N E W S
OF THE NATIONAL ACADEMY OF SCIENCES OF THE REPUBLIC OF KAZAKHSTAN
SERIES CHEMISTRY AND TECHNOLOGY
ISSN 2224-5286 **https://doi.org/10.32014/2020.2518-1491.60**
Volume 4, Number 442 (2020), 22 – 29

UDC 577.1;547.91

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BIOSYNTHESIS AND DYNAMICS OF ACCUMULATION OF SESQUITERPENE LACTONES IN *ARTEMISIA GLABELLA* KAR. ET KIR.

Abstract. The study of biosynthetic processes in plant cells and methods for their regulation is an important aspect in the development of biotechnological methods for the production of valuable medicinal compounds. The identification and establishment of enzyme structures involved in the formation of secondary metabolites in plants by using molecular genetic methods is one of the relevant ways in the study of their biosynthesis. For a successful search, it is necessary to use plant organs with their quantitative accumulation.

In this work for the first time, a quantitative assessment of the content of sesquiterpene lactones arglabin and argolide in CO_2-extracts of *Artemisia glabella* Kar. et Kir. individual organs were performed at different stages of the growing period (start of regrowth, end of regrowth, budding, flowering, fruiting); using electron scanning microscopy, the leaves surface morphology was determined. It was found that the quantitative accumulation of arglabin is observed during budding stage and its amounts are 1.90% in leaves and 1.56% in buds. On the surface of leaves *Artemisia glabella* Kar. et Kir., there are capitate glandular trichomes of an oval shape, the sizes of which vary between 70-80 microns in length and 33-38 microns in width.

Based on the obtained experimental data, we assume that the leaves and buds of intact plant *Artemisia glabella* Kar. et Kir. during the budding stage will be the optimal samples to search for genes involved in the biosynthesis of sesquiterpene lactones.

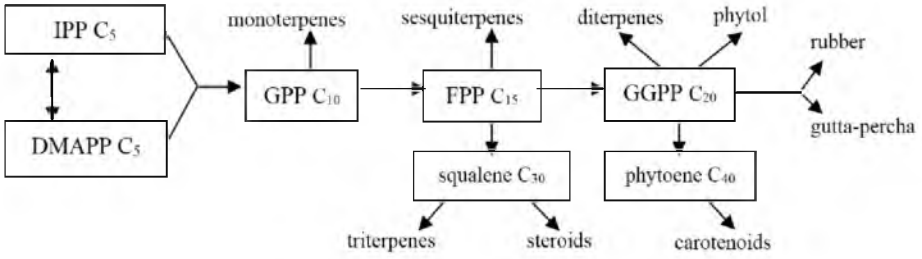
Key words: sesquiterpene lactones, arglabin, biosynthesis, trichomes, supercritical fluid extraction, *Asteraceae*, *Artemisia glabella* Kar. et Kir.

Introduction. The study of biosynthetic processes of plant cells and methods of their regulation is one of the current trends in biological chemistry. The biosynthesis of isoprenoids, a very large group of natural compounds that are diverse in structure and function, has been studied by many foreign researchers [1-15]. It was established that the biosynthetic pathway of all isoprenoids begins with two branched phosphorylated precursors – isopentenyl pyrophosphate and dimethylallyl pyrophosphate (figure 1), which are formed in living organisms along one of two pathways [1]:

1) *mevalonate pathway (classic, Bloch-Lynen pathway, MVA-pathway)* - from mevalonic acid, occurring under the action of enzymes located in cytosol;

2) *non-mevalonate pathway (alternative, Rohmer pathway, MEP- pathway*) - from methylerythritol phosphate occurring under the action of enzymes localized in plastids.

Under the action of isopentenyl pyrophosphate isomerase enzyme, a double bond shift occurs in isopentenyl pyrophosphate (IPP) and an isomeric compound, dimethylallyl pyrophosphate (DMAPP), is formed. Further formation of terpenes occurs by attaching IPP residues to a DMAPP molecule to produce geranyl pyrophosphate or to a growing chain of isoprenoid with the formation of farnesyl pyrophosphate, etc. (figure 1).



