DE GRUYTER OPEN

Current Issues in Pharmacy and Medical Sciences

Formerly ANNALES UNIVERSITATIS MARIAE CURIE-SKLODOWSKA, SECTIO DDD, PHARMACIA

journal homepage: http://www.curipms.umlub.pl/

Cytotoxic activity of ethanolic extracts of *Eleutherococcus* species cultivated in Poland on HL60 leukemia cell line

Daniel Zaluski[1*], Helena Danuta Smolarz[2], Anna Bogucka-Kocka[2]

[1] Chair and Department of Pharmacognosy, Jagiellonian University, Collegium Medicum, Medyczna 9, 30-568 Cracow, Poland
[2] Chair and Department of Pharmaceutical Botany, Medical University of Lublin, Chodzki 1, 20-093 Lublin, Poland

ARTICLE INFO

Received 09 December 2013
Accepted 10 January 2014

Keywords:
Eleutherococcus,
eleutherosides,
HL60,
cytotoxicity,
apoptosis.

ABSTRACT

The *Eleutherococcus* species including 40 species native to Asia are medicinal plants widely used in traditional medicine. Some of these species are cultivated at the botanical gardens in Europe. On the basis on our earlier studies it was concluded that the extracts of analyzed species act as antioxidants, inhibitors of MMPs, and cytotoxic against Jurkat 45 leukemia cell line. In this study, the anti-leukemic potential of roots and leaves from six *Eleutherococcus* species cultivated in Poland was determined.

The *in vitro* cytotoxic activity towards human promyelotic leukemia cell line HL60 using trypan blue assay was evaluated. The induction of apoptosis in stimulated leukemia cells was determined by AnnexinV method. Morphological changes in treated cells were observed by microscopic investigations.

The results showed that ethanolic extracts from the roots and the leaves of *E. senticosus*, *E. setchuensis*, *E. sessiliflorus*, *E.gracilistylus*, *E. henryi* and *E. divaricatus* exhibit cytotoxic effect towards leukemic HL60 cells. The received IC50 values for roots ranged from 49-208 μg/mL and for the leaves from 116-518 μg/mL. The ethanol extract from the roots of *E. divaricatus* showed the highest cytotoxic and proapoptotic effect on HL60 human lymphoid leukemia cell line.

INTRODUCTION

Medicinal plants are the ingredients of many currently important pharmaceutical drugs with broad biological activity. There has been a growing interest in studying plant secondary metabolites as they represent a tremendous library of potentially useful leading compounds for new drug development. In fact, many modern drugs are compounds isolated from plants, which are used in the treatment of many diseases including cancer, e.g. taxol from yew tree, or vincristine from periwinkle. Pharmaceutical industry is still searching for new drugs with anticancer potency. Since there are no effective drugs to treat most cancers, it seems a good solution to produce the anti-leukemic drugs with natural origin. Some natural products are mostly composed of complex compounds, which act synergistically [5, 10].

Eleutherococcus is a genus of the Araliaceae family, which includes approximately 40 species native to North Russia and Asia. The plants from *Eleutherococcus* genus have been used in medicine, especially in Chinese medicine for many years. The roots of *Eleutherococcus senticosus* have been traditionally used as a folk remedy for many diseases including diabetes, hypertension and cancer. The leaves are used as a tonic, as a functional beverage commercially marketed for reducing liver damage and accelerating alcohol detoxicification.

There is an increasing interest in alternative or herbal medicine for the prevention and treatment of various illnesses and an increasing number of scientific research is focusing on these species. As the *Eleutherococcus* species have wide pharmacological properties, there is a need to carry out research towards anti-leukemic properties. The abundance and variety of chemical compounds give a large hope for new drugs [10, 12-13].

The main chemical compounds present in these species are glycosides, known as eleutherosides. Eleutherosides are the derivatives of coumarins, lignans, sterols and triterpenic acids. A special attention is given to eleutherosides B, E and E1, the compounds, which according to some authors, play a dominant role in the treatment of many diseases. Our previous phytochemical investigations have shown that eleutehrosides B, E and E1 are present in all the investigated species cultivated in Poland [1].

