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Comparative Analysis of Chemical Composition and Biological Activities of *Ajuga genevensis* L. in *in vitro* Culture and Intact Plants

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Abstract**—**One of the tasks in contemporary biotechnology, pharmacology and other fields of human activities is to obtain biologically active substances from plants. They are very essential in the treatment of many diseases due to their actually high therapeutic value without visible side effects. However, sometimes the possibility of obtaining the metabolites is limited due to the reduction of wild-growing plants. That is why the plant cell cultures are of great interest as alternative sources of biologically active substances. Besides, during the monitored cultivation, it is possible to obtain substances that are not synthesized by plants in nature. Isolated culture of *Ajuga genevensis* with high growth activity and ability of regeneration was obtained using MS nutrient medium. The agar-diffusion method showed that aqueous extracts of callus culture revealed high antimicrobial activity towards various gram-positive (*Bacillus subtilis* A1WT; *B. mesentericus* WDCM 1873; *Staphylococcus aureus* WDCM 5233; *Staph. citreus* WT) and gram-negative (*Escherichia coli* WKPM M-17; *Salmonella typhimurium* TA 100) microorganisms. The broth dilution method revealed that the minimal and half maximal inhibitory concentration values against *E. coli* corresponded to the 70 μg/mL and 140 μg/mL concentration of the extract respectively. According to the photochemiluminescent analysis, callus tissue extracts of leaf and root origin showed higher antioxidant activity than the same quantity of *A. genevensis* intact plant extract. *A. genevensis* intact plant and callus culture extracts showed no cytotoxic effect on K-562 suspension cell line of human chronic myeloid leukemia. The GC-MS analysis showed deep differences between the qualitative and quantitative composition of callus culture and intact plant extracts. Hexacosane (11.17%); n-hexadecanoic acid (9.33%); and 2-methoxy-4-vinylphenol (4.28%) were the main components of intact plant extracts. 10-Methylnonadecane (57.0%); methoxyacetic acid, 2-tetradecyl ester (17.75%) and 1-Bromopentadecane (14.55%) were the main components of *A. genevensis* callus culture extracts. Obtained data indicate that callus culture of *A. genevensis* can be used as an alternative source of biologically active substances.

Keywords**—***Ajuga genevensis*, antibacterial activity, antioxidant activity, callus cultures.

I. Introduction

PLANTS are an excellent source of biologically active substances which are used in biotechnology, pharmacology, food, cosmetics etc. The plant origin substances are essential for the treatment and prevention of many diseases or disorders without visible side effects. The role, purpose and significance of the secondary metabolites in plants have always been in the focus of many scientists for many years [1], [2]. Now, the demand for the substances of plant origin is also very high [3], [4]. However, due to the chemical composition complexity, low-yield, demand for high purity in secondary origin substances, their chemical synthesis is challenging. At the same time, biological activity of some substances is revealed in combination with the others [5]-[7].

Plant cell cultures became an alternative object for obtaining biologically active substances due to the reduction of wild-growing plant sources. Plant cell culture systems represent a potential renewable source of valuable medicinal compounds, flavors, fragrances and colorants which cannot be produced by microbial cells or obtained via biochemical manipulations [7], [8].

Armenian flora is very rich in many plants of pharmacological value. Genus Ajuga includes over 50 species among which 4 species grow in Armenia. They are considered to be valuable because of their pharmacological interest due to the content of valuable metabolites. These plants contain terpenoids [9], [10], iridoids [11], ecdysteroids [12], [13], flavonoids, fatty acids, glycosides, steroids [10], [14], oligosaccharides [15] which have different effects on the human body [16]-[19]. Some species exhibit insecticidal, particularly antimalarial action [20].

The importance and relevance of microbial strain resistance against different antibiotics and searching for new sources of substances with antimicrobial effects should also be noted today [21]. The available data show the presence of antibacterial, antifungal and antiviral activity of some species of this genus [10], [22]. Over the last years, the interest in the antioxidant activity of plants has also become important [23], [24], because of the fact that free radicals can be the reason for a number of diseases such as heart diseases, stroke, arteriosclerosis and cancer as well as aging processes [25]. Thus, based on the family peculiarities as well as the fact that *A. genevensis* was widely used in folk medicine, it was considered to be perspective source of biologically active substances. In this paper, we investigate some features of *A. genevensis in vitro* cultivated plants and *in vivo* and *in vitro* metabolism of this plant.