IPP – isopentenyl pyrophosphate; DMAPP – dimethylallyl pyrophosphate;
GPP – geranyl pyrophosphate; FPP – farnesyl pyrophosphate; GGPP – geranylgeranyl pyrophosphate

Figure 1 – The main pathways of terpenoids formation

It should be noted that the cytoplasmic (MVA) and chloroplast (MEP) pathways differ only in the early stages: since the formation of the C_5-unit of IPPP, there are no differences in the stages of biosynthesis [2, 3]. In addition, higher plants and some microorganisms are characterized by the use of both pathways of isoprenoid biosynthesis, and although both pathways in the plant cell function simultaneously, they are physically isolated from each other [1].

To date, the biosynthetic pathways of the formation of individual sesquiterpene lactones in a number of plants of the *Asteraceae* family have been studied (costunolide in *Cichorium intybus* L. [4], *Helianthus annuus* L. и *Lactuca sativa* L. [5]; parthenolide in *Tanacetum parthenium* (L.) Sch. Bip. [6]; artemisinin in *Artemisia annua* L. [7, 8, 9] and in some others). The key to determining biosynthetic genes in many studies has been the identification of plant organs in which quantitative accumulation of sesquiterpene lactones occurs [9, 10]. On the surface of most of them, there are exogenous specialized terpenoid-containing secretory structures - glandular trichomes [11], which show the highest concentration of sesquiterpene lactones and quantitative content of TpGAS synthase (a gene encoding the first stage of germacranolide biosynthesis) [8, 9, 12]. Their presence and morphological diversity for a number of plants *Asteraceae* family are confirmed by electronic scanning microscopy [13, 14, 15]. It was glandular trichomes that were used in many studies to isolate RNA and its sequencing in order to determine the nucleotide sequences of genes encoding the structures of terpenoid biosynthesis enzymes in plants.

The aim of this work was the quantitive evaluation of the content of sesquiterpene lactones in individual organs of *Artemisia glabella* Kar. et Kir. at different stages of the growing period and determining the size of glandular trichomes on their surface in order to select optimal samples for the search for genes involved in biosynthesis of arglabin.

Materials and methods of the research. The object of research in this work was the plant of the flora of Kazakhstan *Artemisia glabella* Kar. et Kir. of *Asteraceae* family. Aerial parts of *Artemisia glabella* Kar. et Kir. were collected in the Karkaraly district of the Karaganda region in accordance with the phenospectrum for the main stages of growing season of the plant: start of regrowth – 01.06.2018; end of regrowth – 22.06.2018; budding – 18.07.2018; flowering – 15.08.2018; fruiting – 18.09.2018). Plant samples were placed in the herbarium fund of the laboratory of botany and biotechnology of JSC "IRPH "Phytochemistry".

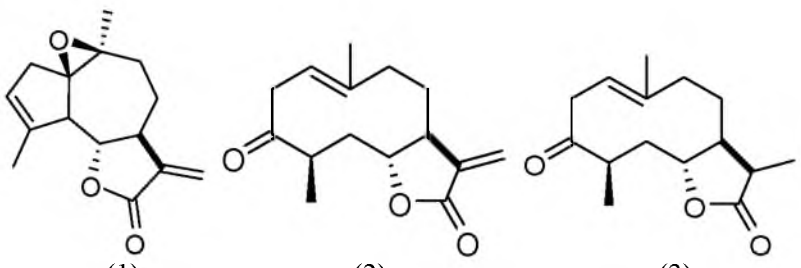
The collected raw materials were divided into individual organs (stems, leaves were separated and, depending on the developmental stage, buds/flowers/fruits), also whole shoots were left for study. All plant sampes were dried by the air-shadow method.