Corresponding author
* e-mail: daniel.zaluski@uj.edu.pl
tel./fax: +48 12 742 37 02

DOI: 10.2478/cipms-2014-0011

E. senticosus is the best-known species of all species belonging to *Eleutherococcus* genus. Its monograph can be found in several Pharmacopoeias; e.g. European, American or Polish. According to Pharmacopoeias the roots of *E. senticosus* should contain at least 0.08% of the sum of eleutheroside B and E. Apart from eleutherosides also polysaccharides, essential oil, eleutherans, phenolic acids and diketopiperazines have been found. The medicinal properties of *E. senticosus* are compared to *Panax ginseng* or *Aralia mandshurica*. This species is classified as adaptogenic plant, which can increase the ability of the organism to adapt to environmental factors and to avoid damage from such factors. It is worth noting, that the extracts from this species were used after nuclear explosion in Chernobyl in order to reduce the effects of radiation. *E. senticosus* is also used in the treatment of bacterial and viral infections. In addition, it has been reported, that *E. senticosus* has antiinflammatory, immunostimulatory and immunomodulatory properties [1, 7-9, 19].

Apart from *E. senticocus,* the Chinese Traditional Medicine has also used *E. gracilistylus, E. sessiliflorus* and *E. setchuensis*. The extracts and isolated compounds have been shown to have various levels of activity such as antibacterial, antiinflammatory, anti-gout, anti-hepatitis, anti-hyperglycemic, anti-oxidant, anti-pyretic, choleretic, radioprotectant, hemostatic, immunostimulatory and hypocholesterolemic effects. The bark from the roots of *E. gracilistylus* described in Chinese Pharmakopoeia as *Cortex Acanthopanacis* (Wujiapi) is used in the treatment of rheumatism. The products containing the root bark are recommended as ”therapeutic wine” [11-12, 16].

The anti-leukemic properties of *Eleutherococcus* species cultivated in Poland have not been researched yet. World literature reports mainly on one species, *E. senticocus*. Anti-leukemic activity of these species is unknown, in this case the main aim of studies was to establish these properties and to explain, whether they induce apoptosis or necrosis in HL60 leukemia cells.

MATERIAL AND METHODS

PLANT MATERIAL

The roots, spring and autumn leaves of *E. senticosus* (Rup. et Maxim.) *E. setchuensis* (Harms) Nakai, *E. sessiliflorus* (Rupr. et Maxim.) S. Y. Hu, *E. gracilistylus* (W. W. Smith) S. Y. Hu, *E. henryi* Oliv. and *E. divaricatus* (Siebold & Zucc.) S. Y. Hu were obtained from arboretum in Rogów (Poland). The spring leaves were collected in June 2010, while the roots and autumn leaves in October 2010. Voucher specimens were deposited at the Chair and Department of Pharmaceutical Botany, Medical University of Lublin, Poland, and was numbered as E01, E02, E03, E04, E05, E06, respectively.

PREPARATION PLANT SAMPLES

The air-dried and powdered roots and leaves (15 g) were soaked for 24 h in 150 mL of 75% ethanol, in a round-bottomed flask. After that, the samples were sonicated in an ultrasonic bath (Polsonic, Warsaw, Poland) at room temperature for 15 min. The liquids were carefully filtered and the plant material was re-extracted for 15 min. with 100 mL of the same solvent. The filters, on which the samples were filtered, were sonicated afterwards, with 100 mL 75% ethanol. There was obtained 350 mL of each extract. The solvents were evaporated under reduced pressure at 45°C and subjected to lyophilisation.

BIOASSAYS

Cell culture

Leukemic cells (HL60 cell line, ECACC, cat. no. 980 701 06) were incubated at the concentration of 5×10^5 cells/mL in 5% CO_2 atmosphere for 24 h at 37°C. An RPMI 1640 medium (Sigma, St. Luis, USA) , with 15% fetal bovine serum (Sigma), 2 mM L-glutamine and antibiotics [100 U/mL penicillin, 100 µM/mL streptomycin and 2.5 µg/mL amphotericin B (Gibco, Carlsbad, USA) served as a growing medium.

