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ISOLATED CULTURE OF *AJUGA GENEVENSIS* L. AS A POTENTIAL SOURCE OF BIOLOGICAL ACTIVE SUBSTANCES

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Abstract

Biochemical traits of isolated cultures of bugle-weed Genevan (*Ajuga genevensis*), which is widespread in Armenian flora and commonly used in folk medicine, were studied. High antibacterial activity of calli and their water and ethanol extracts against various gram-positive and gram-negative microorganisms, both pathogenic and non-pathogenic, was revealed. These extracts did not show cytotoxicity to the cell line of monocitar human leukemia (cell line L-41). Dependences of synthesis of secondary metabolites with antibacterial activity on the terms of cultivation during single passage, quantity of passages and hormonal composition of nutrient medium were established. Total quantity of soluble amino acids in isolated cultures was determined. The antioxidant properties of intact plants and obtained callus cultures were studied. The content of ecdysteroidal fraction and 20-hydroxyecdysone in intact plants and their isolated cultures was revealed by method of high performance liquid chromatography (HPLC).

Keywords: *Ajuga genevensis*, callus, isolated culture, antibacterial activity, antioxidant activity, ecdysteroids.

Introduction

Obtaining biological active substances from plants is one of the actual tasks of contemporary biotechnology and pharmacology. In particular, these preparations are very promising for treatment of many diseases due to their actually high therapeutic value without side effects. The latter are no exception in case of chemical preparations, which consist of non-natural substances [*Hammerman A. et al., 1983*]. During the investigation of different biological active substances the research is usually aimed at determination of phytoecdysteroids and plant-producers of these metabolites.

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Ecdysteroids are polyhydroxilated sterols, relating to terpenoids and structurally similar to the molting hormones of insects [*Plant Physiology 2007*; *Nosov A., 1998*; *Alekseeva L. et al, 1998*]. For the first time some of the substances of this family (ecdyson and 20-hydroxyecdysone) were discovered in silkworm about 50 years ago. It was found that these substances were responsible for moulting and metamorphose of arthropoda [*Volodin V. et al. 2007*]. Later similar substances were discovered in plants as well [*Volodin V., Kovler L., 1998*].

Until present, the physiological role of phytoecdysteroids in plants is not entirely understood. Some authors do not consider these substances as phytohormones because of their inability to

regulate directly the growth processes of plants. Being the most hydrophilic sterols, which are transported easily in actively proliferated plant tissues, they can create favorable growth conditions [*Phytoecdysteroids*, *2003; Plant Physiology 2007*], whereas the substances of ecdysteroidal nature could display high physiological activity in mammals and humans [*Hikino H. et al., 1972; Lafont R.. et al., 1988; Lafont R.., Dinan L., 2003; Volodin V. et al., 1998*]. They provide the influence on mineral, carbohydrate, lipid and protein metabolism [*Uchiyama M., Yoshida T., 1974; Syrov V. et al., 1975; Catalan R. et al., 1985; Kholodova Yu. et al., 1997*], show antioxidant [*Osinskaya L. et al., 1992; Kuzmenko A. et al., 1999*], antimicrobial [*Phytoecdysteroids*, *2003*] and wound-healing properties [*Darmogray V. et al., 1996*]. In medical practice ecdysteroid-containing preparations are used as restorative and stimulant remedies for treatment of cardiovascular, central nervous and reproductive system injuries, in cases of mental and physical tiredness and decrease of capacity for work, impotencies [*Darmogray V. et al., 1998; Medicine and BAAs, 2003; Phytoecdysteroids*, *2003*]. In the sport and military medicine ecdysteroid-based preparations could be used for adaptation and rising of the capacity for work under conditions of limiting factors [*Timofeev N., Ivanovski A., 1996; Chermnik N., 1998; Seyfulla R. et al., 1999*].

