The Journal of Medicinal Plants and By-Products

Original Article

Tissue Culture of *Ungernia Victoris* Vved. EX Artjush. (Amaryllidaceae J.ST.-HIL.) and *Ptelea Trifoliata* L. (Rutaceae JUSS.) as the Sources of Acetylcholinesterase Inhibitors

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Article History: Received: 19 September 2024/Accepted in revised form: 04 December 2024

ABSTRACT

Several alkaloids extracted from diverse plant species across different botanical groups are recognized for their acetylcholinesterase-inhibitory properties. Among these, *Ungernia victoris* (UV), a rare endemic species from the western spurs of Pamir-Alay, is a significant source of galanthamine - a compound used in drugs for treating early- to mid-stage Alzheimer's disease, poliomyelitis, and other neurological disorders. Similarly, *Ptelea trifoliata* (PT), a North American species widely cultivated in botanical gardens, demonstrates anti-inflammatory, antioxidant, anti-renal fibrosis, and acetylcholinesterase-inhibitory activities. This study aims to establish in vitro propagation protocols for *U. victoris* Vved. ex Artjush. (Amaryllidaceae J.St.-Hil.) and *P. trifoliata* L. (Rutaceae Juss.) as sources of acetylcholinesterase-inhibiting compounds.

Using tissue culture techniques, including callus induction and regeneration, alkaloid biosynthesis was evaluated. Callus cultures of UV exhibited the highest galanthamine content, reaching 5.10% DW in a medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin, whereas in vitro-regenerated leaves contained lower levels (1.41% DW) and bulbs lacked galanthamine entirely. Naturally collected and botanical garden samples showed lower galanthamine levels, while seeds contained moderate amounts. The total alkaloid content in UV leaves collected from natural habitats reached up to 0.5%, comprising galanthamine (0.14–0.21%) and lycorine (0.059%), while the bulbs contained up to 0.96%, with galanthamine (0.24%) and lycorine (0.31%).

For PT, callus cultures produced the highest kokusaginine content (1.12% DW) in Murashige & Skoog medium with 2,4-D and kinetin, surpassing levels in botanical garden samples (0.54–0.78% DW). However, kokusaginine biosynthesis was suppressed in *in vitro*-regenerated plants, where it was undetectable.

This study demonstrates that callus cultures are a reliable and efficient source of galanthamine and kokusaginine, highlighting the potential of tissue culture methods to enhance the production of acetylcholinesterase-inhibiting alkaloids from medicinal plants.

Keywords: Biological active components, Tissue culture, Acetylcholinesterase inhibitors, Galanthamine, Kokusaginine, Alzheimer's disease

INTRODUCTION

According to World Health Organization projections, three-quarters of the population aged 60 or over will be living in developing countries by 2025 [1]. The number of people affected by dementia will increase from 42 million people in 2020 to 81 million people in 2040 [2]. Several types of dementia are known, among them Alzheimer's disease is the most common cause of dementia (60%), followed by vascular dementia (20%) and dementia with Lewy bodies (15%) [3]. Cholinesterase activates the hydrolysis of the neurotransmitter acetylcholine into choline and acetic acid allowing inactivation of a cholinergic neuron [4]. This process involves

two types of enzymes: acetylcholinesterase and butyrylcholinesterase. Several anticholinesterase inhibitors are known, including drugs, natural toxins, pesticides, chemical warfare agents inhibiting the cholinesterase enzyme from breaking down, and by this way increasing the level and duration of the neurotransmitter action [4, 5].

Several alkaloids extracted from the species of different plant groups are known as acetylcholinesterase inhibitors: lycorine, tazettine, crinine, 3-epi-hydroxybulbispermine, 2-demethoxy-montanine, and galanthamine extracted from the species of Amaryllidacae family [6-10], conypododiol - Asparagaceae family [11], bulbocapnin, corydaline - Fumariaceae family [12], N-methylasimilobine - Nelumbonacae family [13, 14], annotinine, annotine, annotine N-oxide, lycodoline, lycoposerramine M, anhydrolycodolin, gnidioidine, lycofoline – Lycopodiaceae family [15, 16], buxakarachiamine, buxakashmiramin, buxahejramine, cyclomicrophylline-A, cyclovirobuxeine-A, cycloprotobuxine-C - Buxaceae family [6, 17], stepharanine, cyclanoline, and N-methyl stepholidine – Menispermaceae family [18], juliflorine - Fabaceae family [5, 6], dehydroevodiamine, kokusaginine - Rutaceae family [6, 19, 20], (–)-huperzine A - Lycopodiaceae family [6, 21], trigonelline - Leguminosae family [22, 23], isotalatizidine hydrate - Ranunculaceae family [15, 24].

The galanthamine is an alkaloid, which is most common for plants of Amaryllidaceae family, a selective and competitive acetylcholinesterase inhibitor. The galanthamine is used as a substance in the production of the drugs used for the treatment of early- to mid-stage Alzheimer's disease, poliomyelitis, and other neurological diseases. This biological active component is produced by extraction from plants such as daffodils (*Narcissus pseudonarcissus* (L.) cv. Carlto), snowflake (*Leucojum aestivum* L.), red-tubed lily (*Lycoris radiate* (L'Hér.) Herb.), and Viktor's ungernia (*Ungernia victoris* Vved. ex Artjush.), and alternatively by chemical synthesis [28].

Kokusaginine, a quinoline alkaloid primarily found in plants of the Rutaceae family (*Ptelea trifoliata* and *Zanthoxylum* spp.), has demonstrated potential as an acetylcholinesterase (AChE) inhibitor in several studies; this property positions kokusaginine as a candidate for therapeutic applications, particularly in neurodegenerative diseases like Alzheimer's disease, where AChE inhibitors are used to enhance cholinergic transmission [26-28]. The kokusaginine was isolated from *Ruta graveolens* L. [29], *Tinospora malabarica* (Lour.) Merr. [30], *Orixa japonica* Thunb [31], *Platydesma campanulata* Mann. [32], *Acronychia baueri* Schott [33], *Balfourodendron riedelanum* Engler [34], *Ptelea trifoliata* L., and others [35].