Cytotoxicity test-trypan blue assay

The *in vitro* cytotoxicity assay was carried out using trypan blue assay. The HL60 cell line in concentration 5×10^5 cells/mL was treated with different concentrations of testing extracts and incubated for 24 h at 37°C in air atmosphere humidified by 5% CO_2. At the end of this period, the medium was removed from each plate by aspiration. Next, the cells were washed with PBS and centrifuged at 800 rpm for 10 min, and then PBS was removed by aspiration. Than 10 µL suspension cells were incubated for 5 min with the 10 µl 0.4% trypan blue solution (Sigma). The samples were analyzed using an Olympus BX41 light microscope. The cells were stimulated with the ethanol extracts from the roots and leaves dissolved in DMSO at the final concentration 2; 25; 100; 300; 600 µg/mL of cell culture. The final concentration of DMSO in incubating mixture was 1%. Every assay was performed in triplicate.

Apoptotic test-Annexin V assay

The annexin-V-Fluos assay was used to estimate the number of cells in the stage of apoptosis. After 24h cell cultures were centrifuged at 800 rpm for 10 min. and the culture medium was removed. Than they were incubated for 5 min in the buffer comprising 10 mM Hepes [N-(2-hydroxyethyl) piperazine-N’-(2-ethanesulfonic acid) hemisodium salt]/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$, annexin V labeled with 0.65 µg/mL of FITC and propidium iodide at the concentration of 12 µg/mL. Thereafter, the samples were analyzed by an Olympus BX41 light and fluorescence microscope for the presence of: a) viable cells-annexin V negative, PI negative, b) earlyapoptotic cells – annexin V positive, PI negative, c) late apoptotic/secondary necrotic-annexin V positive, PI positive.

The number of apoptotic cells per sample was determined as the percentage of annexine V positive cells per sample. HL60 cell line without the extract was used as a positive control. Cell morphology was examined using a BX41 Olympus fluorescence microscope. Data were processed according to the Multi Scan software.

RESULTS

On the basis of the obtained results, it was concluded that all the examined extracts show cytotoxic activity against leukemic cells. The IC50 values summarized up in Table 1 are differentiated, depending on the type of raw material. The IC50 value ranged between 49 and 522 µg/mL. The highest cytotoxicity effect was observed in the case of the extract from roots. The most active was the extract from the roots of *E. divaricatus* (IC50; 49 µg/mL).

Table 1. IC50 [µg/mL] of extracts from *Eleutherococcus* spp.

Species	Roots*	Spring leaves*	Autumn leaves*
E. senticosus	**208** ± 0.005	**312** ± 0.1	**299** ± 0.004
E. divaricatus	**49** ± 0.008	**223** ± 0.005	**270** ± 0.005
E. setchuensis	**135** ± 0.06	**522** ± 0.2	**518** ± 0.3
E. sessiliflorus	**128** ± 0.009	**400** ± 0.07	**325** ± 0.04
E. gracilistylus	**206** ± 0.01	**100** ± 0.003	**100** ± 0.005
E. henryi	**185** ± 0.007	**116** ± 0.09	**209** ± 0.08

* Results in terms of mean ± standard deviation

The obtained results show that IC50 values for the extracts from spring and autumn leaves are similar for the particular species. The lowest IC50 was obtained for the leaves of *E. gracilistylus* and *E. henryi* (100-209 µg/mL), the least active was the extract from the spring leaves of *E. setchuensis* (522 µg/mL). The percentage of living, apoptotic and necrotic leukaemic cells after 24 h stimulation with extracts from roots at concentration IC50 is presented in Fig. 1.