The discovery of phytoecdysteroids led to search of ecdysteroid-containing plants, including the *Ajuga genus* (bugle-weed). Many representatives of this genus are known to have high productivity of valuable secondary metabolites, which is also testified by the wide use of these plants in folk medicine. Due to content of ecdysteroids and some other secondary metabolites of terpenoidal nature, they have antifidant properties to the insects-phytophages and show considerable physiological activity towards mammalians and humans [*Alekseeva L. et al., 1998*]. Some species of mentioned genus, such as *A. decumbens, A. incise, A. iva, A. japonica, A. nipponensis, A. reptans, A. turkestanika* were studied in order to find out 20-hydroxyecdysone (20-E) [*Lafont R. et al., 1998*]. Some species of this genus were described to exist in different regions of Armenia. Among them is also *A. genevensis* [*Zolotnitskaya S., 1965; Sokolov P., 1974; Flora of Armenia, 1987*]. From the medical point of view, these species have a valuable role. Thus, *A. genevensis* contains iridoids, flavonoids, glycosides, terpenoids, tannins, sterols, and exhibits anti-inflammatory, homeostatical and wound-healing features. However, in available literature we found no evidence concerning the research carried out for revealing ecdysteroids in bugle-weed Genevan and, moreover, in the isolated culture obtained from it.

As a rule, the processes of secondary substances formation in cultures *in vitro* very often differ from those in intact plants, whereas data necessary for the establishment of conformities when the synthesis of metabolites occurs in isolated cell cultures are contradictory [*Nosov A., 1994; Kunakh V., 1999*]. During the cultivation, changes of qualitative and quantitative composition of active metabolites may occur. The composition of biologically active substances also changes even during one growing cycle. Usually the intensification of the secondary metabolites synthesis under conditions *in vitro* is observed at the end of growth process when cell proliferation reduces or ceases [*Kunakh V., 1999*]. However, the study on growth regularity as well as metabolic activity of isolated culture is necessary, because there may be possible exceptions.

Methods

The plants *A. genevensis* were collected from slopes of Aragats mountain during the flowering phase. Leaf origin callus cultures were obtained using the well-known method in Murashige-Skoog nutrient medium (MS). Further callus tissue stable growth was supported in both MS medium, and MS medium modification, which

differs by the composition of phytohormones and conditionally was marked as N7. Unlike MS medium, the modified version contained no indolyl acetic acid (IAA), glycine, but contained hibberellic acid (0.2 *mg/l*), α-naphtyl acetic acid (NAA) (0.5 *mg/l*), 6-benzylaminopourine (1 *mg/l*) and kinetine, the concentration of which achieved 1 *mg/l*.

In order to induce organogenesis the calli were transferred to the medium with 0.1 *mg/l* NAA and 2 % sacharose. Root origination process of formed shoots took place in medium without cytokinin, and the concentration of NAA remained unchanged.

The antibacterial activity of calli, mericlones and intact plants was determined by dropping their extracts on the test-microorganisms lawn and measuring "formed" diameters of growth absence zone around the wells containing extracts. For extracts preparation 1 *g* tissue was extracted with 10 *ml* liquid (ethanol, methanol or water) during 20 *hours* on magnetic stirrer/shaker at 4°C. The obtained suspension was centrifuged during 10 *min* (5000 *r/min*), and then the supernatant was used. The experiments were repeated 6-fold. The statistical analysis of obtained data was carried out using SPSS program. The subspecies of microorganisms from the Republic Center of Depositing Microorganisms, as well as microorganisms from the Department of Microbiology and Biotechnology of Plants and Microorganisms of the Yerevan State University were used as test-organisms.

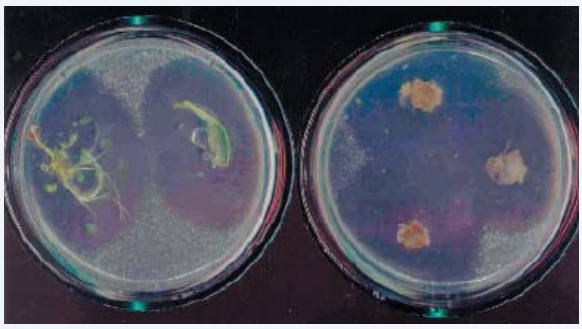

Figure 1. ***Zones of growth absence around the calli (a) and mericlones (b) of A. genevensis L. (test-organism: Bacillus subtilis).***

The citotoxicity was determined in monocytic human leukemia cell line (cell line L-41). The essence of this method is as follows: water extracts of intact plants and callus cultures of bugle-weed at concentration 100 *μl/ml* were added to the daily cell culture of leukemia. After the 24-hour incubation the number of living cells in 1 *ml* of suspension was counted with the use of vital staining by trypan blue [*Verpoorte R. et al., 1994*].

