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EXTRACTION OF VARIOUS ALKALOIDS FROM *IN VITRO* CULTURES AND INTACT PLANTS OF *LEUCOJUM AESTIVUM*

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Abstract

Leucojum aestivum (summer snowflake), a valuable ornamental and medicinal herb, is the native plant of the south of Europe, Turkey, Bulgaria and the north of Iran. This bulbous plant, belonging to the Amaryllidaceae family, is on the verge of extinction. The present study was aimed at detecting the alkaloids available in intact plants and *in vitro* cultures of *Leucojum aestivum*. The plantlets were produced with direct organogenesis from the scale explant, and then the taken extract from both plantlets, obtained from *in vitro* cultures and intact plants, was injected to GC/Mass. Finally, lycorine was detected as the main alkaloid in both *in vitro* cultures and intact plants of *Leucojum aestivum.*

Keywords: *Leucojum aestivum,* tissue culture, alkaloid, lycorine, GC/Mass.

Introduction

Plants are a valuable source of several chemicals, including aromatic components, pigments, pharmaceuticals, and food. (Benzineb *et al*., 2019; Arifin *et al*., 2019). Many of these materials, without regard to their chemical differences **,**are placed in the vast metabolic group named secondary metabolites. Plenty of secondary metabolites are synthesized at a special stage of the plant's life, making it difficult to study the biosynthesis stages of secondary metabolites. Furthermore, sometimes the amount of secondary metabolites synthesized is too low; and hence the detection of their biosynthesis sites for biochemical studies becomes very hard **.**

Over the last two decades, the tendency to use tissue culture techniques for producing secondary metabolites has sharply increased, since the production of these precious components from intact plants has met a number of difficulties, including the limited plant sources available, high costs, and so on. It seems that using *in vitro* culture techniques such as tissue culture and cell suspension can solve many of the above-mentioned problems. It is presumed that the amount of secondary metabolite production is higher *in vitro* cultures (Asna Ashari and Khosroshahli 1388).

The plants belonging to the Amaryllidaceae family produce pharmaceutical active alkaloids with acetylcholinesterase inhibitory, cytotoxicity and antitumor activities.

In various genera of the Amaryllidaceae family, including *Narcissus, Galanthus,* and *Leucojum,* there are well-known alkaloids, that galanthamine and lycorine are two of which (Bogdanova *et al*., 2009).

Galanthamine has been effectively employed in the treatment of a handful of diseases. For instance, poliomyelitis, post-polio syndrome, myasthenia gravis, facial nerve paralysis, traumatic brain injuries, schizophrenia, and other neuromuscular disorders have responded well to treatment with galanthamine (Paskov, 1955; Irwin and Smith, 1960).

Lycorine, an alkaloid available in a number of species of the Amaryllidaceae family, has also been investigated for its medicinal properties, including inhibitory activities against HIV-1, poliovirus, measles virus, and herpes simplex virus type 1 (Ieven *et al*. 1983; Szlávik *et al*., 2004; Ahmed *et al*., 2019). In addition, lycorine has antimalarial activity and is considered as an antiviral and a strong inhibitor of cell division in higher plants (Ghosal *et al*., 1958).

Material and methods

Plant material

Plants of *Leucojum aestivum* were collected from its natural habitat, Anzali lagoon, in March **-**April and transferred to Tehran. All plants, having leaves and flowers at that time, were grown in pots and gradually used as a source of explant in the laboratory. First, roots, leaves and old and dry scales of the bulbs were removed, surface sterilized with 50% benomyl for 5 hours and then kept in the refrigerator at the 3°C for 5 weeks until the need for a cold was obviated and the dormancy was broken.

Extraction of alkaloids

To detect pharmaceutical important alkaloids available in *Leucojum aestivum,* samples were transferred to the Institute of Medicinal Plants.

