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Extraction of bioactive compounds from conifers growing in the Windsor Great Park and other arboretums

D. I. Ivanova[1]*, R. P. Adams[2], J. Anderson[3], A. N. Tashev[4], P. T. Nedialkov[5], Z. K. Kokanova-Nedialkova[5], Y. E. Ilieva[6], T. N. Atanassova[5], G. I. Kalotova[1], G. Angelov[1], H. M. Najdenski[6]

[1] *Institute of Chemical Engineering, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria*
[2] *Biology Department, Baylor University, Waco, TX 76798, USA*
[3] *Crown Estate Office, Windsor Great Park, SL4 2HT Windsor, UK*
[4] *University of Forestry, Department of Dendrology, 1756 Sofia, Bulgaria*
[5] *Faculty of Pharmacy, Medical University, 1000 Sofia, Bulgaria*
[6] *The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria*

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The present study is aimed at identification of antioxidant and antiproliferative compounds in extracts of conifers originating from the Windsor Great Park (WGP), UK, and other arboretums. Species of the genera *Juniperus, Cupressus, Chamaecyparis* and *Taxus* were studied. Rare representatives of *J. indica, J. squamata, J. pingii, J. recurva, J. davurica* etc., as well as widely distributed species and cultivars were analyzed. Antioxidant activities were evaluated by total polyphenol content (TPC) and half-maximum DPPH-radical scavenging concentrations (DPPH-SC_{50}) of the extracts. Antiproliferative activities were determined by half-maximum growth-inhibitory concentrations (IC_{50}) obtained after MTT-assay of NB4 acute promyelocytic leukemia cells treated with the corresponding extracts. In this group of studied conifers, *J. indica* leaves extract was determined as the best antioxidant agent with DPPH-SC_{50} 52 μg/ml and TPC 320±10 GAE mg/g extract. The best antiproliferative properties were demonstrated by the leaves extracts of *J. virginiana* cultivars with NB4-IC_{50} in the range of 0.27-0.31 μg/ml. Remarkable cytotoxic activity was found also for *J.* × *pfitzeriana, J. pingii* var. *wilsonii* and *T. baccata* leaves extracts. Rare species, such as *J. indica* and *J. recurva* "Embley Park", also showed high antiproliferative activity. Podophyllotoxin was identified in the best cytotoxic extracts obtained from *J. virginiana* and *J.* × *pfitzeriana* cultivars. Identification of other metabolites in the efficient bioactive extracts is in progress. The present results revealed various conifers as potential sources of cytotoxic and antioxidant lead compounds for prevention of the living organisms from oncogenic, degenerative or other radical-induced diseases.

Keywords: Antioxidants, Antiproliferative activity, *Chamaecyparis* Spach., *Cupressus* L., *Juniperus* L., *Taxus* L.

INTRODUCTION

Conifers belong to the Pinophyta (Coniferophyta) division of the Plantae kingdom. They are cone-bearing gymnosperm plants, growing as magnificent trees or small shrubs. Various conifers are natural sources of highly efficient bioactive molecules. The essential oils from various conifers have been used from ancient times and nowadays, due to their efficient antioxidant, antimicrobial, cytotoxic and other bioactive properties. Cedar oil is obtained from conifers belonging to Cupressaceae (*Juniperus, Cryptomeria, Cupressus, Calocedrus, Chamaecyparis, Austrocedrus* and *Thuja* species) and Pinaceae (*Cedrus* and *Pinus* species) families [1]. In the present study, extraction of antioxidant and antiproliferative compounds as potential pharmaceutical agents from representatives of the genera *Juniperus*, *Cupressus*, *Chamaecyparis* and *Taxus*, growing in the Windsor Great Park and other arboretums, were investigated.