The antioxidant activity of both intact plants and obtained calli was determined by photochemoluminescent analyzer PHOTOCHEM (AnaliticJena Co., Germany). In order to determine the antioxidant activity of a studied extract the standard solution containing 10 *nM* ascorbic acid was used. Such analysis is used as a sensitive method for monitoring and detection of the inhibiting influence of investigated components on free radicals, which form as a result of photo-induced chemoluminescence of luminol [*Bajinyan S. et al., 2005*].

The amino acid composition in intact plants and callus cultures was revealed by amino acid analyzer (AAA T-339 M).

Analyses for revealing ecdysteroids and 20-E were carried out by HPLC method under the following conditions: chromatographic system ProStar (Varian, USA); eluent: acetonyl/water (100/20), the elution speed: 1.5 *ml/min*, wavelength: 242 *nm,* column: 150 x 4 *mm* Diasorb 130 C_{13}/T, size of particles: 7*μm* (BioChimMak, Russia, Moscow).

Results and discussions

Test results of obtained isolated cultures of *A. genevensis* L. showed high antibacterial activity. Large sterile zones formed around the calli and mericlones of *A. genevensis* are shown in the Figure 1, which evidence the high level of synthesis of substances with antibacterial features.

Testing of extracts obtained from calli and different parts of mericlones also revealed the high antibacterial activity of these cultures in relation to various gram-positive and gram-negative

Table 1.

Inhibition of test-organisms growing under the influence of extracts from calli of *A. genevensis*

Test-organisms	Passage number	Growth absence zone diameters (cm) of test-organisms			
		Days of calli growth			
		5	10	20	35-40
1. *Bacillus subtilis* 205	4	0.35 ± 0.005	1.6 ± 0.2	2.7 ± 0.14	3.4 ± 0.1
	18	0.6 ± 0.002	1.9 ± 0.14	4.1 ± 0.2	3.4 ± 0.07
	39	0.8 ± 0.14	2.4 ± 0.1	4.2 ± 0.14	3.45 ± 0.2
2. *B. subtilis* 1759	4	0.35 ± 0.07	1.6 ± 0.14	3.35 ± 0.07	2.9 ± 0.14
	18	0.45 ± 0.07	1.8 ± 0.1	3.8 ± 0.14	3.0 ± 0.2
	39	0.5 ± 0.14	2.5 ± 0.1	4.0 ± 0.14	3.1 ± 0.1
3. *B. mesentericus*	4	-	1.3 ± 0.2	3.3 ± 0.1	3.1 ± 0.14
	18	0.55 ± 0.2	1.5 ± 0.2	3.75 ± 0.07	3.55 ± 0.07
	39	0.85 ± 0.1	1.6 ± 0.14	4.0 ± 0.07	3.7 ± 0.13
4. *S. citreus*	4	-	0.85 ± 0.14	3.1 ± 0.14	2.4 ± 0.14
	18	-	1.45 ± 0.11	3.6 ± 0.1	2.55 ± 0.07
	39	0.55 ± 0.07	1.75 ± 0.07	4.2 ± 0.07	3.35 ± 0.07
5. *S. aureus* 209	4	-	2.3 ± 0.14	2.9 ± 0.14	2.45 ± 0.2
	18	0.4 ± 0.14	2.3 ± 0.1	2.9 ± 0.07	2.45 ± 0.07
	39	0.85 ± 0.14	2.4 ± 0.14	3.0 ± 0.1	2.7 ± 0.14
6. *Escherichia coli* 5009	4	-	-	2.1 ± 0.14	-
	18	-	1.35 ± 0.07	2.1 ± 0.1	-
	39	0.4 ± 0.14	1.85 ± 0.07	2.75 ± 0.07	-
7. *E. coli* 205	4	-	1.9 ± 0.14	2.9 ± 0.1	-
	18	-	2.1 ± 0.1	3.3 ± 0.14	-
	39	-	2.15±0.07	3.55 ± 0.06	-
8. *Salmonella typhimurium* 1474	4	-	-	1.0 ± 0.22	1.75 ± 0.07
	18	-	-	1.3 ± 0.3	1.95 ± 0.16
	39	-	-	2.2 ± 0.14	2.2 ± 0.1