Having been removed the roots, both bulblets obtained from *in vitro* cultures free of any hormones and those obtained from intact plants were dried by the oven at 60°C for 2-3 days and were finely powdered. The powdered samples (250 mg each) were dissolved in 5 ml of methanol and placed in the shaker for 12 hours, 5 ml of methanol was added to the solution twice, mixed and filtered using filter paper and then methanol was removed by vacuum rotary evaporation.

The dry extract was re-dissolved in 8 ml of 3% sulfuric acid and defatted three times with 10 ml of diethyl ether by aqueous-aqueous method. Then the solution was adjusted to pH 9-10 with 25% ammonia, and the extraction of alkaloids was carried out with 10 ml of chloroform three times,

followed by dehydration of the organic solvent by anhydrous sulfate sodium, and chloroform was completely removed by vacuum rotary evaporation. The remaining extract was collected by 0.5 ml of methanol with HPLC grade, sterilized with a syringe filter (0.2 µm), and injected into GC-MS.

The main specifications of the GC/MS used are described in Table 1.

Table 1: Specifications of GC/MS.

GC brand	Agilent 6890
Mass brand	Agilent 5973
column type	BPX5
Column's length	30 m
Column's internal diameter	0/25 µ
initial temperature of the column	50
final temperature of the column	300
carrier gas	He
Detector	Mass

The column specifications of the chromatography, Agilent 6890, used are follows: 30m in length, 0.25 µm in internal diameter, with a thickness of 0.25 µm, BPX5.

Statistical analysis

A factorial experiment with a completely randomized design was used for conducting all experiments. The analysis of data was carried out by IBM SPSS and mean comparisons were performed by Duncan multi-domain test (DMRT) at the P<1% or p<5%. MS Excel was employed for plotting.

Results and Discussion

Results of injecting the extracted alkaloid into GC-MS

- **Identification of alkaloids:**

Until now, industrial synthesis and employing the plants belonging to the Amaryllidaceae family, such as snowflake and Narcissus, have been two sources for obtaining important alkaloids like galanthamine and lycorine. However, using the latter source has two main drawbacks: first, there is a need for about one tone bulb to extract one kg galanthamine, and second, the increasing demand of pharmaceutical market may bring about the complete extinction of summer snowflake because of its limited natural source**.** This was the reason why the present study was conducted, the main goal was to see if there were any alkaloids in intact plants and *in vitro* cultures, if so, their contents could be increased in the upcoming researches.

After they were extracted, prepared, and injected into GC-MS, several alkaloids were identified (Table 2). The plants belonging to the Amaryllidaceae family produce pharmaceutical active alkaloids with remarkable medicinal properties such as acetylcholinesterase inhibitory, cytotoxicity and antitumor activities (Bastida *et al*., 2006*;* Raul Colque *et al*., 2009). In the various genera of the Amaryllidaceae family, including *Narcissus, Galanthus,* and *Leucojum,* galanthamine and lycorine are two of the most famous alkaloids (Bogdanova *et al*., 2009).

One of the most important alkaloids produced *in vitro* and in intact plants was lycorine, with 0.71 DW and 0.57 DW, respectively. The reason why lycorine was produced *in vitro* cultures more than intact plants could be the presence of the sucrose, the source of carbon. Alkaloids are macromolecules, and therefore some of which cannot evaporate in the gas chromatography because of their high molecular weight. This, therefore, can explain why the detection of galanthamine ended in failure. It is important to say that the failure of detection galanthamine should not be interpreted for its absence in the plant.