II. Materials and Methods

A. Plant Material

Ajuga is a genus of Lamiaceae family herbaceous flowering

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plants growing in Europe, Asia, Africa and Australia. In Armenia the investigated plant *A. genevensis* L. is common in Shirak, Aragatsotn, Tavush, Lori regions as well as in Yerevan. Plants were collected in the slopes of Mountain Aragats (2000-2500 m, above sea-level) during the flowering phase. Plants were identified at the Institute of Botany of the National Academy of Sciences, Armenia.

B. Cultures and Nutrient Media

Callus cultures of leaf origin were obtained using Murashige - Skoog (MS) [26] nutrient medium (with addition of glycine (2.0 mg/L), indole-3-acetic acid (IAA) (2.0 mg/L) and kinetine (6-furfurylaminopurine) (0.2 mg/L)) and sterilizing with mixed solution of cetylpyridinium chloride (660 mg/L) and mercuric chloride (330 mg/L) for 7 min. The Petri dishes with explants (approx. diameters of leaf-origin explants were 0.8 to 1.2 cm, root origin – 1.0 cm in length) were placed in thermostat under the thermal conditions of 22-25 °C (for the initiation of proliferation processes). Afterwards the formed primary callus tissues were placed in the flasks (50 mL), then replaced in both thermostat (22-25 °C, in dark conditions) and room conditions (22 ± 1 °C) under the light illumination (150 µmol quanta m^{-2} s^{-1}).

The medium both without cytokinins and with NAA (0.1 mg/L) and sucrose (20 g/L) was used for the root origination.

Callus cultures of root origin were obtained from the formed roots of *in vitro* plantlets without sterilization. All of the *in vitro* plantlets were incubated at 22-25 °C with the photoperiod of 16 h (natural daylight, supplemented with artificial light (approx. 150 µmol quanta m^{-2} s^{-1}) provided by a white fluorescent lamp (Philips Inc., 36 W).

The pH of the all culture media was 5.8 before adding agar and media were sterilized by autoclaving at 120 °C for 20 min.

C. Plant Material Extraction

1 g dried (at 60 °C, for 12 h), powdered material (intact plants or *in vitro* plantlets or callus tissue) was extracted with 10 mL water or 40% ethanol or methanol during 20 h on magnetic shaker at room temperature. The obtained suspension was centrifuged (10 min at 5000 rev/min), the supernatant was isolated. Precipitate was extracted 4 times and the combined supernatant was dried by evaporation at room temperature. For further investigations, the evaporated mass was dissolved in appropriate solvents in different concentrations.

D. Antibacterial Activity Determination

Agar diffusion method was used for this investigation [27], [28]: the wells in 8 mm diameter were cut out on agar, inoculated with the different Gram-positive (*Bacillus subtilis* A1WT; *B. mesentericus* WDCM 1873; *Staphylococcus aureus* WDCM 5233; *Staph. ctreus* WT) and Gram-negative (*Escherichia coli* WKPM M-17; *Salmonella typhimurium* TA 100) test-microorganisms (200 µl of microbial cell suspension in 20 mL agar medium). Meat-peptone agar (MPA) was used for microbial growth. The level of antibacterial activity was estimated by dropping extracts (100 µL) on the wells and measuring "formed" diameters of growth absence zones around the wells after the incubation at 37 °C for 24 h. The sterile distilled water or 40 % ethanol or methanol was used as a negative control and ampicillin at the concentration of 50 µg/µL was used as positive control (Table I).

Minimal inhibitory concentration (MIC) and half maximal inhibitory concentration (IC_{50}) of extracts were determined by the following scheme: Several tubes containing the same volume of culture medium (MPB-meat-peptone broth) inoculated with the test-bacteria *E. coli* VKPM M-17 (10^4 - 10^6 bacterial cells per mL) were used. The extracts of the following concentrations were added to the above mentioned bacterial suspension: 280; 140; 70; 35 and 16.5 µg/mL. After 24 h of incubation (36-37 °C) the results were evaluated by measuring the absorbance of bacterial suspension (wavelength 560 nm) using a spectrophotometer (Genesys 10S UV-Vis, Thermo Scientific, USA) [29], [30].

E. Antioxidant and Cytotoxic Activity Determination

The antioxidant activity of aqueous extracts (10 µL of extract in the concentration of 70 µg/mL) was determined by photochemiluminescent analyzer (PHOTOCHEM, Analytic Co., Jena, Germany). The positive control was a standard solution containing 10 nM ascorbic acid [31].