The extraction of secondary metabolites from the aerial part and individual organs of the plant was carried out on a supercritical fluid extraction unit USFE-5/2 [16] with the following parameters (for all types of raw materials): pressure 16 MPa; temperature 60^0C; duration 180 minutes. For each extraction, 100 g of raw material collected in 2018 during the corresponding growing period was used.

The quantitative content of components in the CO_2 extracts was determined by high-pressure reversed-phase HPLC with a UV-detector on a Hewlett Packard Agilent 1100 Series chromatograph in isocratic mode. Analysis conditions: analytical column 4.6×150 mm, Zorbax SB-C18 sorbent with a particle size of 5 μm, column temperature - room temperature, mobile phase acetonitrile:water mixture (1:1), mobile phase velocity 0.5 ml/min, injection load 20 μl, detection at a wavelength of 204 nm. Data was calculated using ChemStation software.

The study of surface morphology of individual plant organs was carried out using optical microscopy methods. MBR-1A biological microscope was used to inspect the surface. Scanning electronic microscope of high-spatial resolution Mira 3 LMU Tescan was used to detect the size of terpenoid-containing structures. Air-dried samples of *Artemisia glabella* Kar. et Kir. leaves with a sputtering of 25 nm thick gold were prepared for analysis.

Results and discussion. Among secondary metabolites of *Artemisia glabella* Kar. et Kir., there are sesquiterpene lactones arglabin (1) and argolide (2) - biologically active compounds with a proven pharmacological effect and promising for chemical modifications, as well as dihydroargolide (3).



(1) (2) (3)

The major component of the *Artemisia glabella* extract is sesquiterpene lactone arglabin - 1(10)-epoxy-5,7α,6β(H)-guaia-3(4),11(13)-dien-12,6-olide, the average content of which is 1.49% for air-dry raw materials, the rate of recovery by supercritical fluid extraction reaches 92.4% [17]. It has an inhibitory effect on a number of transplantable tumors (Pliss's lymphosarcoma, Walker's carcinosarcoma, RMC-1 breast cancer, alveolar liver cancer, P-388 and D-1120 leukemia), as well as on tumor strains resistant to standard chemotherapeutic drugs (fluorouracil, sarcolysin, prospidin, rubomycin), is used as an immunomodulating agent [18]. There was also revealed its hypolipidemic effect in hepatoma cells [19]. The water-soluble derivative of dimethylaminoarglabin hydrochloride [18] is the active substance in the unique antitumor drug "Arglabin", which, according to a number of characteristics, has no analogues in the world, has passed clinical studies and is used in antitumour therapy. Arglabin is promising for chemical modification; more than 70 derivatives are obtained on its basis [20].

Microscopic examination of the surface of *Artemisia glabella* Kar. et Kir. organs allowed us to establish that the epidermal glandular structures (trichomes) of the aerial parts *Artemisia glabella* (figure 2) are represented by two forms: glandular hairs and capitate glandular trihomes (both are multicellular). On both sides of the leaves and on the lower part of flower *Artemisia glabella*, glandular trichomes predominate (Figure 2c, 2d), which have an oval in shape. The size of glandular trichomes on the leaves surface varies between 70-80 microns in length and 33-38 microns in width (figure 3).

a b c d e

a – anthodium of *Artemisia glabella*; b – flowers from anthodium;
c – lower part of the flower; d – leaves on the stem; e – leaf (view from above)

Figure 2 – The presence of terpenoid-containing structures
on the surface of aerial parts *Artemisia glabella* Kar. et Kir.