The genus *Juniperus* L. (Cupressaceae) includes about 50-67 species and more than 220 cultivars [2, 3]. About 580 juniper secondary metabolites have been identified – cytotoxic podophyllotoxin (PPT) and other lignans, sesquiterpenes, diterpenes, flavonoids, etc. [4]. The present sources of PPT [*Sinopodophyllum hexandrum* (Royle) T. S. Ying, *Podophyllum peltatum* L.] are already considered as endangered species because of their intensive industrial exploitation in the synthesis of efficient anticancer drugs Etoposide, Teniposide, etc. That is why, new sources of PPT are necessary.

In the context of the conquest of cancer, new sources of antioxidant compounds are also required as active agents against excessive accumulation of deleterious free radicals in the cells. We have found recently that *J. sibirica* Burgsd. and *J. excelsa* M. Bieb. leaves extracts have demonstrated the best antioxidant activity among plenty of juniper species from the Balkan region [5].

* To whom all correspondence should be sent:
E-mail: dianadoc@abv.bg

In this study, more efficient antioxidant properties were found for other conifer representatives – *J. indica* Bertol., *J. recurva* "Embley Park", *C. arizonica* Greene, *J. squamata* Buch.-Ham. ex D. Don, etc.

The genus *Cupressus* includes about 16-25 species and a wide variety of cultivars. *Cupressus sempervirens* L. leaves extract and essential oil have demonstrated remarkable radical scavenging activity [6]. Quercetin, rutin, caffeic acid, and *p*-coumaric acid have been isolated from *C. sempervirens* leaves [7]. The bark extract of *Cupressus lusitanica* Mill. (Mexican white cedar) has shown high cytotoxicity on MCF-7 (estrogen receptor positive breast carcinoma) cells [8].

Chamaecyparis Spach is a small genus with about 5-7 species. *Chamaecyparis nootkatensis* D. Don (Alaskan yellow cedar, disputed now to be classified back in the genus *Cupressus*), containing diterpene constituents termed nootkastatins, has demonstrated efficient cytotoxic effects [9]. Thujaplicins (tropolone-related compounds) were identified in the woods of *Chamaecyparis obtusa* (Siebold & Zucc.) Endl. (Japanese cypress) and are known for their antioxidant and other bioactive properties [10]. The lignan chamaecypanone C is a novel microtubule inhibitor from the heartwood of *Chamaecyparis obtusa* var. *formosana* (Taiwan hinoki) that has shown high cytotoxic activity in nanomolar ranges on various cancer cells [11]. In addition, hot-water leaves extract of this Taiwan endemic conifer has exhibited high radical scavenging activity, attributed to several constituents (catechin, quercetin, quercetin-3-O-α-rhamnoyranoside, myricetin-3-O-α-rhamnoyranoside, vanillic acid, and 4-hydroxybenzoic acid). Extracts from the bark of this cypress have also shown efficient antioxidant activity [12].

Representatives of genus *Taxus* L. (Taxaceae), such as *T. baccata* L., *T. brevifolia* Nutt., *T. cuspidata* Siebold & Zucc., etc., are sources of taxine alkaloids, which are precursors for the synthesis of powerful anticancer drugs like Paclitaxel [13], Docetaxel [14], etc. Cytotoxic lignans have been isolated from the heartwood of *T. baccata* (common yew) and have shown antioxidant and other activities [15]. The comparison of the bioactivity of extracts of *Taxus baccata* bark and *Juniperus sabina* fruits have revealed their similar cytotoxicity on different cancer cells [16]. These findings showed that junipers are also perspective sources of efficient cytotoxic and antioxidant agents for treatment of various malignancies and other diseases.

In response to the requirements of the pharmacy in invention of new efficient bioactive agents, the present study is aimed at extraction and identification of antioxidant and antiproliferative substances as potential pharmaceutical agents from species of the genera *Juniperus* L., *Cupressus* L., *Chamaecyparis* Spach and *Taxus* L., growing in the Windsor Great Park, UK, and other arboretums.