The cytotoxicity of the aqueous extracts was determined using K-562 suspension cell line of human chronic myeloid leukemia incubated in the RMPI-1640 nutrient medium (Sigma) with the addition of 10% fetal calf serum. The essence of this method was as the following: 100 µL of *A. genevensis* intact plants and callus cultures extracts at the concentration of 70 µg/mL were added to the daily cell culture of leukemia. After 24 h incubation, the number of living cells in 1 mL of suspension was counted with the use of vital staining by trypan blue [32].

F. Determination of Intact Plant and Callus Extract Chemical Composition

Gas chromatography–mass spectrometry (GC-MS) of the extracts was performed using a Hewlett–Packard 5890 Series II gas chromatograph, fitted with a fused silica HP – 5MS capillary column (30 m x 0.25 mm, in thickness 0.25 µm). The oven temperature varied from 40 to 250 °C with the scanning rate of 3 °C/min. Helium (purity 5.6) was used as a carrier gas at a flow rate of 1 mL/min. The GC was equipped with Hewlett–Packard 5972 Series MS detector. The MS operating parameters were ionization voltage 70 eV and ion source temperature 250 °C. The extracts (1/100, v/v in HPLC methanol) of 1 µL were injected manually. To avoid the GC column overloading, the essential oils were diluted 1:100 (v/v) in methanol. The identification of peaks was tentatively carried out based on National Institute of Standards and Technology (NIST) database NIST– 2013 [33].

G. Data Processing

The experiments were repeated at least 6 times. The obtained data were processed using SPSS Statistics 17.0 software package (SPSS Inc., Chicago, IL, USA).

III. Results and Discussion

The proliferation began on the 7th-8th days on MS medium and the primary callus was formed on the 18th-20th days of cultivation. Callus tissue of *A. genevensis* had a dense consistency and light color when growing in dark conditions (Fig. 1 (a)) and intensive green – under the light (Fig. 1 (c)).

The root formation occurred on the medium with 0.1 mg/L NAA. Generally, the growth cycle of calli ended on the 27th-28th day, but in case with *in vitro* plantlets it was over on the 35-40th day of cultivation. Generation of shoots and leaves were observed on the MS medium (Fig. 1 (b)). Subsequently shoots were separated from each other and transferred to fresh medium with 0.1 mg/L NAA and 20 g/L sucrose under the light (Figs. 1 (d) and (e)). Root formation process in the liquid medium was equal to that in agar nutrient medium (Fig. 1 (f)). *A. genevensis* isolated culture possessed high-frequency regeneration ability (index of micro-propagation was 10^5-10^6). In most cases *in vitro* plants reached t blooming phase during May-June period which corresponded to the natural cycle of development. 20-25% of obtained *in vitro* plantlets was successfully acclimatized in a soil substrate (at the stage when they reached 3-5 cm high) (Fig. 1 (g)).

Testing of *A. genevensis* calli aqueous extracts revealed a high antimicrobial activity against various Gram-positive and Gram-negative microorganisms (Table I).

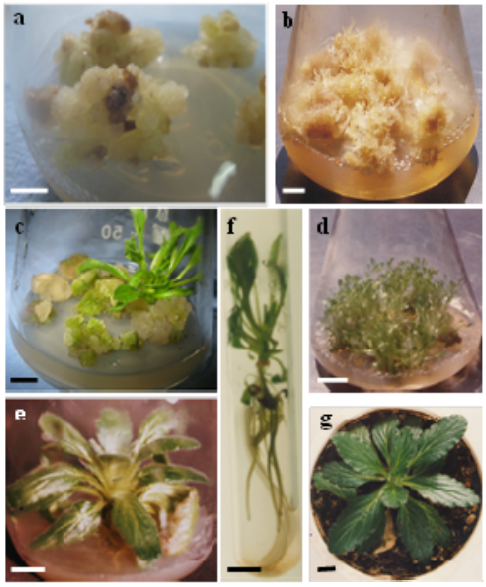

Fig. 1 Plant regeneration from callus cells of *A. genevensis* and micropropagation (a) Callus culture of *A. genevensis* in dark conditions (MS medium). (b) Callus culture growing and shoot-organogenesis under the light conditions (MS medium). (c, d, e) Micro-propagation (nutrient medium with 0.1 mg L^{-1} NAA). (f) Root formation in the liquid nutrient medium with 0.1 mg L-1NAA. (g) Plants grown in a soil substrate in greenhouse conditions. Scale bars are 1 cm

TABLE I
Inhibition of the Test Microorganisms Growth Affected by *A. genevensis* Callus Tissue Extracts