$p < 0.0005$

microorganisms. It has been found out that synthesis of substances with antibacterial properties depend on different conditions: growing phases, passage number, and composition of nutrient medium. The dependence of synthesis of substance with antibacterial activity on growth phase and common age of culture is shown in Table 1, from which it is obvious that during one cycle of growing the peak of synthesis of investigated metabolites was registered on the 20th day of calli cultivation. This regularity preserved regardless (apart) from the passage. Synthesis of substances with antimicrobial activity increased with increase of the common age of a culture (quantity of passages): the greatest zones of growth absence were revealed around the calli of passages 39 – 40.

The fact that antimicrobial activity of investigated substances increased by the 35–40th day of cultivation might testify to possible disintegration and/or use of these substances from culture cells at the end of cultivation.

The most sensible microorganisms to the antimicrobial substances of calli culture were *B. subtilis*, *B. mesentericus*, *St. citreus* and *E. coli*, whereas these microorganisms showed lower sensibility to metabolites from intact plants.

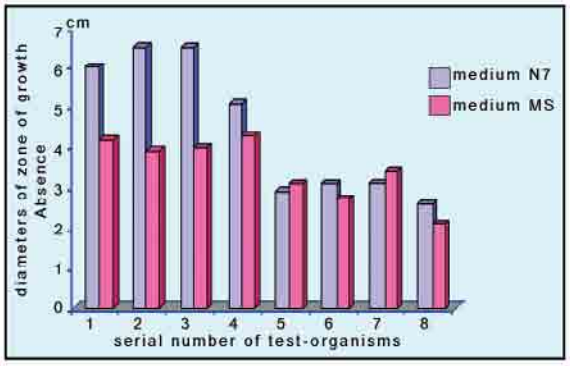

Figure 2. *Inhibition of the test microorganisms growth with the help of A. genevensis extracts of calli tissues of the 39th passage grown in MS and N7 nutrient media. P<0.001*
Note: the ordinal numbers of test organisms are shown in Table 1.

The value of P was below 0.0005 in comparison with the antibacterial activity of twenty-day callus cultures with five-day activity in relation to *B. subtilis* 205 and *B. subtilis* 1759 of all passages, as well as *B. mesentericus*, *S. citreus*, *S. aureus* 209 and *E. coli* 5009 of the 39th and/or 18th passages. In all other cases calli begin to display antibacterial activity only starting from the 10th day of cultivation.

The advantage of the use of cultures *in vitro* is the possibility of control, as well as the purposeful alterations of the metabolism. To increase the

Table 2.

Inhibition of pathogenic microorganisms growth under the influence of extracts from isolated cultures of *A. genevensis*

Test-organisms	Diameters of growth absence zone of test-organisms (cm)			
	Culture used			
	Overground parts of mericlones	Roots of mericlones	Calli cultures	Intact plants
Yersinia pestis	1.5 ± 0.1	3.75 ± 0.35	5.75 ± 0.35	---
Y. enterocolitica	4.1 ± 0.14	4.05 ± 0.8	---	---
Brucella abortus	---	---	3.25 ± 0.4	---
Bacillus anthracoides	1.1 ± 0.4	0.85 ± 0.2	3.3 ± 0.4	---

antimicrobial activity of the tissue, the influence of different combinations of phytohormones was tested. It was clarified that the cytokinin summary quantity increased as well as the auxin-type change in the nutrient medium N7 promoted the considerable increase in the synthesis of the substances with antimicrobial activity in tissues of isolated cultures of *A. genevensis* L. (Figure 2).