EXPERIMENTAL

Materials

Chemicals and reagents. Podophyllotoxin, DPPH (2,2-diphenyl-1-picrylhydrazyl), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazo-lium bromide], Folin-Ciocalteu's reagent (2N), gallic acid, formic acid, RPMI 1640 medium were purchased from Sigma-Aldrich Co. (Saint Louis, MO, USA). Fetal calf serum for cell culture was delivered by Biochrom GmbH (Berlin, Germany), DMSO was from Fluka Chemie AG (Buchs, Switzerland). LC-MS grade solvents were purchased from Fisher Scientific (USA) and Sigma-Aldrich (USA).

Plant material. Juniperus representatives and *Cuprocyparis notabilis* were delivered in June 2018 from the Windsor Great Park, London, UK. Their specimen numbers are given in Table 1. *Taxus baccata*, *Cupressus arizonica*, *Chamaecyparis lawsoniana* and *Chamaecyparis pisifera* were obtained in February 2018 from the Arboretum of the University of Forestry, Sofia, Bulgaria. Voucher specimen of the plants of Bulgarian origin were deposited in the Herbarium (SOM) of the Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences. The plant species were authenticated by R. P. Adams and A. N. Tashev (Table 1).

Experimental procedures

Experimental procedures were carried out as it has been described previously (Ivanova *et al.* [5]) and are described here in brief.

Determination of total polyphenol content (TPC). The TPC of the extracts was determined by Folin-Ciocalteu method with minor modifications [17] and was expressed in Gallic Acid Equivalents (GAE) according to the formula:

$$C = c.V/m,$$

where C is concentration of phenolic compounds in mg GAE per gram dry extract; c – gallic acid concentration [mg/ml], calculated from the calibration curve; m – weight of the dry plant extract [g]; V – plant extract volume [ml].

Table 1. Summary of the specimens of conifers of different origin, total polyphenol content, antioxidant and antiproliferative activity of their leaves extracts.

№	Specimen number	Conifer species	Arboretum	TPC [GAE mg/g DE]	DPPH-SC_{50} [μg/ml]	NB4-IC_{50}±SD [μg/ml]
1	1999-6149	*Juniperus virginiana* "Glauca" × "Grey Owl"	WGP	107 ±6	190	0.27±0.03
2	1999-6045	*Juniperus virginiana* "Canaertii"	WGP	134±4	151	0.31±0.03
3	2001-2555	*Juniperus* × *pfitzeriana* "Saybrook Gold"	WGP	86±1	282	2.3±0.2
4	2000-1308	*Juniperus pingii* var. *Wilsonii*	WGP	151±4	112	2.6±0.2
5	2000-1308	*Juniperus indica* Bertol.	WGP	320±10	52	43±2
6	1999-2968	*Juniperus recurva* "Embley Park"	WGP	213±3	71	25±1
7	1999-5914	*Juniperus davurica* "Expansa Aurea"	WGP	84±2	250	85±11
8	1999-6163	*Juniperus squamata* Buch.-Ham. ex D.Don	WGP	209±9	78	139±14
9	1999-5996	*Juniperus sargentii* "Glauca"	WGP	97±12	191	184±17
10	SOM 177 249	*Taxus baccata* L.	UFA	127±16	141	9±1
11	SOM 177 250	*Cupressus arizonica* Greene	UFA	196±4	76	62±7
12	SOM 177 251	*Chamaecyparis lawsoniana* Parl.	UFA	152±1	106	87±11
13	SOM 177 252	*Chamaecyparis pisifera* (Siebold & Zucc.) Endl.	UFA	134±4	134	145±22
14	1999-3346	*Cuprocyparis notabilis* (A. F. Mitchell.) Farjon	WGP	174±6	118	111±12

Abbreviations: WGP – Windsor Great Park, London, UK; BAS – Bulgarian Academy of Sciences; UFA – University of Forestry Arboretum, Sofia, Bulgaria; TPC – total polyphenol content; DE – dry extract; GAE – milligrams gallic acid equivalents per gram DE. Lower IC_{50} and SC_{50} values denote higher activity.

The TPC of each extract was determined by two independent analyses and was given as an average value ± SD (standard deviation).