Test-microorganism	The growth absence zones (Ø mm)	
	Callus culture extracts (280 μg mL^{-1})	Positive control (Ampicillin, 50 μg mL^{-1})
Bacillus subtilis A1 WT[1]	65.0 ± 1.0	40.0 ± 0
Bacillus mesentericus WDCM 1873	64.0 ± 0 .7	34.0 ± 0
Staphylococcus aureus WDCM 5233	28.0 ± 0.6	26.0 ± 0
Staphylococcus citreus WT	51.0 ± 0.7	41.0 ± 0
Escherichia coli VKPM M-17	31.0 ± 0.5	31.0 ± 0
Salmonella typhimurium TA 100	25.0 ± 0.6	NT[2]

P<0.001; WT[1]- wild type; NT[2] - not tested

According to our results the Gram-positive microorganisms were more sensitive to the extracts with antibacterial activity. The synthesis of substances with antibacterial properties depended on different conditions: growth phases, passage number and composition of nutrient medium.

In one cycle of growth the peak of investigated metabolites synthesis was registered on the 20th day of calli cultivation. This regularity was followed regardless from the passage. Synthesis of substances with antibacterial activity increased with the growth of the culture general age (quantity of passage): the greatest zones of growth absence were revealed around the extracts from the calli of 18th-20th passages. In spite of callus culture rather high antibacterial activity, the extracts of intact plants showed lower activity against the same test-microorganisms. The antibacterial activity of the isolated culture extracts also depended on the nature of extracting liquid. Aqueous extracts showed the highest activity in comparison with methanol or ethanol [34].

Investigations revealed that OD of bacterial suspension in control tubes after 24 h of cultivation increased about 5-6 times. Approximately a five-fold increase of bacterial suspension OD was observed in case of two lowest concentrations of the extract addition (35 μg/mL and 16.5 μg/mL), whereas OD values remained unchanged (at the concentration of 70 μg/mL) or decreased by almost a half (at the concentrations of 140 μg/mL, 280 μg/mL) in other cases. Thus, these findings allow us to suppose, that MIC corresponds to the extract concentration of 70 μg/mL. Based on these data it is possible to insist that IC_{50} value corresponds to the extract concentration of 140 μg/mL.

A. genevensis culture demonstrated also high antioxidant activity. Indeed, 10 μL extract of the intact plant showed antioxidant activity equal to 65.5±2.7 nM ascorbic acid. The same quantity of callus tissue extract of leaf and root origin showed antioxidant activity equal to 67.5± 2.9 nM and 95.5±5.4 nM ascorbic acid, respectively (Fig. 2). These properties raise the value of callus cultures as potential producers of biologically active substances.

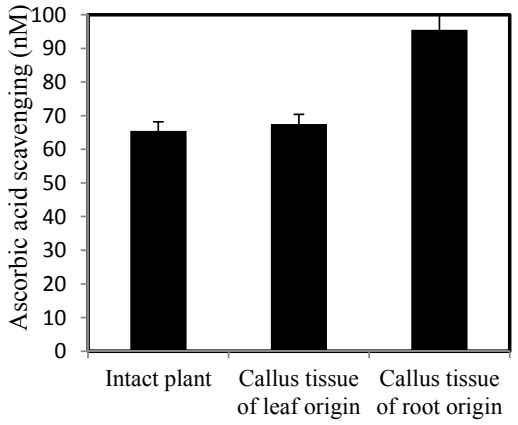

Fig. 2 Comparative antioxidant (free radical scavenging) activity of *A. genevensis* intact plant, callus tissues of leaf and root origin (the positive control was ascorbic acid). See the text

The absence of *A. genevensis* plants cytotoxicity as well as their callus culture extracts was established in relation to the K-562 suspension cell line of human chronic myeloid leukemia.

Deep differences in the composition of the extracts are also the basis of these kind distinctions in biological activity.

The GS-MS analyses showed that the main components of intact plant extracts were hexacosane (11.17%); n-hexadecanoic acid (9.33%) and 2-methoxy-4-vinylphenol (4.28%). While the concentration of 10-Methylnonadecane (57.0%) was prevalent upon the other substances of *A. genevensis* callus culture extracts. So, based on obtained data it is possible to introduce the relevance of obtaining *in vitro* cultures of *A. genevensis* and to study their metabolic features.

IV. Conclusions

During the cultivation process some changes in qualitative and quantitative composition of active metabolites may take place. This property is important in terms of purposeful change of *in vitro* culture metabolism. This makes a significant basis to study the growth and metabolic features of pharmacologically valuable plant isolated cultures.

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