The antibacterial activity of isolated cultures of *A. genevensis* L. was revealed also in some pathogenic microorganisms. Thus, due to collaboration with the Center of Prophylaxis of Especially Dangerous Infections after S. Mkrtchyan (Ministry of Health, Republic of Armenia), it was clarified that pathogenic microorganisms like *Yersinia pestis*, *Y. enterocolitica*, *Brucella abortus* and *Bacillus anthracoides* showed high sensitivity to water extracts of the overground parts, roots of mericlones, as well as *A. genevensis* L. calli tissues (Table 2), while the extracts of intact plants showed no antimicrobial activity.

The antibacterial activity level of the extracts of isolated cultures also depended on the nature of extractant. Water extracts showed the maximal activity.

Thus, data obtained by us indicate a rather high antimicrobial action of water extracts from isolated cultures of *A. genevensis* in relation to different gram-positive and gram-negative pathogenic and non-pathogenic microorganisms.

In addition to high antibacterial activity, the culture of *A. genevensis* showed also high antioxidant activity. Thus, 10 *μl* extract of the intact plant showed antioxidant activity equal to that of 65.5 *nM* ascorbic acid. The same quantity of extract quantity of callus tissue of leaf origin showed antioxidant activity corresponding to 67.5 *nM* ascorbic acid, whereas the extract of callus culture of root origin showed the mentioned activity corresponding to 95.5 *nM* ascorbic acid. This property raises the value of callus cultures as potential producers of biologically active substances.

Table 3.

Analysis of ecdysteroidal fraction of *A. genevensis* L. plants

	Callus of leaf origin	Callus of root origin	Intact plant
Retention Time, *min*	2.250	-	-
	2.699	-	-
	-	2.727	2.728
	2.887	-	2.902
	-	-	3.185
	3.303	-	-
	3.667	-	-
	-	4.083	4.133
	4.459	-	-
	-	-	4,544
	-	4.948	4.935
	-	-	(5.5)20E
	6.107	**6.056**	**6.101**
	-	**7.530**	**7.586**
	7.849	-	-
	-	9.698	-
	-	-	9.866
	11.930	-	-
	-	**12.395**	**12.448**
	14.155	-	-
	16.030	-	-

Note: The values of intense peaks are given in extended Bold type.

The absence of cytotoxicity in extracts of *A. genevensis* plants as well as callus cultures obtained from them is established in relation to the human monocytar leukemia cell line (cell line 41).

In order to determine ecdysteroid contents in our cultures free amino acid content serving as a source of biologically active compounds' formation, was established. According to literature data as a source of acetyl CoA, which is the precursor of sterols and ecdysteroids synthesis, may be carbohydrates, fatty acids, and amino acids. Acetyl CoA is synthesized from alanine, serine, cystine, or cysteine via piruvic acid [*Alieva M. et al., 2002*].

In our experiments, alanine content in callus tissue of leaf origin exceeded its content in intact plant almost two times and was 0.217 *mg/g* of dry