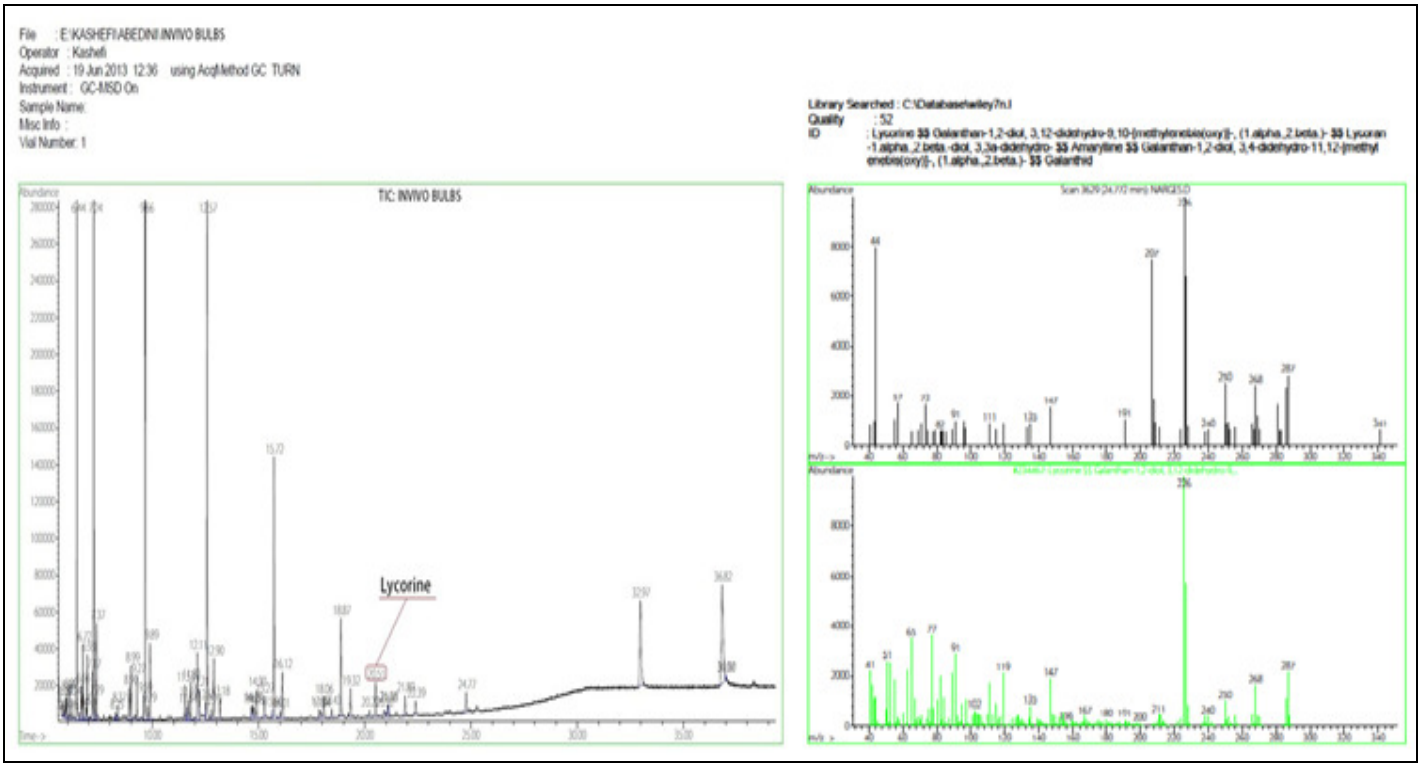

Fig. 1: Gas chromatography–mass spectrometry of lycorine, the bulbs of intact plants.