Due to the increased demand by the pharmaceutical companies and the limited availability of plant sources, the biosynthesis of acetylcholinesterase inhibitors by plant *in vitro* systems has been considered as an alternative approach for sustainable production of these biological active compounds in several studies: callus cultures of *Narcissus* spp., *Leucojum aestivum*, *Hippeastrum* spp., *Lycoris radiata* as the source of galanthamine [36 - 43], and *Ptelea trifoliata* L., *Zanthoxylum* spp., and other Rutaceae family plants are the primary sources of kokusaginine [44 - 48].

This study aims to establish protocols for the *in vitro* propagation of two species, *Ungernia victoris* Vved. ex Artjush. (Amaryllidaceae) and *Ptelea trifoliata* L. (Rutaceae), as sources of acetylcholinesterase-inhibiting compounds. Galanthamine is derived from *U. victoris*, while kokusaginine is obtained from *P. trifoliata*. The investigation compares these compounds' production across tissue cultures, *in vitro*-regenerated plants, plants introduced into the Tashkent botanical garden, and plants collected from natural habitats.

MATERIAL AND METHODS

Ungernia victoris Vved. ex Artjush. (Amaryllidaceae), Victor's ungernia (UV). The plant is a perennial species endemic to the Southwest Pamir-Alay. Its growth cycle begins in February, with the leaves emerging early in the year and completely withering by late May to early July. The peak accumulation of galanthamine occurs in the leaves around mid-April. Flowering begins in early August, characterized by yellowish-pink flowers borne on nearly equal pedicels. The perianth leaflets are initially yellowish but turn rosy upon drying. The fruits, measuring 3–4 cm in diameter, mature by September [49, 50] (Fig. 1).

Fig. 1 *Ungernia victoris.* Mature plants observed near the village of Nilu, Surkhandarya region, Uzbekistan. Photo by D. Turdiev, taken on 27 March 2022

Ptelea trifoliata L. (Rutaceae), common hoptree or water ash (PT). The plant is a small tree or often a shrub with a few spreading stems, typically reaching a height of 6–8 m and forming a broad crown. The fully grown leaves are dark green and glossy on the upper surface, with a paler green underside. In autumn, the leaves transition to a rusty yellow color. The flowers are small, measuring 1–2 cm across, and have 4–5 narrow, greenish-white petals. The fruit is a round, wafer-like, papery samara, 2–2.5 cm in diameter, light brown in color, and contains two seeds. The fruit ripens in October and remains on the tree until high winds dislodge them in early winter [51 - 56] (Fig. 2).

Fig. 2 *Ptelea trifoliata.* Specimen from the Tashkent Botanical Garden, Tashkent. Photo by A.T. Khazratov, taken on 23 August 2024

Explants

Ungernia victoris. For the induction of callus, parts of germinated seeds, specifically the hypocotyl, were used as explants. Freshly collected seeds were stored in mesh bags (20 × 30 cm) in a freezer at -10 °C for over 30 days. The seeds were then sterilized to remove contaminants. The sterilization process involved washing under running water, treating with a 20% v/v solution of the sterilizing soap "Domestos" with constant stirring at 150 rpm on a shaker, rinsing with distilled water, immersion in 70% ethanol for 90 seconds, further washing with distilled water, and soaking in a 4% sodium hypochlorite solution for 20 minutes. The seeds were finally washed thoroughly with distilled water.

After sterilization, 25–30 seeds were placed in Petri dishes containing 25 mgl⁻¹ of 25% MS nutrient medium with sucrose (7.5 gl⁻¹), agar (7 gl⁻¹), and streptomycin (0.2%, v/v), but without phytohormones. The Petri dishes were hermetically sealed with elastic stretch film and stored in the dark at +5 °C for 1–4 weeks. Subsequently, the seeds were transferred to 50% MS nutrient medium (25 mgl⁻¹) in 0.5 L sterile jars, supplemented with sucrose (15 gl⁻¹) and agar (7 gl⁻¹), without antibiotics or phytohormones. These jars were placed in a culture room maintained at +24±2°C with a 16/8-hour light/dark photoperiod. Segments approximately 0.5 mm long, including cotyledons, hypocotyls, and radicles from the germinated seeds, were used as explants for callus induction (Fig. 3).

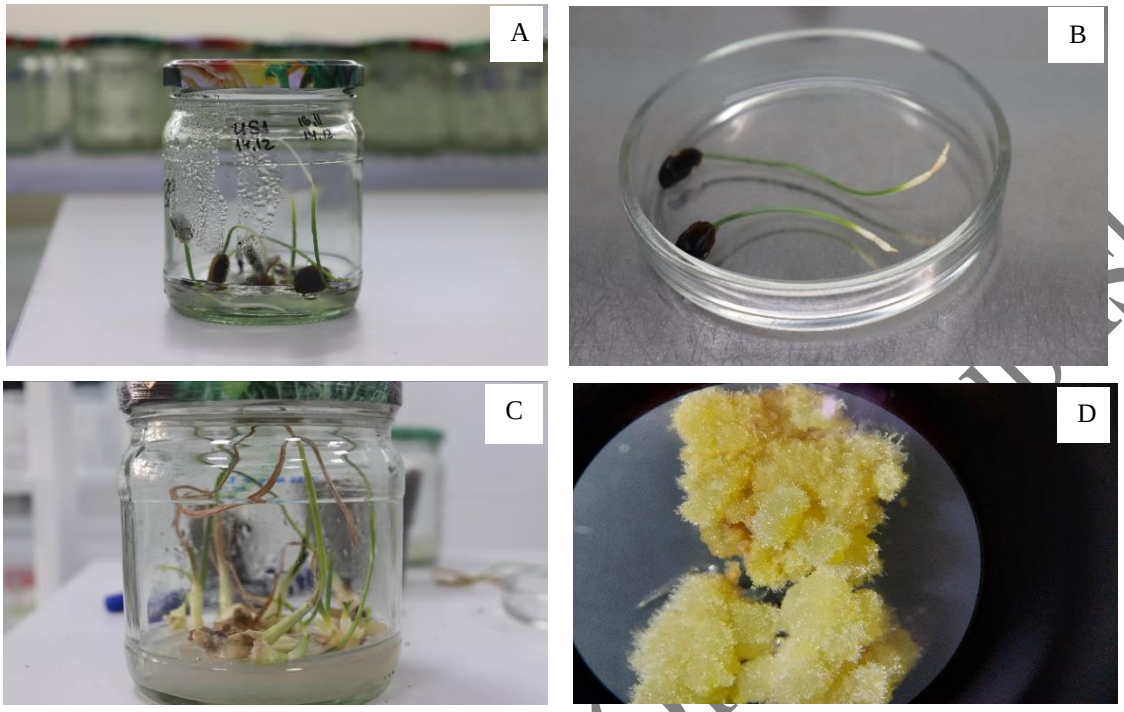

Fig. 3 *Ungernia victoris.* A. Seeds germinated on MS nutrient medium. B. Germinated seed used as an explant. C. *In vitro* regenerated plants. D. Callus generated on MS medium supplemented with 0.5 mgl⁻¹ BAP. Photo by F.U. Mustafina, 2023