Figure 1 presents that all the samples contribute to activation of apoptosis process in leukemia cells. The number of apoptotic cells ranged between 43% and 32%, necrotic cells between 17% and 9%. The most apoptotic cells were determined in *E. divaricatus* (43%) whereas the number of necrotic (toxic effect of the extracts) cells was the highest in *E. gracilistylus* (17%). The percentage of apoptotic cells in controls was lower than 5%.

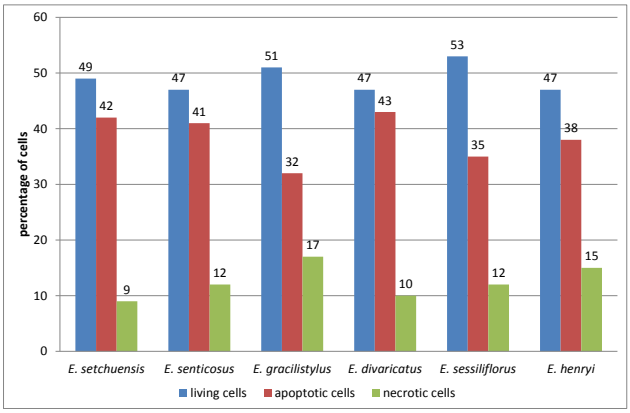

Figure 1. Living, apoptotic and necrotic HL 60 cells (24 h culture) after stimulation with ethanolic extracts from the roots of *Eleutherococcus* spp. The concentration of the extracts equalled IC50

DISCUSSION

Eleutherococcus spp. has been a subject of many studies for many years. Its secondary compounds have shown to have a wide range of pharmacological activities.

It is thought that eleutherosides are responsible for the medical activity of *Eleutherococcus* spp. Based on studies on the extracts and their ingredients, including eleutherosides, it appeared that they are very well absorbed in human intestines and accumulated in plasma, heart, kidney and liver. Unfortunately, these species are native of eastern Asia and currently are cultivated only in European botanic gardens. The chemical analysis performed by the authors has shown that the investigated species contain eleutherosides B and E, whose content is higher than the requirements of pharmacopoeia. Our earlier studies have shown that eleutherosides B, E and E1 are in all roots and fruits, but not in the leaves [1, 2, 17].

In the present study, HL-60 cell line was used as an *in vitro* model to examine the cytotoxic and apoptotic properties of the ethanolic extracts from the roots of spring and autumn leaves of the species belonging to *Eleutherococcus* genus cultivated in Poland. These species are cultivated at the botanical garden in Rogów, which lies in the Central Polish Lowlands region with geographic data such as 51°49'N and 19°53'E. The vegetative period lasts for 212 days and the average annual precipitation is 596 mm, of which 80% occurs during the vegetative period. The average annual air temperature is 7.2°C. The average, long-term temperature is – 20.1°C, what classified the garden to the 6bth sub-climate (according to "USDA Frost Hardiness Zones") and to the second zone according to the Kórnik category. These plants are grown on the acidic, luvic and sand soils [14].

Leukemia is one of the most frequently occurring diseases among young people to 30 years of age. For many years, the increase in the incidence of leukemia has been observed, especially in children and young people. Because of growing resistance to drugs, the treatment of leukemia is very difficult. New drugs with antileukemic or strengthening body properties are still being searched for. One of the most intensely studied areas of research has been the investigation of plants with anticancer action.

World scientific literature refers mainly to the research on one species, namely *E. senticosus*. The earlier studies performed by Hacker and Medon revealed cytotoxic effect of *E. senticosus* water extract against L1210 leukemia cells with ED50 value of 75 µg/mL [4]. Other studies performed by Yoon *et al.* [15] have shown anti-metastatic activity of a water extract of *E. senticosus*. The extract inhibited lung metastasis of colon 26-M3.1. An *in vitro* analysis showed that the extract affects the growth of colon 26-M3.1 cells at the concentration higher than 1000 µg/mL [15]. Additional studies performed by the above authors have shown that the glycoprotein fraction is responsible for the anti-metastatic activity of the extract . Based on the obtained results it appeared that the glycoproteins fraction showed higher anti-metastatic activity and higher stimulation of the proliferation of splenocytes compared to the water extract [17]. In turn, the performed studies with the use of other cell lines, such