Determination of antioxidant activity. The radical scavenging activity of the extracts was determined by the DPPH-method [18]. The percentage of the DPPH-inhibition by the corresponding extract was calculated according to the formula:

$$\%\ inhibition\ =\ [(Ac - As)/Ac] \times 100,$$

where *Ac* is the absorbance of the DPPH solution in the control sample without extract and *As* is the absorbance of the DPPH in the sample containing the corresponding plant extract.

The antioxidant activity of the plant extracts was analyzed by their DPPH-SC_{50} (half-maximum DPPH-scavenging concentration of the extracts) – concentration of the extract that decreased the initial DPPH concentration by 50%.

An UV-1600PC spectrophotometer (VWR int.) was used for Folin-Ciocalteu and DPPH-assays.

Cell culture and MTT-test for antiproliferative activity of the plant extracts. NB-4 cells were purchased from the DSMZ (Germany). MTT-tests were carried out using a microplate reader Labexim

LMR1s. The antiproliferative activity of the corresponding extract was determined by analysis of its NB4-IC_{50} (half-maximum growth-inhibitory concentration in NB4 cells) – concentration of the extract that decreased the initial NB4 cells growth by 50%. Positive control for MTT-test was podophyllotoxin standard with NB4-IC_{50} 0.005±0.001 μg/ml. Cell proliferation was determined by MTT-assay as it was described elsewhere [19].

Data processing and statistics. The MTT data were fitted to sigmoidal concentration–response curves and the NB4-IC_{50} values were calculated using non-linear regression analysis (GraphPad Prism software). Statistical processing exploited Student's t-test with $p \leq 0.05$ set as the lowest level of statistical significance. TPC and DPPH-SC_{50} statistics were calculated using 'Excel 2013' software.

UHPLC/HRMS for podophyllotoxin identification. Ultra high-performance liquid chromatography (UHPLC) coupled to high-resolution mass spectrometry (HRMS) was performed on a Thermo Scientific Dionex Ultimate 3000 RSLC system connected to Thermo Scientific Q Exactive Plus mass spectrometer (Bremen, Germany), equipped with a heated electrospray ionization (HESI-II) probe (Thermo Scientific). The tune parameters in positive mode were as follows: spray voltage 3.5 kV; sheath gas flow rate 38; auxiliary gas flow rate 12; spare gas flow rate 0; capillary temperature 320 °C; probe heater temperature 320 °C and S-lens RF level 50. Acquisition was acquired at Full-scan MS and Data Dependent-MS^2 modes ($ddMS^2$). Full-scan spectra were obtained over the *m/z* range 100-1500 at a resolution of 70000, authomatic gain control (AGC) target and maximum ion injection time (IT) were set to $3e^6$ and 100 ms, respectively. The instrument parameter settings for $ddMS^2$ mode were as follows: resolution 17500, AGC target $1e^5$, maximum IT 50 ms, loop count 5 (TOP5), isolation window 2.0 m/z, stepped normalized collision energy (NCE) 10, 30, 60 eV. Data acquisition and processing were carried out with Xcalibur 4.0 software (Thermo Scientific). Fragmentation pathways were simulated with Mass Frontier 7.0 (Thermo Fisher Scientific). Prior to injection, samples were subjected to solid-phase purification by Sep-Pak C_{18} cartridges (Waters, Ireland), using 80% (v/v) methanol. Chromatographic separation was achieved on AkzoNobel Kromasil Externity XT-1.8-C18 (Bohus, Sweden) narrow-bore column (2.1×100 mm, 1.8 μm) with Phenomenex Security Guard ULTRA UHPLC EVO C18 (Torrance, USA) at 40°C. The mobile phase consisted of systems A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). The following gradient was used: the mobile phase was held at 5% B for 0.5 min, gradually turned to 60% B over 22.5 min, kept at 60% B for 2 min, followed by a gradual increase to 85% B over 2.5 min, kept at 85% B over 2 min and the system was turned to the initial condition of 5% B in 0.5 min. The system was conditioned at 5% B for 4.5 min before injection. The flow rate and injection volume were 300 μL/min and 2 μL, respectively.