Ptelea trifoliata. One-year-old sprouts and fresh leaves were used as the source of explants. The leaves were removed from the branches and underwent a sterilization process as follows: they were washed under running tap water, immersed in soapy water for 15–20 minutes, and treated with a fungicide solution containing difenoconazole (Score 250EC, 23.3% v/v) for 5–7 minutes. After this, the leaves were rinsed with distilled water, placed in a 20% solution of "Belizna" (containing 18% sodium hypochlorite) with 5–7 drops of Tween20 for 10 minutes, and washed again with distilled water. The leaves were then immersed in 70% ethanol for 20 seconds and finally washed thoroughly with distilled water.

Nutrient Media

The ready-made nutrient media from Duchefa Biochemie B.V (https://www.duchefa-biochemie.com) according to the MS [57], N6 [58, 59], B5 [60], and WPM [61] media were used. Antibiotic streptomycin was added to the nutrient media at a concentration of 0.2% v/v. The nutrient medium by Vollosovich (1979) (Vch) [62] was prepared with reagents and biochemicals from Duchefa Biochemie B.V (https://www.duchefa-biochemie.com). Among tested nutrient media, the best results were observed on MS media: up to 60±2% of the explants were introduced in *in vitro* culture, following with WPM (56±2%) and Vch (45±2%) media.

Callusogenesis of *U. victoris* and *P. trifoliata*

Callusogenesis of UV and PT was induced on the MS medium with the compositions of phytohormones 2.4-D 0.5 mgl^{-1} + BAP 0.5 mgl^{-1}, 2.4-D 0.5 mgl^{-1} +Kin 0.5 mgl⁻¹, and BAP 0.5 mgl⁻¹. The first subculture of the callus was on the MS medium of the same composition and concentration of components as it was used for induction of *in vitro* culture: 2.4-D 0.5 mgl^{-1} + BAP 0.5 mgl⁻¹, 2.4-D 0.5 mgl⁻¹+Kin 0.5 mgl⁻¹, and BAP 0.5 mgl⁻¹. The second subculture of the callus for proliferation was on the MS medium with 2.4-D 0.5 mgl⁻¹ + Kin 0.5 mgl⁻¹.

Organogenesis

Ungernia victoris: Organogenesis of the UV was achieved according to the protocol by Mustafina & al. (2024). For UV, the following combinations of phytohormones induced organogenesis: IAA 0.5 mgl^{-1} + BAP 0.5 mgl^{-1}; NAA 0.5 mgl^{-1} + BAP 0.5 mgl^{-1}; NAA 0.5 mgl^{-1} + Kin 0.5 mgl^{-1}, and IAA 0.5 mgl^{-1} + Kin 0.5 mgl^{-1}. When the callus was placed on the 100% MS medium, the development of indirect organogenesis was observed. The first subculture of the callus was on the media with the same content and concentration of phytohormones: IAA 0.5 mgl^{-1} + BAP 0.5 mgl^{-1}, NAA 0.5 mgl^{-1} + BAP 0.5 mgl^{-1}, NAA 0.5 mgl^{-1} + Kin 0.5 mgl^{-1}, and IAA 0.5 mgl^{-1} + Kin 0.5 mgl^{-1}. The second subculture was on a 100% MS nutrient medium with a ratio of auxin to cytokinin of 5:1 for the proliferation of the microbulbs: IAA 2.5 mgl^{-1} + BAP 0.5 mgl^{-1}, NAA 2.5 mgl^{-1} + BAP 0.5 mgl^{-1}, NAA 2.5 mgl^{-1} + Kin 0.5 mgl^{-1}, and IAA 2.5 mgl^{-1} + Kin 0.5 mgl^{-1}. The third subculture was on a 50% MS nutrient medium to stimulate the development of the root system: NAA 2.5 mgl^{-1} + BAP 0.5 mgl^{-1} + TDZ 0.3 mgl^{-1}. Further subcultures were on a 50% MS nutrient medium with NAA 0.5 mgl^{-1}, which stimulated the development of the root system and prepared regenerated plants for adaptation to the soil.

Ptelea trifoliata: *In vitro* propagation of the PT was achieved on MS media with the composition of the phytohormones BAP 0.2 mgl^{-1}; BAP 0.1 mgl^{-1} + NAA 0.05 mgl^{-1}; BAP 0.2 mgl^{-1} + NAA 0.05 mgl^{-1}. After 2-3 subcultures of the explants, the lateral shoots started to propagate with 3-4 leaves. When the lateral shoots developed well enough, they were subcultured to the media with the IBA 0.5 mgl^{-1}. Next subcultures were also done on the MS media with IBA 0.5 mgl^{-1}. Well propagated plantlets were transferred to the soil for further adaptation (Fig. 4).