as MOLT-4F (IC50; 14.29 µg/mL), PC-3, HCT-15, SW-620, ACHN and A549 have showed the similar cytotoxic activity of extracts for all cell lines. The obtained IC50 values were on the level nearly 30 µg/mL. The extracts of the *E. sessiliflorus* seedlings have cytotoxic action towards Calu-6 and SMU-601 cell lines. The IC50 values were 25 µg/mL and 196.7 µg/mL, respectively [2, 19, 6]. Other studies showed that the water extract from the stem bark of *A. senticosus* induced apoptosis in human stomach cancer KATO III cells [4]. Unfortunately, there is still little information on the antileukemic activity of the other investigated species growing in Asia.

An important problem in the evaluation of the cytotoxic properties of extracts is to establish the way of their action. Based on the presented results, it has appeared that *Eleutherococcus* species cultivated in Poland possess cytotoxic properties and act via apoptosis process. The extract from the roots of *E. divaricatus* showed the highest mortality (IC50, 49 µg/mL). Significantly, large differences in IC50 value between the roots and the leaves from *E. divaricatus* and *E. setchuensis* have been observed. During apoptosis, the morphological features of cells such as compaction and margination of nuclear chromatin, cytoplasmic condensation and membrane blebbing and cell shrinkage were observed. Taking into account the results of own studies, it appears that some species from the Polish origin have stronger cytotoxic activity than those from Asia.

The most of the studied species have a significant biological activity that is probably due to the presence of phenols, called as eleutherosides. To the best of our knowledge, neither phytochemical nor biological detailed studies have been performed for a majority of the investigated species. According to Załuski et al. these compounds are present only in the roots and fruits. Using a validated HPTLC-densitometric method, it appeared that the roots of the investigated species have high eleutherosides content [1, 16, 18]. The amount of eleutheroside B ranged between 0.5 mg/g and 3.4 mg/g; eleutheroside E between 0.5 mg/g and 1.2 mg/g; and eleutheroside E1 between 0.2 and 0.9 mg/g of the sample, dry weight. The highest concentration of eleutherosides B and E was detected in *E. henryi* (3.4 mg/g and 1.2 mg/g, respectively), and of eleutheroside E1 in *E. divaricatus* (0.9 mg/g). According to statistical analysis, positive correlation between cytotoxic activity and eleutherosides content was not found, which could be the result of the assay conditions or the chemical structure of the eleutherosides.

These first comprehensive studies during the apoptosis process have been researched with use of the eighteen extracts from six *Eleutherococcus* species. There is still great interest in finding more effective and safe cancer process inhibitors, so these plants are very promising source of biologically active substances. Based on the obtained results and discussion, we can say that *E. senticosus* cultivated in Polish climatic conditions possesses strong pharmacological properties in *in vitro* model. To develop our research, it is necessary to isolate and identify the active compounds.

CONCLUSIONS

All these comparative studies for different parts of *Eleutherococcus* brought useful information on their potential use. The roots of *E. divaricatus* contain phytochemicals with the cytotoxic activity against leukemia cells. In the further research, the main attention will be focused on the isolation of single compounds and their cytotoxic activity against leukemic cells and normal human lymphocytes. Keeping in mind their rich biological properties, consumption, especially of the fresh fruits or the roots of extract products can act protectively against leukemia.

ACKNOWLEDGEMENTS

Authors are thankful to Piotr Banaszczak, director of the Botanical Garden in Rogów (Poland) for the *Eleutherococcus* samples.

The paper was developed using the equipment purchased within the Project ,,The equipment of innovative laboratories doing research on new medicines used in the therapy of civilization and neoplastic diseases" within the Operational Program Development of Eastern Poland 2007-2013, Priority Axis and Modern Economy, Operations 1.3 Innovation Promotion.