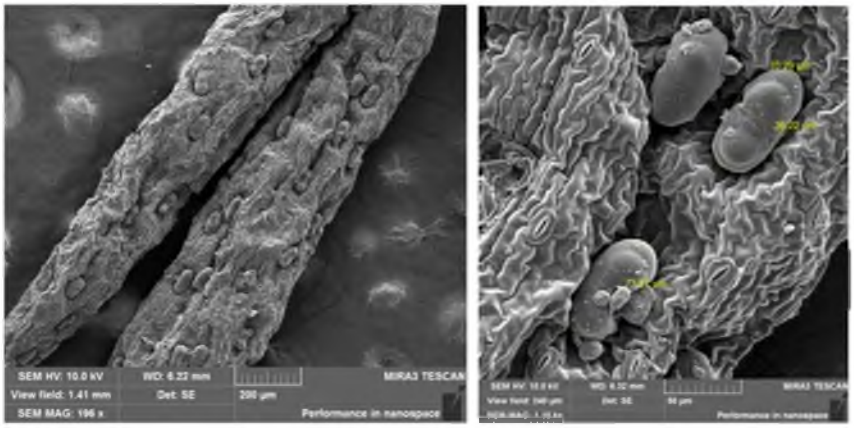


Figure 3 – Size of glandular trichomes on the surface of *Artemisia glabella* Kar. et Kir. leaves

To study the dynamics of accumulation of sesquiterpene lactones in individual organs of *Artemisia glabella* Kar. et Kir. a series of CO_2-extraction of raw materials collected at different stages of the growing period was carried out. The results of chromatographic analysis of the obtained CO_2-extracts are presented in table 1.

Table 1 – Results of HPLC analysis of the quantitative content of arglabin and argolide in CO_2-extracts of *Artemisia glabella*

№	Type of plant raw material	Extract mass	Content, % per extract		Content, % on air-dry materials	
			arglabin	argolide	arglabin	argolide
1	2	3	4	5	6	7
I	*SR – start of regrowth (raw material collection 01.06.2018)*					
1	whole shoots	7,05	15,98	1,95	1,12	0,14
II	*ER – end of regrowth (raw material collection 22.06.2018)*					
2	whole shoots	5,71	13,75	3,86	0,78	0,22
3	shredded stems	1,85	11,74	2,91	0,21	0,05
4	leaves	9,58	13,13	3,71	1,26	0,35
III	*B - budding (raw material collection 18.07.2018)*					
5	whole shoots	7,15	20,76	5,56	1,50	0,40
6	shredded stems	2,90	13,91	2,91	0,40	0,08
7	leaves	7,44	25,56	6,83	1,90	0,51
8	buds	8,28	18,84	4,65	1,56	0,39
IV	*FL – flowering (raw material collection 15.08.2018)*					
9	whole shoots	6,25	8,73	2,09	0,55	0,13
10	shredded stems	2,50	7,83	1,69	0,20	0,04
11	leaves	5,70	8,12	1,72	0,46	0,10
12	inflorescences	6,60	10,25	2,32	0,68	0,15
13	young whole shoots (regrowth)	8,18	9,37	1,93	0,77	0,16
V	*FR– fruiting (raw material collection 18.09.2018)*					
14	whole shoots	3,57	11,12	2,31	0,39	0,08
15	shredded stems	1,66	8,04	1,26	0,13	0,02
16	leaves	7,35	15,43	3,29	1,13	0,24
17	multiple fruit + leaves	7,00	12,86	3,15	0,90	0,22
18	young whole shoots (regrowth)	7,40	15,96	3,41	1,18	0,25

At the *start of regrowth* stage, the yield of CO_2-extract from whole shoots (leaves and stems) is 7.05% on air-dry materials with an average concentration of arglabin of 1.12% on air-dry materials.