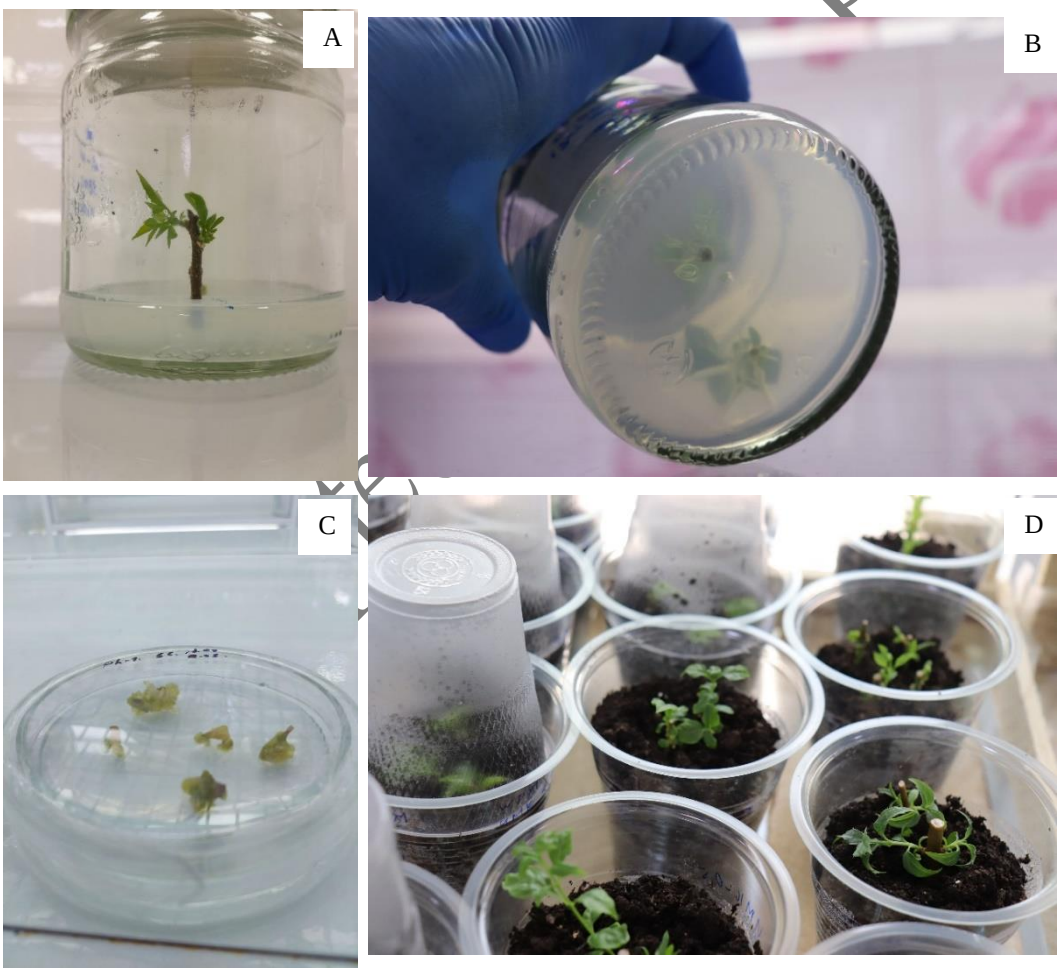

Fig. 4 *Ptelea trifoliata*. A. Explant introduced to *in vitro* culture. B. Rhizogenesis. C. Callussogenesis. D. Adaptation to soil

Extraction

The samples of callus and regenerated plants were dried in the “Binder” (Tuttlingen, Germany) incubator at +50°C for three days. The dried and powdered samples of plant material and callus were successively extracted

using 99% methanol with a solid-to-solvent ratio of 1:20 (w/v) at room temperature (2 times at 3-day intervals, totaling 6 days). The extracts were filtered through filter paper (No. 2, Whatman Co., Maidstone, Kent, UK) and evaporated at +40°C to dryness using a rotary evaporator (Eyela N-1300, Tokyo Rikakikai Co., Ltd., Tokyo, Japan). The extracts were then dissolved in dimethyl sulfoxide (DMSO) and stored at -20 °C until used in subsequent experiments.

DPPH Free Radical Scavenging Assay

The DPPH free radical scavenging assay was developed according to the methodology proposed by Shah & al. (2019) with slight modifications, using 2,2-diphenyl-1-picrylhydryl free radical (DPPH) (Sigma Aldrich, St. Louis, MO, USA). A total of 200 µL of 150 µM DPPH in methanol was added to the samples, as much as 20 µL with various concentrations in a 96-well microplate. Ascorbic acid was used as a standard; all measurements were performed in triplicate. After 30 min, absorbance was determined at 517 nm using a UV spectrophotometer (Molecular Devices, San Jose, CA, USA).

ABTS Free Radical Scavenging Assay

2,2-Azinobis (3-ethylbenzothiazoline)-6-sulphonic acid (ABTS) (Sigma Aldrich, St. Louis, MO, USA) assay was carried out according to the previously reported method [63]. The ABTS solution was prepared by mixing 7 mM ABTS and 2.45 mM potassium persulfate (final concentration) and then incubated in the dark at room temperature for 16 h. ABTS solution was diluted with distilled water for the absorbance to reach 0.70 ± 0.02 at 740 nm. Ten microliters of different concentrations of the extracts were added to 190 µL of ABTS solution. Antioxidant activity measurements were carried out in triplicate. Ascorbic acid was taken as a standard.

Statistical Analysis

The analysis of data focused on summarizing the distribution, mean, and standard deviation of measurements obtained from triplicate experiments (n = 3). Statistical methods involved applying one-way ANOVA, followed by Tukey's post hoc test to identify significant differences, with a 95% confidence level ($P < 0.05$) for datasets conforming to normality criteria.

RESULTS AND DISCUSSION

Ungernia victoris (UV)

The galanthamine content (%, DW) was evaluated in plant material introduced into the Tashkent botanical garden, collected from natural populations, derived from callus cultures, and found in seeds (Table 1-3). The highest galanthamine content, up to 5.1% DW, was observed in callus cultures of UV, with significantly higher levels in Vch medium compared to MS medium. In MS medium, galanthamine levels reached 1.05% DW with the combination of 2.4-D 0.5 mgl^{-1} + Kin 0.5 mgl^{-1} and 1.01% DW with IAA 0.5 mgl^{-1}. In Vch medium, the content ranged from 2.46% to 5.10% DW. Lower galanthamine levels were detected in plants from natural populations and the botanical garden. Galanthamine was absent in the bulbs of *in vitro* regenerated plants but present in their leaves.

The galanthamine content varied significantly across different sources and cultivation conditions, reflecting the influence of origin, tissue type, and nutrient medium. Naturally collected materials exhibited relatively low content, ranging from 0.28% DW in UV_1 (Sangardak, Surkhandarya region) to 0.64% DW in UV_4 (Sovuqbuloq, Surkhandarya region). The highest galanthamine content among natural samples was found in material from Sovuqbuloq (UV_4), while it was undetected in UV_2 collected near Nilu village, Surkhandarya region (Table 2).