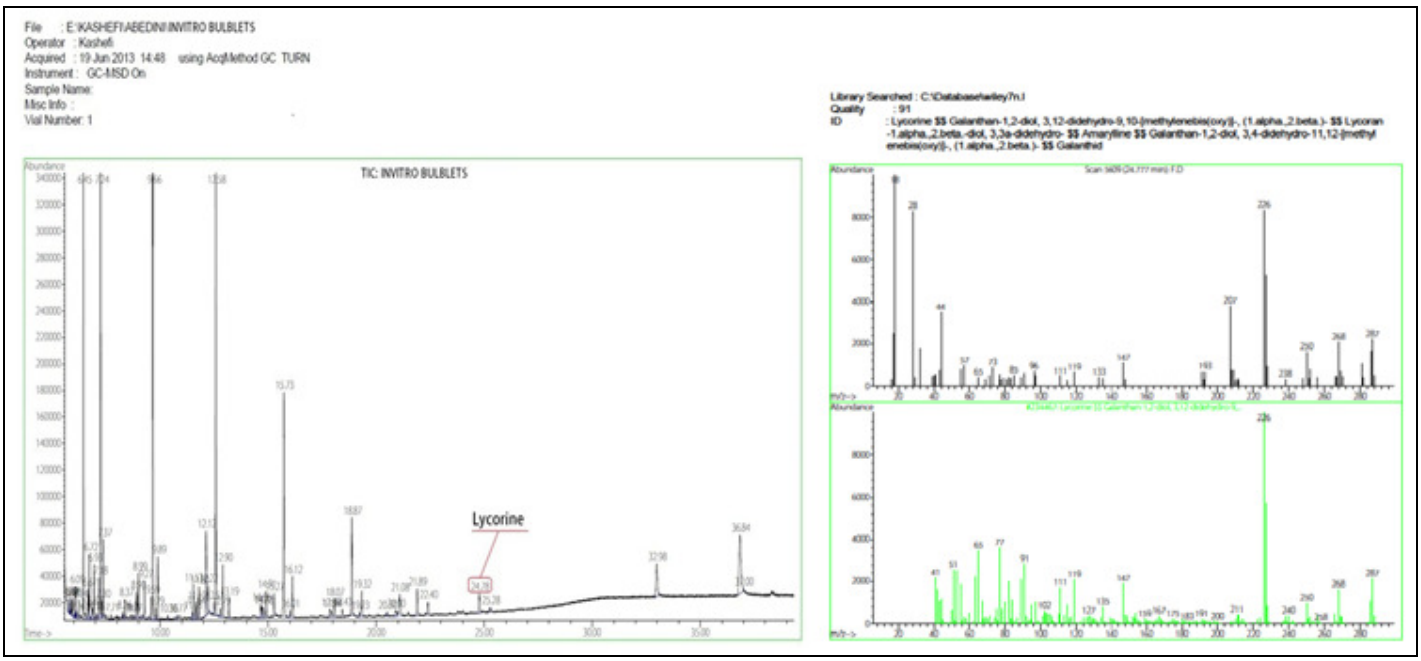

Fig. 2: Gas chromatography–mass spectrometry of lycorine, the bulbs of *in vitro cultures.*

Table 2: Identification of alkaloids in intact plants and *in vitro* cultures by GC-MS.

	Medium	R.T(min)	Name
1	invitro	5.85	Indole-2-one, 2,3-dihydro-N-hyd
2	invitro	8.26	5-Acetamido-4,7-dioxo-4,7-dihydrobenzofurazan
3	invitro	20.5	3-Pyridinol, 6-methyl
4	invitro	20.5	Lycorenan-7-one, 8,9,10-trimeth
5	invitro	20.5	Lycorenan-7-one, 1-methyl-9,10
6	invitro	24.77	Lycorine - Galanthan-1,2-diol
1	invivo	8.68	2-Methyl-6-(5-methyl-2-thiazoli)
2	invivo	8.77	3,4-dihydro-6,7-dimethoxyisoqui
3	invivo	8.77	4,5,6-Trimethoxyindole
4	invivo	10.77	6,7-Dimethoxyisatin 1H-Indol
5	invivo	13.19	4H-1,3-Thiazin-2-amine, N
6	invivo	20.5	2-Ethylacridine
7	invivo	24.78	Lycorine
8	invivo	25.28	2-Ethylacridine

According to Diop *et al*. (2007), the samples of the roots and bulbs taken from intact plants and *in vitro* cultures showed no galanthamine, whereas the bulblets formed from *in vitro* cultures free of any hormones produced 1.14*10-3 D.W galanthamine. The bulblets grown *in vitro* cultures supplemented with 10 µM NAA and 0.5 µM BA produced 6.79* 10-3 D.W galanthamine and those grown on the medium containing 0.5 µM NAA and 5 µM BA had 4.3*10-3 D.W galanthamine.