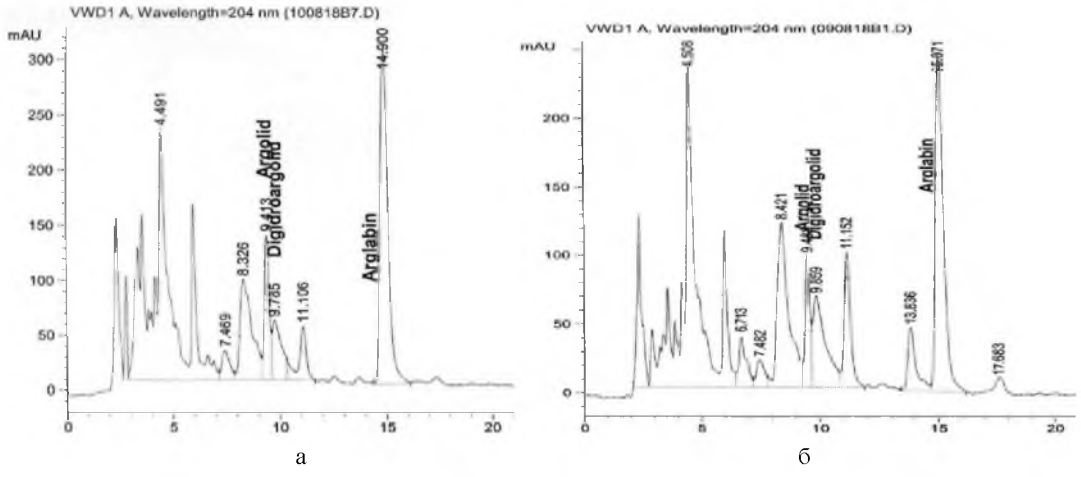
At the *end of regrowth* stage, there is a slight decrease in the yield of extract to 5.70% on air-dry material, which is most likely due to the relative increase in the proportion of stems in the total weight of the raw material, the extract yield of which (as can be seen from the data in Table 1) is insignificant and is 1.85% on air-dry material. Also, in this phase, an increase in argolide concentration is observed by almost 2 times (up to 3.86 in the extract and 0.22 on air-dry materials) with a slight decrease in the concentration of arglabin (from 1.12% to 0.78%).

A quantitative yield of CO_2-extract is observed during *budding* stage (on average, within 7.15% on air-dry material). During this stage, the highest concentration of arglabin and argolide in the obtained extracts was noted. Thus, the highest content of arglabin was recorded in the leaves (25.56% in the extract and 1.90% on air-dry material), it is slightly lower in buds (18.84% in the extract and 1.56% on air-dry material). This fact is completely correlated with the presence on the surface of precisely these *Artemisia glabella* organs of capitate glandular trichomes - structures where the formation and accumulation of terpenoid nature substances occur.

The chromatogram of the CO_2-extract of raw material *Artemisia glabella* collected during budding stage shows the presence of three additional peaks of components with retention times of 6.7139, 13.836 and 17.683 min, which form and accumulate in buds (figure 4b).

During the *flowering* stage, the yield of the extract decreases to 6.25% on air-dry material, there is an almost three-fold decrease in the concentration of arglabin and argolide (up to 0.55% and 0.13% on air-dry material respectively).

For the *fruiting* stage, the extract yield is minimal (3.57% on air-dry material) and the content of sesquiterpene lactones arglabin and argolide in whole shoots is 0.39 and 0.08% on air-dry material.



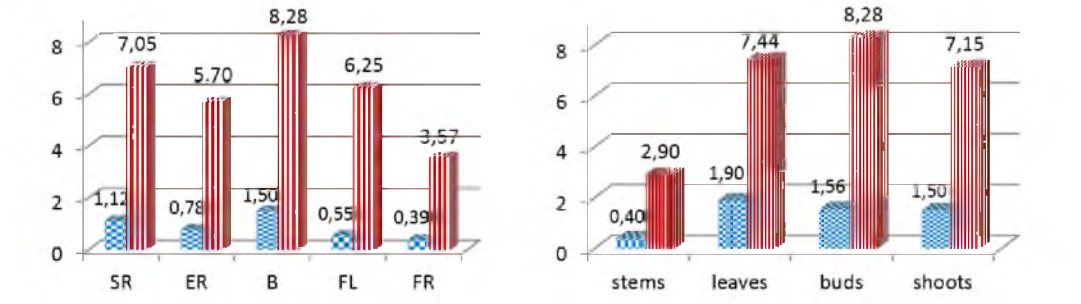
а б

a – leaves extract; b – buds extract

Figure 4 – Chromatograms of the component composition of the CO_2-extract of individual organs of *Artemisia glabella* during budding stage

From the data in table 1 it is also seen that the biosynthesis of arglabin continues in young whole shoots even with the repeat regrowth, which is observed in the 2-3rd decade of August. Moreover, the content of arglabin in them remains quite high (1.18% on air-dry material during the fruiting stage).