In *in vitro* regenerated plants, galanthamine was found in the leaves, with the highest content of 1.41% DW in UV_5, but it was absent in the bulbs (e.g., UV_1B, UV_2B, UV_3B, UV_4B). Botanical garden samples exhibited variability in galanthamine content, with the lowest level at 0.04% DW in UV_6 (Sovuqbuloq) and the highest at 1.43% DW in UV_9 (Pojaz). Material from Nilu village (UV_8), introduced into the botanical garden, also showed relatively high galanthamine content of 1.27% DW.

Table 1 Analysis Results for *Ungernia victoris*: DPPH and ABTS antioxidant activities and galanthamine content

No.	Code	Place of collection	Yield of Extracts [%]	Antioxidant activity DPPH IC_{50} [$\mu g l^{-1}$]	Antioxidant activity ABTS IC_{50} [$\mu g l^{-1}$]	Content Galanthamine [%]
Plant material collected from nature						
1	UV_1	Sangardak. Polgasay. Surkhandarya region. Collected in 10.04.2023.	10.5	2397.7	751.30	0.28
2	UV_2	Nilu. Surkhandarya region. Collected in 15.04.2023.	7.67	1367.9	533.82	-
3	UV_3	Pojaz. Surkhandarya region. Collected in 17.04.2023.	7.76	1992.4	716.23	0.56
4	UV_4	Sovuqbuloq. Surkhandarya region. Collected in 17.04.2023.	3.08	1803.8	471.23	0.64
In vitro regenerated plant material (leaves)						
5	UV_5	*in vitro* (UV Sovuqbuloq). The plantlets developed in *in vitro* conditions.	8.58	1542.7	823.83	1.41
In vitro regenerated plant material (bulbs)						
6	UV_1B	Sangardak. Polgasay. Surkhandarya region. Collected in 10.04.2023.	1.25	-	-	-
7	UV_2B	Nilu, Surkhandarya region Collected in 15.04.2023.	7.18	8989.0	1580.1	-
8	UV_3B	Pojaz, Surkhandarya region. Collected in 17.04.2023.	5.34	4409.0	1496.6	-
9	UV_4B	Sovuqbuloq, Surkhandarya region. Collected in 17.04.2023.	7.07	3556.0	1081.0	-
Plant material collected from Botanical Garden						
10	UV_6	Botanical Garden. The plants were collected from Sovuqbuloq, Surkhandarya region. Collected from nature in 04.2022.	4.27	1580.4	538.95	0.04
11	UV_7	Botanical Garden. The plants were collected in 2021 from Sangardak. Polgasay. Surkhandarya region.	6.3	2389.9	591.30	0.28
12	UV_8	Botanical Garden. The plants were collected in 2021 from Nilu, Surkhandarya region.	10.96	1299.3	537.34	1.27
13	UV_9	Botanical Garden. The plants were collected in 2021 from Pojaz, Surkhandarya region.	9.77	685.70	490.03	1.43
14	UV_10	Botanical Garden. The plants were collected in 2021 from Sovuqbuloq, Surkhandarya region.	9.02	923.46	603.82	0.11
15	UV_10-1	Botanical Garden. The plants were collected in 2021 from Sovuqbuloq, Surkhandarya region.	15.45	2297.7	729.71	-
Callus						
16	UV_C1	Vch 2.4-D 0.5 mgl^{-1} +BAP 0.5 mgl^{-1}	8.41	955.09	2.15	2.46
18	UV_C2	MS 2.4-D 0.5 mgl^{-1} + Kin 0.5 mgl^{-1}	6.62	944.75	53.53	1.05
19	UV1_C3	Vch 2.4-D 1.0 mgl^{-1} +Kin 1.0 mgl^{-1}	13.08	1465.5	11.33	5.10
20	UV_C4	Vch 2.4-D 0.5 mgl^{-1} +BAP 0.5 mgl^{-1}	15.22	825.57	4.91	4.82
21	UV1_C5	Vch 2.4-D 0.5 mgl^{-1} + Kin 0.5 mgl^{-1}	18.79	782.59	1.84	4.40
22	UV_C6	MS IAA 0.5 mgl^{-1}	11.36	1585.1	25.35	1.01
Seeds						
23	UV1	Sovuqbuloq. Surkhandarya region. Collected in 17.04.2023.	16.23	3595.1	3538.8	1.03
24	UV2	Gissar range. Sangardak river, Sangardak village.	14.75	-	4413.3	2.18

Table 2 Comparative results on galanthamine content from different sources of *Ungernia victoris*

Source	Range of galanthamine content (% DW)	Highest content (%)	Key observation
Plant material from nature	0.28–0.64	0.64 (UV_4)	Moderate galanthamine content, with the highest detected in Sovuqbuloq samples.
In vitro regenerated plants (leaves)	1.41	1.41 (UV_5)	The highest galanthamine content observed among all sources.
In vitro regenerated plants (Bulbs)	Not detected	-	Galanthamine not detected in bulbs of regenerated plants.
Plant material from Botanical garden	0.04–1.43	1.43 (UV_9)	Higher galanthamine levels in plants introduced from Pojaz.
Callus cultures	1.05–5.10	5.10 (UV1_C3)	Callus cultures exhibited the highest galanthamine content, influenced by media composition and hormones

Callus cultures demonstrated the highest galanthamine content among all sample types, ranging from 1.01% DW (UV_C6) to 5.10% DW (UV1_C3). Vch medium consistently produced higher galanthamine levels, up to 5.10% DW, compared to MS medium, which yielded lower levels of 1.05% DW (UV_C2) and 1.01% DW (UV_C6). Seeds contained moderate galanthamine levels, with 1.03% DW in UV1 (Sovuqbuloq) and 2.18% DW in UV2 (Gissar range).

Callus cultures are the most promising source of galanthamine, with the highest observed content (up to 5.10% DW) in Vch medium with 2.4-D and kinetin. *In vitro* regenerated leaves provide potential as a secondary source, with a maximum content of 1.41% DW. Naturally collected materials and botanical garden samples exhibit lower levels of galanthamine. Seeds offer moderate galanthamine content, serving as an auxiliary source. Optimizing nutrient media and hormone combinations, particularly Vch medium with auxins and cytokinins, can enhance galanthamine production and demonstrates potential for biotechnological applications.

