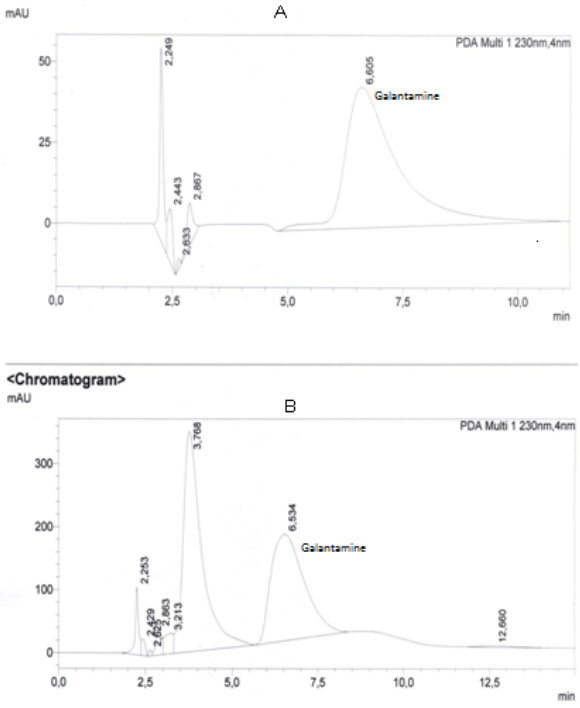

Fig. 2. HPLC chromatograms of Galantamine standard solution panel **A** and L. aestivum plant extract panel **B**.

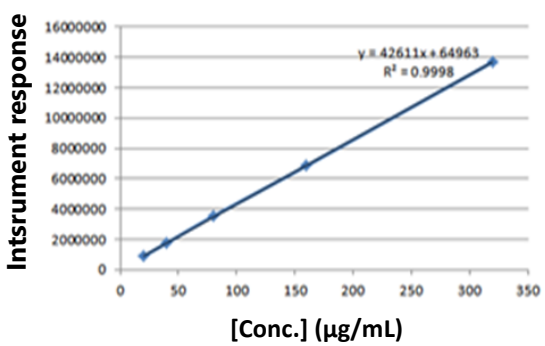

Fig. 3. Calibration curve used for Galantamine quantification extracted from L. aestivum.

Conclusions The plant, collected in a small island near the Buna river banks, was identified as *Leucojum aestivum* L. (Amaryllidaceae), because of macroscopic characteristics and the alkaloid galantamine presence. TLC chromatograms of methanol extracts from plant bulbs showed eight well separated alkaloids, with the galantamine as the main component. Galantamine was identified also in the leaf extracts. The application of high performance liquid chromatography, using the mixture acetonitrile : methanol : buffer 4.5 (10:10:80) as mobile phase and the C_{18} column, led to an efficient separation of galantamine from the other compounds present in the extracts. The calibration curve showed linearity from 20 to 320 µg/ml, with correlation factor 0.9998. The estimated galantamine content in bulbs from plants gathered in two different periods of vegetation, bloom and fructification, did not vary widely. The average galantamine content was found to be 0.14 and 0.13% respectively, with reference to the dried substance. Given the special value of Leucojum aestivum as plant raw material for galantamine isolation as well as growing market demand, we propose further studies in the natural habitats where the plant grows.

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