Comparative data on the content of target component (arglabin) for different stages of the growing period and in individual organs at the budding stage are presented on diagrams (figure 5).



a b

a – in whole shoots along the stages of growing period; b - in individual organs in the budding stage; blue chessboard pattern - yield of extract, % on air-dry material; red stripe pattern - arglabin content, % on air-dry material

Figure 5 – Arglabin content and CO_2-extract yield on dried raw materials of *Artemisia glabella* Kar. et Kir.

The results of an experiment on CO_2-extraction of secondary metabolites from raw materials of *Artemisia glabella* correlate with previously obtained by chloroform extraction data on the quantitative accumulation of sesquiterpene lactones precisely at the budding stage in leaves and buds [21, p. 79, 89]. It should be noted that the percentage content of arglabin in the *Artemisia glabella* population growing in the natural habitat (Karkaraly district) is almost 3 times higher. However, this difference can also be associated with the use of various methods for its extraction from plant materials (chloroform and supercritical fluid extraction).

Conclusion. As a result of the studies, it was found that the presence of sesquiterpene lactone arglabin is observed in all individual organs of *Artemisia glabella* Kar. et Kir. and during all stages of the growing period. Terpenoid-containing organs are leaves and buds, in which its quantitative accumulation during budding stage is noted (within 1.56–1.90% according to HPLC analysis of CO_2 extracts). On the surface of these organs there are specialized terpenoid-containing structures - glandular trichomes, in which the biosynthesis and accumulation of sesquiterpene lactones and other terpenoids under the action of sesquiterpene synthases occur. According to electronic scanning microscopy data, the glandular trichomes on the surface of *Artemisia glabella* Kar. et Kir. leaves have an oval shape, their sizes vary between 70-80 microns in length and 33-38 microns in width. Thus, we assume that the leaves and buds of an intact plant are optimal samples to search for genes involved in the biosynthesis of sesquiterpene lactones.

Acknowledgments

The research work has been carried out in the frame of project AP05134198 granted by Ministry of Education and Science of the Republic of Kazakhstan.

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ARTEMISIA GLABELLA KAR. ET KIR. ӨСІМДІГІНДЕГІ СЕСКВИТЕРПЕНДІ ЛАКТОНДАРДЫҢ ЖИНАҚТАЛУ ДИНАМИКАСЫ ЖӘНЕ БИОСИНТЕЗІ

Аннотация. Жұмыстың мақсаты – арглабин биосинтезіне қатысатын гендерді іздеу үшін оңтайлы үлгілерді таңдау мақсатында вегетациялық кезеңнің түрлі сатысында *Artemisia glabella* Kar. et Kir. өсімдігінің жекелеген мүшелеріндегі сесквитерпенді лактондардың мөлшерін сандық бағалау және олардың бетіндегі безді трихомалардың көлемін анықтау.

Бұл жұмыста алғаш рет вегетациялық кезеңнің түрлі сатысында (өсу үдерісінің басы, аягы, бүрлеу, гүлдеу, жеміс беру) *Artemisia glabella* Kar. et Kir. өсімдігінің жекелеген мүше CO_2-сығындысындағы арглабин мен арголид сесквитерпенді лактондарының мөлшеріне сандық бағалау жүргізілді; электронды сканерлеу микроскопия әдісімен жапырақ бетінің морфологиясы анықталды.

Зерттеу нәтижесінде арглабин сесквитерпенді лактоны *Artemisia glabella* Kar. et Kir. өсімдігінің барлық мүшесінде және вегетаңиялық кезеңнің барлық сатысында байқалады. Оның сандық жинақталуы бүрлеу кезеңінде айқындалды және жапырақта 1,90%, гүлшанақта 1,56% көрсетті. Арглабин биосинтезі тамыз айының 2-3-онкүндігінде байқалатын жас өскіннің қайта өсу үдерісінде де жалғасады. Бұл ретте олардың құрамындағы арглабиннің мөлшері салыстырмалы түрде жоғары болады (жеміс беру сатысында 1,18%).