The DPPH activity (IC_{50}, μgl^{-1}) varied widely across samples (Table 3). The strongest antioxidant activity (lowest IC_{50}) was observed in callus UV1_C5 grown on Vch medium with 2.4-D 0.5 mgl^{-1} + Kin 0.5 mgl^{-1} (IC_{50} = 782.59 μgl^{-1}). The weakest activity was found in the bulbs of *in vitro* regenerated plants (UV_2B) from Surkhandarya region (IC_{50} = 8989.0 μgl^{-1}). Callus cultures and seeds generally exhibited lower IC_{50} values, indicating superior antioxidant activity compared to plant materials collected from nature or *in vitro* regenerated plants.

Table 3 Antioxidant activity of the studied material of *Ungernia victoris* according to DPPH and ABTS methods

Methods	Strongest Antioxidant Activity	Weakest Antioxidant Activity
DPPH	Callus culture grown on Vch medium with 2.4-D 0.5 mgl^{-1} and Kin 0.5 mgl^{-1} UV1_C5 (IC_{50} = 782.59 μgl^{-1})	*In vitro* regenerated plant bulb from the Nilu village exhibited the weakest activity UV_2B (IC_{50} = 8989.0 μgl^{-1})
ABTS	Seeds collected from the vicinity to Nilu village, demonstrated the highest inhibition UV2 (IC_{50} = 4413.3 μgl^{-1})	Callus culture grown on Vch medium with 2.4-D 0.5 mgl^{-1} and Kin 0.5 mgl^{-1} UV1_C5 (IC_{50} = 1.84 μgl^{-1})

Extract yields varied significantly by media type and phytohormone combinations. Vch medium produced the highest yields (18.79% with 2.4-D 0.5 mgl^{-1} + Kin 0.5 mgl^{-1}), outperforming MS medium, which had the lowest yields (6.62% with the same hormone combination). Vch medium consistently demonstrated stronger antioxidant potential (lower DPPH IC_{50}) compared to MS medium, regardless of the hormone combination. Kinetin paired with 2.4-D enhanced DPPH activity and extract yields, while IAA showed moderate ABTS activity but weaker DPPH results, suggesting media and hormone combinations influence secondary metabolite production pathways.

ABTS activity (IC_{50}, μgl^{-1}) also exhibited significant variation. The highest ABTS activity was observed in seeds from Nilu village (UV2, 4413.3 μgl^{-1}), while the lowest was found in callus UV1_C5 grown on Vch medium (1.84 μgl^{-1}). Seeds demonstrated the strongest ABTS activity, followed by *in vitro* regenerated plant leaves, with

callus cultures generally showing lower values. Notable exceptions included UV_C6 (25.35 μgl⁻¹). These trends highlight distinct metabolic profiles and antioxidant mechanisms among the sample types.

Natural plant materials exhibited moderate antioxidant activity, with DPPH IC_{50} values around 2397.7 μgl⁻¹ and ABTS activity ranging from 471.23 μgl⁻¹ to 716.23 μgl⁻¹. Leaves of *in vitro* regenerated plants (UV_5) showed stronger antioxidant properties than bulbs (UV_4B), likely due to stress-induced production of secondary metabolites. Botanical garden samples varied widely in antioxidant activity, influenced by plant origin and storage conditions.

The discrepancy between DPPH and ABTS activities suggests the involvement of different antioxidant compounds. DPPH primarily measures hydrophilic compounds, while ABTS captures both hydrophilic and lipophilic antioxidants. Callus cultures and seeds demonstrated higher DPPH activity, suggesting controlled conditions enhance specific pathways. Seeds exhibited superior ABTS values, likely due to reserves of phenolics and tocopherols [66].

Callus cultures and seeds stand out as promising sources of antioxidants, depending on the desired activity. Vch medium with 2.4-D 0.5 mgl⁻¹ + Kin 0.5 mgl⁻¹ is optimal for maximizing extract yield and DPPH activity, while MS medium shows higher ABTS activity. Stress induction and optimized growth conditions can further enhance antioxidant potential. These findings suggest targeted applications of different sample types in pharmaceutical and nutraceutical development, leveraging their distinct antioxidant profiles.

The contradictory results for the same sample, UV1_C5, which shows comparatively strong antioxidant activity in the DPPH assay (IC_{50} = 782.59 μgl⁻¹) but weak activity in the ABTS assay (1.84 μgl⁻¹), can be explained by the fundamental differences in these two methods and the types of compounds they assess. The DPPH assay primarily detects hydrophilic antioxidants. Compounds with high solubility in the aqueous phase, such as phenolic acids or certain flavonoids, effectively reduce the DPPH radical. UV1_C5 likely contains a high concentration of hydrophilic antioxidants, leading to its strong activity in the DPPH assay. The ABTS assay measures the activity of both hydrophilic and lipophilic antioxidants. The low inhibition observed in the ABTS assay suggests that UV1_C5 has a low concentration of lipophilic antioxidants or a limited variety of antioxidants capable of interacting with the ABTS radical. To reconcile the results, further analysis of the specific antioxidant compounds in UV1_C5 (e.g., phenolics, flavonoids, lipophilic antioxidants) and their distribution between hydrophilic and lipophilic phases is recommended.

Ptelea trifoliata (PT)

The content of the standard component kokusaginine was analyzed in plant material introduced into the botanical garden, callus cultures, and leaves of *in vitro* regenerated plants (Table 4-6).

Kokusaginine content in botanical garden samples ranged from 0.54% to 0.78% DW. The highest kokusaginine content was detected in botanical garden sample PT_3 (0.78%), followed by PT_1 (0.56%) and PT_4 (0.54%). No kokusaginine was detected in PT_2, indicating potential variability among individual trees. Botanical Garden samples demonstrated moderate kokusaginine levels, suggesting consistent but non-optimized production of the compound under natural conditions (Table 5).

Callus cultures exhibited a broader range of kokusaginine content, from 0.11% to 1.12% DW. The highest content (1.12%) was recorded in PT_C2 (MS medium with 2.4-D 0.5 mgl⁻¹ + Kin 0.5 mgl⁻¹), followed closely by PT_C6 (1.07%) on MS medium with IAA 0.5 mgl⁻¹. The lowest content (0.11%) was observed in PT_C1 (MS medium with 2.4-D 0.5 mgl⁻¹ + BAP 0.5 mgl⁻¹), likely due to differences in hormonal composition or the early stage of culture development. Callus cultures generally produced higher kokusaginine levels compared to botanical garden samples, indicating enhanced biosynthesis under controlled conditions.