RESULTS AND DISCUSSION

Correlation of the total polyphenol content with the antioxidant activity of various conifers – determination of J. indica extract as superior antioxidant agent

According to the requirements of the pharmacy for identification of new efficient antioxidant agents, the present work studied the activity of extracts of widely distributed species, as well as rare representatives of *J. indica* Bertol. (black juniper), *J. squamata* Buch.-Ham. ex D. Don (Himalayan juniper, flaky juniper), *J. pingii* W. C. Cheng (Chinese juniper), *J. recurva* Buch.-Ham. ex D. Don (Himalayan juniper, drooping juniper), *J. davurica* Pall., etc., growing in the Windsor Great Park and other arboretums. The Windsor Great Park has been created in the 13th century. At present, it covers about 2020 hectares of lands, in which a large part is a conservation area with recognized value of biodiversity.

Conifers of different origin, total polyphenol content, antioxidant and antiproliferative activity of their leaves extracts are presented in Table 1. As it could be seen from the examined specimens, lowest DPPH-SC_{50} values were determined for *J. indica* Bertol. (52 μg/ml), followed by *J. recurva* "Embley Park" (71 μg/ml), *C. arizonica* Greene (76 μg/ml) and *J. squamata* Buch.-Ham. ex D. Don (78 μg/ml) extracts. These findings corresponded to the highest values of their TPC. Data about the total polyphenol content of the studied juniper leaves extracts are presented in Figure 1.

A polynomial function was derived in order to describe the correlation between the TPC and half-maximum DPPH-radical scavenging concentrations of the corresponding extracts (Figure 2). Lower DPPH-SC_{50} values denote higher activity and correspond to higher TPC values. In summary, the comparison of the polyphenol content and radical-scavenging activities of the extracts revealed *J. indica* (the black juniper) leaves extract as a superior antioxidant agent, exhibiting best TPC and DPPH-SC_{50} values among the studied species.

Moderate radical scavenging activity and cytotoxicity have been observed previously for *J. recurva* Buch.-Ham. ex D. Don extracts [20].

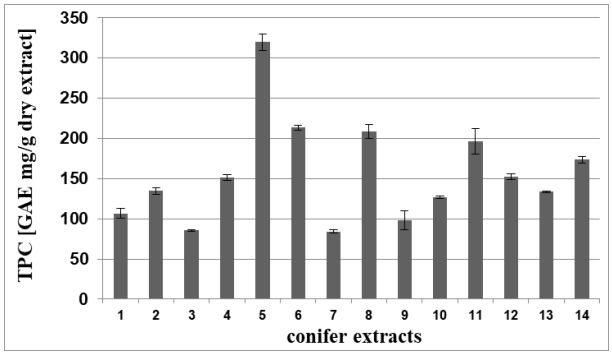

Figure 1. Comparison of the total polyphenol content of the studied conifer leaves extracts. Conifer extracts (**1-14**) correspond to the numbers in Table 1.

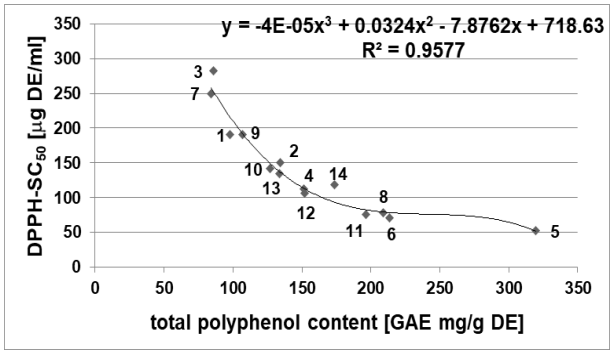

Figure 2. Graphical presentation of the polynomial function derived as a correlation of the total polyphenol content of the studied conifer leaves extracts with their DPPH-radical scavenging half-maximum concentrations. Conifer extracts (**1-14**) correspond to the numbers in Table 1.