Artemisia glabella Kar. et Kir. өсімдігінің жерүсті бөлігінде терпеноидты құрылымдардың екі түрі анықталды: безді түкше және бас тәрізді безді трихома. Тықыр жусан жапырақтарының екі жағында және гүлдің төменгі бөлігінде бас тәрізді безді трихомалар басым келеді. Электронды сканерлеу микроскопиясының деректері бойынша тықыр жусан жапырақтарының бетіндегі безді трихомалар сопақ пішінді болып келеді, өлшем ұзындыгы 70-80 мкм және ені 33-38 мкм шегінде өзгереді.

Алынған эксперименттік деректердің негізінде сесквитерпенді лактондардың биосинтезіне қатысатын гендерді іздеу үшін оңтайлы үлгілер ретінде бүрлен фазасындағы *Artemisia glabella* Kar. et Kir. интактілі өсімдігінің жапырагы мен гүлшанағы таңдалды.

Түйін сөздер: *Asteraceae*, *Artemisia glabella* Kar. et Kir., сесквитерпенді лактондар, арглабин, биосинтез, трихомалар, жоғары критикалық флюидті экстракция.

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БИОСИНТЕЗ И ДИНАМИКА НАКОПЛЕНИЯ СЕСКВИТЕРПЕНОВЫХ ЛАКТОНОВ В *ARTEMISIA GLABELLA* KAR. ET KIR.

Аннотация. Цель работы – количественная оценка содержания сесквитерпеновых лактонов в отдельных органах *Artemisia glabella* Kar. et Kir. на разных стадиях вегетационного периода и определение размеров железистых трихом на их поверхности при выборе оптимальных органов растения для поиска генов, участвующих в биосинтезе арглабина.

Впервые проведена количественная оценка содержания сесквитерпеновых лактонов арглабина и арголида в CO_2-экстрактах отдельных органов *Artemisia glabella* Kar. et Kir. на разных стадиях вегетационного периода (начало отрастания, конец отрастания, бутонизация, цветение, плодоношение). Методом электронной сканирующей микроскопии определена морфология поверхности листьев – одного из основных органов биосинтеза сесквитерпеновых лактонов.

Установлено, что присутствие сесквитерпенового лактона арглабина наблюдается во всех органах *Artemisia glabella* Kar. et Kir. и на всех стадиях вегетационного периода. Количественное его накопление отмечается в период бутонизации и составляет 1,90% в листьях и 1,56% в бутонах. Биосинтез арглабина продолжается и при повторном отрастании молодых побегов, которое наблюдается во 2-ой - 3-ей декадах августа. При этом содержание арглабина в них остается сравнительно высоким (1,18% в период плодоношения).

На поверхности листьев и бутонов *Artemisia glabella* Kar. et Kir. установлено наличие двух форм терпеноидсодержащих структур: железистых волосков и головчатых железистых трихом. На обеих сторонах листьев и нижней части цветка у полыни гладкой преобладают головчатые железистые трихомы. По данным электронной сканирующей микроскопии, железистые трихомы на поверхности листьев полыни гладкой имеют овальную форму, размеры варьируются в пределах 70-80 мкм в длину и 33-38 мкм в ширину.

На основании полученных экспериментальных данных в качестве оптимальных органов для поиска генов, участвующих в биосинтезе сесквитерпеновых лактонов, выбраны листья и бутоны интактного растения *Artemisia glabella* Kar. et Kir. в фазе бутонизации.

Ключевые слова: *Asteraceae*, *Artemisia glabella* Kar. et Kir., сесквитерпеновые лактоны, арглабин, биосинтез, трихомы, сверхкритическая флюидная экстракция.

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