Kokusaginine content in *in vitro* regenerated plants was not detected in any sample (PT_R1 to PT_R4). Despite advanced regeneration processes, the absence of kokusaginine suggests that its biosynthesis is suppressed or not triggered under the tested conditions. This indicates that *in vitro* regenerated plants do not contribute to kokusaginine production, likely due to environmental or metabolic limitations.

Callus cultures consistently demonstrated the highest kokusaginine content, particularly PT_C2 (1.12%) on MS medium with 2.4-D (0.5 mgl⁻¹) and Kin (0.5 mgl⁻¹), and PT_C6 (1.07%) on MS medium with IAA (0.5 mgl⁻¹). These results emphasize the influence of specific hormonal combinations on kokusaginine biosynthesis. Botanical garden samples showed moderate kokusaginine levels, with PT_3 (0.78%) as the highest. This suggests

that natural conditions support kokusaginine production but not to the extent achieved in optimized callus cultures.

Table 4 *Ptelea trifoliata*. The results of DPPH and ABTS analyses, and kokusaginine standard component analyses

No.	Code	Place of collection	Yield of Extracts [%]	Antioxidant activity		Contents
				DPPH	ABTS	Kokusaginine [%]
				IC_{50} [μgl^{-1}]		
Plant material collected from Botanical Garden						
1	PT_1	Tashkent Botanical Garden collection. Tree #1.	3.4	1387.77	342.30	0.56
2	PT_2	Tashkent Botanical Garden collection. Tree #2.	6.4	1164.95	243.82	-
3	PT_3	Tashkent Botanical Garden collection. Tree #3.	3.4	1292.43	546.23	0.78-
4	PT_4	Tashkent Botanical Garden collection. Tree #4.	2.1	1293.88	533.23	0.54
In vitro regenerated plant material (leaves)						
5	PT_R1	Regenerated plant #1.	2.1	-	-	-
6	PT_R2	Regenerated plant #2.	3.4	7654.00	6657.19	-
7	PT_R3	Regenerated plant #3.	5.4	3121.07	8843.65	-
8	PT_R4	Regenerated plant #4.	2.4	2132.05	3214.04	-
Callus						
9	PT_Cl	MS 2.4-D 0.5 mgl^{-1} +BAP 0.5 mgl^{-1}	12.0	544.09	20.15	0.11
10	PT_C2	MS 2.4-D 0.5 mgl^{-1} + Kin 0.5 mgl^{-1}	8.6	643.16	55.38	1.12
11	PT_C3	MS 2.4-D 1.0 mgl^{-1} +Kin 1.0 mgl^{-1}	7.6	567.75	78.53	0.05
12	PT_C4	MS 2.4-D 0.5 mgl^{-1} +BAP 0.5 mgl^{-1}	10.7	876.51	43.33	1.04
13	PT_C5	MS 2.4-D 0.5 mgl^{-1} + Kin 0.5 mgl^{-1}	8.7	675.57	54.91	0.43
14	PT_C6	MS IAA 0.5 mgl^{-1}	5.4	848.59	31.84	1.07
15	PT_C7	MS 2.4-D 0.5 mgl^{-1} +BAP 0.5 mgl^{-1}	7.8	946.19	48.35	0.56

Table 5 Overall comparison of kokusaginine content in different sources of *Ptelea trifoliata*

Source	Range of kokusaginine content (% DW)	Highest content (%)	Key observation
Botanical Garden	0.54–0.78	0.78 (PT_3)	Moderate levels; variability observed between trees.
Callus Cultures	0.11–1.12	1.12 (PT_C2)	Highest kokusaginine production; hormone composition significantly influences yield.
In vitro regenerated plants	Not detected	-	No kokusaginine accumulation in regenerated plants.

The antioxidant activity of PT samples, measured using DPPH (IC_{50}, μgl^{-1}) and ABTS (IC_{50}, μgl^{-1}) assays, varied significantly across botanical garden samples, *in vitro* regenerated plants, and callus cultures (Table 6).

Table 6 Antioxidant activity of the studied material of *Ptelea trifoliata* according to DPPH and ABTS methods

Methods	Strongest Antioxidant Activity	Weakest Antioxidant Activity
DPPH	Callus sample PT_Cl (IC_{50} = 544.09 μgl^{-1}).	Regenerated plant PT_R2 (IC_{50} = 7654.0 μgl^{-1}).
ABTS	Regenerated plant PT_R3 (IC_{50} = 8843.65 μgl^{-1}).	Callus sample PT_Cl (IC_{50} = 20.15 μgl^{-1}).

DPPH activity (IC_{50}, μgl^{-1}) analyses revealed that callus cultures exhibited the strongest antioxidant activity. PT_C1 had the lowest IC_{50} value (544.09 μgl^{-1}), followed by PT_C3 (567.75 μgl^{-1}) and PT_C2 (643.16 μgl^{-1}).

This strong activity indicates that controlled growth conditions and hormonal treatments enhance secondary metabolite production. Botanical garden samples demonstrated moderate DPPH activity, ranging from 1164.95 μgl^{-1} (PT_2) to 1387.77 μgl^{-1} (PT_1). *In vitro* regenerated plants had the weakest DPPH activity, with PT_R2 (7654.00 μgl^{-1}) and PT_R3 (3121.07 μgl^{-1}) showing poor performance.

ABTS activity (IC_{50}, μgl^{-1}) showed different trends. *In vitro* regenerated plants exhibited the highest ABTS activity, particularly PT_R3 (8843.65 μgl^{-1}) and PT_R2 (6657.19 μgl^{-1}). Botanical garden samples showed moderate ABTS activity, ranging from 243.82 μgl^{-1} (PT_2) to 546.23 μgl^{-1} (PT_3). Callus cultures demonstrated much lower ABTS activity, with PT_C1 (20.15 μgl^{-1}) being the weakest and PT_C3 (78.53 μgl^{-1}) the strongest among callus samples.

These findings suggest that callus cultures primarily produce hydrophilic antioxidants, as demonstrated by their superior DPPH activity, while *in vitro* regenerated plants are more effective in producing compounds with high ABTS activity, possibly due to stress-induced metabolic changes. Botanical garden samples exhibited balanced antioxidant properties but were less potent compared to optimized callus cultures and regenerated plants.