REFERENCES

1. Cieśla Ł. et al.: HPTLC – densitometric method for determination of eleutherosides B, E and E1 in different *Eleutherococcus* species. *J. Chromatog. Sci.*, 49 (3), 182-186, 2011.
2. Feng S., Hu F., Zhao J.: Determination of eleutheroside E and eleutheroside B in rat plasma and tissue by high-performance liquid chromatography using solid-phase extraction and photodiode array detection. *Eur. J. Pharm. Biopharm.* 62, 315-320, 2006.
3. Ha E.S. et al.: Anti-metastatic activity of glycoprotein fractionated from *Acanthopanax senticosus*, involvement of NK-cell and macrophage activation. *Arch. Pharm. Res.* 27(2), 217-224, 2004.
4. Hacker B., Medon P.J.: Cytotoxic effects of *Eleutherococcus senticosus* aqueous extracts in combination with N6-(delta 2-isopentenyl)--adenosine and 1-beta-D-arabinofuranosylcytosine against L1210 leukemia cells. *J. Pharm. Sci.* 73(2), 270-272, 1984.
5. Hibasami H. et al.: Induction of apoptosis by *Acanthopanax senticosus* Harms and its component, sesamin in human stomach cancer KATO III cells. *Oncol. Rep.* 7, 1213-1216, 2000.
6. Chon S.U. et al.: Total phenolics level, antioxidant activities and cytotoxicity of young sprouts of some Traditional Korean Salad Plants. *Plant. Foods Hum. Nutr.*, 64, 25–31, 2009.
7. Jang M.H. et al.: Protective effect of Acanthopanax senticosus against ethanol-induced apoptosis of human neuroblastoma cell line SK-N-MC. *Am. J. Chin. Med.* 31, 379-388, 2003.
8. Kurkin V.A. et al.: Antidepressant activity of some phytopharmaceuticals and phenylpropanoids. *Pharm.Chem. J.* 40 (11), 614-619, 2006.
9. Li Z.F. et al.: Two diketopiperazines from *Acanthopanax senticosus* Harms. *J. Asian Nat. Prod. Res.* 12, 51–55, 2010.
10. Liu W. (2011). Traditional herbal medicine research methods. New Jersey:John Wiley&Sons.
11. Panossian A.: Stimulating effect of adaptogens: An overview with particular reference to their efficacy following single dose administration. *Phytother. Res.* 19(10), 819-838, 2005.
12. Shan B.E. et al.: Chinese medicinal herb, *Acanthopanax gracilistylus*, extract induces cell cycle arrest of human tumor cells *in vitro*. *Clin. Exp. Immunol.* 118, 41-8, 1999.
13. Sun Y.L., Liu L.D., Hong S.K.: *Eleutherococcus senticosus* as a crude medicine: Review of biological and pharmacological effects. *J. Med. Plants Res.*, 5(25), 5946-5952, 2011.
14. Tumiłowicz J., Banaszczak P.: Drzewa i krzewy z rodziny *Aquifolaceae* w arboretach w Rogowie i Glinnej. *Rocznik Dendrologiczny* 55, 41-56, 2007.

15. Yoon T.J. et al.: Anti-metastatic activity of *Acanthopanax senticosus* extract and its possible immunological mechanism of action. *J. Ethnopharmacol*. 93(2-3), 247-253, 2004.
16. Załuski D., Smolarz H.D., Szpilewska M.: Eleutherosides in aerial parts of *Eleutherococcus* species cultivated in Poland. *Journal of AOAC International*, 94 (5), 1422-1425, 2011.
17. Załuski D., Smolarz H.D.: *Eleutherococcus senticosus* – przykład rośliny adaptogennej. *Post. Fit.* 4, 240-246, 2008.
18. Załuski D., Smolarz H.D., Gawlik-Dziki U.: Bioactive Compounds and antioxidative, antileukemic and anti-MMPs activity of *Eleutherococcus* species cultivated in Poland. *Nat. Prod. Commun.* 7(11), 1483-1486, 2012.
19. Zhao W.M. et al.: Constituents from the roots of *Acanthopanax setchuenensis. Fitoterapia* 70, 529-531, 1999.