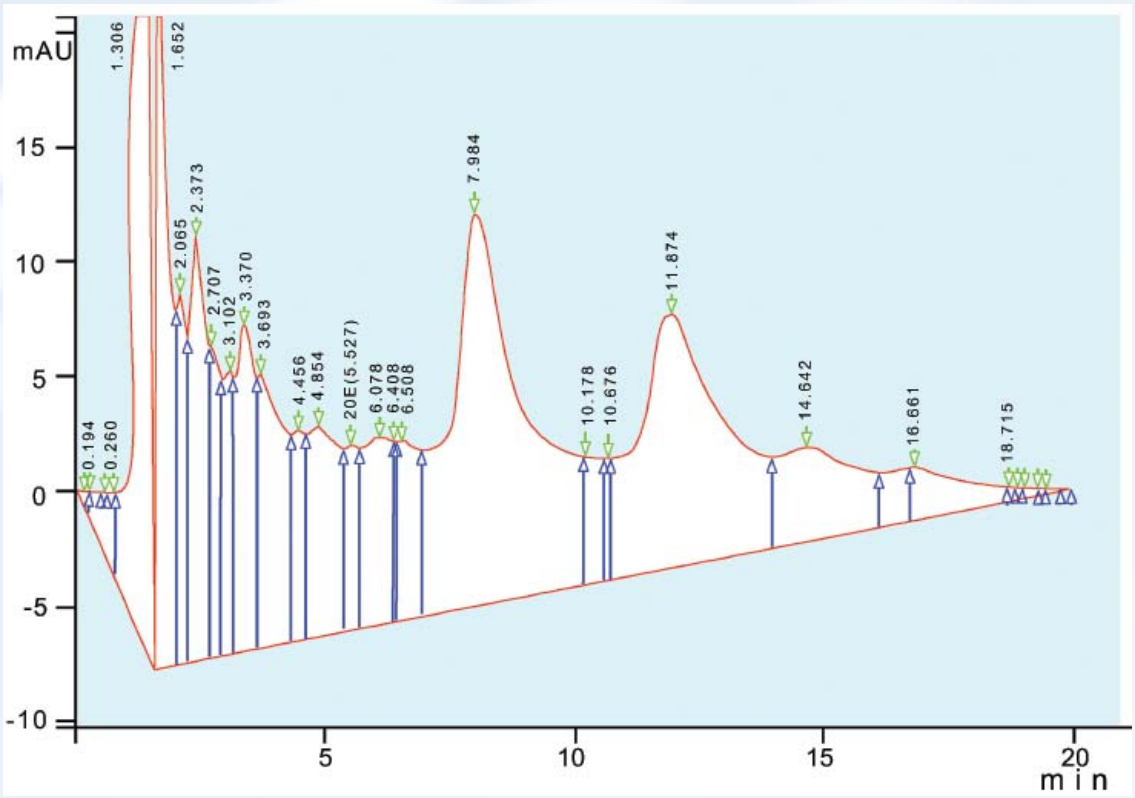

Figure 3. *Chromatogram of ecdysteroidal fraction of intact plants A. genevensis*

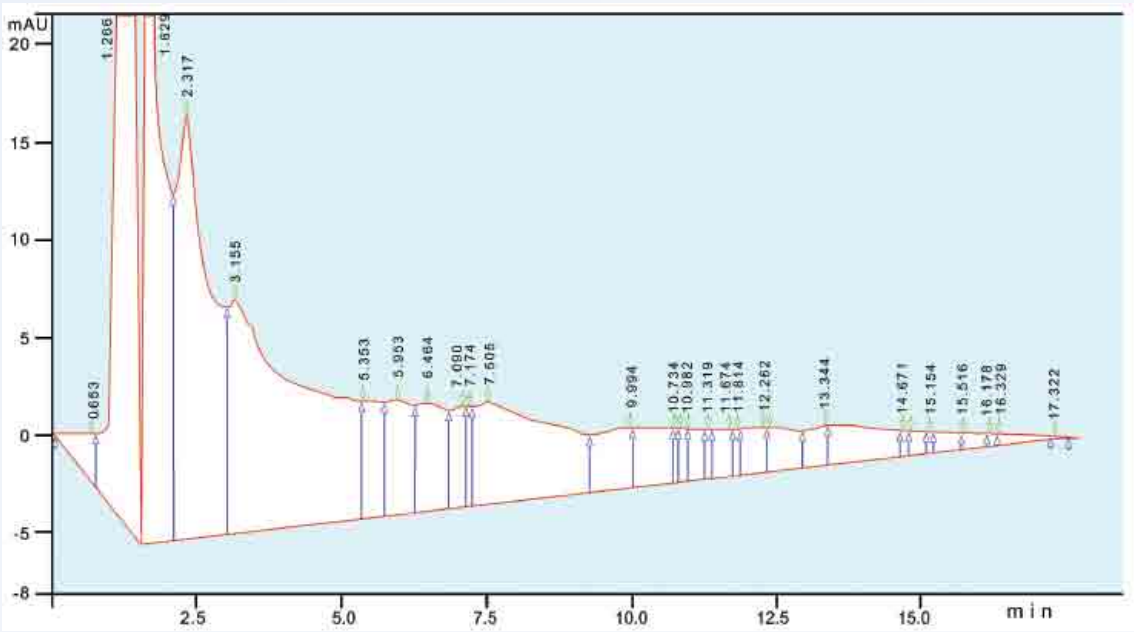

Figure 4. *Chromatogram of ecdysteroidal fraction of A.genevensis' calli culture of leaf origin.*