They concluded that *in vitro* organogenesis caused to cumulate galanthamine in the bulblets. Although in the present study the bulbs of intact plants contained lycorine, no galanthamine identified. Since in this study GC-MS was used for injecting the alkaloid instead of HPLC, which had been used in the study of Diop *et al.* (2007) so it can be realized that the underlying reason for the difference in the results between two studies must be the difference in the devices employed.

In another study, after injecting the samples, extracted from the leaves and bulblets of *L. aestivum* produced *in vitro* cultures, into HPLC, Bogdanova *et al*. (2009) identified several alkaloids, including galanthamine, lycorine, norgalanthamine homolycorinee, galanthamine, and ungiminorine. They also claimed that two important alkaloids, galanthamine and lycorine, presented in the leaves and bulblets of all three populations. Seeing the profiles of various alkaloids in *L. aestivum.* Stefanove (1990) calculated the content of galanthamine 0.1-0.56% DW, he also pointed out to three different geographical chemotypes having important alkaloids such as galanthamine, lycorine, and lycornin.

Berkove *et al*. (2005) inferred that since CGS-MS was able to detect 8 types of alkaloids extracted from the calli, clumps, and bulblets of *L. aestivum,* so it could be confirmed that *in vitro* culture had the potential of producing alkaloids. Nevertheless, according to Diop *et al.* (2007), there are only a few reports for producing little or no galanthamine in *L. aestivum.* Bogdanova *et al.* (2009) suggested that the biosynthesis of alkaloids in plants, particularly their galanthamine content, depended only on latitude and the characteristics of the soil that populations grow in.

Berkov *et al*. (2009) conducted an extensive review of the articles related to the production of alkaloids in the Amaryllidaceae family. They reported that other alkaloids like N-demethylgalanthamine and narwedine could be the precursor of galanthamine; the amount of galanthamine

depended on the geographical location of the plant population. And the alkaloids synthesized in the population of *L. aestivum* included different structural types such as galanthamine, lycorinee, homolycorinee and haemanthamine.

The snowflake's populations available in the southwest of Bulgaria are rich in galanthamine, whereas their counterparts in Romania are rich in lycorine. However, the populations of the snowflake in Japan have leucotamine and methylleucotamin alkaloids**.**

Three alkaloids having the inhibition of the Acetylcholinesterase (AChE) enzyme activity more than galntamin, meaning N -allylnorgalanthamine, sanguinine andN-14)-methyllallyl **(**norgalanthamin, have extracted from the snowflake.

In a study, the bulbs of Narcissus obtained from thousands of cultivars were evaluated and of about 0.1% galanthamine was reported **.**A cultivar named Ice Follies contained 70mg 100/ g galanthamine in dry weight of its bulbs.

In another research using GC-MS, 58% of total alkaloid content of Narcissus bulbs was composed of galanthamine. When studying on Lycoris radata, the native plant of Japan, China, and Korea, 22 alkaloids were detected that one of the most important of them was lycorine. On studying Ungenia victoris, the perennial bulbous plant, 10 alkaloids were extracted from its leaves and bulbs**.**

To evaluate the potential of *in vitro* cultures for producing alkaloids, only two plant species, Narcissus and snowflake, have been studied. Although callus induction is the key point to produce alkaloids, the differentiation rate of cells *in vitro* cultures is one of the influencing factors on the amount of galanthamine. The highest and lowest galanthamine content has been reported in calli and bulblets, respectively.

Having evaluated the amount of alkaloids in three species, *Leucojum aestivum,* Narcissus pseudonarcissus, and Galanthus elwesii from the Amaryllidaceae family, Tahchy *et al.* (2011) succeeded to detect two important alkaloids, lycorine, and galanthamine, by GC-MS. They tried different concentrations of sucrose (30, 90, 60, and 120 g/l) as secondary metabolite elicitors.

The results confirmed the effect of sucrose on the metabolism of alkaloids in cell cultures.