Callus cultures are the most promising source for hydrophilic antioxidants, while *in vitro* regenerated plants hold potential for lipophilic antioxidant production. Botanical garden samples provide moderate antioxidant activity and kokusaginine levels suitable for natural collection. Further optimization of growth conditions, hormonal treatments, and stress-inducing protocols in callus cultures and regenerated plants could significantly enhance kokusaginine biosynthesis and antioxidant potential.

CONCLUSION

Biological active compounds can be extracted from naturally grown plants, but their industrial production is confined by environmental, climatic, seasonable, anthropogenic and many other factors. The plants take several years to grow and reach the point for desired compounds. An alternate method to surpass such situation is to use plant tissue culture techniques for the production of biological active compounds efficiently within a short time for commercial use. It approvals the bulk propagation of plants in controlled environmental conditions without any seasonal constraints. The results of this research showed that *in vitro* callus could serve as the source of the biologically active compounds which can be used in production of the substances for use in pharmaceutics and medicine thereby reducing the anthropogenic pressing on natural populations of this valuable plant species.

Significant variation in galanthamine content and antioxidant activity across different plant materials, cultivation conditions, and media types was demonstrated in this study. Callus cultures proved to be the most promising source of galanthamine, with the highest observed levels (up to 5.10% DW) achieved using Vch medium supplemented with 2.4-D and kinetin. *In vitro*-regenerated leaves also showed potential as a secondary source of galanthamine, while naturally collected materials and botanical garden specimens exhibited lower levels. Seeds, with moderate galanthamine content, offer potential as an auxiliary source for extraction.

Antioxidant activities, measured using DPPH and ABTS assays, revealed distinct metabolic profiles across samples. Callus cultures and seeds demonstrated strong DPPH activity, indicating a high presence of hydrophilic antioxidants, whereas seeds exhibited the highest ABTS activity due to their reserves of both hydrophilic and lipophilic antioxidant compounds. The optimized use of Vch medium consistently enhanced antioxidant activity and extract yields compared to MS medium, highlighting the influence of nutrient composition and hormone combinations on secondary metabolite production.

The findings underscore the importance of controlled cultivation conditions for maximizing the production of galanthamine and antioxidant compounds, with potential applications in pharmaceutical and nutraceutical development. To fully harness the biotechnological potential of these plant materials, further research is recommended, focusing on stress induction, detailed compound characterization, and the optimization of media formulations.

Significant variations in kokusaginine content and antioxidant activity across botanical garden samples, callus cultures, and *in vitro* regenerated plants were demonstrated in this research. Callus cultures demonstrated the highest kokusaginine production, with optimized MS media containing specific hormone combinations, such as 2.4-D and kinetin, significantly enhancing biosynthesis. These findings emphasize the potential of controlled conditions in maximizing secondary metabolite production.

Botanical garden samples exhibited moderate kokusaginine levels, indicating natural biosynthesis but limited by environmental factors. In contrast, *in vitro* regenerated plants failed to produce detectable kokusaginine, suggesting that specific biosynthetic pathways are suppressed or require environmental triggers absent during regeneration.

Antioxidant activity analyses revealed that callus cultures excelled in DPPH activity, indicating their strong hydrophilic antioxidant potential. *In vitro* regenerated plants demonstrated the highest ABTS activity, likely due to the stress-induced production of lipophilic antioxidants. Botanical garden samples provided balanced antioxidant properties but were less potent compared to callus cultures and regenerated plants.

Overall, callus cultures emerge as the most promising source for kokusaginine and hydrophilic antioxidants, while *in vitro* regenerated plants show potential for producing lipophilic antioxidants. Botanical garden samples offer moderate levels of both, suitable for natural collection.

Future studies should focus on optimizing hormonal treatments, stress induction, and environmental factors to enhance galanthamine and kokusaginine biosynthesis and antioxidant activity in all sample types, particularly in callus. These advancements could support biotechnological applications in pharmaceutical and nutraceutical industries [67, 68].

Author Contributions

Conceptualization, F.U.M. and S.K.A., Investigation, H.K.k.J, M.A.Sh, Formal Analysis, H.K.k,J., A.T.o.H., A.J.J., V.U.A., Supervision, H.-J.K., F.U.M., Funding Acquisition, C.-S.N., F.U.M., Investigation, M.-S.L. and Y.-J.O., M.F.U., Project Administration, N.K.A., S.K.A., and J.T.L. All authors have read and agreed to the published version of the manuscript.

Funding

Research and publication of the manuscript were supported by (1) “Eurasian Seed Conservation Network” project of the Baekdudaegan National Arboretum, Republic of Korea, (2) the project A-FA-2021-146 “The development of the technology of organization and propagation of medicinal plant species with the use of *in vitro* methods” of the Institute of Botany of the Academy of Sciences of the Republic of Uzbekistan, and (3) the state program “Developing of the scientific basis of the sustainable *in vitro* propagation of valuable samples of the collection of Botanical Garden” of the Tashkent Botanical Garden named after acad. F.N. Rusanov of the Academy of Sciences of the Republic of Uzbekistan.

Acknowledgments

The research was conducted with the cooperation between Baekdudaegan National Arboretum, Republic of Korea, and Tashkent Botanical Garden named after academician F.N. Rusanov of the Institute of Botany of the Academy of Sciences, Republic of Uzbekistan.

Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

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Abbreviations

2.4-D - 2.4-Dichlorophenoxyacetic acid
ABTS – 2,2-Azinobis (3-ethylbenzothiazoline)-6-sulphonic acid
atm. – atmosphere, air pressure measurement at sea level at a temperature of 15°C.
B5 – nutrient media by Gamborg & al. (1968)
BAC – Biologically active compounds
BAP - 6-Benzylaminopurine
DPPH – 2,2-diphenyl-1-picrylhydryl
DW – Dry weight
IAA - Indole-3-acetic acid
IBA- indole 3-butyric acid
Kin - Kinetin
l - litr
masl – meters above sea level
mg – milligram
mM - millimol
MS - Murashige & Skoog, nutrient media by Murashige & Skoog (1962)
N6 – nutrient media by Chu & al. (1975)
NAA - α-Naphthaleneacetic acid
nm – nanomol
Vch –nutrient medium by Vollosovich (1979)
WPM - Woody Plant Medium, nutrient media by Lloyd G. and McCown (1980)