The fruit oil of *Cupressus arizonica* Greene and extracts from the bark of *Chamaecyparis lawsoniana* (A. Murray) Parl. (Lawson cypress) have shown also remarkable antioxidant activity [21, 22]. To our knowledge, this study is the first observation about the remarkable antioxidant activity of *J. indica*. The black juniper is a rare plant, grown in the Windsor Great Park, but it exists naturally at high-altitudes of the Himalayas (as high as 5200 m a.s.l.) and the Tibetan Plateau.

Identification of conifers with efficient antiproliferative activity in NB4 APL cells.

The anticancer drug precursor podophyllotoxin (PPT) with efficient antiproliferative properties has been detected in many *Juniperus* species, such as *J. virginiana* L. [23], *J. × media* Pfitzeriana (Spath) Schmidt [24], *J. horizontalis* Moench, *J. scopulorum* Sarg. [25], etc. In this study, the antiproliferative activity of the studied conifer extracts was analyzed by MTT tests after treatment of NB4 APL (acute promyelocytic leukemia) cells, bearing t(15;17) PML-RARA fusion gene with oncogenic properties. The comparative analysis of the NB4-IC_{50} values of the studied leaves extracts revealed the best antiproliferative activity of *J. virginiana* cultivars ('Canaertii', 'Glauca × Grey Owl') with NB4-IC_{50} values in the range of 0.27-0.31 μg/ml. In addition, high activity in NB4 cells was found also for the extracts of *J. × pfitzeriana, J. pingii* var. *wilsonii* and *T. baccata* representatives with IC_{50} values in the range of 2-9 μg/ml. This study revealed that the leaves extracts of *J. virginiana, J. × pfitzeriana cultivars* and *J. pingii* var. *wilsonii*, distinguished here as the best cytotoxic agents among the studied species, showed even higher antiproliferative activity in comparison with the *T. baccata* leaves extract (Table 1). Rare species of the Windsor Great Park, such as *J. indica* and *J. recurva* "Embley Park" also showed remarkable antiproliferative activity.

Podophyllotoxin was identified in the extracts of *J. virginiana* and *J. × pfitzeriana* cultivars, which demonstrated the best antiproliferative activity in this study. Using UHPLC- HRMS, the exact mass of the protonated molecule $[M+H]^+$ of PPT was detected in the full scan spectrum at *m/z* 415.1385 (the calculated *m/z* for $C_{22}H_{23}O_8$ is 415.1387), while its characteristic ion fragments at *m/z* 397.1267, 313.1068, 282.089 and 247.0603 appeared in the MS^2 spectrum [26]. Taxine alkaloids were identified in the *T. baccata* leaves extract in correspondence with literature data [27]. Identification of other bioactive metabolites in the most efficient antioxidant and cytotoxic extracts is in progress.

CONCLUSIONS

The Windsor Great Park is an area of recognized value of biodiversity, making it a perspective source of rare species and cultivars of scientific interest. To our knowledge, this research is the first study of the bioactivity of conifers growing in the Windsor Great Park. Their activity and bioactive metabolites were analyzed in comparison with conifers from other arboretums.

This study outlined for the first time *J. indica* leaves extract as a natural agent with superior total polyphenol content and antioxidant properties.

Highly efficient antiproliferative activity was observed for the first time in NB4 acute promyelocytic leukemia cells for the leaves extracts of *J. virginiana* ('Canaertii', 'Glauca x Grey Owl'), *J. × pfitzeriana* cultivars and *J. pingii* var. *wilsonii*. These agents showed excellent cytotoxic activity that was even at higher values in comparison with the activity of the *T. baccata* leaves extract containing taxine alkaloids. Rare species of the

WGP, such as *J. indica* and *J. recurva* "Embley Park", also showed remarkable antiproliferative activity. Podophyllotoxin was identified in the extracts of *J. virginiana* and *J.* × *pfitzeriana* cultivars. Identification of other metabolites and quantitative analysis of the bioactive substances, identified in the best cytotoxic and antioxidant extracts, are in progress. The conifer extracts, selected in this study as efficient antioxidant and antiproliferative agents, are potential natural sources of lead compounds for the drug industry.

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