Among all species evaluated, the lowest alkaloid variation was seen in the shoots of *L. aestivum* grown on MSB supplemented with 10 µM-2,4 D. Tahchy *et al.* (2011) was able to identify 4 alkaloids, including Gal, crinine, lycorinee, and demethylmaritidin. After adding NAA and picloram to the medium, they detected only two alkaloids, meaning the effect of them on the variation of detected alkaloids was not considerable.

It is worth mentioning that the effect of sucrose and auxins on the variation of alkaloids in the Amaryllidaceae family depends on the species itself. Overall, the highest alkaloid variation was seen in G.elwesii cultured on 30 g/l sucrose supplemented with NAA and picloram, and N.pseudonarcissus cultured on 30 g/l sucrose supplemented with NAA, and *L. aestivum* cultured on 30 g/l sucrose supplemented with 2, 4-D.

Tahchy *et al.* (2011) also achieved interesting results in relation to alkaloid contents in intact plants among the above-mentioned three species. The alkaloids, trispheridine, anhydrolycorinee and crinine, extracted from the intact plant bulbs of *N.pseudonarcissus* showed the highest variation in comparison to the other two species**.**

In general, with only detecting crinine, the least variation of the alkaloids was reported in intact plant bulbs of *L.aestivum*. However, in this study, alkaloid variation was also observed in the bulbs of intact plants. Nevertheless, in both *in vitro* cultures and intact plant bulbs, the most well-known alkaloid was lycorine with a little higher amount *in vitro* cultures, perhaps due to sucrose according to the results of other reports.

Georgiev *et al*. (2009) reported the highest content of alkaloid in Narcissus and snowflake in the medium containing 60 and 180 g/l sucrose.

Ivanov *et al*. (2010) employed the shoots obtained from the medium supplemented with 2 mg/l BA and 0.15g/l NAA, and achieved to extract galanthamine after extracting and injecting alkaloid to HPLC.

Extracting alkaloid from the bulbs obtained from *in vitro* cultures and intact plants of *L. aestivum* and injecting it into GC-MS, Georgieva *et al.* (2007) were able to identify lycorinee, galanthamin, sanguinine, narwedine, N-formylnorgalanthamin, epinorhalanthamine, homolycorinee-8, O **-**demethylhomolycorinee-11, hydroxyvitattin, haemanthamin, and hordenine.

The explants of *L. aestivum,* having the same conditions, were subcultured on the MS supplemented with BAP and NAA for 6 years by Stanilova *et al*., (2010), and then galanthamine and lycorine were successfully extracted from the bulblets**.**

Pigni *et al*. (2012) used the whole intact plant of Narcissus serotinus for extracting alkaloid, and after injection it into GC-MS, they detected eleven alkaloids, including masonine galanthine-1, O-acetyl-3-Omethylnarcissidine, incartin , hippeastrine-3, O-methylnarcissidine-11, hydroxygalanthine narseronine-2, O-methylclivonine, -2 methoxypratosine, -1 O**-** acetyl-3-O-methyl-6oxonarcissidine. In the present study, no similar alkaloids to those of Pigin *et al*'s study were detected.

Conclusions

To evaluate the potential of *L. aestivum* for producing valuable alkaloid, the alkaloid available in the bulblets of *in vitro* cultures and intact plants was extracted and injected into GC-MS, and finally, the most important alkaloid identified was lycorine.

The results revealed that the plantlets obtained from *in vitro* cultures free of any hormones had normal growth. Non-use of hormones for mass production of plants is commercially important and cost-effective, especially when the plant is therapeutically desirable, and needs its alkaloids to be extracted extensively.

Nonetheless, it is important to take it into consideration that the presence of the hormone in the medium acts as an elicitor and increases alkaloid production in the plant. On the whole for *in vitro* propragation of *L. asetivum*, using its bulbs as the explant is both practical and useful. It is vitally

important to have in mind that this species is on the verge of extinction and its protection is crucial.

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