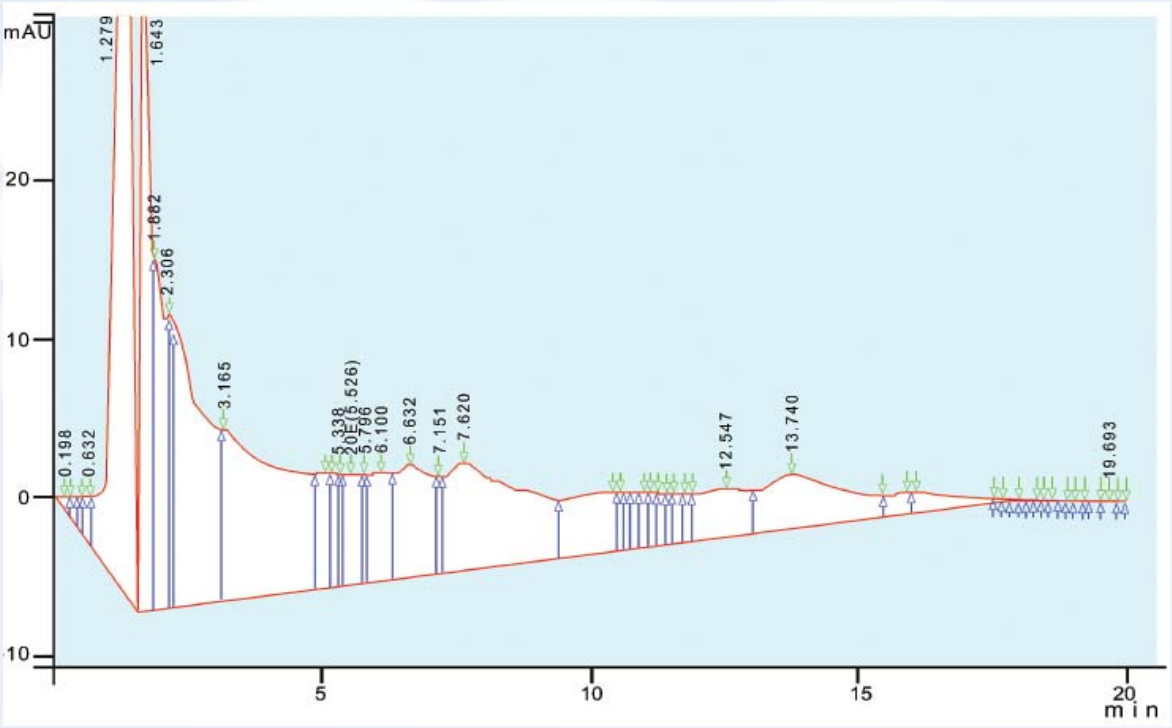

Figure 5. *Chromatogram of ecdysteroidal fraction of A.genevensis' calli culture of root origin*

weight, whereas serine content in both patterns was very high (in comparison with literature data [*Alieva M. et al., 2002*]) achieving about 0.98 *mg/g* of dry weight. In the final analysis, the total free amino acid content was rather high in both callus culture and intact plant. The above mentioned allowed us to suppose the possible presence of phytoecdysteroids in the given plant.

The assumption mentioned above was confirmed by the fact of revealing high content of non-identified compound of ecdysteroid nature in some patterns of callus cultures and intact plants, using HPLC method. The peaks of ecdysteroidal subtances in callus cultures of leaf origin were observed in 3.667, 6.107, 7.849 and 11.930 *min* (Figure 4; Table 3); in callus culture of root origin peak time was registered in 6.056, 7.530, 12.395 *min* (Figure 5; Table 3) and in intact plants retention time was 6.101, 7.586, 12.448 *min* (Figure 4; Table 3).

The content of 20-hydroxiecdysol, the basic ecdysteroid of *A. genevensis* L. plant, was revealed only in overground parts of intact plant achieving 0.05%.

Thus, it was established that callus cultures of *A. genevensis* preserve the ability to synthesize metabolic active substances with antibiotic and antioxidant activities as well as different phytoecdysteroids. It should be also mentioned that qualitative and quantitative content of metabolites differs in favour of *in vitro* cultures.

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